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Mono-allelic and bi-allelic ENPP1 deficiency promote post-injury neointimal hyperplasia associated with increased C/EBP homologous protein expression

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Abstract

Objective—Bi-allelic function-inactivating ENPP1 mutations cause artery media calcification (AMC) with associated severe myointimal hyperplasia in generalized arterial calcification of infancy (GACI), whereas mono-allelic ENPP1 deficiency is phenotypically normal. Here, we tested if ENPP1 deficiency promotes abnormal vascular smooth muscle cell (VSMC)-driven responses to injury, with or without calcification. The ER stress mediator C/EBP homologous protein (CHOP) affects neointimal hyperplasia and atherosclerosis, and has paradoxical effects on bone formation. Hence, we assessed relationships between ENPP1 and CHOP in VSMCs.

Methods—We studied ENPP1-deficient mice and control littermates subjected to left carotid artery ligation, and isolated and studied VSMCs from these and *Chop*^{-/-} mice, or with CHOP siRNA treatment.

Results—Normal *Enpp1*^{-/+} mice, in addition to *Enpp1*^{-/-} mice prior to AMC development, had accelerated neointimal hyperplasia in response to carotid artery ligation at 7-8 weeks age. Neointimal hyperplasia was linked with robust artery media CHOP expression *in situ*, but with marked AMC only in injured *Enpp1*^{-/-} arteries. Cultured, ENPP1-deficient and CHOP-deficient VSMCs had increased migration and proliferation to PDGF. Cultured *Chop*^{-/-} VSMCs demonstrated increased P_i donor-induced calcification. CHOP was significantly increased in P_i donor treated *Enpp1*^{-/-} and *Enpp1*^{-/+} cultured VSMCs. CHOP siRNA treatment of *Enpp1*^{-/-} VSMCs increased calcification, associated with elevated expression of tissue nonspecific alkaline phosphatase and the master osteoblastic transcription factor RUNX2.

Conclusions—Both mono-allelic and bi-allelic ENPP1 deficiency promote dysregulated VSMC function, with robust lesion CHOP expression and enhanced neointimal hyperplasia after injury *in vivo*, but marked post-injury calcification limited to *Enpp1*^{-/-} mice. Intimal hyperplasia in GACI appears regulated by biologic effects of ENPP1 deficiency other than calcification, including ER stress. VSMC CHOP excess in ENPP1 deficiency may primarily function to limit VSMC calcification.

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Keywords

Artery media calcification; Generalized arterial calcification of infancy; ER stress; Unfolded protein response; CHOP

Introduction

Artery media calcification (AMC), in association with variable degrees of myointimal hyperplasia and fibrosis, becomes common in end stage renal disease (ESRD), diabetes, and aging (1,2). AMC decreases arterial compliance (3,4) and, along with fibrosis, promotes hypertension and arterial obstruction (2). AMC is associated with increased risk of limb amputations and mortality in diabetic arteriopathy and ESRD. Health care costs of arterial disease in diabetes and ESRD are high. Effectively targeting AMC, and associated myointimal hyperplasia and fibrosis, is a major unmet need.

Bi-allelic function-inactivating ENPP1 mutations cause AMC and associated severe myointimal fibrosing hyperplasia of large and medium-sized muscular arteries in Generalized Arterial Calcification of Infancy (GACI), an often lethal disease of infancy (5,6). ENPP1 is selectively expressed by VSMCs and other cells with robust synthetic function (e.g., chondrocytes, osteoblasts, plasma cells, and hepatocytes) (7-9). Because most GACI is a single gene disorder (bi-allelic ENPP1 deficiency (6)), that can help our understanding of the pathogenesis in intimal hyperplasia associated with AMC.

Intimal hyperplasia is promoted by dysregulated VSMC differentiation and function, including increased migration, MMP activation, and both proliferation and apoptosis (10). Oxidative stress and inflammatory stimuli (eg, PDGF, IL-1 β , IL-6) are among mediators of abnormal VSMC function in intimal hyperplasia (11). However, deposited basic calcium phosphate crystals in AMC can promote macrophage activation and cell proliferation (12), and AMC promotes arterial stiffness and hypertension. Hence, we need to understand if myointimal hyperplasia in GACI is driven by antecedent effects of AMC and/or other biologic effects from ENPP1 deficiency.

ENPP1 hydrolyzes ATP and other nucleoside phosphates to PP_i and AMP (8). PP_i is a requisite physiologic inhibitor of calcification, by effects including inhibition of hydroxyapatite crystal growth (8). However, ENPP1 and PP_i also modulate cell differentiation. For example, ENPP1, PP_i, and the PP_i transporter ANK suppress osteochondral differentiation switching in VSMCs and mesenchymal progenitor cells (9,13), and deficiency of ENPP1 and PP_i increase expression of pro-fibrotic and pro-mineralizing type I collagen (7). Moreover, ENPP1 deficiency is associated with decreased soluble RAGE production by VSMCs and increased expression of the RAGE ligand S100A11 *in vitro*, and RAGE knockout inhibits AMC in *Enpp1*^{-/-} mice (14). RAGE is an essential mediator of injury-induced neointimal hyperplasia (15,16). RAGE ligand-induced signaling stimulates dysregulated ER stress responses (17), one of multiple inflammatory mechanisms by which RAGE signaling can promote diabetic organ complications.

Induction of C/EBP homologous protein (CHOP) is among consequences of ER stress (17,18). CHOP restores protein translation depressed in the course of the unfolded protein response (UPR) (18). However, excess CHOP promotes oxidative stress and apoptosis (18,19). Apoptosis of not only endothelial cells but also of VSMCs, promotes injury-induced neointimal hyperplasia (10). Significantly, CHOP knockout reduce both post-arterial injury neointimal hyperplasia and progression of atherosclerosis (19, 20).

AMC is mediated by ectopic bone and cartilage development processes (1,2). In this context, CHOP has paradoxical effects on bone formation. Specifically, constitutive CHOP expression promotes osteoblast maturation in response to bone morphogenetic protein (BMP) treatment in a cell-type-dependent manner, thereby supporting physiologic calcification in bone *in vivo* (21,22). However, CHOP also heterodimerizes with C/EBP β and thereby limits C/EBP β DNA-binding and RUNX2-binding activities, thereby inhibiting osteoblast differentiation (21). Moreover, transgenic bone-specific CHOP overexpression limits bone formation *in vivo* (23). Here, we observe that both mono-allelic and bi-allelic ENPP1 deficiency promote neointimal hyperplasia responses to arterial injury, in association with dysregulated VSMC function and increased VSMC expression of CHOP, whose consequences we elucidate in VSMCs.

Methods

Mice

We studied *Enpp1*^{-/-} mice on C57BL/6 background (9). *Enpp1*^{-/+} breeders generated congenic *Enpp1*^{-/-}, *Enpp1*^{-/+}, and *Enpp1*^{+/+} littermates for experiments. CHOP knockout mice (22), from Jackson Laboratories, were bred onto C57BL/6 background for more than 10 generations. All animal procedures were humanely performed, with institutionally approved protocol.

Carotid Artery Ligation

Left carotid artery ligation was performed as described (16), in 7-8 weeks old mice. Carotid arteries in euthanized animals were fixed with 4% paraformaldehyde (PFA) in PBS for morphological analysis or frozen for protein extraction and analysis by tissue SDS-PAGE Western blot. Radii were measured on elastin stained sections by Verhoeff-van Gieson protocol (16).

Immunohistochemistry

For immunohistochemistry, mice carotid artery paraffin-embedded sections, with endogenous peroxide activity blocked with 0.3% H₂O₂, and nonspecific antibody binding blocked using 5% normal goat serum, were incubated with primary antibodies for 18 h at 4°C (16). We used primary rabbit antibody to CHOP (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit antibody to PARPp85 (Promega, Fitchburg, WI). Secondary antibody (biotinylated goat anti-rabbit IgG, Invitrogen, Carlsbad, CA) treatment for 30 minutes at 22°C, was followed by 30 min treatment using Histostain Plus kit (Invitrogen). Washed sections were incubated with 3,3'-diaminobenzidine (DAB) substrate for 2-5 min, and counterstained with hematoxylin. Images were captured using an Olympus BX51

microscopic DP71 Digital Camera System. Calcification *in situ* was assessed via von Kossa staining kit (NovaUltra, IHC World, Woodstock, MA). Section area was measured using NIH Image J software.

Primary mouse aortic VSMC Isolation, Culture, and Function and SDS-PAGE/Western blotting Studies

Primary VSMCs were isolated via collagenase and elastase digestion from aortae of euthanized 7-8 weeks old mice (16). VSMCs seeded in 0.1% gelatin pre-coated 6-well plates and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, and expanded up to passage 6, with VSMC purity confirmed, as described (16). VSMC migration (4 replicates/experiment) was measured by Transwell assay for ENPP1 studies (16) and by a modified approach (24) for the CHOP knockout VSMC studies. VSMC proliferation (8 replicates/experiment) was measured using CellTiter 96 Aqueous One Solution (Promega) (16). Transfection of VSMCs was by Nucleofection (Amaxa, Gaithersburg, MD), with the manufacturer protocol optimized for primary VSMCs, yielding a transfection efficiency of 60-80%; siRNA for CHOP was from Ambion (Austin, TX). SDS-PAGE and Western blotting were performed as described in detail (16). Antibody sources were: CHOP (Biolegend, San Diego, CA), GRP78 and RUNX2, GAPDH (Cell Signaling, Boston, MA), and β -actin (Sigma-Aldrich, St. Louis, MO).

For *in vitro* calcification studies, 10 mM β -glycerolphosphate with 50 µg mM L-ascorbate (Sigma-Aldrich) was added to VSMCs, with medium changes every 3 days, and Alizarin Red S staining performed and solubilized calcium quantified, as described (25). For CHOP knockdown in ENPP1 deficiency VSMC studies, passage 1 VSMCs in a 24-well plate (2×10^5 cells/well), were cultured until confluency and transfected with CHOP siRNA (100 nmoles) or siRNA control (100 nmoles) (Santa Cruz Biotechnologies) using X-tremeGENE siRNA reagent (Roche, South San Francisco, CA), per manufacturer protocol, and transfections repeated every 4 days for 15 days total, with culture medium replaced every 3 days.

Quantitative RT-PCR

Total RNA was extracted from cells by RNeasy column (Qiagen, Valencia, CA), and total RNA (100 ng) was reverse-transcribed with random and anchored Oligo-DT primers (Transcriptor First Strand cDNA synthesis kit (Roche)). Resulting cDNA was used as template (2 µL) in 20 µL real-time PCR reactions with SYBR Green PCR Master Mix (Roche). Optimized primers were used in the real-time PCR reactions on LightCycler Roche480: mouse CHOP forward 5'-CTGCCTTTCACCTTGAGAC-3', and reverse 5'-CGTTTCCTGGGGATGAGATA-3'; mouse RUNX2 forward 5'-TAAGAAGAGCCAGGCAGGTG-3', and reverse 5'-GGTTGGGTGCTTACGTGAT-3'; mouse TNAP forward 5'-AACCCAGACACAAGCARRCC-3' and reverse 5'-GAGAGCGAAGGGTCSGTCAG-3' and mouse GAPDH forward 5'-TGTGTCCGTCGTGGATCTGA-3' and reverse 5'-CCTGCTTCACCACTTCTTGA-3' (10 µM, 2 µL). Relative quantitative analyses ($2^{-\Delta\Delta C_t}$) normalized to GAPDH (ΔC_t). Initial

(pre-induced) gene expression levels (dCt) for each gene of interest were used as calibrators (ddCt) for fold-changes compared to unstimulated control cells or siRNA control cells.

Statistical analyses

Results were expressed as mean $\pm$ SEM. Statistical differences between group means were determined by one-way ANOVA with post hoc Tukey testing, or, where specifically indicated, by two-way ANOVA with post hoc Bonferroni testing, using GraphPad PRISM 5 software. P values < 0.05 were considered statistically significant.

Results

Mono-allelic and bi-allelic ENPP1 deficiency promoted neointimal hyperplasia in response to arterial injury

We first tested if left carotid artery injury-induced myointimal hyperplasia promotes AMC. To do so, we studied 7-8 weeks old *Enpp1*^{-/-} mice, since this is a time point before the spontaneous AMC appears, which typically is between 14 and 25 weeks of age in our mice, established on C57BL/6 background (9). In parallel, we studied phenotypically normal *Enpp1*^{+/-} mice. Accelerated neointimal hyperplasia developed in both *Enpp1*^{-/-} and *Enpp1*^{+/-} mice within 14 days (**Figure 1A-B, Table 1**). Within 21 days, we detected development of robust AMC in only in ENPP1-deficient mice, and the AMC was significantly greater in the injured *Enpp1*^{-/-} mice carotid arteries compared to *Enpp1*^{+/-} arteries (**Figure 2**).

ER stress responses increased *in situ* in both the injured *Enpp1*^{+/-} and *Enpp1*^{-/-} carotid arteries, compared to wild type carotid arteries. This was evidenced by more robust increase in CHOP expression, and ER stress-related GRP78 *in situ* in injured ENPP1-deficient carotid arteries than in wild type arteries, at 14 and 21 days after injury, in tissue Western blots (**Figure 3**). In contrast, there was little gross difference in cleaved caspase-3 in protein extracts of injured ENPP1-deficient carotid arteries compared to wild type injured carotid arteries (**Figure 3**). Next, we studied the arterial lesion media *in situ* by Immunohistochemistry, as early as 3 days after injury, before manifestation of the accelerated neointimal hyperplasia in ENPP1-deficient mice. In comparison to wild type, we observed more robust *de novo* media cell expression *in situ* of CHOP (**Figure 4A**), and apoptosis marker PARPp85 as early as 3 days after carotid ligation injury in *Enpp1*^{+/-} and even more so *Enpp1*^{-/-} mice (**Figure 4B**).

Dysregulated function of *Enpp1*^{-/-} VSMCs and effects of CHOP

To examine ENPP1 deficiency and CHOP in VSMC function independent of calcification, we first cultured VSMCs under non-calcifying conditions, under which we saw no spontaneous calcification, unlike *Enpp1*^{-/-} mice VSMCs treated with P₁ donor (data not shown). Compared to *Enpp1*^{+/+}, both *Enpp1*^{-/-} and *Enpp1*^{-/+} cultured aortic VSMCs demonstrated increased migration and proliferation in response to PDGF (**Figure 5**). In *Chop*^{-/-} VSMCs, there also was increased migration and proliferation in response to PDGF, compared to congenic *Chop*^{+/+} (**Figure 6**).

Chop^{-/-} VSMCs manifested increased calcification at 15 and 21 days in response to β -glycerolphosphate/ascorbate-phosphate, compared to congenic *Chop*^{+/+} VSMCs (**Figure 7**). CHOP expression was significantly increased in P_i donor-treated *Enpp1*^{-/-} and *Enpp1*^{+/-} VSMCs, compared to *Enpp1*^{+/+} VSMCs, and also increased in untreated *Enpp1*^{-/-} (but not *Enpp1*^{+/-}) VSMCs (**Supplemental Figure 1**). Hence, we concluded the study by focusing on the role of CHOP in calcification by *Enpp1*^{-/-} VSMCs. We observed increased P_i donor-induced calcification, with associated increased RUNX2 and TNAP expression, after CHOP siRNA knockdown in *Enpp1*^{-/-} VSMCs (**Figure 8**).

Discussion

Our results reveal that ENPP1 deficiency, accelerates neointimal hyperplasia in response to arterial injury, beyond known promotion of matrix calcification associated with ectopic osteochondral differentiation in pluripotential cells (9). Both mono-allelic and bi-allelic ENPP1 deficiency promoted neointimal hyperplasia within 3 weeks after carotid artery ligation injury. Post-injury AMC developed in *Enpp1*^{-/-} as well as *Enpp1*^{+/-} mice, but was robust only in *Enpp1*^{-/-} mice. These findings reinforce that arterial injury can induce and accelerate AMC.

Dysregulated VSMC function is central to neointimal hyperplasia. We observed increased migration and proliferation responses to PDGF in cultured *Enpp1*^{-/-} and *Enpp1*^{+/-} VSMCs. ENPP1 deficiency also was linked with increased expression of ER stress mediators GRP78 and CHOP *in situ* in both *Enpp1*^{-/-} and *Enpp1*^{+/-} carotid arteries after injury. Robust expression of CHOP and apoptosis marker PARP85 was detected in artery media in *Enpp1*^{+/-} and *Enpp1*^{-/-} mice prior to progression of neointimal hyperplasia. These findings on detection of pro-apoptotic lesion CHOP excess and of PARP85 are pertinent partly because VSMC apoptosis (as well as endothelial cell apoptosis) promote progression of neointimal hyperplasia (10). Hence, early arterial injury-induced apoptosis of VSMCs within artery media could be enhanced by ER stress-modulated apoptosis in ENPP1 deficiency. However, increased VSMC oxidative stress, MMP activity, proliferation and migration also mediate neointimal hyperplasia (11), and we did not globally examine VSMC functions here.

CHOP expression was increased in untreated *Enpp1*^{-/-} VSMCs, and P_i donor-treated *Enpp1*^{-/-} and *Enpp1*^{+/-} VSMCs. CHOP knockout diminishes neointimal hyperplasia in response to femoral artery cuff injury *in vivo* (20). CHOP promotes ER oxidative stress *in vitro* (18) and CHOP knockout limits atherosclerosis lesion progression *in vivo* (19). CHOP knockout also impairs physiologic bone calcification (22), but biologic effects of CHOP excess indicate paradoxical CHOP functions. Specifically, transgenic CHOP excess limits bone formation (23), putatively mediated by known capacity to promote osteoblast apoptosis and limit RUNX2 expression (21). Here, we observed that CHOP knockout increased PDGF-induced migration and proliferation *in vitro*, and increased calcification in VSMCs. In addition, CHOP siRNA knockdown in P_i donor-treated *Enpp1*^{-/-} VSMCs increased calcification, in association with increased RUNX2 and TNAP expression.

Taken together, our results suggest that CHOP excess, linked to heightened ER stress in ENPP1 deficiency, may function to limit ENPP1 deficiency-associated increases in proliferation and migration responses of VSMCs to injury, and suppress calcification in response to the organic P_i donor β -glycerolphosphate with ascorbate-phosphate. However, results for CHOP function in VSMCs in this study, tied either to specific stimuli or to CHOP excess in ENPP1 deficient VSMCs *in vitro*, are not generalizable. For example, lentiviral CHOP shRNA knockdown suppressed calcification responses to TNF α and a cytotoxic concentration of the inorganic P_i donor sodium phosphate, but this was done in the SV40-immortalized VSMC cell line MOVAS-1 (26). As such, the nature of arterial wall disease, the sources and levels of artery wall P_i excess, and the roles of basal and excess levels of CHOP expression, could have distinct roles in pathways leading to neointimal hyperplasia and AMC. In this context, P_i triggers many pro-calcifying pathways, and high P_i induces varying degrees of apoptosis and autophagy in VSMCs, mediated by P_i uptake and oxidative stress (27). P_i induction of autophagy is a major endogenous inhibitory mechanism for calcification by cultured VSMCs, via inhibiting procalcifying functions of release of matrix vesicles, cell fragments that promote apatite crystal growth more potently than do apoptotic bodies (27). This renders the known capacity of CHOP to promote not only apoptosis but also autophagy (28) potentially pertinent to our finding of CHOP limiting calcification by P_i donor-treated VSMCs (28).

Study limitations included that the ENPP1 knockout is global; we have not yet floxed ENPP1 or examined tissue-specific ENPP1 deficiency. Examination of CHOP knockout animals *in vivo*, and double ENPP1 and CHOP deficient mice also were beyond study scope, and potentially limited by combining defects in long bone calcification in both ENPP1 and CHOP knockout mice. Moreover, we did not determine the root cause of increased VSMC ER stress in ENPP1 deficiency, and whether increased RAGE signaling is a factor (14).

This mouse study suggests inherited partial human VSMC ENPP1 deficiency can promote vascular disease even without AMC. In obesity with diabetes, *ENPP1* K121Q polymorphism is associated with elevated cardiovascular events (29), coronary artery calcification, and higher aortic stiffness in dialysis (30), though these findings have not been broadly replicated. We linked *ENPP1* K121Q to modestly decreased ENPP1 enzymatic activity (6), but *ENPP1* K121Q also can increase insulin resistance (31). Acquired ENPP1 deficiency, or related extracellular PP_i deficiency due to decreased ANKH-mediated PP_i transport (32), also can develop in chronic vascular disease. For example, pro-atherogenic IL-1 β and TNF α decrease ENPP1 and/or ANKH expression and PP_i levels (33,34). Plasma PP_i levels, and arterial expression of ANKH, are depressed in human end stage renal disease (33,34), findings potentially physiologically significant, since systemic PP_i treatment limits experimental uremic AMC (35).

In conclusion, both mono-allelic and bi-allelic ENPP1 deficiency promote abnormal responses to arterial injury, associated with dysregulated VSMC function and increased VSMC CHOP that may act to limit calcification. These results point to novel effects of ENPP1 on VSMC homeostasis independent of osteochondral differentiation switching and matrix calcification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ENNP1	ectonucleotide pyrophosphatase phosphodiesterase 1
CHOP	C/EBP homology protein
RUNX2	runt-related transcription factor 2
VSMCs	vascular smooth muscle cells
GRP78	glucose-regulated protein 78kDa
AMC	artery media calcification
MMP	matrix metalloproteinase

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Figure 1. Both ENPP1 mono- and bi-allelic deficiency are associated with increased intimal hyperplasia in response to arterial injury

A. Left carotid ligation surgery, proximal to the carotid bifurcation, used 6-0 nylon silk, in 7-8 weeks old mice ($n = 7-9$ of each genotype). Shown are representative elastin stained sections (Verhoef-van Gieson method) at 14-21 days after left carotid ligation. **B-C.** Intima/media area (I/M) ratios showed accelerated neointimal hyperplasia in *Enpp1*^{+/-} and *Enpp1*^{-/-} mice at 14 days and 21 days (statistics: 2-way ANOVA, post hoc Bonferroni testing). Scale bar: 200 μ m.

Figure 2. Media calcification in response to carotid artery ligation injury in ENPP1 haploinsufficient and homozygous knockout mice

Enpp1^{+/+}, *Enpp1*^{+/-}, and *Enpp1*^{-/-} mice underwent left carotid ligation at 7-8 weeks of age.

A. Representative von Kossa staining (black areas in media) in sections of ligated left carotid arteries are shown at 21 days post-procedure. In the injured, but not uninjured, carotid arteries, the *Enpp1*^{-/-} mice developed both intimal hyperplasia and robust von Kossa staining by 21 days post-ligation (Arrows). Media calcification was slightly increased, relative to congenic wild type, in *Enpp1*^{-/-} mice at 21 days post-ligation. (40X magnification for all). **B.** Quantification of von Kossa lesion staining, using NIH Image J software. One-way ANOVA, post hoc Tukey testing.

Figure 3. Increased expression of CHOP and GRP78 ER stress markers in injured ENPP1deficient carotid arteries analyzed by tissue SDS-PAGE/Western blot
 Left carotid artery tissue Western blots for ER stress markers and other comparators, at 0, 14, and 21 days after ligation. Results show increased CHOP, and GRP78 in *Enpp1*^{+/-} and *Enpp1*^{-/-} mice, well out of proportion to differences in cleaved caspase-3, or the housekeeping gene control GAPDH.

Figure 4. Early increased CHOP and apoptosis marker PARPp85 *in situ* in response to injury in ENPP1-deficient left carotid artery media

Representative cross-sections of left carotid arteries obtained from *Enpp1*^{+/+}, *Enpp1*^{+/-}, and *Enpp1*^{-/-} mice 0, 3, 14, and 21 days after ligation injury, immunohistochemically stained for CHOP (A) and PARPp85 staining (B), as described in the Methods. Scale bar: 200 μ m. Arrows: positive cellular CHOP (A), PARPp85 staining (B).

Figure 5. Increased migration and proliferation in both cultured *Enpp1*^{-/-} and *Enpp1*^{-/+} VSMCs (A) VSMCs of the indicated genotypes were seeded in Transwell plates for 18 h and then treated with vehicle or PDGF (10 ng/ml) for another 24 h, and numbers of migrating cells measured, as described in the Methods. (B) VSMC proliferation was measured, as described in the Methods. n=4 experiments; **p* < 0.05, ** *p* < 0.01, *** *p* < 0.001 vs. *Enpp1*^{+/+}. Statistics: 1-way ANOVA, with post hoc Tukey testing.

Figure 6. Increased migration and proliferation in cultured *Chop*^{-/-} VSMCs

A. VSMCs of the indicated genotypes were seeded in Transwell plates for 18 h and then treated with vehicle or PDGF (10 ng/ml) for another 24 h. As described in the Methods, migrating cells were measured using a protocol modified from that of Figure 5. **B.** VSMC proliferation was measured as above. n=4 experiments; statistics by 1-way ANOVA, with post hoc Tukey testing.

Figure 7. CHOP knockout increases calcification in cultured VSMCs

Aortic VSMCs from 8 weeks old congenic *Chop*^{+/+} and *Chop*^{-/-} mice were cultured for (A) 15 days and (B) 21 days, with addition of P_i donor 10 mM β-glycerolphosphate with 50 μg/ml ascorbate-2-phosphate, where indicated (50 μg/ml). (A,B) Calcification was determined by Alizarin Red S staining, with quantification as described in the Methods (n=3 experiments, statistics by 1-way ANOVA, post hoc Tukey testing).

Figure 8. CHOP siRNA knockdown increases calcification, and TNAP and RUNX2 expression, in cultured *Enpp1*^{-/-} VSMCs

(A) VSMCs of indicated genotypes, where indicated, were treated with β -glycerolphosphate/ ascorbate-2-phosphate or medium control for 15 days; and CHOP knockdown was done, as described in the Methods. Quantification of calcification is shown, based on 3 experiments.

(B) RUNX2 expression, assessed by Western blotting of VSMC lysates, was increased by CHOP siRNA knockdown in *Enpp1*^{-/-} VSMCs (n=4 experiments) as were (C) RUNX2 and

TNAP mRNA, normalized to GAPDH. (n=4 experiments) Statistics by 1-way ANOVA, with post hoc Tukey testing.

Table 1

Detailed morphologic measurements of left carotid artery lesions at 14 and 21 days after ligation injury, in the genotypes indicated.

	14 Days			21 Days		
	<i>Enpp1</i> ^{+/+} (n=8)	<i>Enpp1</i> ^{+/-} (n=8)	<i>Enpp1</i> ^{-/-} (n=7)	<i>Enpp1</i> ^{+/+} (n=9)	<i>Enpp1</i> ^{+/-} (n=8)	<i>Enpp1</i> ^{-/-} (n=9)
Lumen Area, mm²	0.28±0.04	0.26±0.04	0.10±0.02 **	0.30±0.04	0.13±0.02 **	0.03±0.001 ***
Media Area, mm²	0.37±0.03	0.43±0.04	0.37±0.02	0.35±0.03	0.32±0.03	0.31±0.04
Intimal Area, mm²	0.09±0.01	0.17±0.02 *	0.21±0.03 **	0.21±0.03	0.32±0.04 *	0.34±0.02 **
I/M Ratio	0.24±0.03	0.42±0.05	0.63±0.07 *#	0.66±0.09	0.96±0.08 *	1.06±0.09 **

Data here are expressed as mean ± SEM.

Statistics: 2-way ANOVA, post hoc Bonferroni testing.

*
p < 0.05

**
p < 0.01

p < 0.001, vs. *Enpp1*^{+/+}.

p < 0.05 vs. *Enpp1*^{+/-}.