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Apelin Protects Against Angiotensin II-Induced Cardiovascular Fibrosis and Decreases PAI-1 Production

Khandaker Siddiquee, PhD.¹, Jessica Hampton¹, Susan Khan², Dan Zadory², Linda Gleaves³, Douglas E. Vaughan, MD.³, and Layton Harris Smith, PhD¹

¹Sanford Burnham Medical Research Institute at Lake Nona, Orlando, FL 32827

²The Scripps Research Institute, Jupiter FL 33458

³Northwestern University School of Medicine, Chicago, IL 60611

Abstract

Objective—To test the hypothesis that apelin protects against AngII-induced cardiovascular fibrosis and vascular remodeling.

Methods and Results—Wild type mice administered apelin or apelin plus Ang II exhibited less cardiovascular fibrosis and decreased PAI-1 gene expression than mice receiving Ang II, L-NAME, apelin plus L-NAME or apelin plus AngII plus L-NAME. *In vitro* analysis using a luciferase construct driven by 3.1Kb of the human PAI-1 promoter revealed that apelin blocks Ang II-mediated PAI-1 gene expression. Immunoblotting for phosphorylated myosin phosphatase subunit and myosin light chain revealed that apelin blocked Ang II activation of the Rho kinase pathway, which is associated with induction of PAI-1 gene expression by Ang II. In addition, treatment of human aortic smooth muscle cells with apelin reduced PAI-1 mRNA and protein production in the presence and absence of Ang II. Conversely, L-NAME treatment attenuated the down-regulation of PAI-1 by apelin in cells.

Conclusions—Apelin protects against cardiac fibrosis and vascular remodeling through direct regulation of PAI-1 gene expression. This protective effect is mediated through the synergistic inhibition of Ang II signaling and increased production of NO by apelin. Our data extend previous findings and provide new insight into the molecular mechanisms by which apelin elicits a cardio-protective effect.

Keywords

fibrosis; apelin; angiotensin II; plasminogen activator inhibitor type-1 (PAI-1)

Introduction

Apelin is the endogenous peptide ligand for the apelin receptor (APJ), both of which are expressed by endothelial cells [1]. Apelin is synthesized as a 77-amino acid preproprotein that is sequentially cleaved into at least four circulating active peptides, apelin-36, apelin-17, apelin-13, and apelin-12 [2]. In cells, the order of potency in inhibiting forskolin-induced increased cAMP production is inversely proportional to peptide size, with apelin-12 >

Corresponding Author: Layton Harris Smith, PhD Assistant Professor, Sanford Burnham Medical Research Institute at Lake Nona, 6400 Sanger Rd., Orlando FL 32827, Phone: 407-745-2064, FAX: 407-745-2001, lhsmith@sanfordburnham.org.

Conflict of Interest: None

Disclosures:

None

apelin-13 > apelin-17 > apelin-36 [3–5]. Apelin has both direct and indirect effects on cardiovascular physiology, including endothelial-dependent vasodilatation [6] and a potent inotropic effect [7–10], which are mediated via binding to the APJ receptor on endothelial cells, vascular smooth muscle cells, and cardiac myocytes.

Apelin and APJ are emerging as a novel endogenous counter-regulatory mechanism of angiotensin II (Ang II). APJ-deficient mice, which are normotensive at baseline, are more sensitive to exogenous Ang II than wild type mice [11]. The baseline mean arterial pressure (MAP) in double-knockout mice lacking both APJ and the Ang II type 1 receptor (AT_{1a}) is greater than in AT_{1a}-receptor-deficient mice, suggesting that apelin/APJ elicits compensatory vasorelaxation to counter angiotensin II-mediated vasoconstriction [11]. Additionally, administration of apelin decreases the extent and severity of atherosclerotic lesions and the formation of abdominal aortic aneurysms exacerbated by administration of Ang II to apolipoprotein E (ApoE)^{−/−} mice [12]. A substantial body of evidence suggests that activation of the renin-angiotensin system (RAS) increases the risk of ischemic cardiovascular events independent of its pressor effects [13]. Ang II contributes to cardiovascular injury by mediating inflammatory and oxidative pathways, stimulating growth and proliferation of smooth muscle cells [14], and increasing extracellular matrix formation [15]. Ang II promotes cardiac fibrosis and vascular remodeling by inducing plasminogen activator inhibitor-1 (PAI-1) and transforming growth factor-beta (TGF-β) expression [16–18] by vascular smooth muscle cells, [19–21] endothelial cells, [20, 22] and cardiomyocytes [21]. PAI-1 enables fibrosis by blocking activation of the matrix metalloproteinases (MMPs) that hydrolyze collagenous proteins and by interrupting the clearance of extracellular matrix by plasmin [23].

These observations prompted the hypothesis that apelin protects against cardiac fibrosis and vascular remodeling by blocking Ang II-induced PAI-1 gene expression. To investigate this hypothesis, we treated wild type mice with the combination of apelin plus Ang II, apelin plus N^G-nitro-L-arginine methyl ester (L-NAME) or apelin plus Ang II plus L-NAME and compared them to wild type mice treated with apelin, Ang II, or L-NAME alone. Here we report the protective effect of apelin and the relationship between apelin and PAI-1 expression in cardiovascular fibrosis and remodeling.

Methods

Animals

Animal care was provided in accordance with the Laboratory Animal Welfare Act, the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 78–23. Revised 1978), and the Sanford-Burnham Institute for Medical Research guidelines and policies for the use of laboratory animals for research. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the Sanford-Burnham Institute for Medical Research. Mice were maintained in a temperature-controlled (20°C–22°C) environment with a 12h light-dark cycle. Sterile water and standard chow diet were available *ad libitum*. Eight-week-old C57Bl/6j mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

Experimental Design

Male mice were divided into 7 groups (n=6–8/group). Saline, Ang II (1.0 µg/kg/day), apelin-13 (15.0 µg/kg/day), or the combination of Ang II (1.0 µg/kg/day) plus apelin-13 (15.0 µg/kg/day) were administered for three weeks via osmotic mini-pump (Alzet minipumps #1004, Cupertino, CA), surgically implanted subcutaneously between the scapulae under isoflurane anesthesia. L-NAME was administered via drinking water at a

concentration of 1.0 mg/mL (Sigma, St. Louis, MO). Although there are other vasoactive forms of apelin, we used apelin-13 because it has been shown to be the most potent vasodilator *in vivo* [24]. Blood pressure and heart rate were measured via non-invasive tail cuff as described [25]. The volume pressure recording method utilized has been demonstrated to most accurately measure systolic blood pressure (SBP) in unanesthsized mice; Therefore we performed our analysis on SBP only [26]. Blood was drawn from the mandibular vein at baseline, day 14, and at the end of the study. All peptides used in this study were purchased from Bachem (King of Prussia, PA).

Histology

Histopathology was assessed by a single investigator who was unaware of the study protocol. After extensive perfusion with saline, hearts were harvested from each mouse, bisected at the equator of the ventricles and fixed in 10% formalin. Paraffin sections (5 μ m) were stained with Masson's trichrome and scanned with ScanScope XT (Aperio Technologies, Vista, CA) using bright field imaging at 20x magnification and stored as 24-bit RGB TIFF file format with a resolution of 1 μ m/pixel using the ImageScope software (Aperio). The area of total cardiac and perivascular fibrosis was quantified using automated color co-localization and deconvolution analysis algorithms [27] from at least 15 sections taken from the apex to the base of the heart from each mouse.

Plasma Markers of Cardiac Fibrosis

Plasma levels of osteopontin (OPN), and tissue and inhibitor of metalloproteinase-1 (TIMP-1) were measured with commercially available ELISA Kits used according to manufacturer protocols (R&D systems, Minneapolis, MN). Plasminogen activator inhibitor type-1 (PAI-1) antigen was also measured with a commercially available ELISA Kit (Molecular Innovations, Novi, MI).

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and RNeasy Mini Kit (Qiagen, Valencia, CA) and made into cDNA using SuperScript III (Invitrogen). qPCR was performed on the Realplex⁴ Real Time PCR Detection System (Eppendorf, Westbury, NY) using TaqMan probe (Applied Biosystems, Foster City, CA) sets (supplementary digital content Table 1). Experimental cycle threshold (Ct) values were normalized to β -actin or GAPDH measured on the same plate, and fold differences in gene expression were determined using the $2^{-\Delta\Delta C_t}$ method [28]. Total RNA from formalin fixed paraffin embedded (FFPE) tissue was extracted by using RecoverAll total nucleic acid kit (Ambion, Austin, TX) and analyzed as described above.

Cell Culture and Luciferase Reporter Assay

Human aortic smooth muscle (HASM) cells were cultured in Medium 231 supplemented with growth factors (SMGS) specific for the HASM cells (Invitrogen). Chinese hamster ovary (CHO) cells were cultured in F-12 medium (Hyclone, Thermo scientific) supplemented with 10% fetal bovine serum (FBS). CHO cells were transfected with 0.150 μ g of 3.1kb PAI-1/pGL2 luciferase vector [29] and 0.060 μ g of the constitutively active control plasmid pRLTK (Promega, Madison, WI) per well using Lipofectamine LTX and Plus reagent (Invitrogen). After 48h cells were incubated with apelin, AngII, L-NAME or the combination of apelin plus AngII or apelin plus L-NAME for additional 24h. Cells were lysed, and firefly and *Renilla* luciferase activities were assayed using a Dual Glow luciferase assay kit (Promega). The *Renilla* luciferase activity then served as a measure of normalized luciferase activity for each sample. The data from each experiment is expressed as mean ($\pm$ S.E.M) relative fold induction of normalized luciferase activity.

Western Blotting

Rho kinase activity induced by Ang II was assessed by determining phosphorylation at threonine 853 (Thr853) of myosin phosphatase subunit (MYPT) and at serine 19 (Ser19) of myosin light chain (MLC) by immunoblotting with a rabbit polyclonal or a mouse monoclonal antibody against the phospho-MYPT and phospho-MLC (Cell Signaling Technologies, Boston, MA). Nitric oxide synthase (NOS) protein in HASM and CHO cells was confirmed using a pan-species, universal antibody against NOS (Sigma, St. Louis, MO). Immunoreactive bands were visualized with specific antibodies using IRDye conjugated goat anti-rabbit or anti-mouse antibodies (Li-Cor Biosciences, Lincoln, Nebraska). All images were captured and analyzed on the Li-Cor Odyssey scanner.

Statistics

Statistical analyses of data were performed using Prism 5 software (Graph Pad, San Diego, CA). All experiments were repeated three times unless otherwise specified. Reported data were analyzed by ANOVA using the Bonferroni correction for multiple comparisons where appropriate.

Results

Hemodynamic Analysis

Mice received Ang II, apelin, and L-NAME alone, or in combination for 3 weeks. At the end of the study, SBP was significantly elevated in mice receiving Ang II alone (189.7 ± 2.4 mmHg), L-NAME alone (170.0 ± 3.0 mmHg), the combination of apelin plus L-NAME (185.5 ± 3.5 mmHg), and apelin plus Ang II plus L-NAME (164.24 ± 3.0 mmHg) compared with saline controls (130.7 ± 2.4 mmHg). SBP in mice receiving apelin (135.8 ± 6.9 mmHg) or apelin plus Ang II (134.8 ± 6.7 mmHg) was not significantly different from controls (Supplemental Digital Content Figure 1).

Cardiac and Perivascular Fibrosis

Total cardiac fibrosis was significantly higher in the hearts of mice administered Ang II or L-NAME than in mice that received apelin. This effect was attenuated in mice administered apelin with Ang II but not with L-NAME or the combination of apelin plus Ang II plus L-NAME (Figure 1A, B). The extent of perivascular fibrosis also was significantly greater in mice that received Ang II, L-NAME, the combinations of apelin plus Ang II, apelin plus L-NAME and apelin plus Ang II plus L-NAME, compared with saline controls or apelin alone. Mice that received apelin plus Ang II exhibited less perivascular fibrosis than animals receiving Ang II alone, but more extensive perivascular fibrosis than mice that received apelin or saline control (Figure 2A, B). The effect of apelin on Ang II-induced fibrosis is consistent with the observed effects of apelin plus Ang II on SBP. However, apelin alone only decreased the severity and extent of Ang II-induced fibrosis, while it abolished Ang II-induced hypertension.

Apelin Reduces PAI-1 Gene Expression

The effects of apelin, Ang II, and L-NAME administered individually and in combination on the cardiac expression of PAI-1 and other profibrotic genes were assessed by qPCR. Cardiac PAI-1 mRNA was significantly increased in mice that received Ang II alone, L-NAME alone, or the combination of apelin plus L-NAME or apelin plus Ang II plus L-NAME compared with mice that received apelin plus Ang II, apelin alone, or saline (Figure 3A). The same was true for collagen (Col1a1), tissue inhibitor of metalloproteinases (TIMP-1), fibronectin (FN1), and integrin- β -1 (ITGB1) mRNA. The mRNA levels of these targets were not increased in mice that received apelin plus Ang II (Supplemental Digital Content Figure

2A). Of note, apelin alone significantly reduced PAI-1 mRNA levels compared to saline, and this effect was even more pronounced in the presence of Ang II. In addition, plasma levels of OPN and TIMP-1 were significantly increased in mice that received Ang II, but were not changed in mice that received apelin, L-NAME, apelin plus Ang II, apelin plus L-NAME, apelin plus Ang II plus L-NAME or saline control (Supplemental Digital Content Figure 3A, B).

We next assessed the effects of apelin on Ang II-induced expression of these same genes *in vitro*. HASMs were incubated with Ang II or apelin, alone and in combination for 24 hours. Consistent with the effects of apelin in the heart, qPCR showed significantly higher levels of PAI-1 mRNA in cells incubated with Ang II than in cells incubated with apelin plus Ang II, apelin alone, or saline vehicle (Figure 3B). In addition, apelin significantly increased the mRNA of the collagen degrading enzyme MMP-2 (Supplemental Digital Content Figure 2B).

The effects of apelin on PAI-1 expression were confirmed by using a luciferase reporter construct under the control of 3.1kb of the human PAI-1 promoter. In addition we further determined the effect of apelin on PAI-1 mRNA and protein production. Both Ang II and L-NAME increased PAI-1 promoter activity, mRNA synthesis, and protein production. Apelin blocked the effects of Ang II on PAI-1 expression. In contrast, the effects of apelin on PAI-1 expression were abolished by pre-treatment of cells with L-NAME (Figure 3B–D). Because PAI-1 is regulated by NO [30, 31] and apelin appears to regulate PAI-1 in part by its effects on NO production, we confirmed the presence of nitric oxide synthase (NOS) in the HASM and CHO cells used in this study (Supplemental Digital Content Figure 4).

To further characterize the molecular mechanism by which apelin mediates down regulation of PAI-1, we explored the effect of apelin on Ang II signaling through the Rho-kinase pathway. As before HASM cells were incubated with apelin or Ang II, alone and in combination. Cells incubated with Ang II exhibited significantly greater amounts of phosphorylated myosin phosphatase subunit (p-MYPT) and myosin light chain (p-MLC) (more than 2-fold increase, as measured by densitometry) than cells incubated with either apelin or vehicle. Conversely, addition of apelin reduced the Ang II-mediated phosphorylation of MYPT and MLC (Figure 4A, B).

Based on these findings, we re-examined the tissues from our *in vivo* study to determine the effect of apelin on NOS expression. The relative level of NOS mRNA was significantly increased in the hearts of mice receiving apelin than in those of mice receiving Ang II, L-NAME, or vehicle. The addition of apelin plus Ang II increased NOS mRNA even further. Conversely, NOS mRNA was significantly decreased in the hearts of mice receiving apelin plus L-NAME or apelin plus AngII plus L-NAME, compared to all other treatments (Figure 5).

Discussion

Recent studies have demonstrated that apelin counter-regulates the effects of Ang II [7–9, 12], and that genetic variants within apelin and APJ may be linked to increased risk of hypertension [32]. Herein we report that apelin protects wild type mice against Ang II-induced hypertension and cardiovascular fibrosis. The antifibrotic effects of apelin are similar to those reported for angiotensin II receptor blockers (ARBs) [33], and are mediated through at least two distinct mechanisms: inhibition of PAI-1 production and changes in the expression of matrix proteins and degrading enzymes. Several lines of evidence support this conclusion. First, co-administration of apelin with Ang II abolished the pressor effects of Ang II and decreased the extent and severity of Ang II-induced cardiovascular fibrosis.

Second, apelin significantly decreased the cardiac expression of PAI-1 as well as several other Ang II-target genes that contribute to the development of fibrosis. Third, apelin alone increased the expression of the collagen degrading enzyme MMP-2, while simultaneously reducing the expression of collagen. Fourth, apelin decreased PAI-1 promoter activity and production of mRNA and protein in vascular smooth muscle cells in response to Ang II. Taken together, these data suggest that the cardioprotective effects of apelin occur through direct regulation of PAI-1 and are in part independent of its effects on Ang II.

Down-regulation of PAI-1 is central to the anti-fibrotic effects of apelin. As in the present study, reduction or inhibition of PAI-1 by genetic or pharmacological manipulation protects against fibrosis in a variety of models, including chronic infusion of Ang II [30, 34]. The role of PAI-1 and the fibrinolytic system in vascular biology extends beyond thrombosis and into vascular tissue housekeeping. In the vessel wall, increased PAI-1 decreases plasmin production and MMP activation leading to reduced matrix remodeling capacity. We observed that apelin elicited the opposite effect on these important profibrotic genes. Indeed, the combined effect of decreased PAI-1, TIMP-1 and Colla1 mRNA with increased MMP-2 mRNA suggests that apelin may exert a protective effect on the vessel wall by accelerating matrix remodeling [25]. The protective role of apelin on matrix balance is further supported by our observation that apelin decreased plasma OPN, another profibrotic cytokine regulated by Ang II and associated with PAI-1 and fibrosis [34, 35].

The vasodilatory effects of apelin are well established and have been shown to be mediated through a nitric oxide (NO)-dependent pathway [8, 36, 37]. In this study, we sought to dissect the anti-fibrotic effect from the vasodilator effect of apelin by co-administering apelin with the inhibitor of NO synthase, L-NAME. Mice receiving apelin plus L-NAME, or apelin plus Ang II plus L-NAME exhibited significant cardiovascular fibrosis. The extent of cardiovascular fibrosis in these mice was similar to mice receiving either Ang II or L-NAME without apelin. Further, *in vivo* analysis revealed that apelin significantly induced NOS expression, an effect that was eliminated by treatment with L-NAME. Interestingly, apelin plus L-NAME appeared to reduce eNOS mRNA to a greater extent than L-NAME alone. Although this difference was not statistically significant, it does suggest that apelin may regulate eNOS through a NO-dependent manner. This finding should be confirmed and explored in future studies of apelin and eNOS. Together, these observations led us to conclude that the anti-fibrotic effect of apelin represents the additive actions of activating an NO-dependent pathway, as well as inhibition of Ang II signaling.

It was recently reported that apelin antagonizes Ang II signaling in a mouse model of atherosclerosis and abdominal aortic aneurysm formation [12]. In addition to reducing the extent and severity of aneurysms induced by Ang II infusion, that study revealed that apelin interfered with Ang II-induced signaling through the formation of heterodimer complexes between the Ang II-type 1 receptor and APJ. Consistent with this model, we similarly demonstrated that apelin blocked Ang II-induced PAI-1 promoter activity and decreased phosphorylated MLC and MYPT, two targets downstream of Rho kinase. Given that Ang II stimulates PAI-1 through this pathway, it is possible that inhibition of PAI-1 expression by apelin results from antagonism of Ang II signaling through the Rho-kinase pathway. Despite the observed effects of apelin on MLC phosphorylation, our data conflict with another report showing apelin increased phosphorylation of MLC [38]. These conflicting results may be attributed to experimental differences. First, we used a 10-fold lower concentration of apelin-13 (100 nM), while the other investigators used 1.0 μ M of pyr-Apelin-13. Thus, it is possible that higher concentrations of apelin may be required to stimulate $G_{i/o}$ -mediated phosphorylation of MLC or to activate non-G-protein signaling, such as the MAPK/ERK pathway. Second, our investigation utilized aortic smooth muscle cells from humans at a

higher passage. The different cell culture conditions likely affect APJ expression and signal transduction, which may account for the differing results.

Although the precise mechanism by which apelin decreased PAI-1 was not elucidated in this study, our results together with those previously reported suggest that apelin may impede Ang II signaling through the formation of a heterodimer between the apelin and AngII receptors [12]. This mechanism explains the repression of Ang II signaling and subsequent decrease in PAI-1 expression observed in our study, but not those effects observed when apelin is administered alone. Collectively our results prompt a broader mechanistic hypothesis in which the cardioprotective effects of apelin are mediated through the simultaneous and synergistic inhibition of Ang II signaling and activation of NO-dependent pathways. This expanded mechanistic interaction warrants further research.

In summary, we observed that apelin protected against Ang II-induced hypertension and cardiovascular fibrosis in wild type mice. The anti-fibrotic effects of apelin are mediated through antagonism of Ang II signaling and increased NOS expression, resulting in decreased profibrotic gene expression, including PAI-1. Together these data confirm and extend previous findings showing that apelin plays an important role in cardiovascular function. More importantly, our studies provide critical insight into the molecular mechanisms by which apelin can elicit cardioprotective effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Apelin reduces cardiac fibrosis

(A) Co-administration of apelin plus Ang II blocks fibrosis. Upper panels show representative macroscopic views of the entire cardiac section. Lower panels show interstitial fibrosis within the myocardium (20X). (B) Quantitative analysis of cardiac fibrosis (n=5 sections/mouse, 6–8 mice/group) assessed by measuring collagen content using digital image analysis as described in materials and methods. The data from each experiment is expressed as mean ($\pm$ S.E.M.). * $P < 0.001$ vs saline, † $P < 0.001$ vs apelin, ‡ $P < 0.001$ vs Ang II by one-way ANOVA with Bonferroni's Multiple Comparison Test.

A)

B)

Figure 2. Apelin reduces perivascular fibrosis

(A) Representative coronary artery sections stained with Masson's trichrome. (B) Quantification of the area of perivascular fibrosis was determined as described in materials and methods from a minimum of 15 arteries/mouse, 6–8 mice/group). The data from each experiment is expressed as mean ($\pm$ S.E.M). * $P < 0.001$ vs saline, † $P < 0.001$ vs apelin, ‡ $P < 0.001$ vs Ang II by one-way ANOVA with Bonferroni's Multiple Comparison Test.

Figure 3. Apelin regulates PAI-1 *in vivo* and *in vitro*

(A) Apelin decreases PAI-1 mRNA in FFPE cardiac sections from mice receiving either apelin alone, or apelin plus Ang II. (B) Apelin decreases PAI-1 promoter activity at baseline and induced by Ang II, but had no effect on L-NAME-induced PAI-1 promoter activity. CHO cells were transfected with a PAI-1 promoter reporter construct, apelin receptor expression vector, and transfection control plasmid pRLTK. After 48 hours, cells were incubated with apelin (100 nM), Ang II (100 nM), L-NAME (100 μ M), or the combination of apelin plus Ang II or apelin plus L-NAME. (C) Apelin decreases PAI-1 mRNA *in vitro*. HASM cells were treated with Ang II or apelin alone, or in combination for 24h. Results are presented as the relative increase in PAI-1 mRNA compared to saline controls, and normalized to β -actin. (D) Apelin decreases PAI-1 protein production *in vitro*. Active PAI-1 in HASM cells incubated as described was quantified by ELISA. The data from each experiment is expressed as mean ($\pm$ S.E.M). * P <0.001 vs vehicle, † P <0.001 vs apelin, ‡ P <0.01 vs Ang II by one-way ANOVA with Bonferroni's Multiple Comparison Test.

Figure 4. Apelin antagonizes Ang II stimulation of the Rho kinase pathway

(A) Western blotting for phospho-MYPT and phospho-MLC demonstrate that apelin blocks the activation of Rho kinase signaling by Ang II (*lane 3 vs lane 4*). Quantification of band intensity by fluorescence intensity revealed significantly greater pMYPT in cells treated with Ang II than in cells treated with apelin, apelin + Ang II, or vehicle. (B) The amount of pMLC was significantly less in cells treated with apelin plus Ang II, or apelin alone than in cells treated with Ang II. * $P < 0.05$ vs vehicle, $^{\dagger}P < 0.05$ vs apelin, $^{\ddagger}P < 0.01$ vs Ang II by one-way ANOVA with Bonferroni's Multiple Comparison Test. *Lane 1*: vehicle; *Lane 2*: apelin; *Lane 3*: AngII; *Lane 4*: apelin plus Ang II. The relative fluorescence intensity from each experiment is expressed as mean ($\pm$ S.E.M).

Figure 5. Apelin induces eNOS genes expression *in vivo*

Apelin significantly increases eNOS mRNA in FFPE tissues from mice treated with apelin. L-NAME significantly decreased eNOS mRNA in both the presence and absence of apelin. For post-hoc comparisons, * $P < 0.01$ vs saline, † $P < 0.05$ vs apelin, ‡ $P < 0.001$ vs Ang II, § $P < 0.001$ vs L-NAME by one-way ANOVA with Bonferroni's Multiple Comparison Test.