Calpain-1 Regulation of Matrix Metalloproteinase 2 Activity in Vascular Smooth Muscle Cells Facilitates Age-Associated Aortic Wall Calcification and Fibrosis

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Abstract — Age-associated central arterial wall stiffness is linked to extracellular matrix remodeling, including fibrosis and vascular calcification. Angiotensin II induces both matrix metalloproteinase 2 (MMP2) and calpain-1 expression and activity in the arterial wall. However, the role of calpain-1 in MMP2 activation and extracellular matrix remodeling remains unknown. Dual histo-immunolabeling demonstrates colocalization of calpain-1 and MMP2 within old rat vascular smooth muscle cells. Overexpression of calpain-1 induces MMP2 transcripts, protein levels, and activity, in part, by increasing the ratio of membrane type 1 MMPs to tissue inhibitor of metalloproteinases 2. These effects of calpain-1 overexpression-induced MMP2 activation are linked to increased collagen I and III production and vascular calcification. In addition, overexpression of calpain-1 also induces transforming growth factor-β1/Smad signaling, elastin degradation, alkaline phosphatase activation, and total calcium content but reduces the expression of calcification inhibitors, osteopontin, and osteonectin, in cultured vascular smooth muscle cells in vitro and in carotid artery rings ex vivo. Furthermore, both calpain-1 and collagen II increase with aging within human aortic intima. Interestingly, in aged human aortic wall, both calpain-1 and collagen II are highly expressed in artherosclerotic plaque areas compared with grossly normal areas. Cross-talk of 2 proteases, calpain-1 and MMP2, leads to secretion of active MMP2, which modulates extracellular matrix remodeling via enhancing collagen production and facilitating vascular calcification. These results establish calpain-1 as a novel molecular candidate to retard age-associated extracellular matrix remodeling and its attendant risk for hypertension and atherosclerosis. (***Hypertension*. 2012;60:1192-1199.**) ● **Online Data Supplement**

Key Words: aging ■ matrix metalloproteinase 2 ■ vascular calcification ■ calpain-1 ■ fibrosis

It is well known that aging induces several changes in arterial structure and function and is the major independent risk factor for cardiovascular diseases, that is, hypertension, atherosclerosis, and stroke.[1–5] An age-associated increase in central arterial wall stiffness, which is manifested as increases in pulse wave velocity, is attributable to extracellular matrix (ECM) remodeling, including increased collagen content and cross-linking, elastin fragmentation,[1,6,7] and vascular calcification (VC).[8–15] ECM remodeling that accompanies advancing age is initially induced by a phenotypic shift of vascular smooth muscle cells (VSMCs) within the arterial wall from the contractile to synthetic state[16] in vivo and is associated with arterial wall inflammation.

VC is a very common salient feature of age-associated ECM remodeling. Accumulating evidence indicates that VC is not a passive phenomenon but rather is controlled by a tightly regulated balance of factors that promote and inhibit calcification.[11,12] Synthetic VSMCs express many of the calcification-regulatory proteins commonly found in bone.[17,18] Among these are transforming growth factor-β1 (TGF-β1),[19,20] bone morphogenetic protein-2,[21] collagen II, elastin,[13,14] osteopontin (OPN), and osteonectin (ON).[22]

Angiotensin II (Ang II) and its downstream molecules, that is, matrix metalloproteinase 2 (MMP2),[23,24] TGF-β1,[25] and calpain-1,[16] are involved in VSMC phenotype transition. ECM alterations appear to be closely linked to exaggerated arterial wall MMP2 activation.[26] We have shown previously that activated MMP2 is increased within the aged rat,[24] nonhuman primate,[23] and human[26] aorta. Increased MMP2 facilitates TGF-β1 activation, and these 2 molecules form part of a signaling loop to induce collagen production in the central arterial wall.[25] MMP2-mediated elastin degradation also triggers VC in the media of the central arterial wall.[14]

Calpain-1 is a Ca^{2+}-activated, intracellular cysteine protease that carries out limited proteolytic cleavage of a diverse range of cellular substrates.[27] Our previous studies have shown that rat aortic wall calpain-1 transcripts, protein, and activity increase with aging.[16] That calpain-1 plays an important role in Ang II-induced VSMC MMP2 activation and invasion[16] suggests a possible link

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among the VSMC intracellular protease, calpain-1, and MMP2, a major secreted protease. However, the role of calpain-1 in age-associated ECM remodeling has not been investigated. In the present study, we hypothesize that calpain-1 activity contributes to ECM remodeling that accompanies advancing age by inducing MMP2 activation, which facilitates induction of collagen I, II, III, TGF-β1, elastin fragmentation, and reduction of OPN and ON.

Methods

See the online-only Data Supplement.

Results

Calpain-1 Induces MMP2 Activity by Modifying the Membrane Type 1 Matrix Metalloproteinase/Tissue Inhibitor of Matrix Metalloproteinase 2 Ratio

We first investigated whether calpain-1 regulates MMP2 activation in early passage old rat VSMCs. Dual histo-immunolabeling shows that calpain-1 colocalizes with MMP2 (Figure 1A). Overexpression of calpain-1 by adenovirus harboring calpain-1 (CANP1) infection increases VSMC MMP2 transcripts (Figures S1A in the online-only Data Supplement), protein (Figure 1B), and activity (Figure 1C). The respective increases in young (8-month) VSMCs were 34%, 107%, and 20% ($P<0.05$) above the control adenovirus harboring Green fluorescent protein (GFP) and are similar to levels in old (30-month) untreated VSMCs. Overexpression of calpastatin by adenovirus harboring calpastatin infection, the endogenous calpain inhibitor, reduces MMP2 activities in both young and old VSMCs (Figure 1D).

To further clarify the role of calpain-1 in the regulation of MMP2 activation, we studied the calpain-1 effects on the MMP2 activation inhibitor tissue inhibitor of metalloproteinase 2 (TIMP2) and activator membrane type 1 matrix metalloproteinase (MT1MMP).[28] Real-time polymerase chain reaction shows that overexpression of calpain-1 in young

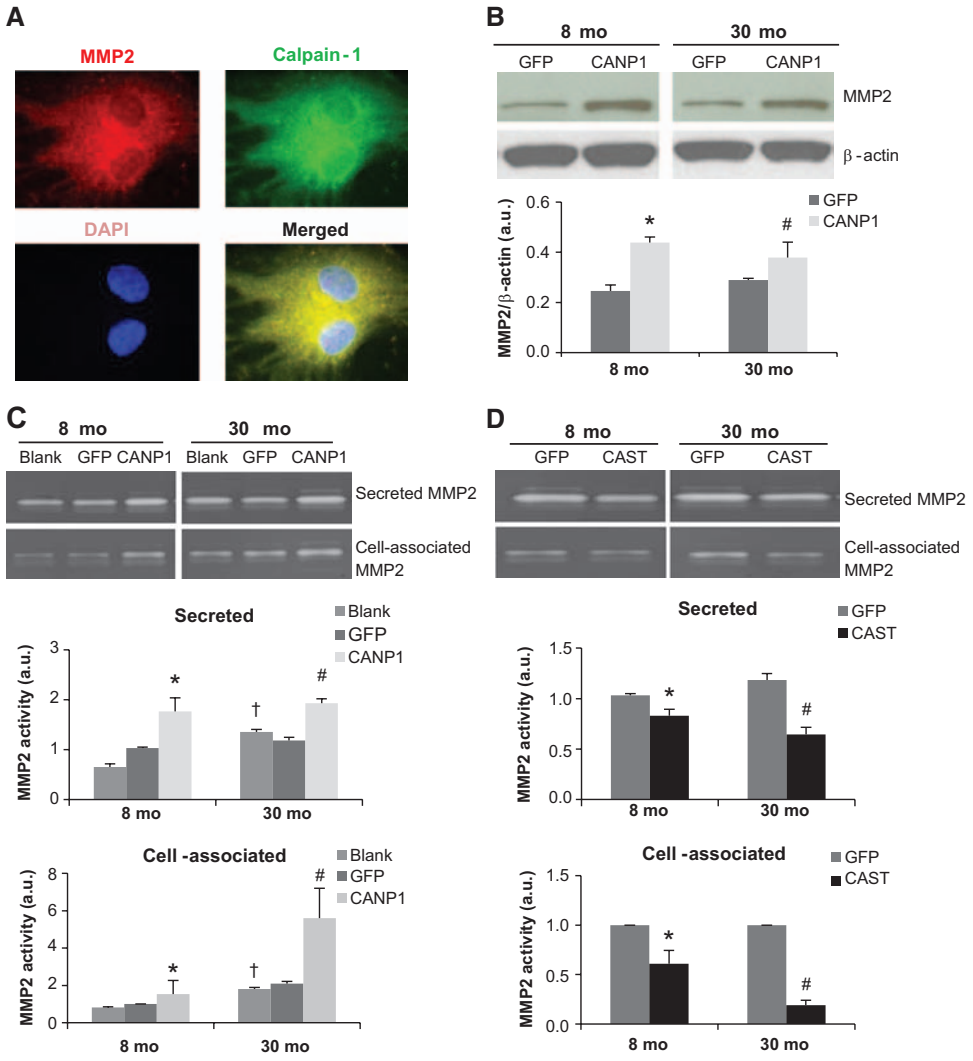

Figure 1. Calpain-1 induces matrix metalloproteinase 2 (MMP2) activation in vascular smooth muscle cells (VSMC). **A**, Photomicrographs of dual labeling for calpain-1 (green), MMP2 (red) and merged image (**lower right**) within old rat VSMC. Nuclei are counterstained with 4',6-Diamidino-2-phenylindole (DAPI) (blue). Magnification, ×400. **B**, Representative Western blot and average MMP2 protein levels in young (8-month) and old (30-month) rat VSMC infected with green fluorescent protein (GFP) or calpain-1 (CANP1) adenoviruses for 48 hours. **C**, Representative gelatin zymograph and average MMP2 activity from young (8-month) and old (30-month) rat VSMC infected with GFP or CANP1 for 48 hours. **D**, Representative gelatin zymograph and average MMP2 activity from young (8-month) and old (30-month) rat VSMC infected with GFP or calpastatin (CAST) adenoviruses. *$P<0.05$ compared with GFP-infected young VSMC, n=3. †$P<0.05$ compared with young blank VSMC, n=3. #$P<0.05$ compared with GFP-infected old VSMC, n=3.

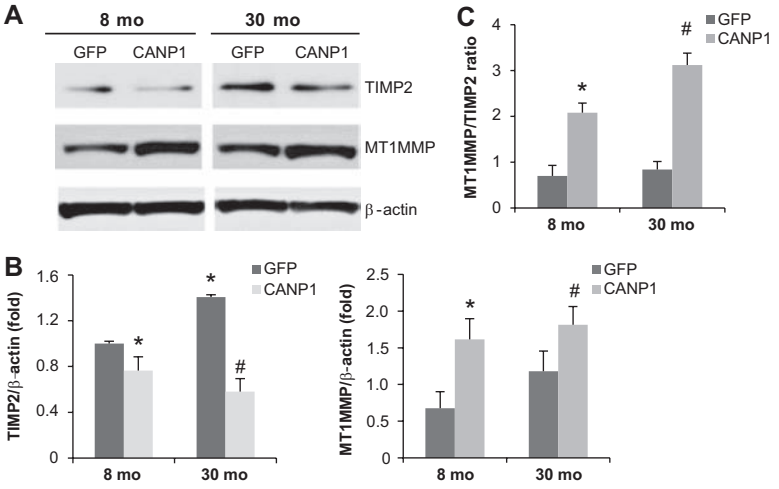

Figure 2. Calpain-1 induces membrane type 1 matrix metalloproteinase (MT1MMP)/tissue inhibitor of metalloproteinase 2 (TIMP2) ratio in vascular smooth muscle cells (VSMC). Representative Western blot (**A**) and average protein levels of TIMP2 and MT1MMP from VSMC infected by GFP or CANP1 adenoviruses for 48 hours. *P<0.05 compared with GFP-infected 8-month VSMC, n=3. #P<0.05 compared with GFP-infected old (30 month) VSMC, n=3.

VSMCs decreases TIMP2 transcripts by 38% and increases MT1MMP transcripts by 32% (Figure S1A). Furthermore, overexpression of calpain-1 decreases TIMP2 protein levels by 31% and increases MT1MMP protein by 48% (Figure 2A and 2B).Thus, overexpression of calpain-1 increases the MT1MMP/TIMP2 ratio (Figure 2C), which induces MMP2 activation. Interestingly, that calpain-1 is augmented by MMP2 inhibition in vitro and in vivo (in the online-only Data Supplement) suggests that MMP2 is downstream of calpain-1 signaling.

Overexpression of Calpain-1 Induces Collagen I, II, and III Expression and Reduces Elastin

Fibrosis, attributed mainly to collagen overdeposition, is a major characteristic of the ECM central arterial wall remodeling with aging. Transcript levels of collagen type I (Col I, *Col1a1*) and type III (Col III, *Col3a1*) in old rat aortic wall (30-month) increase by 29% and 60%, respectively, compared with the young (8-month) (Figure S2A). Compared with control GFP virus, overexpression of calpain-1 by CANP1 infection in young rat VSMCs increases Col I and Col III transcripts levels by 54% and 59%, respectively (Figure S2B) and protein levels of Col I and Col III to 2.18- and 1.99-fold, respectively (Figure 3A), and these protein levels are comparable with those of old control cells.

Collagen II is the major collagen within cartilage.[29] Interestingly, histo-immunostaining shows that collagen II levels are increased in old compared with the young aortic wall (Figure 3B). Overexpression of calpain-1 in young VSMCs enhances collagen II levels (Figure 3C) and reduces elastin (Figure 3D).

Calpain-1 Induces VSMC Calcification

Compared with the GFP control (Figure 4A, top left), overexpression of calpain-1 (Figure 4A, middle left) induces young VSMC calcification in high phosphate containing medium, and the degree of calcification achieved is similar to the old GFP infected control (Figure 4A, bottom left). That overexpression of TIMP2 abolishes the calpain-1 effects on VSMC calcification (Figure 4A, middle right) suggests that the calpain-1 effect to increase calcification is mediated via its effect to increase MMP2 activity.

We next investigate calpain-1 effects on factors that regulate calcification. Previous studies indicate that calcification markers alkaline phosphatase and total calcium content are both increased in the aged aortic wall.[29–32] Overexpression of calpain-1 in young VSMCs increases secreted alkaline phosphatase activity by 35% (0.248±0.039 versus 0.183±0.021 IU/L; P<0.05; n=3) and total calcium content by 72% (7.92±0.45 versus 4.60±0.26 μg Ca^{2+}/mg of total protein; P<0.05; n=3), that is, up to the levels observed in old VSMCs (6.774±1.44 μg Ca^{2+}/mg of total protein; n=3). Overexpression of calpain-1 in young rat VSMCs also induces TGF-β1 (Figure 4B) and smad 2/3 activation (Figure 4C), up to the levels in old control cells. Overexpression of calpain-1 in young VSMCs, however, has no effects on bone morphogenetic protein-2 expression (Figure S3).

OPN and ON are 2 major inhibitors of calcification. In early passage-old (30-month) rat VSMCs, both OPN and ON protein levels are reduced by 39% and 36%, respectively, compared with the young (8-month) (Figures 5A and 5B). Overexpression of calpain-1 in young VSMCs decreases OPN by 75% and ON by 39%, resulting in similar levels to those in old rat VSMCs. Conversely, infection of young VSMCs with calpastatin, an endogenous inhibitor of calpain-1, increases OPN and ON by 52% and 55%, respectively (Figures 5A and 5B). These results indicate that increased calpain-1 overrides the effects of calcification inhibitors in the vessel wall. Interestingly, overexpression of TIMP2 can partially block calpain-1 effects on ON but not on OPN (Figure 5C and 5D).

Calpain-1 Induces ECM Remodeling in Carotid Aortic Rings Ex Vivo

Overexpressions of calpain-1 and TIMP2 are validated by Western blot in cultured rat carotid artery rings (Figure 6A). Overexpression of calpain-1 induces collagen I, II, III, TGF-β1, and Smad 2/3 but reduces elastin, OPN, and ON (Figure 6). Interestingly, overexpression of TIMP2 blocks, in part, calpain-1 effects on these proteins (Figure 6).

Both Calpain-1 and Collagen II Levels Increase in Human Aortic Intima With Aging, Particularly the Atherosclerotic Areas

Both calpain-1 and collagen II levels are significantly increased in aged versus young human aortic intima (Figure 7A).

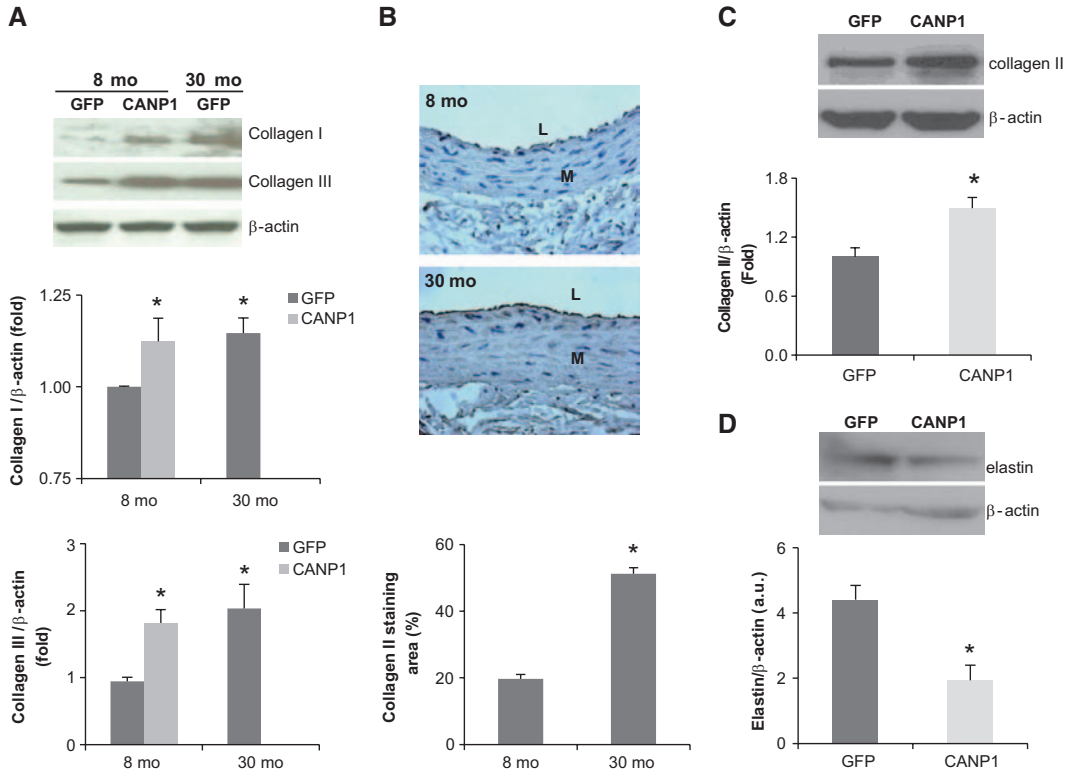

Figure 3. Overexpression of calpain-1 induces collagen type I, II, and III and reduces elastin expression levels in vascular smooth muscle cells (VSMC). **A**, Representative Western blots of collagen I and collagen III in VSMC after infection by GFP or CANP1 adenoviruses for 48 hours. **B**, Photomicrographs (×400) of collagen II protein staining (brown color) from young (8-month) and old (30-month) rat aortic walls. Nuclei are counterstained with hematoxylin (blue color). L indicates lumen; M, media. **C** and **D**, Representative Western blots and average protein levels of collagen II (**C**) and elastin (**D**) in young VSMC infected with GFP or CANP1 for 48 hours. *P<0.05 compared with GFP-infected 8-month VSMC, n=3.

Furthermore, in atherosclerotic areas of aged human aortas, in which calcification is distinguished by Alizarin red staining, both calpain-1 and collagen II are also expressed highly (Figure 7B). Importantly, calpain-1 activity also significantly increases within atherosclerotic areas compared with grossly normal areas (Figure 7C).

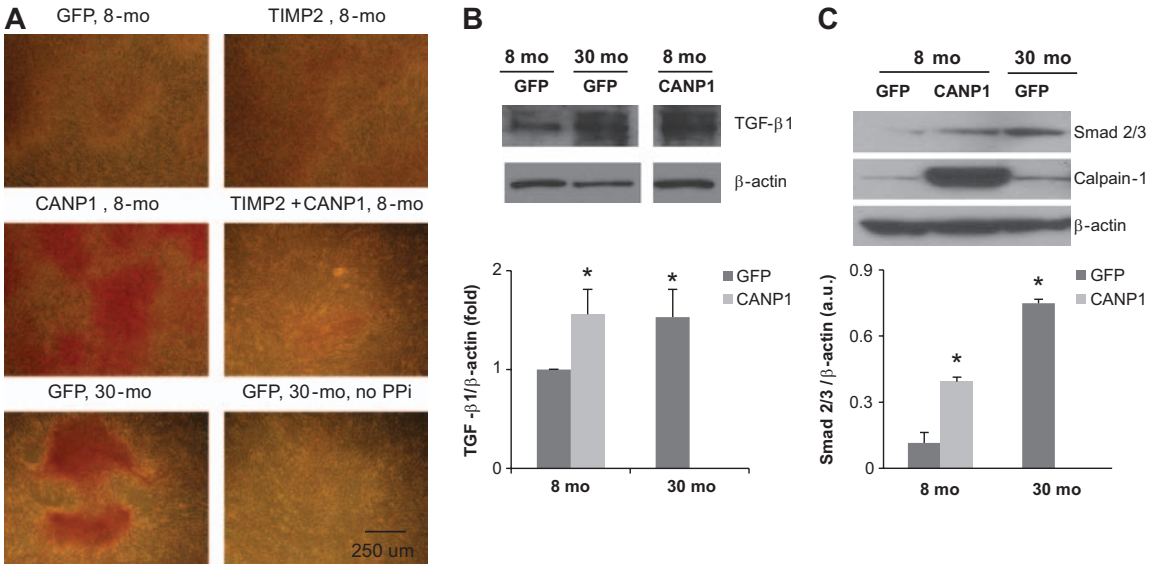

Figure 4. Overexpression of calpain-1 induces vascular smooth muscle cells (VSMC) calcification. **A**, Representative Alizarin red S staining showing that compared with GFP control (**top left**) overexpression of calpain-1 induces local calcification in young (8-month) VSMC (**middle left**) in high-phosphate (2.0 mmol/L) calcification medium. Resultant levels in these treated young VSMC are comparable with the levels of old GFP control (30-month, **lower left**). Calpain-1-induced calcification is inhibited by tissue inhibitor of metalloproteinase 2 (TIMP2) (**lower right**). Red areas indicate deposits of phosphate-containing mineral. GFP-infected VSMC without phosphate (**lower right**) is a negative control. Representative Western blots and average protein levels of transforming growth factor-β1 (TGF-β1) (**B**), and Smad 2/3 (**C**) in young (8-month) and old (30-month) VSMC infected with GFP or CANP1 for 48 hours. *P<0.05 compared with GFP-infected 8-month VSMC, n=3.

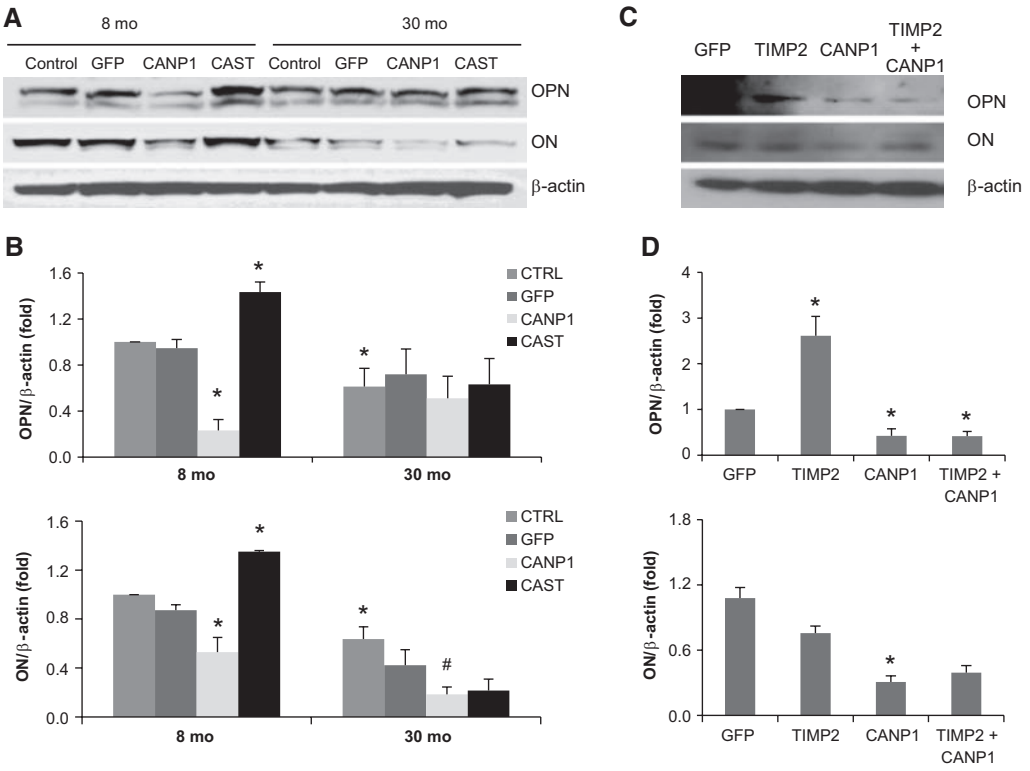

Figure 5. Overexpression of calpain-1 down-regulates osteopontin (OPN) and osteonectin (ON) levels in vascular smooth muscle cells (VSMC), whereas overexpression of calpastatin (CAST), a specific inhibitor of calpain-1, has the opposite effects. Representative Western blot (**A** and **C**) and average protein levels (**B** and **D**) of OPN and ON in VSMC infected by GFP or CANP1 or CAST or tissue inhibitor of metalloproteinase 2 (TIMP2) adenoviruses for 48 hours. **P*<0.05 compared with GFP-infected 8-month VSMC, n=3. #*P*<0.05 compared with GFP infected old VSMC, n=3. CTRL indicates control.

Discussion

The major findings of our study indicate a role of calpain-1 to increase vascular calcification and fibrosis via an effect to increase MMP2 activation (Figure S4).

The first novel finding is that calpain-1 induces MMP2 activation, in part, by modifying the MT1MMP/TIMP2 ratio. MMP2 activity is dramatically elevated in the central arterial wall with advancing age[1–6,23,24] and plays a key role to facilitate age-associated ECM remodeling. MT1MMP is the most potent activator, and TIMP2 is the most important inhibitor of MMP2 activity.[28] Our previous studies showed that calpain-1 facilitates an Ang II signaling-induced increase in VSMC migration and that this effect can be blocked by MMP2 inhibition.[16] The present study demonstrates that calpain-1 induces MMP2 transcripts, protein, and activities in rat VSMCs, and that calpastatin, the endogenous inhibitor of calpain-1, reduces both cell-associated and -secreted MMP2 activities in VSMCs. The present results, for the first time, also show that overexpression of calpain-1 increases MT1MMP and reduces TIMP2 levels in VSMCs, resulting in an increased ratio of MT1MMP/TIMP2, which favors induction of MMP2 activation.

The second novel finding is that overexpression of calpain-1 in young VSMCs increases Col I and III transcripts and proteins levels. This result is consistent with a previous report showing that overexpression of calpastatin prevents Ang II-dependent perivascular fibrosis by collagen fibrils.[33] The ECM structural framework is essential for the functional properties of blood vessels.[6] Our previous work demonstrated that the intima of the older aorta is markedly and diffusely thickened compared with that in younger rat aorta and harbors increased levels of MMP2 and Col I and III proteins.[28] Ang II signaling triggers Col I and III expression and MMP2 activation,[18] and calpain-1 is one of the important signal transducers of Ang II signaling, which regulates MMP2 activation.[16] The present studies extend these findings in the aortic wall in vivo to VSMCs in vitro and carotid aortic rings ex vivo. Unlike Col I and III, Col II is a main component of cartilage matrix proteins and a marker for cartilaginous metaplasia in arteries.[15] The present study also shows that Col II is increased with aging in both the rat aortic wall and human aortic intima, particularly within atherosclerotic areas.

The third novel finding of our study is that calpain-1 accelerates vascular calcification and that this effect is counteracted, at least in part, by TIMP2 overexpression. Our results also show that calpain-1 induces expression of molecules and factors that promote calcification, including TGF-β1/Smad elevation, elastin degradation, and Col II production, and reduces expression of molecules that inhibit calcification, including OPN and ON.

A growing body of evidence suggests that VC is complex, and involves multiple steps and mediators.[9] With advancing age, VC is triggered by inflammatory cytokines.[8] New pathways regulating calcification, including TGF-β1, OPN, and ON, are the focus of many current studies.[11,12] Overexpression of calpain-1 enhances secretion of activated alkaline phosphatase, total calcium content, and Col II, hallmarks of VC.[29–31]

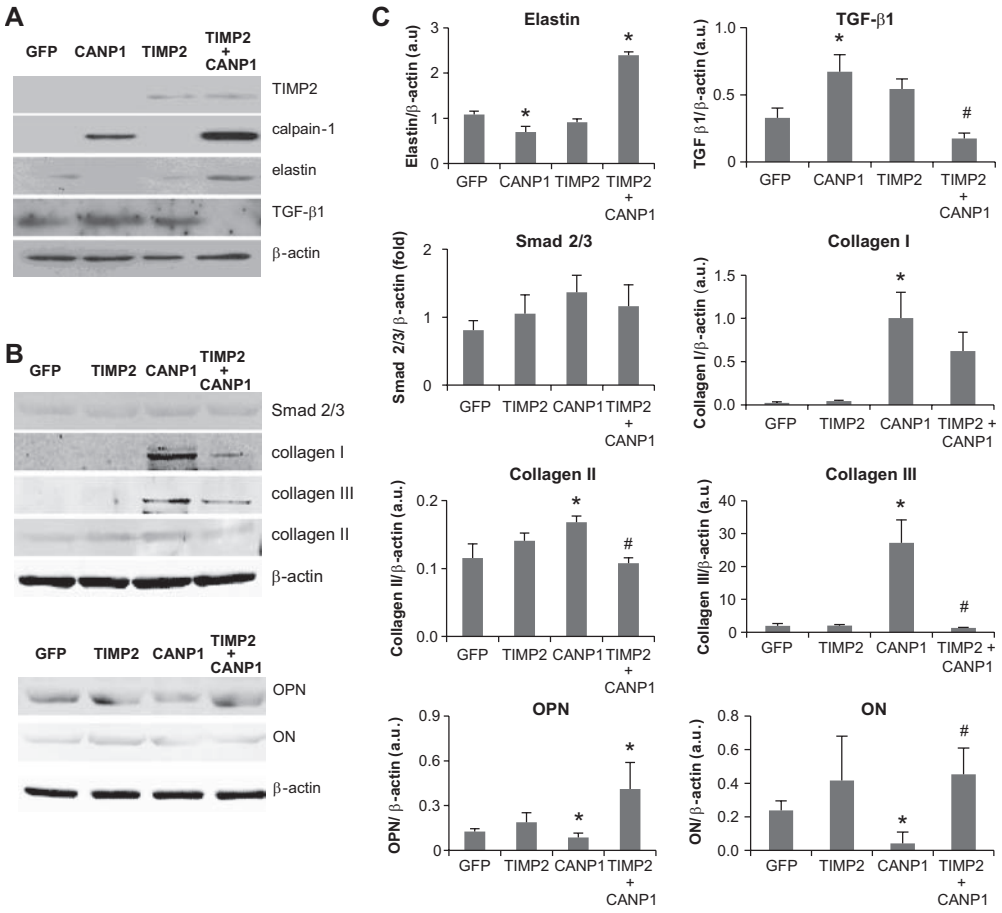

Figure 6. Overexpression of calpain-1 regulates extracellular matrix remodeling, particularly fibrosis and vascular calcification in cultured carotid artery rings. Representative Western blot of tissue inhibitor of metalloprotease 2 (TIMP2), calpain-1, elastin, transforming growth factor-β1 (TGFβ1), (**A**) Smad 2/3, collagen I, II, III, (**B**) and osteopontin (OPN) and osteonectin (ON) (**C**) in cultured carotid artery rings isolated from 8-month-old rat after infection by GFP or CANP1 or TIMP2 adenoviruses for 48 hours.

The present results are consistent with the previous observation that calpain-1 activation is involved in atherosclerotic lesions of the human carotid artery,[34] a highly potent calcification locus, and also rodent osteoblastic cell differentiation.[35]

Medial calcification is also associated with elastic fiber fragmentation, appearing initially as linear deposits along elastic lamellae.[13,14] Our finding that calpain-1 induces elastin degradation, via induction of MMP2 activation, supports the idea that MMP-mediated elastic fiber degradation contributes to calcification. Furthermore, the calpain-1-induced increases in Col I and III provide fertile soil for calcium deposition.

It is known that TGF-β1 plays a crucial role in bone matrix production and many fibrocalcific lesions, including calcific aortic valve stenosis.[19,20] Calpain releases mature TGF-β from the latency-associated protein (LAP) in test tube experiments.[36] In the aged arterial wall, TGF-β1 interaction with MMP2 creates a proinflammatory niche.[25] We have demonstrated previously that MMP2 increases active TGF-β by cleaving TGF-β-LAP also. The present results, which demonstrate that calpain-1 induces TGF-β1/Smad signaling, suggest that a new interactive signaling module, involving calpain-1/MMP2/TGF-β1, participates in calcification within aged VSMCs.

OPN and ON inhibit mineralization via direct binding to crystal surfaces to prevent any further propagation.[22] OPN is an inhibitor of crystal growth during VSMC calcification. Our results demonstrate that calpain-1 prevents inhibitory effects of OPN and ONs on VC and that this inhibition is partially inhibited by MMP2 inhibition. Interestingly, overexpression of the calpain-1 inhibitor calpastatin induces both OPN and ON in young VSMCs.

Age-associated arterial remodeling results, in part, from proinflammatory Ang II signaling cascades, which involve calpain-1, MMP-2/9, monocyte chemoattractant protein-1, TGF-β1 activation, and milk fat globule epidermal growth factor-8.[4] The major focus of the present study is to perturb VSMCs isolated from young rats to determine the extent to which this shifts their phenotype to that observed in untreated cells with advancing age. However, aging is associated with a complex remodeling of the arterial wall and is composed of changes in multiple steps of multiple signaling pathways over a prolonged period. Thus, overexpression of one molecule in young vascular cells may not, per se, mimic completely quantitatively or qualitatively the chronic changes that occur and vary over time in numerous pathways. For example, when changes occur in one pathway, compensations may occur in other pathways to counteract the effects.

Age-associated arterial stiffness is, to a large extent, attributable to ECM remodeling, including fibrosis and VC.

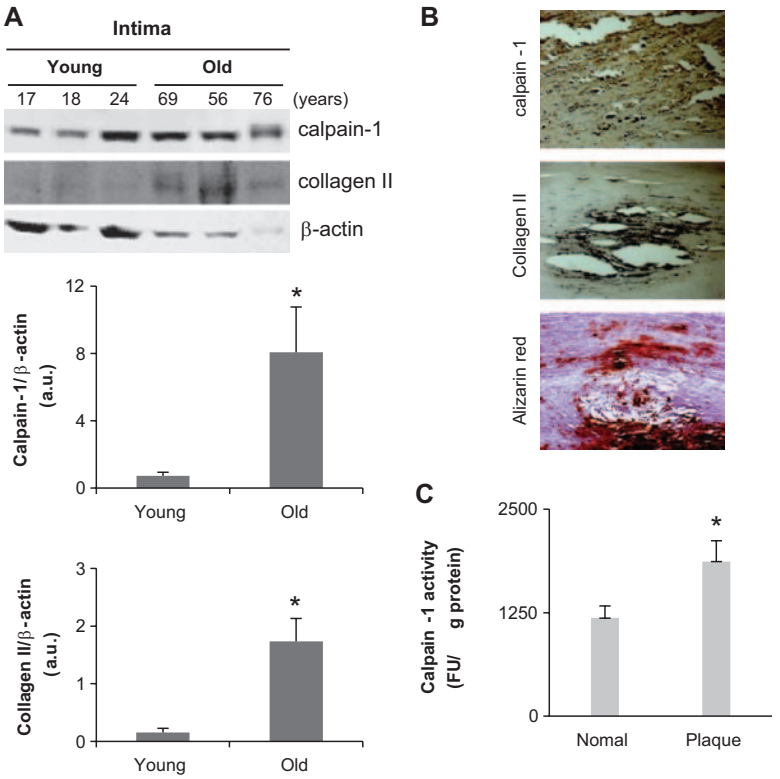

Figure 7. Both calpain-1 and collagen II are increased in human aortic intima and arthrosclerotic area. **A**, Representative Western blot and average protein levels of calpain-1 and collagen II in human aortic intima. **B**, Photomicrographs (×400) of calpain-1 and collagen II protein staining (brown color) within arthrosclerotic area from old human aortic wall. Nuclei were counterstained with hematoxylin (blue color). Representative Alizarin red S staining showing local calcification within the same atherosclerotic area. **C**, Calpain-1 activity is significantly increased in the atherosclerotic area compared with normal area in human aortic wall. **P*<0.05 compared with young ones (**A**) or normal area (**C**) in human aortic wall, n=3.

It has been shown that several risk factors, that is, high blood pressure, diabetes mellitus, and smoking, are related to arterial stiffness and its link to cardiovascular diseases. Ang II, the major biologically active component of the renin-angiotensin system, contributes to the regulation of vascular tone and blood pressure. Calpain-1 is induced by Ang II in vitro, ex vivo, and in vivo.[16] The present study extends Ang II/calpain-1 effects on MMP2 activation and remodeling, which are important in hypertension. Arterial wall stiffening in type 2 diabetes mellitus patients has a significant impact on cardiac function and peripheral vascular manifestations. Furthermore, myocardial hypertrophy and fibrosis in diabetic mice are attenuated by a reduction of calpain function.[37] Chronic exposure to cigarette smoke via calpain inhibition attenuates pulmonary artery endothelial cells angiogenesis, tube formation, cell migration, and proliferation and inhibits wound repair.[38] Our data in the arterial wall are consistent with these results and suggest that overexpression of calpain-1 induces ECM remodeling, including fibrosis and VC, not only during aging but also in chronic diseases. Thus, targeted inhibition of calpain represents a potential novel therapeutic strategy for retarding age-associated central arterial remodeling and its attendant high risk for chronic arterial disease.

Perspectives

An age-associated increase in central arterial wall stiffness is, in large measure, attributable to ECM remodeling, including increased collagen content and cross-linking[1,6,7] and VC.[8–15] The present study investigates the role of calpain-1 in age-associated ECM remodeling. Our results indicate that calpain-1, via its effect on MMP2 activity, induces collagen production and participates in multiple steps of VC with advancing age. Calpain-1 induces collagen II, TGF-β1/Smad signaling, elastin degradation, and reduction of VC inhibitors, OPN and ON. Collectively, these observations strongly suggest that calpain-1 plays a pivotal role in central arterial ECM remodeling during aging, at least in part, by regulating MMP2 activation. Thus, calpain-1 inhibition is an attractive novel strategy to retard central arterial ECM remodeling with advancing age and its attendant increased risk for hypertension and atherosclerosis.

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Disclosures

None

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Novelty and Significance

What is New?

- The expression of calpain-1 is increased in human aortic intima with advancing age, particularly within atherosclerotic plaque areas. Calpain-1 induces fibrosis and calcification partially through MMP2 activation in young VSMCs in vitro and in young carotid artery rings ex vivo.

What is Relevant?

- An age-associated increase in central arterial wall stiffness is attributable, in large measure, to extracellular matrix remodeling. This study strongly indicates that calpain-1 plays a pivotal role in MMP2 activation and extracellular matrix remodeling, including fibrosis and vascular calcification.

Summary

Calpain-1 inhibition is an attractive novel strategy to retard vascular extracellular matrix remodeling that underlies age-associated remodeling and its attendant diseases, that is, hypertension and atherosclerosis.