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Christa Caesar, *Emory University*
Alicia N. Lyle, *Emory University*
Giji Joseph, *Emory University*
Daiana Weiss, *Emory University*
Fadi M.F. Alameddine, *Emory University*
Bernard P Lassegue, *Emory University*
Kathy Griendling, *Emory University*
W Robert Taylor, *Emory University*

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Cyclic Strain and Hypertension Increase Osteopontin Expression in the Aorta

Christa Caesar[1,2], **Alicia N. Lyle**[1], **Giji Joseph**[1], **Daiana Weiss**[1], **Fadi M. F. Alameddine**[1], **Bernard Lassègue**[1], **Kathy K. Griendling**[1], and **W. Robert Taylor**[1,2,3]

[1]Division of Cardiology, Department of Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA

[2]Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA 30332, USA

[3]Cardiology Division, Atlanta Veterans Affairs Medical Center, Decatur, GA 30033, USA

Abstract

Hypertension has a direct impact on vascular hypertrophy and is a known risk factor for the development of atherosclerosis. Osteopontin (OPN) has emerged as an important protein mediator of inflammation and remodeling of large arteries. However, its role and mechanism of regulation in the setting of hypertension is still unknown. Our objectives for this study were therefore to investigate the role of OPN in hypertension-induced vascular remodeling and inflammation. OPN Knockout (KO) and wild type (WT) mice were made hypertensive with angiotensin II (Ang II) infusion for seven days. We observed that OPN KO aortas were protected against Ang II-induced medial hypertrophy and inflammation, despite comparable increases in systolic blood pressure (SBP) in both groups. OPN expression was increased in WT aortas from hypertensive mice (induced by either Ang II or norepinephrine). OPN expression was increased in aortic smooth muscle cells (SMCs) subjected to cyclic mechanical strain suggesting that mechanical deformation of the aortic wall is responsible in part for the increased OPN expression induced by hypertension. Finally, we utilized hypertensive transgenic smooth muscle cell-specific catalase overexpressing ($Tg^{SMC\text{-}Cat}$) mice to determine the role of H_2O_2 in mediating hypertension-induced increases in OPN expression. We also found that the hypertension-induced increase in OPN expression was inhibited in transgenic smooth muscle cell-specific catalase overexpressing ($Tg^{SMC\text{-}Cat}$) mice, suggesting that H_2O_2, plays a vital role in mediating the hypertension-induced increase in OPN expression. Taken together, these results define a potentially important role for OPN in the pathophysiology of hypertension.

Address correspondence to W. Robert Taylor, Division of Cardiology, Department of Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA. wtaylor@emory.edu.

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CONFLICT OF INTEREST

Christa Caesar, Alicia N. Lyle, Giji Joseph, Daiana Weiss, Fadi M. F. Alameddine, Bernard Lassègue, Kathy K. Griendling, and W. Robert Taylor have no conflicts of interest to disclose.

ETHICAL APPROVAL

This article does not contain any studies with human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Keywords

Angiotensin II; Norepinephrine; Cyclic strain; Inflammation; Vascular hypertrophy; Smooth muscle cells

INTRODUCTION

The arterial wall is constantly exposed to both humoral and mechanical stimuli that may be relevant to the development of vascular inflammation and subsequent pathophysiology. With the onset of hypertension, cells of the large arteries, including the aorta, respond by secreting pro-inflammatory gene products.[6,19,33] This in turn, stimulates an inflammatory environment within the arterial wall, which involves recruitment of inflammatory cells, accelerates cell proliferation, migration, and remodeling of the extracellular matrix.[7,10] These processes ultimately facilitate the adaptive or maladaptive changes of the vascular wall, that are characteristic of hypertensive vascular disease.

OPN has recently emerged as an important mediator of multiple cardiovascular pathophysiologic states.[37] OPN is a pro-inflammatory, integrin-binding, matricellular protein.[10] OPN is expressed at very low levels in the uninjured artery, but under various pathological conditions, OPN expression significantly increases in endothelial, smooth muscle, and inflammatory cells.[10,12,14,20] OPN is known to play an important role in mediating several cardiovascular disease processes including atherosclerosis,[5,8] abdominal aortic aneurysms (AAAs),[5] angiotensin-II (Ang II) induced tubulointerstitial nephritis,[13] neointimal formation,[20] and medial thickening.[20]

OPN expression has been shown to be specifically upregulated by hydrogen peroxide (H_2O_2), in vascular smooth muscle cells (SMCs).[24] Reactive oxygen species such as H_2O_2 function as homeostatic signaling molecules under normal conditions and are critical mediators in the development and progression of several cardiovascular disease pathologies, including hypertension when produced in excess.[28,30] H_2O_2 is a critical mediator of aortic wall remodeling and the production of H_2O_2 is increased in endothelial and smooth muscle cells from hypertensive animals.[19,41] Furthermore, studies in transgenic mice that overexpress catalase specifically in SMCs demonstrate that H_2O_2 mediates OPN expression in response to hind limb ischemia.[23] These data are supported by studies from our group performed in isolated cells that have defined the mechanisms through which OPN is regulated by H_2O_2.[24] However, it is unknown if OPN is upregulated in hypertension, and if this increase is mediated by H_2O_2. Furthermore, while it is well known that OPN expression is regulated by angiotensin II and other humoral factors implicated in hypertension, the potential contribution of the resultant mechanical forces that occur as a result of elevated blood pressure is unclear. Increased in circumferential wall strain with hypertension has been proposed to initiate a cascade of complex changes to the blood vessel subsequently leading to alterations in vascular compliance and function.[16,25,30]

Therefore, the primary goals of this study were to (a) determine if OPN plays a role in mediating hypertension-induced medial thickening of the aorta (b) to determine if OPN

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expression is increased in the aorta with hypertension, and (c) understand the mechanism by which this increase in OPN is regulated.

MATERIALS AND METHODS

Animals

C57BL/6 (Wildtype, WT) or Osteopontin Knockout (OPN KO) mice were purchased from Jackson Laboratories. Transgenic mice with smooth muscle cell-specific catalase overexpression ($Tg^{SMC\text{-}Cat}$) on a C57BL/6 background were bred in-house in the Department of Animal Resources at Emory University. Our laboratory has previously reported the complete characterization of these mice.[41] All animals used were male and between the ages of eight to ten weeks. All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

Osmotic Minipump Implantation and Blood Pressure Measurement

Mice were anesthetized using 1–2% isoflurane. Ang II (Sigma) at a dose of 0.75 mg/kg/day or NE (Sigma) at 5.6 mg/kg/day were infused *via* a primed mini-osmotic pump (Alzet mini-osmotic pump, Model 2004) as previously described.[38] Systolic blood pressure in mice was measured using tail-cuff plethysmography (Visitech Corporation) prior to and after infusion of Ang II or NE.

Immunohistochemistry and Analysis

Mice were euthanized, tissues pressure perfused with saline and fixed with 10% buffered formalin. Whole aortas were excised and paraffin embedded. Sections (5 lm) were stained with hemotoxylin & Eosin (H&E) for morphology and immunostained with Mac3 antibody (BD Pharmingen) to determine macrophage infiltration. Medial thickness was analyzed using NIH Image J. For the OPN antibody staining, antigen retrieval was performed using protease K (10 μg/mL) for 30 min, before incubation with anti-OPN antibody (R&D), followed by incubation with -anti-goat secondary antibody (Vector Labs) and incubation with Streptavidin QDot 655 (Invitrogen). Images were acquired with a Zeiss Axioskope microscope equipped with an AxioCam camera.

Cell Culture, Cell Strain and Materials

SMCs were harvested by enzymatic elastase/collagenase digestion from rat thoracic aorta, as previously described.[18] SMCs between passage 6 and 14 were cultured on silicone membrane 6-well plates coated with type I collagen (Bioflex © Culture Plates, Flexcell International) and grown as previously described.[24] SMCs were serum starved in 0% calf serum containing DMEM for 48 h prior to stimulation with 10% cyclic strain on a computer controlled cell strain system (FX-5000, Flexcell).

Western Blot Analysis

Aortic tissue samples and VSMCs were lysed in Hunter's Buffer, as previously described.[22] Tissue samples were briefly homogenized using a glass mortar and pestle, and cells were sonicated at 10 W for 10 × 1s pulses. Whole tissue or cell lysates were then boiled and

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subsequently used for immunoblotting. Band intensities were quantified by densitometry analysis and normalized to β-Actin. The OPN antibody was purchased from R&D systems. The β-Actin antibody was from Cell Signaling.

ELISA

Media (DMEM) from SMCs exposed to cyclic strain was harvested and stored with protease inhibitor cocktail (Sigma). Secreted OPN expression was then analyzed using a rodent OPN ELISA kit (Enzo Life Sciences).

RNA Isolation and Quantitative Real-time Polymerase Chain Reaction

RNA was harvested from aortas or cultured SMCs using the RNEasy kit (Qiagen). OPN mRNA levels were measured using amplification of cDNA using an Applied Biosystems thermocycler, primers specific for mouse or rat OPN (QuantiTect Primers, Qiagen), and SYBR green dye. Copy numbers were calculated by from standard curves of genuine templates. OPN copy numbers were then normalized to the 18s.

Measurement of Hydrogen Peroxide

H_2O_2 production was measured using the amplex red assay (Invitrogen) as previously described.[11]

Statistics

All results are presented as mean ± SEM. ANOVA or *t* test was used to determine whether statistical differences exist between the individual treatment groups. A $p < 0.05$ was considered significant.

RESULTS

OPN KO Mice have Attenuated Angiotensin II-Induced Medial Thickening and Inflammation

Representative images of aortic cross sections stained with H&E from WT and OPN KO mice treated with or without Ang II for 7 days are shown in Fig. 1a. Ang II induced a similar increase in blood pressure in WT and OPN KO mice. There was no significant difference in blood pressures between the WT and OPN KO mice at baseline (Fig. 1b).

Hypertension induced a dramatic increase in medial thickness in WT animals as expected. However, in OPN KO animal there was a significant attenuation in Ang II-mediated medial hypertrophy (42.3 ± 1.3 μm in WT vs 30.2 ± 3.2 μm in OPN KO; $p < 0.01$; Fig. 1c). Of note, the hypertensive WT mice also had a thickened adventitia, abundant in collagen and inflammatory cells which was attenuated in OPN KO animals (Figs. 1a, 2). Taken together, these findings suggest that OPN does play a role in mediating the remodeling of the aortic wall in the setting of hypertension.

Hypertension Induces Aortic Expression of Osteopontin

As expected, both Ang II and NE treatment significantly increased SBP (169.5 ± 3.3 mmHg Ang II treated, 148.9 ± 3.8 mmHg NE treated, vs. 112.2 ± 4.0 mmHg in WT controls; Fig. 3). OPN protein as analyzed by Western blot analysis revealed that aortic OPN protein

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expression was significantly increased after induction of hypertension with Ang II or NE treated (Figs. 3b, 3c). The increase in OPN protein expression was primarily localized to the medial layer of the aorta (Fig. 3d). Taking these data together, we conclude that hypertension induced by two different humoral agents dramatically upregulates OPN mRNA and protein expression in the aorta.

Cyclic Strain Increases Osteopontin Expression in VSMC

In order to determine the potential contribution of biomechanical forces to the hypertension-induced upregulation of OPN, we evaluated the effect of cyclic mechanical strain on OPN expression in SMCs in culture. Exposure of SMCs for up to 24 h of 10% cyclic biaxial strain resulted in a marked increase in OPN mRNA and protein expression (Figs. 4a, 4b). A similar increase in secreted OPN was observed in the media surrounding SMCs exposed to cyclic strain as measured by an ELISA (Fig. 4c). These results demonstrate that mechanical strain applied to VSMs induces expression of OPN.

Hydrogen Peroxide is Increased in Vascular Smooth Muscle Cells with Cyclic Strain

Given the known role of H_2O_2 in regulating OPN expression, we sought to determine if cyclic strain alos induced production of H_2O_2. We found that SMCs exposed to cyclic strain for 4 h had a significant increase in H_2O_2 levels (~4-fold, $p < 0.05$) compared to non-strained cells (Fig. 5).

Hypertension-Induced Increase in OPN is Mediated by Hydrogen Peroxide

Given that OPN plays an important role in mediating hypertension-induced remodeling within the aorta, and that its expression is upregulated by both mechanical strain and hypertension, we examined specifically if H_2O_2 plays a causal role in regulating this increase *in vivo*. To test this hypothesis, we treated transgenic smooth muscle cell-specific catalase overexpressing mice ($Tg^{SMC\text{-}Cat}$) and their littermate controls with Ang II or NE for 7 days and assayed OPN expression. We found that WT and untreated $Tg^{SMC\text{-}Cat}$ controls had similar baseline blood pressures, but had a similar significant increase in SBP when treated with either Ang II or NE (Fig. 6a). Importantly, we observed that these hypertensive $Tg^{SMC\text{-}Cat}$ mice had a complete blunting in OPN expression in their aortas, as compared to their hypertensive WT littermate controls (Fig. 6b). Taken together, these results implicate a critical role for H_2O_2 in mediating hypertension-induced increased aortic OPN expression.

DISCUSSION

The results of our study demonstrate that OPN expression is markedly increased in the aortic wall with hypertension, *via* a H_2O_2-dependent mechanism, and that OPN plays a critical role in mediating hypertension-induced vascular remodeling. We further observed that mechanical strain has an independent effect on increasing OPN expression in aortic smooth muscle cells grown in culture. These findings prove a causal role for OPN in the vascular pathophysiology of hypertension and provide insight into the potential importance of H_2O_2 as a signaling molecule in this process.

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Under normal conditions, OPN is typically expressed at very low or even undetectable levels in the aorta, but expression has been shown to be increased in several cardiovascular disease states including dystrophic calcification, atherosclerosis, and aortic valve calcification.[12,20,36] We see a similar response in this study, as we have observed low levels of OPN in aortas obtained from healthy mice, but dramatically increased levels of OPN mRNA and protein in aortas obtained from hypertensive mice. Our results are further validated by other studies that have focused on the role of OPN in a balloon catheter initiated injury model to the adult rat aorta and carotid artery, and demonstrated that its expression was heavily localized to the neointimal SMC during the early proliferative and migratory phase post injury.[12] Similar observations of increased OPN protein and mRNA expression have been reported at sites of dystrophic calcification, atherosclerotic plaques, and macrophages present in calcified aortic valves.[12,20,36]

The cells of the aorta are constantly exposed to multiple humoral and mechanical signals. The mechanical cues include shear stress (experienced predominantly by the monolayer of endothelial cells lining the intima) and strain (experienced by the SMCs of the medial layer). In the setting of hypertension, there is a significant change to this humoral and mechanical milieu, and the cells of the aorta respond in order to compensate for the disturbance induced by this altered environment.[1–3,34,39,41] Several studies have established central roles for oxidative stress, pro-inflammatory proteins such as monocyte chemoattractant protein-1 (MCP-1)[19] and plasminogen activator protein-1 (PAI-1),[35] and the subsequent infiltration of leukocytes and macrophages[28] leading to atherosclerosis[1] or vascular hypertrophy.[15,41] Plasma OPN levels are increased with hypertension in humans[21] and additional studies describe a potentially causal role for OPN in a variety of cardiovascular disease pathologies including atherosclerosis,[8] restenosis,[8] aneurysm formation,[5] myocardial infarction.[9] diabetic vascular disease[27] and vascular calcification.[31,32]

It appears that in hypertension, both mechanical and humoral factors are important stimuli for vascular inflammation and subsequent hypertrophy. Our group has previously shown that Ang II alone can have a direct effect on increasing OPN expression in vascular SMCs.[29] We have demonstrated in this study that mechanical strain can also have a direct effect on OPN expression in SMCs. We employed a cell culture system exposing SMCs to 10% mechanical strain. The average circumferential cyclic strains that the cells of the aorta experiences ranges from 5 to 25% or more, depending on the physiologic state of the organism.[4,17,40] While the 10% strain used in the present study is within the range seen *in vivo*, because of the very artificial *ex vivo* conditions, one has to be cautious about quantitatively translating these findings to the *in vivo* setting. Instead, it is probably more appropriate to interpret the cell culture studies as proof of concept studies indicative of the fact that OPN expression is regulated by mechanical strain. In sum, the increased OPN expression observed in hypertensive mice is likely attributable to humoral factors as well as direct mechanical effects.

To gain further insight into the regulation of OPN with hypertension, we used transgenic smooth muscle cell-specific catalase overexpressing ($Tg^{SMC\text{-}Cat}$) mice. Catalase scavenges hydrogen peroxide by decomposing it into water and molecular oxygen. Previous studies have implicated that vascular smooth muscle cell-derived hydrogen peroxide (H_2O_2),

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mediates vascular hypertrophy in $Tg^{SMC\text{-}Cat}$ with Ang II-induced hypertension[41] and collateral blood vessel formation in the setting of hind-limb ischemia.[23] $Tg^{SMC\text{-}Cat}$ have also been shown to be protected against abdominal aortic aneurysm formation.[26] Our studies demonstrated that catalase overexpression in vascular SMCs in the $Tg^{SMC\text{-}Cat}$ mice significantly attenuated OPN expression, even in the presence of elevated vascular mechanical strain with hypertension. These results demonstrate that in the setting of hypertension that H_2O_2 plays a critical role in mediating aortic OPN expression.

In conclusion, our results support the hypothesis that OPN expression is increased with hypertension in the SMCs of the aorta *via* H_2O_2, and that this increase is a prerequisite for vascular remodeling. This work has provided evidence that OPN is a mechanosensitive protein expressed by SMCs of the aorta. Taken together, these findings give us novel insights into the pathogenesis of inflammation and a deeper understanding of the relation between molecular and mechanical events within the aorta.

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ABBREVIATIONS

OPN	Osteopontin
Ang II	Angiotensin II
NE	Norepinephrine
SMCs	Smooth muscle cells
SBP	Systolic blood pressure
$Tg^{SMC\text{-}Cat}$	Transgenic mice with smooth muscle cell-specific catalase overexpression

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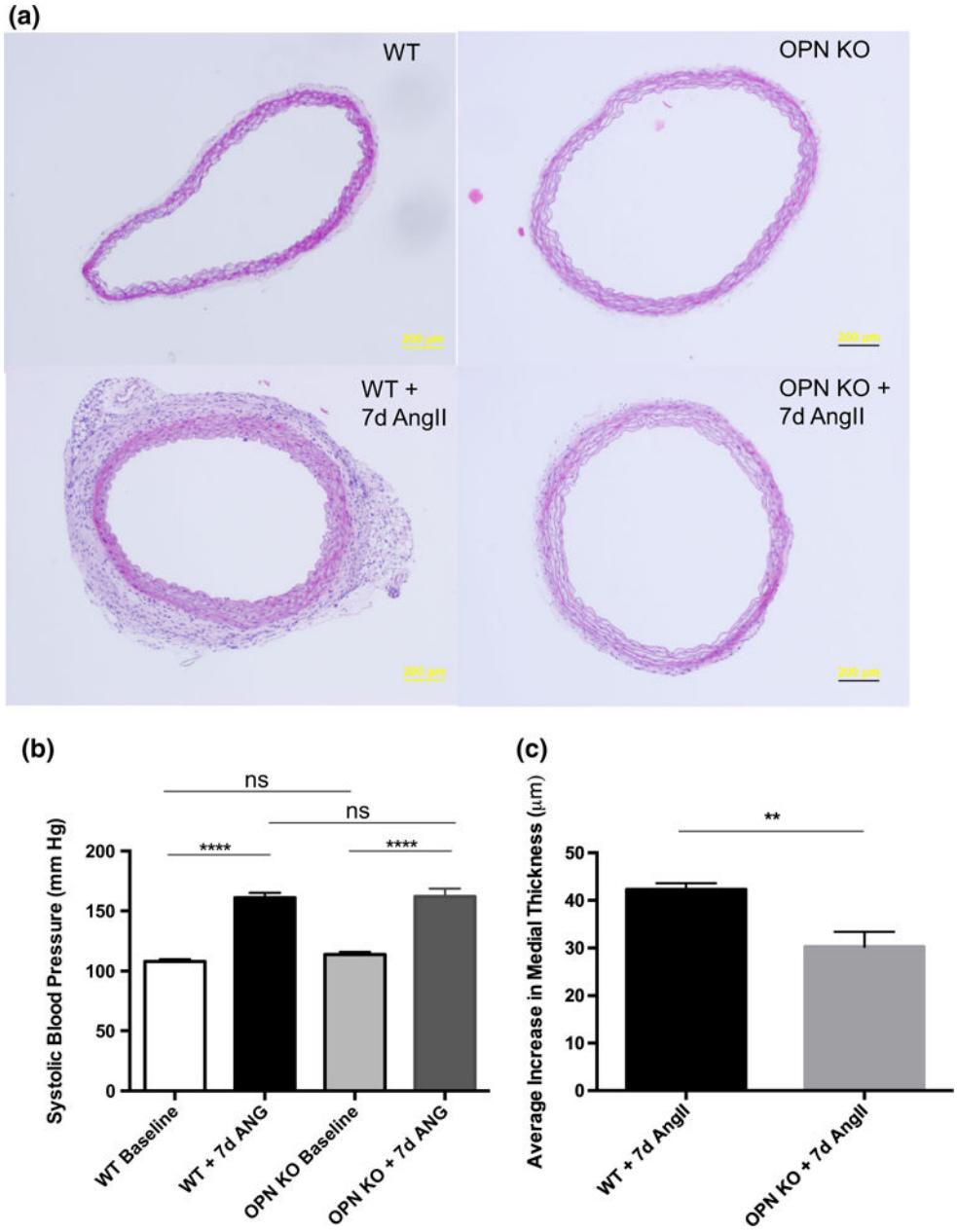

FIGURE 1.

OPN KO mice are Protected against Angiotensin II-induced Medial Thickening (a) Representative micrographs of samples obtained from WT and OPN KO mice at baseline or treated with Ang II for seven days, stained with H&E, N = 6. (b) An increase in systolic blood pressure was observed in both WT and OPN KO mice with 7 days of Ang II treatment as measured using tail-cuff plethysmography. No differences were observed between any other pairs of treatment groups. ****$p < 0.0001$, N = 5–6 (ANOVA) (c) Ang II treated OPN KO aortas were significantly protected against Ang II-induced medial thickening, compared to WT mice. There also appeared to be greater adventitial thickening in the Ang II treated WT mice, compared with Ang II treated OPN KO mice. **$p < 0.001$, N = 6 (t test) for all groups.

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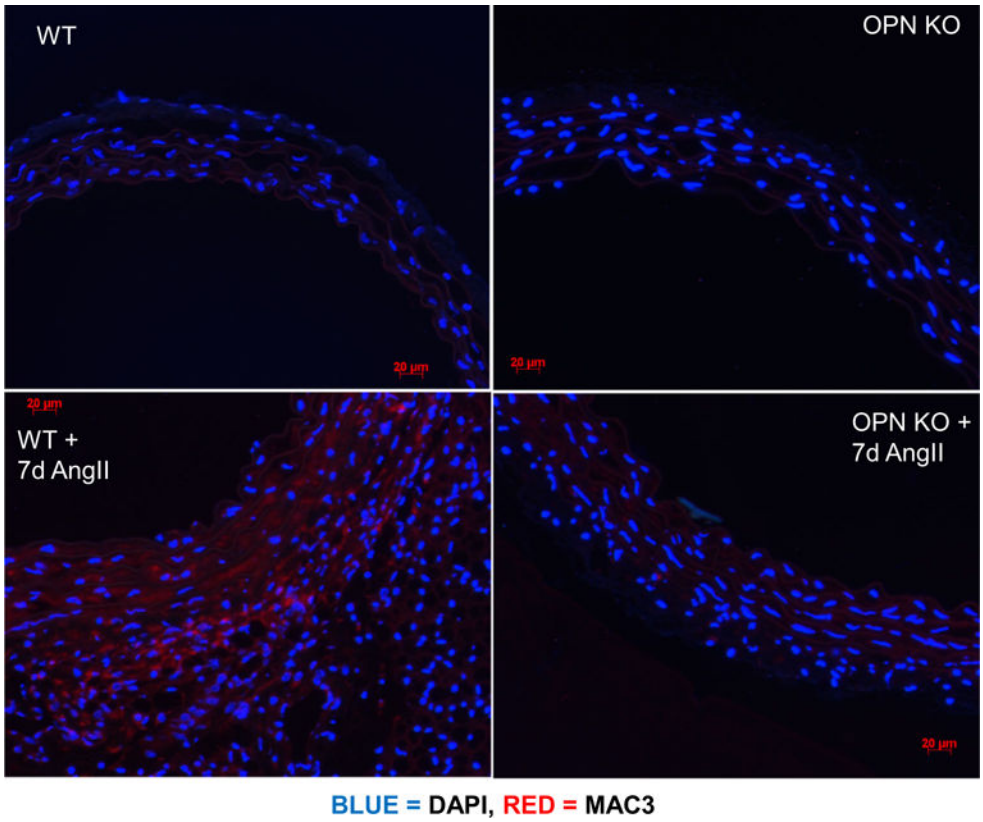

FIGURE 2.

OPN KO mice are Protected against Angiotensin II-induced Inflammation: Representative images of samples obtained from WT and OPN KO mice at baseline or treated with Ang II for 7 days, stained using an anti-mouse Mac3 antibody, N = 5. Ang II treated aortas from OPN KO mice were protected against Ang II-induced inflammation when compared to WT mice.

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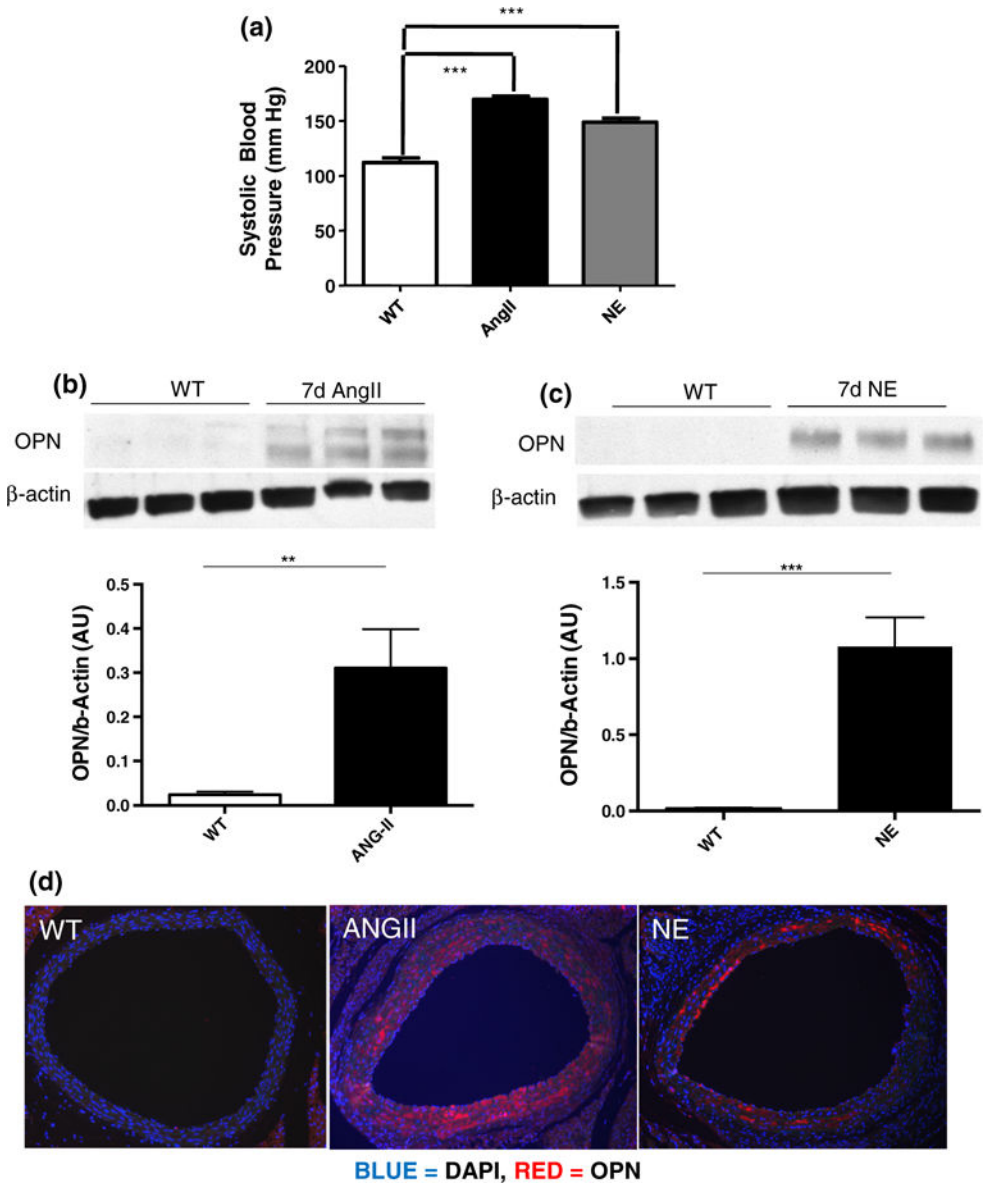

FIGURE 3.

Elevated blood pressure increases OPN expression (a) Increase in systolic blood pressure was observed in mice with 7-days of Ang II or Norepinephrine (NE) treatment as measured using tail-cuff plethysmography ***$p < 0.001$, N = 4 (ANOVA) (b) OPN protein expression was increased in the aortas of mice following 7 days of Ang II treatment as measured by Western blot analysis, ***$p < 0.001$, N = 3 (t test) and (c) A similar significant increase in OPN expression was observed in aortas from mice following 7 days of NE infusion ***$p < 0.001$, N = 3 (t test) (d) Finally, OPN expression is observed to be increased in the medial layer of the aorta in mice after 7-day Ang II or NE infusion compared to WT control, as assessed by immunohistochemistry. RED = OPN, BLUE = DAPI. Representative images are shown and were taken at 10× for an N = 4. For (a–d), bars are mean ± SEM.

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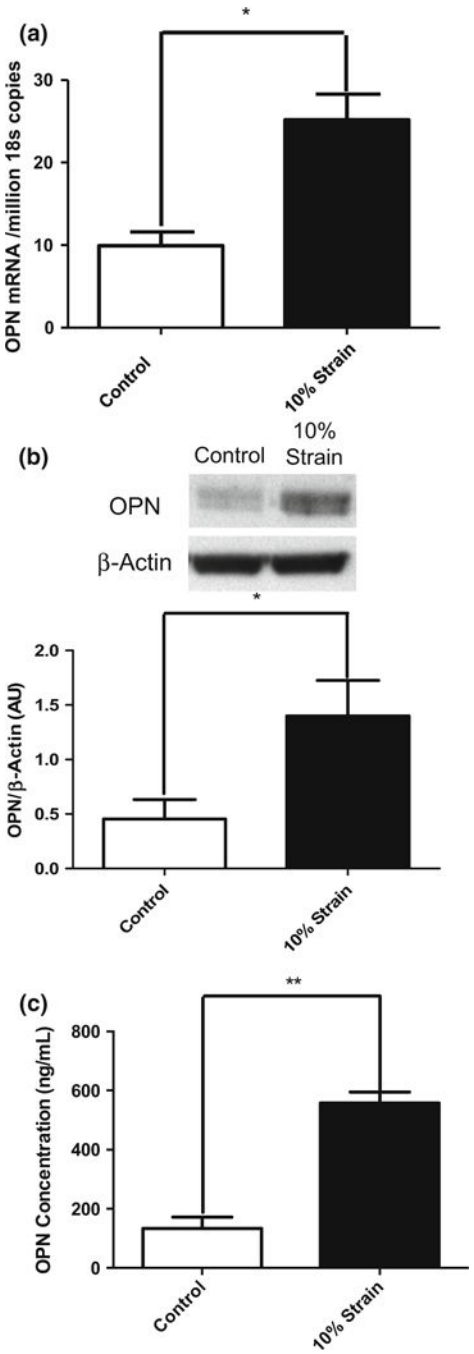

FIGURE 4.

Cyclic strain increases OPN mRNA and Protein Expression (a) Quantitative real-time PCR was used to measure levels of OPN mRNA and were normalized to a million copies of 18s. Samples were obtained by exposing SMCs to cyclic strain for 24 h at 10% equibiaxial strain. $*p < 0.05$ for N = 3 (*t* test). A significant increase in OPN mRNA expression was observed with cyclic strain (b) OPN protein expression was measured *via* Western Blot analysis of SMCs exposed to 24 h of 10% cyclic strain. Protein expression was quantified *via* protein densitometry and is represented in arbitrary units normalized to β-Actin expression $*p <$

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0.0.5 (*t* test), N = 4 (c) Secreted OPN protein expression was measured from media surrounding SMCs also exposed to 10% cyclic strain for 24 h using an ELISA. $^{**}p < 0.01$, N = 3 (*t* test). Significant increases in both cellular and secreted OPN expression were observed with cyclic strain.

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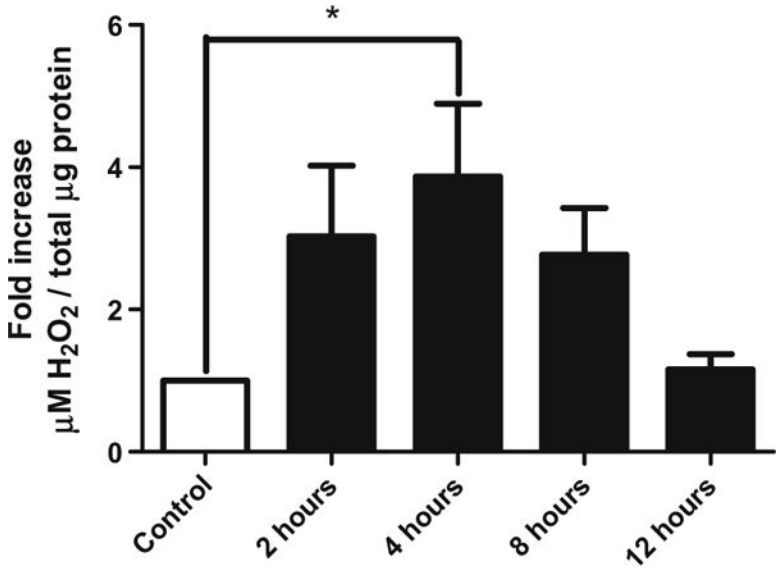

FIGURE 5.

Cyclic strain increases hydrogen peroxide: The amplex red assay was used to measure levels of hydrogen peroxide with cyclic strain. Total concentration of H_2O_2 was normalized to total protein and the fold increase plotted against the control at each time point. Control sample represents smooth muscle cells exposed to no strain for 12 h. All other samples were obtained by straining SMCs for 2, 4, 8, and 12 h at 10% strain. The maximal increase in H_2O_2 was observed after 4 h of cyclic strain. *$p < 0.05$ for N = 4 (ANOVA).

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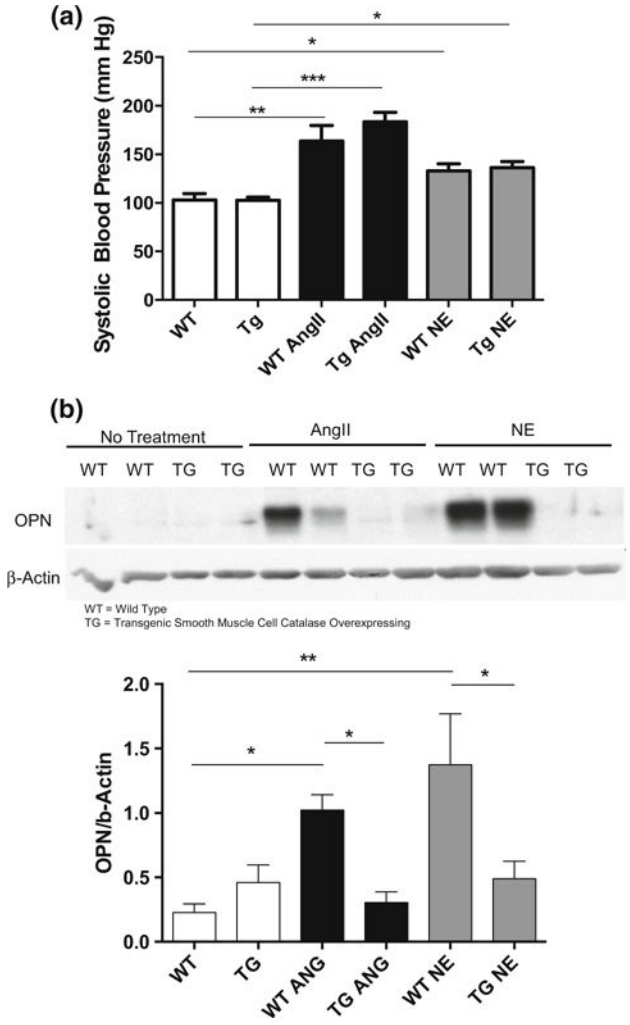

FIGURE 6.

Hypertensive transgenic smooth muscle cell catalase overexpressing mice ($Tg^{SMC\text{-}Cat}$) Showed Blunted Increases in OPN levels (a) Systolic blood pressure was measured in catalase overexpressing (Tg) and wild type (WT) mice following 7 days of Ang II infusion using tail-cuff plethysmography N = 3–4, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ (ANOVA). Tg mice had baseline SBPs similar to WT mice, and had a similar increase in SBP with treatment with either Ang II or NE compared to their WT counterparts. (b) OPN protein was measured using Western Blot analysis in aortas from WT and $Tg^{SMC\text{-}Cat}$ mice made hypertensive using 7-day Ang II or NE infusion for N = 3–4, $*p < 0.05$, $**p < 0.01$ (ANOVA). The hypertension-induced increase in OPN expression was significantly reduced in the Tg mice treated with either Ang II or NE.