Upregulation of the apelin–APJ pathway promotes neointima formation in the carotid ligation model in mouse

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Aims	To investigate apelin–APJ (angiotensin receptor-like 1) signalling in vascular remodelling, we have examined the pathophysiological response to carotid ligation in apelin knockout mice.
Methods and results	Apelin null animals compared with wild-type mice had significantly decreased neointimal lesion area (1.17 ± 0.17 vs. $3.33 \pm 1.04 \times 10^4 \mu\text{m}^2$, $P < 0.05$) and intima/media ratio (0.81 ± 0.23 vs. 1.49 ± 0.44 , $P < 0.05$), averaged over four sites 0.5–2 mm from the ligation. Exogenous apelin infusion rescued the apelin-KO phenotype, promoting neointima formation in the null animals. Apelin null animals showed decreased smooth muscle positive area in the neointima (82.3 ± 2.4 vs. 63.9 ± 8.4 , $P < 0.05$), and a smaller percentage BrdU positive cells in the neointima and media (11.06 ± 1.00 vs. 6.53 ± 0.86 , $P < 0.05$). Apelin mRNA expression increased initially (5.2-fold, $P < 0.01$) followed by increased apelin receptor expression (10.1-fold, $P < 0.05$) in the ligated artery. Cytochemistry studies localized apelin expression to luminal endothelial cells and apelin receptor upregulation to smooth muscle cells (SMC) in the media and neointima. <i>In vitro</i> experiments with cultured rat aortic SMC revealed that apelin stimulation increased migration. In contrast to the increased expression of apelin and apelin receptor in carotid remodelling, expression was not upregulated in the apoE high fat model, and correlated with the known disease-inhibitory effect in this model.
Conclusion	These data suggest that increased apelin receptor expression by SMC provides a paracrine pathway in injured vessels that allows endothelial-derived apelin to stimulate their division and migration into the neointima.
Keywords	Apelin • APJ • Vascular remodelling • Smooth muscle cell • Migration

1. Introduction

The apelin peptide was first identified as an endogenous ligand of APJ (angiotensin receptor-like 1), a previously orphan G protein-coupled receptor (GPCR) with high homology to the angiotensin II (AngII) type 1 receptor (AT1).^{1,2} Apelin and APJ are expressed in the cardiovascular system, with apelin expression primarily restricted to the microvascular endothelium, and APJ being expressed by endothelial cells, myocardial cells, and possibly smooth muscle cells (SMC).^{3–7} In the vasculature, early studies showed that injection of apelin in rat caused a transient decrease in blood pressure, which was abolished by a nitric oxide (NO) synthase inhibitor N(G)-nitro-L-arginine

methylester, suggesting involvement of NO in this vasodepressor effect.^{8–11} Studies with animal models of vascular disease have suggested that apelin–APJ signalling can mitigate the effect of disease-related pathways, through stimulating phosphorylation of Akt and eNOS and resulting increased NO production.¹²

Recently, we reported that apelin inhibited AngII-induced atherosclerosis and abdominal aortic aneurysm formation in apoE knockout mice, with this effect mediated in part by NO, and also possibly by direct APJ and AT1 receptor interactions.¹³ Interestingly, there are also data suggesting that the apelin–APJ pathway may promote vascular disease processes, through signalling in vascular SMC. Apelin produced vasoconstriction in endothelial-denuded, isolated human

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saphenous vein,¹⁴ and increased the phosphorylation of myosin light chain in rat aortic SMC and isolated rat aorta.¹⁵ In addition, Hashimoto *et al.*¹⁶ reported that APJ–apoE double knockout mice had increased superoxide production and aortic atherosclerotic lesions compared with apoE knockout mice. Taken together, these data suggest that apelin–APJ signalling may both inhibit and promote vascular disease, with the overall effect in the vascular wall being determined by poorly understood factors.

In the studies reported here, we have employed a carotid ligation blood-flow cessation model of vascular remodelling and evaluated the influence of apelin–APJ signalling in apelin null (apelin-KO) and wild-type (WT) mice. These studies demonstrate dramatic upregulation of both apelin and APJ receptor expression in the ligated vessel, with decreased neointima formation in the absence of apelin expression. Both *in vivo* and *in vitro* data suggest a SMC mechanism for the observed role of apelin–APJ in neointima formation in this model. The absence of increased APJ expression in the apoE null model of atherosclerosis, where apelin–APJ signalling is known to have a beneficial effect, suggests that upregulation of APJ on SMC may promote vascular disease.

2. Methods

An expanded Materials and Methods section is available in Supplementary material online.

2.1 Animal models

All animal studies were approved by the Stanford University Administrative Panel on Laboratory Animal Care (protocols 10 020, 10 022) and conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Twelve-week-old male WT (SVJ129) and apelin-KO mice⁶ were used. Ligation of the left carotid arteries was performed as described previously.¹⁷ The left common carotid arteries were dissected and ligated completely proximal to their bifurcation. The animals were euthanized 1 or 4 weeks after surgery and the tissues were harvested for RNA, morphology, or histological analysis. In other experiments, apelin-KO mice received 4 weeks infusions of [pGlu]-apelin-13 (2 mg/kg/day, American peptide, Sunnyvale, CA, USA) or saline with osmotic minipump (Alzet osmotic pumps, Cupertino, CA, USA). Carotid artery ligation was performed 24 h after pump implantation and morphological analysis was done after 4 weeks.

To evaluate APJ expression in atherosclerotic lesions in mice, RNA samples of aorta and histology of aortic sinus were prepared from apoE knockout mice.^{13,18} Briefly, apoE knockout mice and C57Bl6/J mice were fed with a high-fat diet. After 24 and 40 weeks of high-fat diet, the mice were euthanized, the aortic sinus tissue fixed for histology, and the whole aorta snap-frozen for RNA isolation.

2.2 Tissue preparation, histology, and lesion quantification

The carotid arteries were fixed and embedded in paraffin. Serial sections (5 µm) were taken at 0.5, 1, 1.5, and 2 mm from the ligation site and stained with haematoxylin and eosin (DAKO, Glostrup, Denmark). Digitized images of the vessels were analysed with Adobe Photoshop Extended version software. We measured the areas enclosed with lumen, internal elastic lamina, and external elastic lamina and calculated the total vascular area, the medial area, the intimal area, and the luminal area.

2.3 RNA extraction and real-time quantitative polymerase chain reaction analysis

RNA was extracted by QIAGEN RNeasy mini kit (Qiagen, Germantown, MD). cDNA was made by Superscript c-DNA synthesis system (Invitrogen, Carlsbad, CA), and quantitative polymerase chain reaction (PCR) performed on a 7900 HT Sequence Detection System with Taqman on Demand Gene Expression Probes (Applied Biosystems, Foster City, CA, USA). Values were normalized to the relative amounts of 18S rRNA for each sample.

2.4 Immunohistochemistry

For immunohistochemistry of SMC and macrophages, sections were incubated with primary antibodies against α -smooth muscle actin (α -SMA, ABCAM, Cambridge, CA, USA) at 1:250 dilution and MAC-3 (BD Pharmingen, San Diego, CA, USA) at 1:250 dilution. Sections were then incubated with biotinylated secondary antibodies followed by avidin–biotin–alkaline phosphatase substrate reaction (Vectastain ABC-AP kit, Vector).

For immunofluorescence of APJ, we used the HOPE Fixation method as per the manufacturer's instructions (Polysciences, Inc., Warrington, PA, USA). Briefly, the samples were incubated in HOPE solutions, and then embedded in low melting paraffin. The sections were incubated with anti-human APJ polyclonal antibody (MBL, Woburn, MA) at 1:250 dilution. After washing, sections were incubated with Alexa fluor 594 goat anti-rat IgG (1:400 dilution, Invitrogen, Carlsbad, CA, USA).

For detection of proliferating cells, BrdU staining was performed. Sections were incubated with an anti-BrdU antibody (ABCAM) at 1:100 dilution, then with secondary antibody (Alexa fluor 488, Invitrogen) at 1:500 dilution. After being washed and mounted in Vectashield with DAPI (Vector), the slides were visualized with a Zeiss fluorescence microscope. For detection of apoptotic cells, TUNEL staining was performed using the Cell Death Detection Kit (Roche, Indianapolis, IN, USA). To evaluate collagen content, Sirius red staining was performed as per the manufacturer's instructions (American Master Tech, Lodi, CA, USA).

2.5 LacZ staining

LacZ staining was performed as described previously.⁶ Briefly, the fixed tissues were immersed in X-Gal substrate at 29°C over night and then embedded in HOPE solution as described above. To verify the cell specificity of lacZ staining, we stained the same sections with for CD31 and X-gal. The sections were blocked, incubated with anti-CD31 antibody (BD Pharmingen, San Diego, CA, USA) at 1:100 dilution using Biocare Medical Rat Detection Kit (Biocare Medical, Concord, CA, USA).

2.6 In situ analysis of cell-specific APJ expression

The APJ localization in carotid arteries was assessed by *in situ* hybridization as described previously.¹⁹ Slides were baked at 60°C for 1 h, the rehydrated sections were treated with 0.2 M HCl, washed, and treated with 10 µg/mL proteinase K for 30 min. The sections were washed and incubated with the murine apelin cRNA probe overnight in a humidified chamber at 65°C. The slides were washed and anti-dig antibody was applied at 1:2000. The colour reaction was carried out using NBT/BCIP as the substrate.

2.7 Cell culture assays

The effect of apelin on SMC migration was assessed by wound healing migration assay and modified Boyden chamber assay²⁰ using rat aortic SMC.

2.8 Statistical analysis

Data are shown as mean $\pm$ SEM. Data were subjected to the Kolmogorov–Smirnov test to determine distribution. Groups were compared using the Students *t*-test for parametric data or the Mann–Whitney

Figure 1 Loss of apelin–APJ signalling is associated with decreased neointima formation and intimal/medial (I/M) ratio in the carotid ligation model. (A) Photomicrographs of representative cross sections from carotid arteries of apelin-KO and WT mice 4 weeks after ligation (H&E staining, 1 mm from ligation site). Bars indicate 100 μm . (B) Morphometric analysis of carotid arteries 4 weeks after ligation, comparing neointimal areas in apelin-KO and WT animals. (C) Morphometric analysis of total vascular areas. Values are shown as the average of sections from four different distances from the ligation site (0.5, 1, 1.5, and 2 mm). (D) I/M ratio is shown for different distances from the ligation site. Values are mean $\pm$ SEM of 10–11 mice in each group; * $P < 0.05$ apelin-KO vs. WT mice.

U test for non-parametric data. A value or $P < 0.05$ was considered statistically significant.

3. Results

3.1 Apelin–APJ signalling promotes neointima formation

Four weeks after the ligation, neointima formation was observed, but there appeared to be significantly less neointima in apelin-KO compared with WT mice (Figure 1A). Morphometric quantitation demonstrated a significantly smaller mean neointimal area in apelin-KO mice compared with WT mice (Figure 1B; 1.17 ± 0.17 vs. $3.33 \pm 1.04 \times$

$10^4 \mu\text{m}^2$, respectively, $P < 0.05$), with no difference in size of the non-ligated carotid arteries. Also, the mean total vascular area was smaller in ligated vessels in apelin-KO mice compared with WT, but this difference did not reach statistical significance (Figure 1C; 8.47 ± 0.68 vs. $10.59 \pm 1.10 \times 10^4 \mu\text{m}^2$, respectively, $P = 0.07$). There was no significant difference in lumen size between apelin-KO and WT mice (2.56 ± 0.24 vs. $2.91 \pm 0.42 \times 10^4 \mu\text{m}^2$, respectively) or medial size (3.73 ± 0.35 vs. $4.09 \pm 0.23 \times 10^4 \mu\text{m}^2$, respectively), and thus the ratio of intimal to medial area was significantly decreased in apelin-KO mice compared with WT mice at 0.5, 1, and 1.5 mm from the ligation (Figure 1D).

This result was surprising, given that exogenous apelin administration in other vascular injury models, including AngII-accelerated

Figure 2 Exogenous apelin administration promotes neointima formation in the carotid ligation model. (A) Photomicrographs of representative cross sections from carotid arteries of apelin-KO mice with apelin or saline infusion 4 weeks after ligation (H&E staining, 1 mm from ligation site). Bars indicate 100 μm . (B) Morphometric analysis of carotid arteries 4 weeks after ligation. Vascular areas for apelin-13 vs. saline infused animals are shown as the average of sections from four different distances from the ligation site (0.5, 1, 1.5, and 2 mm). (C) I/M ratios are shown for apelin-13 vs. saline infused animals, values are mean $\pm$ SEM of seven to eight mice in each group; * $P < 0.05$, apelin vs. saline group.

atherosclerosis and remodelling in a vein graft transplant model, was associated with reduced disease. Thus, to verify these data, we performed a rescue study of the apelin-KO phenotype in the carotid ligation model, asking whether exogenous apelin promotes neointima formation in this model. We administered [pGlu]-apelin-13 (2 mg/kg/day) or saline to apelin-KO mice by osmotic pump and performed carotid ligation. By 4 weeks after ligation, saline-infused animals had almost the same degree of vascular remodelling as control untreated apelin-KO mice. Apelin-infused mice showed more remodelling compared with the saline group (Figure 2A). The neointimal area in apelin-infused animals was significantly higher compared with the saline group (4.37 ± 1.33 vs. $1.95 \pm 0.38 \times 10^4 \mu\text{m}^2$, $P < 0.05$; Figure 2B). Also, the I/M ratio was significantly increased in the apelin infusion group compared with the saline group (1.03 ± 0.2 vs. 0.49 ± 0.05 , $P < 0.05$; Figure 2C). Thus, these studies with administration of exogenous apelin are consistent with the apelin-KO vs. WT mice comparison, and together these two approaches provide strong evidence for an apelin-APJ-mediated increase in disease in this remodelling model.

3.2 Apelin and APJ expression are increased in ligated carotid arteries

To better understand the pathophysiology of the demonstrated effect of apelin-APJ in this model, we next investigated expression of apelin and APJ in the ligated and non-ligated vessels. Quantitative real-time PCR (qRT-PCR) analysis showed very low expression for both

apelin and APJ in non-ligated arteries (Figure 3A). Apelin mRNA expression was significantly increased 5.2-fold in ligated arteries compared with non-ligated arteries in WT mice 1 week after ligation. A two-fold increase remained after 4 weeks ($P < 0.05$), but this difference did not reach statistical significance ($P = 0.07$). APJ mRNA expression was increased 2.5-fold after 1 week, and 10.1-fold after 4 weeks in ligated arteries in WT mice. In apelin-KO mice, APJ expression in unligated arteries was the same as that for WT mice, and increased 3.7-fold in ligated arteries after 4 weeks (Figure 3A). This upregulation of APJ expression in apelin-KO mice was significantly depressed compared with WT mice, suggesting that APJ expression is positively regulated by apelin. To determine the cell specificity of the increased mRNA response of APJ, we performed non-radioactive *in situ* hybridization analysis with representative sections from ligated and control arteries 4 weeks after ligation. As expected, APJ expression was hardly detectable in the non-ligated artery (Figure 3B). In the ligated artery, there was a strong signal in the media, as indicated by blue substrate reaction, with also labelling of cells in the neointima and adventitia.

To investigate further the specificity of cells responding to apelin in this model, we performed immunohistochemistry for APJ using an anti-mouse APJ polyclonal antibody with sections from tissues harvested 1, 2, 3, and 4 weeks after the ligation of WT animals. APJ staining in non-ligated arteries at every time point was very low, consistent with qRT-PCR and *in situ* hybridization studies (Figure 4A). Staining of the small vessels surrounding the carotid artery verified that the antibody assay was functional. There was minimal neointimal hyperplasia in the ligated arteries after 1 and 2 weeks, and there was increased intensity of APJ staining mainly in the media, compared with non-ligated arteries. Co-staining of α -SMA revealed that the increased APJ in the media was localized in SMC. APJ staining was also detected in the adventitia of ligated arteries, and this staining was colocalized with α -SMA, not with MAC-3 staining. After 3 and 4 weeks, APJ staining was also seen in the neointima as well as media (Figure 4A).

To determine the origin of increased apelin expression in the ligated arteries, we performed X-gal assays in the carotid arteries of apelin-lacZ reporter mice. The fidelity of the nuclear-targeted lacZ reporter integrated into the apelin locus has been described previously.⁶ In unligated arteries, the CD31-positive endothelial cell monolayer was visible, but there was no nuclear lacZ staining (Figure 4B, left panel). By 1 week after ligation, robust blue lacZ staining was seen in luminal cells of the carotid artery and cells associated with microcirculation in the adventitia (Figure 4B). CD31 co-staining revealed that lacZ staining luminal cells were endothelial cells.

3.3 SMC in apelin-related disease functions

To investigate the mechanism for decreased neointimal formation in apelin-KO mice, we characterized the cell composition in lesions 4 weeks after ligation. Quantitation of SMC by the measurement of α -SMA positive area identified by immunohistochemistry showed no difference in non-ligated arteries between the apelin-KO and WT genotypes (data not shown), whereas the percent of α -SMA positive area in the neointima of ligated vessels was significantly lower in apelin-KO mice compared with the WT mice (Figure 5A; 82.3 ± 2.4 vs. 63.9 ± 8.4 , $P < 0.05$). Recruitment of inflammatory cells often has an important role in vascular remodelling, so we also analysed macrophage infiltration into the neointimal lesions. There was almost no MAC-3 staining in non-ligated arteries of either genotype (data not shown) and the percent MAC-3 positive area was not

Figure 3 Apelin and APJ mRNA expression are increased in carotid arteries after ligation. (A) Quantitative real-time PCR of apelin and APJ in non-ligated and ligated arteries after 1 and 4 weeks. Apelin and APJ mRNAs were upregulated in ligated arteries, and the upregulation of APJ was attenuated in apelin-KO mice; * $P < 0.05$ vs. non-ligated artery, # $P < 0.05$ vs. ligated artery of WT mice. $n = 5-7$ animals per group. (B) APJ *in situ* hybridization of representative carotid sections is shown by blue non-radioactive substrate reaction. APJ expression was increased in the media, neointima, and the adventitia in the ligated arteries. Bars indicate 100 μm .

significantly different in ligated vessels (Figure 5B). Immunohistochemistry for vascular cell adhesion molecules (VCAM) and inter-cellular adhesion molecule-1 revealed that the expression level of these molecules in the ligated arteries was similar in both genotypes (data not shown). Next, we investigated collagen content by Sirius red staining. The percentage of Sirius red positive area in the neointima was significantly higher in apelin-KO mice (Figure 5C; 25.1 ± 7.2 vs. 13.5 ± 8.9 , $P < 0.05$). Collagen 1 mRNA expression was also increased in ligated arteries from apelin-KO mice compared with WT mice (Figure 5D). Taken together, these data suggest that apelin can promote SMC accumulation and decrease collagen production in the neointima of WT mice, but has no effect on inflammatory cell recruitment.

Proliferation is an important factor for neointima formation in this model. It has previously been reported that apelin stimulates proliferation of several cell types, including vascular SMC.^{16,21} To determine whether SMC proliferation was a feature of the increased neointimal formation in WT mice compared with the apelin-KO mice, *in vivo* BrdU labelling was performed and the number of positive cells counted 1 and 4 weeks after ligation. There were no BrdU positive cells in non-ligated arteries from mice of either genotype (data not shown). The ratio of BrdU positive cells to total cells in the media 1 week after the ligation was significantly lower in apelin-KO compared with WT mice (6.53 ± 0.86 vs. $11.06 \pm 1.00\%$, respectively,

$P < 0.05$), suggesting that SMC proliferation was attenuated in apelin null mice (Figure 5E). This difference was no longer seen by 4 weeks after ligation. It has been reported that apelin suppresses apoptosis via c-Jun N-terminal kinase and PI3-kinase signalling in osteoblasts, so we next investigated whether apoptosis contributed to decreased neointimal formation in apelin-KO mice by TUNEL staining.²² There were few TUNEL positive cells in unligated arteries of either genotype, and there was no significant difference in the ratio of TUNEL positive cells to total cells either 1 or 4 weeks after ligation (Figure 5E).

SMC migration is an important component of neointima formation and remodelling, and apelin has been shown to stimulate migration of other cell types, but apelin-mediated SMC migration has not been reported. To evaluate whether apelin could increase SMC migration, we conducted two types of *in vitro* assays. The wound healing migration assay showed that 100 nM apelin significantly increased the number of migrating cells from the wound edge (Figure 5F, 9.5 ± 2.6 vs. 23.0 ± 4.2 , $P < 0.05$). In the Boyden *trans*-well migration assay, apelin increased SMC migration in a dose-dependent manner. The number of migrating cells with 100 nM apelin treatment was significantly increased compared with control (Figure 5G; 70.7 ± 19.8 vs. 142.7 ± 27.0 , $P < 0.05$). AngII increased SMC migration as previously described,²³ and to the same degree as apelin, but there was no additional increase when the SMC were treated with both apelin

Figure 4 Cell-specific apelin and APJ expression in the ligated carotid artery. (A) Representative photomicrographs of immunohistochemistry with specific antibodies to APJ, α -SMA and the macrophage marker MAC3 are shown. APJ (left panels, red colour) was upregulated in the media and neointima and the staining was colocalized with α -SMA staining (rows 1 and 3, green colour). (B) Representative photomicrographs of carotid artery sections showing nuclear apelin–lacZ reporter expression (blue colour). Sections are evaluated with immunohistochemistry for CD31 (red colour). LacZ reporter expression was increased in luminal and adventitial cells, which are shown to be endothelial cells by colocalization with CD31 staining. Bars indicate 100 μ m.

and AngII. These data suggest that apelin may be important for SMC migration as well as proliferation.

3.4 Apelin and APJ expression in the apoE atherosclerosis model

The inhibition of neointimal formation in apelin-KO mice was surprising because our previous data had shown that a similar deletion of apelin in the apoE null mouse model resulted in greater disease (atherosclerosis) compared with the baseline apoE phenotype.¹³ As the pathological features of the carotid ligation model correlated with increased apelin and APJ expression, possibly resulting in increased APJ activation on SMC, we analysed their expression in atherosclerosis in apoE knockout mice. In sharp contrast to the increased expression observed in the ligated carotid artery, apelin and APJ expression were not significantly different over time for either C57BL/6 or apoE knockout mice. On the other hand, expression of inflammatory molecules such as MCP-1 and VCAM

was increased in the aortas of apoE knockout mice, and greater than that seen in the C57BL/6 mice.

Next, we performed APJ immunohistochemistry in atherosclerotic aortic sinus lesions from apoE knockout mice. Some APJ signal was detected in medial SMC, as demonstrated by co-localization with α -SMA staining, but the expression level appeared less than that of SMC in small vessels in surrounding tissue (Figure 6B). APJ expression was also seen in the atherosclerotic plaque and co-staining with α -SMA revealed that APJ was localized mainly in SMC in these intimal lesions. There was APJ staining of some luminal endothelial cells, as demonstrated by co-localization with CD31 staining.

4. Discussion

These data suggest that local paracrine signalling between endothelial cells and SMC mediate a pathological effect of apelin–APJ in the carotid ligation model. Importantly, this pathophysiological process requires the upregulation of both apelin and APJ, which are expressed at low levels in the carotid vessel wall before ligation. In the vasculature, apelin is synthesized in endothelial cells in capillaries and post-capillary venules, and likely regulates vascular function primarily through an autocrine pathway, with NO being the main effector. Although apelin-like immunoreactivity has been reported in large conduit vessels,⁴ the balance of data suggests that vascular apelin is restricted to the microcirculation under normal conditions.^{6,24} In this study, qRT-PCR and lacZ reporter assays revealed that apelin expression level in the normal carotid artery is very low and that ligation induced apelin expression in the luminal endothelium prior to the upregulation of APJ receptor expression. Recent studies have shown that apelin expression is upregulated with loss of shear forces, and in response to hypoxia, and these are two stimuli that are likely to be functioning in this model.^{6,25–27} Also in this study, APJ was shown to be upregulated with expression being restricted to SMC primarily in the media and neointima. Much less is known about the regulation of APJ expression in SMC. Interestingly, we observed decreased upregulation of APJ in the ligated artery of apelin-KO mice, suggesting that APJ expression is regulated by apelin level, although we could not determine which cell was responsible for this effect. It has been reported that expression of other GPCRs is regulated by their ligand, e.g. AT-1 expression was upregulated by interventions that increased plasma AngII levels.^{28,29} Taken together, these data showing increased expression of apelin by endothelial cells, and APJ expression by SMC provides a paracrine signalling circuit that does not normally exist in the carotid, providing the opportunity for endothelial cells to recruit SMC to the neointima in this vascular remodelling model.

The results presented here are consistent with published data, suggesting that apelin can have a direct stimulatory role for SMC that is supportive of vascular disease processes. Apelin has been shown to produce vasoconstriction in endothelial-denuded, isolated human saphenous vein,¹⁴ and recently, Maguire *et al.*³⁰ clearly demonstrated that apelin-mediated dilatation of the mammary artery was abolished by denudation. Apelin has been shown to increase the phosphorylation of myosin light chain in rat aortic SMC, a response which is associated with SMC contraction and migration and reflects the activation of disease-promoting signalling pathways.¹⁵ In addition, studies by Hashimoto *et al.*¹⁶ with cultured SMC suggested that apelin stimulation leads to increased expression of NADPH oxidase subunits, increased O_2^- production, and O_2^- -mediated cell division.

Figure 5 Role of SMC in modulating apelin-related disease functions. (A) Quantification of the percentage area occupied by α -SMA positive cells in the neointima revealed a relative decrease in SMC infiltration in apelin-KO mice compared with WT. Values are mean $\pm$ SEM of eight mice in each group; * $P < 0.05$ vs. WT mice. (B) Quantification of the percentage area occupied by MAC-3 positive cells in the neointima revealed no difference in macrophage infiltration between the two genotypes. (C) Quantification of the percentage of Sirius red stained area in the neointima revealed collagen content is increased in apelin-KO mice, * $P < 0.05$ vs. WT mice. (D) Collagen 1 mRNA expression 4 weeks after ligation is increased in apelin-KO mice, * $P < 0.05$ vs. WT mice. (E) Quantification of the ratio of BrdU and TUNEL positive to total SMC number in ligated carotid arteries at 1 or 4 weeks after ligation. Values are mean $\pm$ SEM of five to six mice in each group; * $P < 0.05$ vs. WT mice. (F) Migration of SMC in *in vitro* assays. Number of migrating cells in the wound healing assay, values are mean $\pm$ SEM of at least five fields in each group from four replicates; * $P < 0.05$ vs. control. Cells were exposed to media containing [pGlu]-apelin-13 or foetal bovine serum (FBS). (G) Number of migrating cells in *trans-well* migration assay, comparing migration in response to apelin and AngII. Values are mean $\pm$ SEM of at least five fields in each group from four replicates; * $P < 0.05$ vs. control.

Data presented here showed that SMC migrate in response to apelin *in vitro*, and increase their rate of cell division *in vivo* in the presence of apelin. Given the data of Hashimoto *et al.* and our *in vitro* studies presented here, it is apparent that relatively high concentrations (10^{-7} – 10^{-6} mol/L) of apelin are needed for SMC proliferation or migration. Thus, considering the fact that plasma apelin concentration is approximately 10^{-10} mol/L,³¹ SMC in the media must be responding to increased apelin locally in the lesion. We observed that exogenous apelin infusion increased remodelling in apelin-KO mice, and apelin-infused apelin-KO mice showed a trend toward more disease than WT mice, suggesting that high concentrations of apelin can increase neointimal formation if SMC express APJ.

It is well known that AngII contributes to vascular remodelling, and AngII has direct effects on SMC proliferation, hypertrophy, apoptosis,

and synthesis/degradation of collagen.³² Inhibition of AngII signalling by angiotensin I-converting enzyme or AT-1 antagonism inhibits neointimal hyperplasia and collagen and elastin accumulation.^{33,34} Apelin can have direct effects on SMC proliferation and migration similar to AngII. However, these data show that the decreased neointima formation in apelin-KO mice, associated with less SMC accumulation, is also associated with increased collagen content. These data suggest that while apelin has some similar actions as AngII on SMC, it can have very different effects on disease-related SMC, including the regulation of collagen synthesis. Further studies are clearly needed to explore the differences between apelin–APJ and AngII signalling in SMC.

The studies reported here with the carotid ligation model are particularly interesting when considered in the context of our previous

Figure 6 Apelin and APJ expression in atherosclerosis of the apoE null mouse model. (A) mRNA expression of apelin and APJ in the aorta of apoE-KO and WT mice fed a high-fat diet. Apelin and APJ mRNA levels were unchanged, whereas MCP-1 and VCAM-1 were significantly upregulated at the 40-week time point. Values are mean $\pm$ SEM; * $P < 0.05$ vs. WT mice at the same time point. (B) Representative photomicrographs of immunohistochemistry with specific antibodies to APJ, α -SMA, CD-31, and MAC3 are shown. APJ (left panels, green colour) was expressed in medial SMC as well as in the atherosclerotic plaque from aortic sinus lesion of apoE-KO mice. APJ staining in the lesion was mainly colocalized with α -SMA staining (row 1, red colour), and to a lesser extent with CD-31 staining (row 2, red colour). Bars indicate 100 μ m.

studies in other murine disease models showing that apelin can have a beneficial effect in the setting of vascular wall disease.¹³ In these studies, genetic loss of apelin was associated with increased aortic atherosclerosis in the high fat apoE null model, and systemic apelin administration blocked AngII acceleration of disease, including neointima formation in a vein graft model where apelin appeared to inhibit the SMC contribution to disease.¹³ The mechanism for these beneficial effects of apelin–APJ signalling appeared to be increased NO production, with concomitant quenching of reactive oxygen species (ROS). Because of the striking association of increased apelin and APJ expression with the detrimental effect of this pathway on vascular pathology in the carotid ligation model, we assessed APJ expression in one of the previous models that of atherosclerosis in the apoE null mouse. These studies showed that apelin and APJ were not upregulated over the course of 40 weeks of western chow diet. The absence of increased apelin and APJ expression in the apoE null model of atherosclerosis, where apelin–APJ signalling is known to have a beneficial effect, suggests that upregulation of APJ on SMC may promote vascular disease.

The different direction of effect observed in this laboratory between the atherosclerosis and remodelling models may reflect differential activation by apelin–APJ of the endothelial cell vs. the SMC. Apelin–APJ signalling in the endothelial cell stimulates NO production and is primarily vascular protective, whereas signalling in SMC appears to stimulate contraction, migration, NADPH oxidase production of ROS, and the associated vasculopathic consequences of these events. A unifying hypothesis for the apparently conflicting data between models is that a balance exists between the protective effect of apelin-induced EC generation of NO and the harmful effect of apelin-mediated SMC-generated ROS.¹⁶ A disease-promoting milieu could be produced by endothelial dysfunction and loss of APJ expression and thus NO production, or by the upregulation of APJ on SMC with apelin-mediated increases in vascular tone and superoxide production. For instance, the pro-atherosclerotic effects of apelin in the carotid ligation model were associated with a 10-fold increase in APJ expression in medial and adventitial cells. We have recently shown that APJ expression by SMC is highly responsive to ROS (unpublished results), and previous studies have shown upregulation of APJ by other disease-related stimuli such as hypoxia.⁷ Finally, based on these studies with the carotid ligation model, apelin upregulation in endothelial cells that do not normally produce apelin can support SMC pathology.

4. Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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