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Regulatory Peptides 126 (2005) 233–240
REGULATORY PEPTIDES
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Immunocytochemical localisation of the apelin receptor, APJ, to human cardiomyocytes, vascular smooth muscle and endothelial cells

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Received 1 July 2004; received in revised form 6 October 2004; accepted 7 October 2004
Available online 15 December 2004

Abstract

The novel G protein-coupled receptor APJ, recently paired with the proposed cognate peptide ligand apelin, mediates potent vasodilator and positive inotropic actions in rats. Radioligand binding showed apelin receptors in rat and human heart and human large conduit vessels. The specific cell types expressing the receptor, however, have not been determined. Apelin, the cognate receptor ligand, is present in endothelial cells. However, the exact pathway of endothelial apelin synthesis and secretion is not known.

We therefore investigated the cellular distribution of APJ receptor-like immunoreactivity (APJ-LI) in a range of human tissues using immunocytochemistry and fluorescent double staining confocal microscopy. The same techniques were applied to determine the intracellular localisation of apelin-like immunoreactivity (apelin-LI) in cultured human umbilical vein endothelial cells (HUVECs).

APJ-LI is present in endothelial cells, vascular smooth muscle cells and cardiomyocytes. Apelin-LI localises to secretory vesicles and the Golgi complex/endoplasmic reticulum of HUVECs. Apelin-LI does not co-localise with von Willebrand factor in Weibel-Palade bodies, suggesting synthesis of apelin via the constitutive pathway.

The proximity of receptor and ligand in the human vasculature, together with evidence for local vascular apelin synthesis, suggests an important role for APJ/apelin as a paracrine cardiovascular regulator system.
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Keywords: G protein-coupled receptor; Vasodilator; Inotropic; Peptide; Secretion; Constitutive

1. Introduction

The APJ receptor, previously designated an "orphan" G protein-coupled receptor, was first cloned from a human gene by O'Dowd et al. [1] and apelin-36 was proposed as the cognate endogenous ligand [2]. Although apelin-36 was the first apelin peptide discovered, further research has identified a number of shorter forms of the APJ receptor ligand in human tissues and bovine colostrum, which are thought to be produced by posttranslational modification of the 77-amino acid prepropeptide [2,3]. Recently, however, functional assays have provided evidence that the short pyroglutamyl form of apelin, (Pyr[1])apelin-13, may represent the biologically active endogenous ligand [4–7].

Messenger RNA encoding the APJ receptor has been shown to be abundantly expressed in the central nervous system of rats and humans [1,8–10] and the receptor was associated with a role in fluid homeostasis [7,10,11]. The APJ receptor has also been proposed as an essential co-receptor to CD4 in the infection of central nervous system cells with t-tropic or dual-tropic HIV strains [9,12,13]. The main body of evidence, however, supports a role for the APJ/apelin system in the regulation of cardiovascular function. Apelin receptors have been detected in rat and human myocardium as well as in the medial layer of human coronary artery, aorta and saphenous vein using radioligand binding and (Pyr[1])apelin-13 was shown to be a potent vasoconstrictor in endothelium denuded, isolated human

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0167-0115/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.regpep.2004.10.019

saphenous vein [14]. In rats, *in vivo* intravenous administration of apelin leads to a significant decrease in mean arterial blood pressure, a response completely abolished by co-administration of the nitric oxide synthase inhibitor L-NAME [7,10,15,16]. Comparison of mice in which the APJ receptor gene had been deleted with wild type controls revealed a lack of apelin-induced hypotensive actions in the knockout animals. This study also showed apelin-induced over-expression of endothelial nitric oxide synthase in cultured murine endothelial cells from control animals, which was abolished in endothelial cells from APJ receptor knockout mice [17]. Furthermore, apelin has been found to elicit positive inotropic effects in the isolated rat heart [18].

In pathophysiological conditions in humans, left ventricular expression of APJ receptor mRNA was significantly reduced in patients with idiopathic dilated cardiomyopathy[19]. In a similar patient group, the use of microarray technology identified the APJ receptor gene to be one of two genes overexpressed after implantation of a left ventricular assist device, with changes observed in circulating apelin levels in heart failure patients [20]. This may suggest a role for APJ/apelin in the pathogenesis of heart failure.

At present, the mechanism how apelin mediates both endothelium-dependent vasodilator and endothelium-independent vasoconstrictor actions has not been established. The APJ receptor has been detected in human cardiovascular tissue using receptor autoradiography [14], but the precise cellular localisation remains to be determined. Based on our report of apelin being abundantly present in endothelial cells of the human vasculature [21], we have proposed that apelin may elicit vasoconstriction through paracrine activation of APJ receptors on vascular smooth muscle. To explain the vasodilator effects we hypothesised that in the presence of an intact endothelium vasoconstriction is counterbalanced, or even overcome, by apelin-induced release of vasodilator mediators from endothelial cells. To find supportive evidence for this proposed mechanism we investigated the precise cellular distribution of the APJ receptor in human tissues and the intra-cellular localisation of apelin and APJ receptors in both *in situ* and human umbilical vein endothelial cells (HUVECs) using immunocytochemistry and fluorescent double labelling in conjunction with confocal laser scanning microscopy.

2. Materials and methods

2.1. Materials

Unless stated, all chemicals were obtained from Sigma Aldrich (Poole, UK). Rabbit anti-APJ receptor (rat) antiserum and rabbit anti-apelin-12 (rat/human) antiserum used in immunocytochemistry was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA). Mouse-anti-human von Willebrand factor and mouse-anti-human smooth muscle α-actin monoclonal antibodies, secondary antibodies, rabbit-PAP-complex and horseradish-peroxidase-conjugated swine-anti-rabbit antiserum were from Dako (Glostrup, Denmark). AlexaFluor 488 conjugated goat-anti-rabbit serum and AlexaFluor 568 conjugated goat-anti-mouse serum were obtained from Molecular Probes (Leiden, The Netherlands), Vectashield mounting medium containing 4′,6′-diamino-2-phenylindole hydrochloride (DAPI) was from Vector Laboratories (Burlingame, CA, USA) and DePeX-Gurr mounting medium from BDH Laboratory Supplies (Poole, UK). The site-directed rabbit anti-APJ receptor (rat) antiserum was raised against the extreme C-terminus of the APJ receptor (amino acid residues 349–376) sequence, a region differing in only two amino acids in rat and human. Test experiments in human tissues resulted in staining patterns similar to those in rat tissue and consistent with mRNA distribution in human tissue. A BLAST-p search of publicly available human peptide libraries retrieved no other human peptides with significant sequence similarity, making non-specific cross-reactivity of the antiserum unlikely [22].

2.2. Tissue collection

2.2.1. Human tissue sections

Human tissues were obtained with local ethical approval. Left ventricular and atrial myocardium were from patients undergoing heart transplants for cardiomyopathies (n=4) or from donor hearts for which there was no suitable recipient (n=6). Saphenous veins (n=10), radial arteries (n=4) and left internal mammary arteries (n=3) were from patients undergoing coronary artery bypass graft surgery for ischaemic heart disease. Coronary arteries were obtained from patients undergoing heart transplants for ischaemic heart disease (n=2) and donor hearts not required for further transplantation (n=2). Histologically normal kidney (n=4) and lung (n=4) were from patients undergoing nephrectomy and lobectomy respectively for non-obstructive carcinoma. The histologically normal adrenal tissue (n=3) was obtained from two patients undergoing adrenalectomy for phaeochromocytoma. On collection, tissues were snap frozen in liquid nitrogen and stored at −70 °C until further use.

2.2.2. Human umbilical vein endothelial cells

HUVECs were a kind gift from Dr. Jun Wang (Department of Medicine, University of Cambridge), cultured as described previously [23] and grown until they reached subconfluency. Culture medium was aspirated and cells were stored at −70 °C until further use.

2.2.3. Rat tissue

Rat brains (n=3), hearts (n=3) and lungs (n=3) were used to initially characterise the rabbit anti-APJ receptor (rat) antiserum and as positive controls based on previous findings (Lee, 2004). Tissue was obtained from male Sprague–Dawley rats (300–350 g, Charles River, Wilmington, MA, USA) that were euthanized by CO_2 inhalation.

2.3. Immunocytochemistry

Cryostat cut tissue sections (10 μm for human heart, lung, kidney and adrenal, 30 μm for large conduit vessels) were left to dry overnight at room temperature. Tissue was fixed in ice-cold acetone for 10 min. Sections were incubated with 5% non-immunised swine serum (SS) in phosphate-buffered saline (PBS) for 1 h at room temperature to block non-specific protein interaction. Tissues were incubated with rabbit anti-APJ receptor antiserum at a dilution of 1:100 in PBS containing 0.1% Tween-20 and 1% non-immunised swine serum (1% SS PBS/T) for 48 h at 4 °C. In adjacent sections, primary antisera were omitted as a negative control. Tissue sections were then washed three times for 5 min in cold PBS/T before the incubation with swine anti-rabbit antiserum at a dilution of 1:200 in 1%SS PBS/T for 1 h at room temperature. After repeated washing, sections were incubated with rabbit peroxidase/anti-peroxidase complex at a 1:400 dilution in 1% SS PBS/T. Further washing preceded a three minute incubation of tissue sections with a 2.5% solution of 3,3′ -diaminobenzidine in 0.05 M Tris–HCl buffer containing 0.3% hydrogen peroxide. The chromogenic reaction was stopped by immersion of the slides in distilled water. Sections were dehydrated using a graded alcohol series before being submerged in xylene for 1 h to clear. Sections were mounted using DePeX-Gurr mounting medium and then examined using a standard bright field microscope (Olympus UK, London, UK). Images were captured using a U-TV1-X digital camera (Olympus UK) and AnalySis software (Soft Imaging System, Münster, Germany).

2.4. Fluorescent double staining/confocal microscopy

Cryostat cut tissue sections (30 μm) were left to dry overnight at room temperature and fixed in ice-cold acetone for 10 min. Sections were incubated with 5% non-immunised goat serum (GS) in PBS for 1 h at room temperature to block non-specific protein interaction. Tissues were incubated with rabbit anti-APJ receptor antiserum at a dilution of 1:100 and mouse anti-vWF monoclonal antibody at 1:50 dilution in 1%GS PBS/T or mouse anti-smooth muscle α-actin (1:100 dilution) for 48 h at 4 °C. Adjacent sections were incubated with rabbit anti-apelin-12 antiserum (1:200 dilution) and mouse anti-vWF monoclonal antibody (1:50 dilution) or mouse anti-smooth muscle α-actin (1:100 dilution) for 48 h at 4 °C. Tissue sections were washed in cold PBS/T before incubation with the secondary antibody solution. The secondary antibody solution contained both AlexaFluor 488 conjugated goat anti-rabbit serum and AlexaFluor 568 conjugated goat anti-mouse serum at a dilution of 1:100 in 1%GS PBS/T. After repeated washing, sections/cells were mounted using Vectashield mounting medium containing DAPI. The same procedure was carried out on HUVECs using rabbit anti-APJ receptor or rabbit anti-apelin-12 antiserum in combination with mouse anti-vWF antiserum. Confocal imaging was performed using a Leica TCS-NT-UV confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany).

3. Results

3.1. Immunocytochemistry

3.1.1. Human tissue

In sections of human atrial and ventricular myocardium, APJ receptor-like immunoreactivity (APJ-LI) was detected in endothelial cells lining small intramyocardial vessels (Fig. 1a,b), small coronary arteries and in endocardial endothelial cells (data not shown). Lower levels of APJ-LI staining were also localised to cardiomyocytes (Fig. 1d,e) and vascular smooth muscle cells of cardiac blood vessels (Fig. 1a). In coronary adipocytes, nerves and connective tissue APJ-LI was absent or below the level of detection. In the lung, APJ-LI was restricted to endothelial cells of small pulmonary vessels and lower levels to vascular smooth muscle of pulmonary vessels (data not shown). We did not detect APJ-LI in alveolar epithelium or connective tissue. In sections from kidney APJ-LI was present in endothelial cells and in vascular smooth muscle cells of small intrarenal vessels (data not shown). No staining was detected in glomeruli, renal tubular epithelial cells or connective tissue. In the adrenal gland, APJ-LI was confined to endothelial cells of the surrounding arteries, small resistance arteries within the capsular plexus and the central vein (data not shown). In secretory cells of both the adrenal cortex (zona glomerulosa, zona fasciculata, zona reticularis) and medulla, APJ-LI was not detectable. In human large conduit vessels, we observed APJ-LI in endothelial cells lining the walls of saphenous veins (Fig. 1g,h), coronary arteries, radial arteries and left internal mammary arteries and in the vascular smooth muscle cells forming the vessel wall (Fig. 1g,h). Staining was absent in sections where the primary antiserum was omitted as a negative control (Fig. 1c,f,i).

3.1.2. Rat tissue

Sections of rat tissue were used as positive controls and to characterise the primary antiserum. In rat brain we detected APJ-LI in neurones of the lateral cerebellar nucleus (Fig. 1j) and lower levels in endothelial and vascular smooth muscle cells from small cerebral vessels (data not shown). In sections from rat lung, APJ-LI was present in bronchial epithelial cells (Fig. 1k) and lower levels in endothelial and vascular smooth muscle cells from small pulmonary vessels (data not shown). In the rat heart, we detected APJ-LI in endothelial and vascular smooth muscle cells from small intramyocardial vessels and lower levels on cardiomyocytes (data not shown). The vascular smooth muscle of the medial layer of rat aorta and pulmonary artery showed intense staining for APJ-LI (Fig. 1l) Staining was absent in sections where the primary antiserum was omitted as a negative control (Fig. 1m).

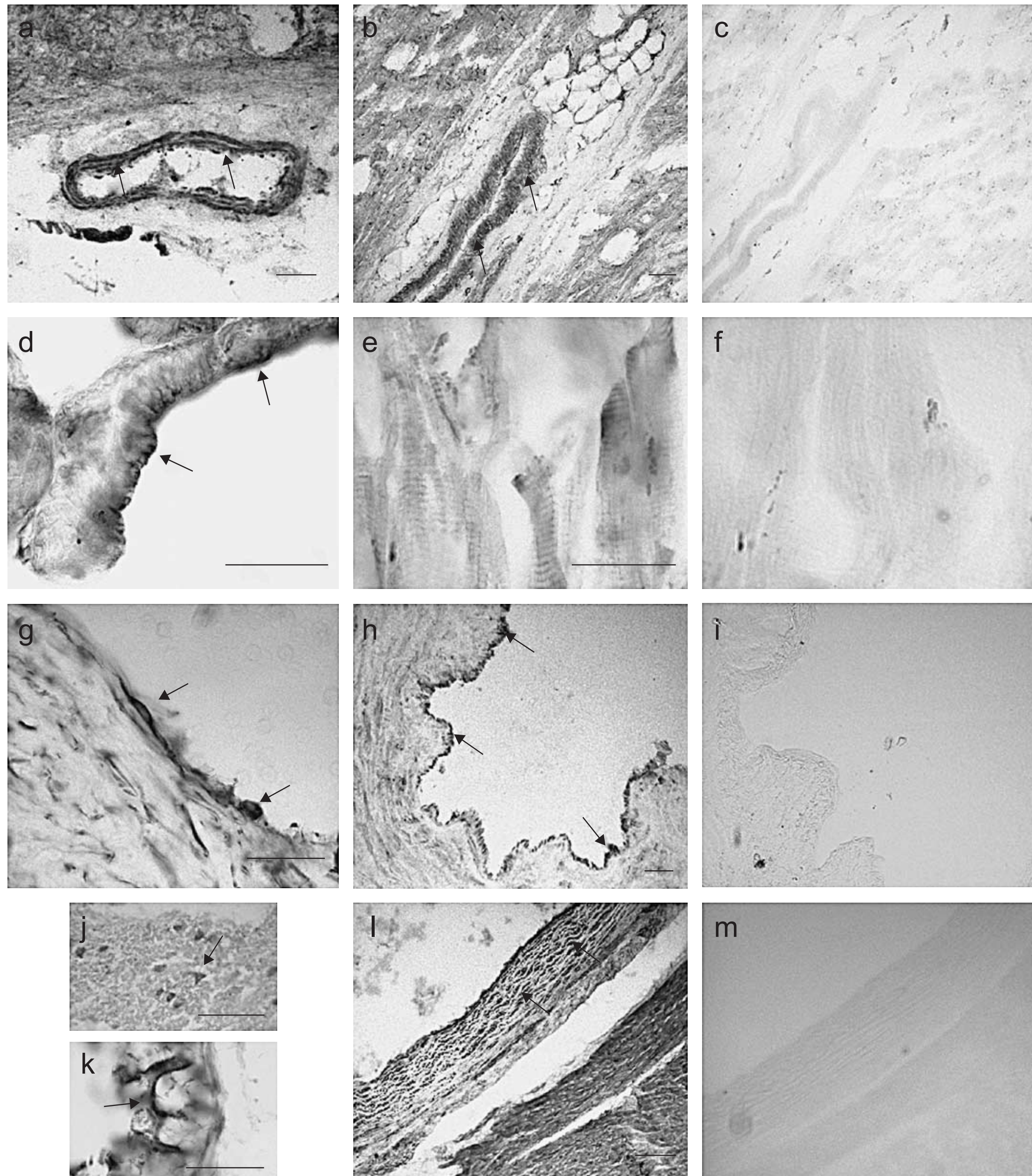

Fig. 1. Representative photomicrographs showing APJ-like immunoreactivity in endothelial cells of human small intramyocardial vessels (a,b), cardiomyocytes (d, e), saphenous vein endothelial and smooth muscle cells (g, h), neurones of rat lateral cerebellar nucleus (j), rat bronchial epithelial cells (k) and smooth muscle cells of rat aorta (l). Photomicrographs in the right column show negative controls of human left ventricle (c, f), human saphenous vein (i) and rat aorta (m). Arrows indicate cells showing positive staining for APJ-like immunoreactivity. Scale bar=50 μm (a–c, g–j, l, m). Scale bar=20 μm (d, e, k).

3.2. Fluorescent double staining/confocal microscopy

3.2.1. Human tissue sections

3.2.1.1. APJ. To confirm our light microscopy findings and to investigate the precise cellular and intracellular distribution of APJ-LI, fluorescent double staining for APJ-LI and the endothelial cell marker vWF or vascular smooth muscle marker α-actin was carried out in tissues showing expression of APJ-LI in standard immunocytochemistry experiments. Confocal laser scanning microscopy confirmed the presence of APJ-LI in vascular smooth muscle cells and endothelial cells of small and large human blood vessels, as APJ was expressed in both cells positive for α-actin (Fig. 2l–d) or vWF (Fig. 2e–l). Within the endothelial cell cytoplasm, APJ-LI was present in vesicle-like structures but did not show spatial co-localisation with vWF in Weibel-Palade bodies (Fig. 2i–l). Vascular smooth muscle cells contained APJ-LI in vesicle-like structures of varying sizes throughout the cytoplasm, which showed partial co-localisation with α-actin (Fig. 2a–d). Confocal microscopy also confirmed the presence of APJ-LI within cardiomyocytes. In cardiomyocytes APJ-LI showed a transversal striated distribution pattern associated with the cell surface membrane (Fig. 2m–p).

3.2.1.2. Apelin. To investigate the precise intracellular distribution of apelin-like immunoreactivity (apelin-LI) in

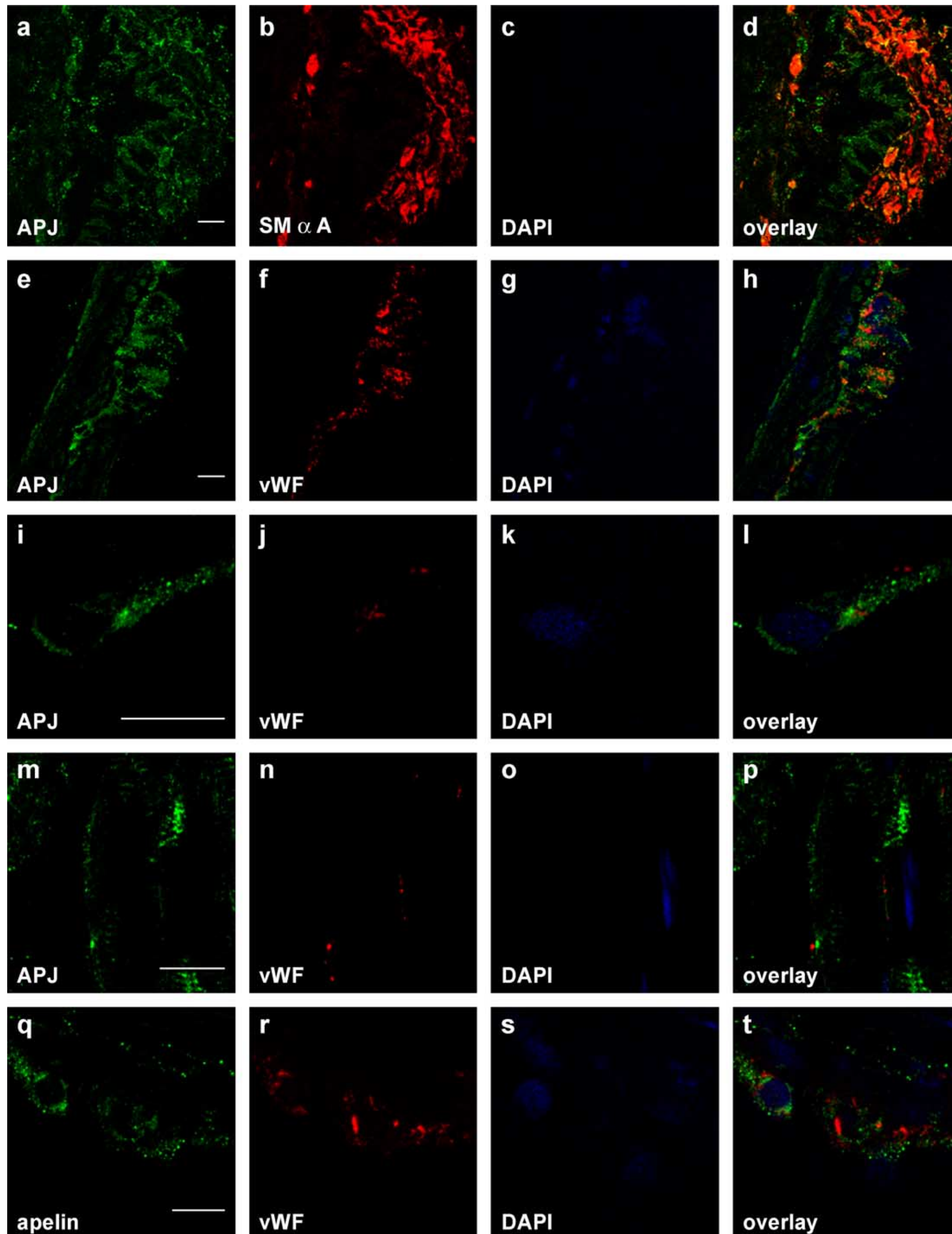

Fig. 2. Representative confocal images of fluorescent double-labelling immunocytochemistry in sections of human left ventricle. Photomicrographs show APJ-LI in small intramyocardial vessels (a–l) and ventricular cardiomyocytes (m–p) and apelin-LI in endothelial cells of small intramyocardial vessels (q–t) as green colour. Red colour represents the vascular smooth muscle marker α-actin (b) or the endothelial marker von Willebrand Factor (vWF, f, j, n, r). DNA specific nuclear staining (DAPI) is shown in blue. Rows show the three individual colour channels and the overlay of those individual channels for one experiment. Scale bar=10 μm.

endothelial cells, we carried out fluorescent double staining for apelin-LI and vWF in a range of human tissues showing endothelial expression of apelin in standard immunocytochemistry experiments [21]. Confocal laser scanning microscopy confirmed the presence of apelin-LI in vascular and endocardial endothelial cells and showed expression of apelin-LI in vesicle-like structures within the cytoplasm. However, there was no spatial co-localisation of apelin-LI and vWF in Weibel-Palade bodies (Fig. 2q–t).

3.2.2. Human umbilical vein endothelial cells

3.2.2.1. APJ. To determine the precise intracellular localisation of APJ-LI in endothelial cells we carried out

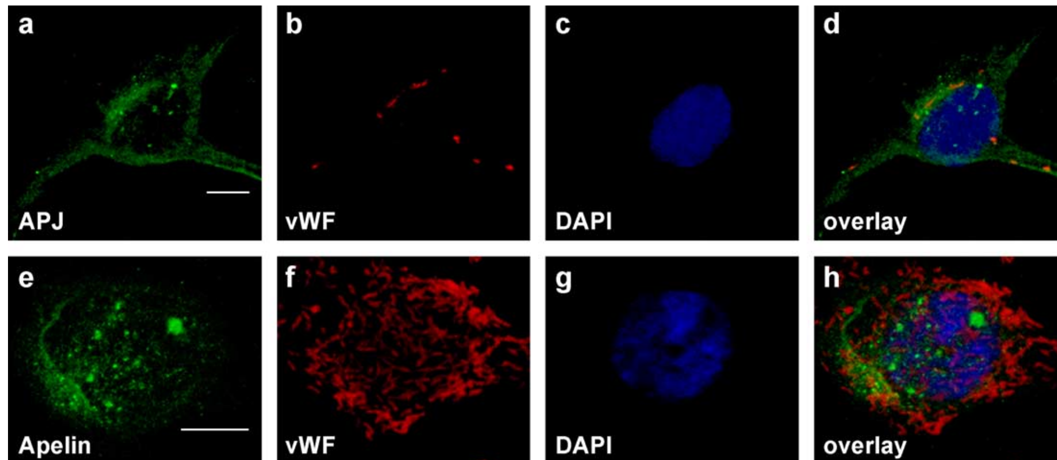

Fig. 3. Representative confocal images of fluorescent double-labelling immunocytochemistry in human umbilical vein endothelial cell (HUVECs). Photomicrographs show maximum intensity projections of APJ-LI (a) and apelin-LI (e) in HUVECs as green colour. Red colour represents the endothelial cell marker von Willebrand factor (vWF). DNA-specific nuclear staining (DAPI) is shown in blue. Rows show the three individual colour channels and the overlay of those individual channels for one experiment. Scale bar=5 μm.

fluorescent double staining for APJ-LI and vWF in HUVECs. Confocal microscopy showed that APJ-LI was abundantly present in the cytoplasm of HUVECs and larger amounts accumulated in structures closely associated to the nuclear surface. APJ-LI did not show spatial co-localisation with vWF (Fig. 3a–d).

3.2.2.2. Apelin. To determine the precise intracellular localisation of apelin-LI in endothelial cells we carried out fluorescent double staining for apelin and vWF in HUVECs. Confocal microscopy detected apelin-LI in small vesicle-like structures (<1 μm) in the cytoplasm of HUVECs and larger amounts accumulated in structures closely associated to the nuclear surface. Apelin-LI did not show spatial co-localisation with vWF (Fig. 3e–h).

4. Discussion

To our knowledge, this is the first report of immunocytochemical detection of APJ-LI in human and rat cardiomyocytes, vascular smooth muscle cells and vascular and endocardial endothelial cells. Investigating the intracellular distribution of APJ-LI and the proposed cognate peptide ligand, apelin, in HUVECs using fluorescent double labelling confocal microscopy showed the presence of low levels of APJ-LI throughout the cytoplasm of endothelial cells. Higher levels of APJ-LI localised to structures close to the surface of the cell nucleus. Apelin-LI was detected in small vesicle-like structures (<1 μm) and in larger structures closely associated to the surface of the cell nucleus. Neither receptor nor ligand showed spatial intracellular co-localisation with vWF. The discovery of APJ-LI in vascular smooth muscle and cardiomyocytes is consistent with the results of micro-autoradiography detecting apelin binding in the heart and blood vessels [14]. The investigation of the intracellular distribution of apelin-LI in cultured endothelial cells is based on the discovery of apelin-LI in human vascular and endocardial endothelial cells [21], a finding consistent with previous reports in rats and humans [15,20].

Apelin mediates positive inotropic actions in the isolated rat heart through activation of the APJ receptor and pre-treatment with pertussis toxin, a selective antagonist of G_i, reduces apelin-induced inotropic effects, confirming that the APJ receptor is coupling to inhibitory G proteins in the rat heart [18]. The role of apelin/APJ in human physiology and pathophysiology, however, remains to be elucidated. In man, a study observed decreased expression of APJ receptor mRNA in the heart of patients with dilated cardiomyopathy. The same study reported a transient increase of plasma apelin in mild cases of heart failure. In severe cases of heart failure, however, circulating apelin levels were significantly decreased [19]. Researchers also observed up-regulated levels of APJ receptor mRNA and apelin in hearts from heart failure patients after implantation of a left ventricular assist device compared to the pre-surgical state [20]. Thus, results may suggest a transient compensatory role of the APJ/apelin receptor system in counterbalancing the development of dilated cardiomyopathy and heart failure. The fact that in cultured rat ventricular cardiomyocytes, the application of cyclic stretch results in the down-regulation of both APJ receptor and apelin mRNA levels [18] may, however, indicate the limitations of this compensatory system which may fail after strong and consistent strain caused for example by chronically increased cardiac afterload.

The proposed cardiovascular effects of apelin, however, can only be mediated if the apelin receptor is expressed in cardiomyocytes. Preliminary molecular biology studies have shown the expression of APJ receptor mRNA in the rat and human myocardium [24,25] and radioligand binding experiments localised apelin binding sites to sections of rat and human heart [14], our results therefore demonstrate the

presence of APJ-LI in human and rat cardiomyocytes. The staining pattern of APJ-LI seen in cardiomyocytes using confocal imaging may also help to understand how apelin mediates positive inotropic effects via APJ-receptors. Staining for APJ-LI showed a transversal striated pattern associated with the surface of cardiomyocytes, indicating localisation of APJ-LI to t-tubules. T-tubules represent transversal membrane invaginations of cardiomyocytes which accommodate L-type Ca^{2+} channels, channels that play a major role in the conductance of the cardiac action potential, the excitation of cardiomyocytes and the regulation of Ca^{2+}-dependent cardiac contraction force [26]. Thus, the interaction of APJ receptor with L-type Ca^{2+} channels in t-tubules may mediate the inotropic effects of apelin, and apelin, present in endocardial endothelial cells, may act as a paracrine inotropic agent released from endocardial endothelial cells to act on the underlying cardiomyocytes.

Apelin is also involved in the regulation of vascular tone. A number of reports suggest potent in vivo endothelium-dependent vasodilator actions for apelin in rats [7,10,15] and studies using APJ receptor knockout mice confirmed that apelin elicits vasodilator actions via activation of the APJ receptor. The observation that endothelial cells from wild-type mice show increased expression of endothelial nitric oxide synthase (eNOS) after being exposed to apelin, a phenomenon not observed in endothelial cells from APJ receptor knockouts, supports the role of endothelial pathways in mediating apelin-induced vasodilatation [26]. The reported endothelial contribution necessary for apelin to elicit vasodilator actions may also explain how apelin constricts endothelium denuded isolated human saphenous vein [14]. Based on the presence of apelin-LI in endothelial cells throughout the human vasculature [21], we hypothesised that endothelial apelin may primarily constrict vascular smooth muscle, an action which may eventually be overcome by autocrine/paracrine activation of endothelial APJ receptors which trigger the release of endothelium-dependent vasodilator substances such as nitric oxide [26]. Vasoconstriction in response to apelin action may occur even though the APJ receptor is reported to couple through inhibitory G proteins, as G_i mediated vasoconstriction is also seen with other receptor systems including α_{1A} adrenoceptors and A1 adenosine receptors, where activation of G_i is thought to result both in increased sensitivity of the contractile apparatus to intracellular calcium and an increase of intracellular calcium via activation of phospholipase C by the $G_{\beta\gamma}$ subunit of the G protein [27,28]. The detection of APJ-LI on vascular smooth muscle cells of both rat and human vessels of different sizes supports this concept, and the fact that we were able to show the presence of APJ-LI on vascular endothelial cells demonstrates the functional substrate for the occurrence of endothelium-dependent apelin actions.

Finally, the reported localisation of apelin-LI in vesicle-like structures of vascular and endocardial cells as well as in structures associated with the nuclear surface, most likely representing the endoplasmic reticulum or Golgi apparatus, supports the concept of endothelial apelin synthesis. In the light of these findings, it is unlikely that apelin is secreted remotely and taken up into endothelial cells from the circulation. The lack of spatial co-localisation of apelin-LI with von Willebrand factor in Weibel-Palade bodies of endothelial cells suggests that apelin is produced via a constitutive synthesis pathway [29]. APJ-LI, present on endothelial cells and proposed to mediate the endothelium-dependent vasodilator actions of apelin, may also regulate endothelial apelin synthesis via a negative feedback loop coupled to G_i. In APJ-knockout mice, administration of angiotensin-II resulted in a significantly greater blood pressure increase compared to wild-type mice [17]. This may suggest that autocrine/paracrine activation of endothelial APJ receptor by endothelial apelin may play a crucial role in the maintenance of vascular tone.

In summary, we report the presence of APJ-LI on rat and human cardiomyocytes, vascular smooth muscle cells and endothelial cells, and the presence of apelin-LI in secretory vesicles of human endothelial cells. The propinquity of APJ-LI and the cognate ligand apelin in the cardiovascular system support an important functional role of APJ/apelin as an emerging cardiovascular regulator system.

Acknowledgements

This work was supported by the British Heart Foundation. Matthias Kleinz was supported by the Cambridge European and Isaac Newton Trusts. We would like to thank Dr. Janet Maguire for discussion and constructive criticism of the manuscript and Rhoda Kuc for excellent technical advice.

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