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ISCHEMIC HEART FAILURE ENHANCES ENDOGENOUS MYOCARDIAL APELIN AND APJ RECEPTOR EXPRESSION

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Abstract: Apelin interacts with the APJ receptor to enhance inotropy. In heart failure, apelin-APJ coupling may provide a means of enhancing myocardial function. The alterations in apelin and APJ receptor concentrations with ischemic cardiomyopathy are poorly understood. We investigated the compensatory changes in endogenous apelin and APJ levels in the setting of ischemic cardiomyopathy.

Male, Lewis rats underwent LAD ligation and progressed into heart failure over 6 weeks. Corresponding animals underwent sham thoracotomy as control. Six weeks after initial surgery, the animals underwent hemodynamic functional analysis in the presence of exogenous apelin-13 infusion and the hearts were explanted for western blot and enzyme immunoassay analysis.

Western blot analysis of myocardial APJ concentration demonstrated increased APJ receptor protein levels with heart failure (1890750±133500 vs. 901600±143120 intensity units, n=8, p=0.00001). Total apelin protein levels increased with ischemic heart failure as demonstrated by enzyme immunoassay (12.0±4.6 vs. 1.0±1.2 ng/ml, n=5, p=0.006) and western blot (1579400±477733 vs. 943000±157600 intensity units, n=10, p=0.008). Infusion of apelin-13 significantly enhanced myocardial function in sham and failing hearts. We conclude that total myocardial apelin and APJ receptor levels increase in compensation for ischemic cardiomyopathy.

Key words: Apelin, G protein coupled receptor, APJ, Inotrope

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Abbreviations used: CHO – Chinese hamster ovary; DTT – dithiothreitol; EDTA – ethylenediaminotetraacetic acid; EIA – enzyme immunoassay; LAD – left anterior descending; SDS – sodium dodecyl sulphate

INTRODUCTION

The orphan 380 amino acid 7-transmembrane domain G-protein coupled APJ receptor was cloned by a PCR based strategy [1]. Apelin was extracted from the bovine stomach and identified as the ligand for the APJ receptor by demonstrating acidification of Chinese hamster ovary (CHO) cells expressing the APJ receptor [2]. Apelin is highly conserved between species [2, 3], consisting of a 77 amino acid pre-proapelin protein that is cleaved by proteases to yield significantly shorter, biologically active forms including apelin-36, apelin-19, apelin-17, apelin-16, apelin-13 and apelin-12 subtypes [2, 4, 5]. Of these, the apelin-13 isoform has the most potent biologic activity [2, 6].

The APJ receptor is present in many tissues including the central nervous system, spinal cord, spleen, liver, stomach, lung and heart [5, 7]. The interaction between apelin and its G-protein coupled APJ receptor has important physiological effects in several homeostatic systems, including regulation of water and food intake, blood pressure regulation, immunoregulatory modulation, pulmonary physiology and myocardial contractility [8-12]. Endogenous expression of apelin may play a regulatory role in heart failure [13, 14]. Exogenous apelin administration has potential therapeutic benefits in enhancing myocardial function in heart failure, as recently demonstrated by our group and others both in vitro and in vivo [15-17].

Alterations in APJ receptor and apelin concentrations as a mechanism of compensation in heart failure are poorly understood. We undertook this study to examine the compensatory changes in myocardial APJ and apelin concentrations in the presence of ischemic cardiomyopathy. A well-described rodent model of ischemic cardiomyopathy was utilized with a focus on the most potent isotype, apelin-13.

MATERIALS AND METHODS

Animal care and biosafety

Adult male Lewis rats weighing 250-300 grams were obtained from Charles River Laboratories (Boston, MA). Food and water were provided ad libitum. The protocol outlining this study was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and conforms with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23).

Induction of heart failure

Male Lewis rats were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg), endotracheally intubated with a 14-gauge angiocatheter and mechanically ventilated (Hallowell EMC, Pittsfield, MA) with 0.5% isoflurane maintenance anesthesia. A left thoracotomy was performed via the fourth interspace, the pericardium was entered, and the left anterior descending (LAD) coronary artery was encircled with a 7-0 prolene suture at the level of the left

atrial appendage. In the heart failure subset, the suture was briefly snared to verify isolation of the LAD and tied, thereby ligating the LAD and inducing an anterolateral infarction of 30-40% of the left ventricle. This method of infarction has been highly reproducible and previously published. Over the course of 6 weeks, the animals predictably progressed into moderate heart failure [18-20]. In sham animals, the suture was removed without LAD ligation, thereby maintaining normal myocardial function. The thoracotomy was closed in 3 layers over a temporary thoracostomy tube, and the animals were allowed to recover.

Western blot analysis of APJ and apelin protein concentrations in ischemic heart failure

Myocardial sections were isolated from the left ventricle of heart failure animals and corresponding regions of sham-operated animals, snap frozen in liquid nitrogen, and homogenized with a mortar and pestle for protein extraction. Homogenized myocardial samples from both the heart failure and sham animals were suspended in protein extraction buffer [5 mM EDTA, 50 mM Tris Cl pH 8, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% Na deoxycholate, 50 mM DTT, protease inihibitors (Roche), phosphatase inhibitors (Sigma)] and sheared with a 25-gauge needle. Protein concentrations were determined by the BioRad protein assay in triplicate. 50 µg of total myocardial protein was loaded on precast 10% SDS-PAGE gels after denaturation in reducing sample buffer for 10 minutes at 95^{o}C. Proteins were transferred to Immobolin-P PVDF membranes (Millipore, Billerica, MA) and subsequently blocked with 5% non-fat dry milk in tris-buffered saline (TBS). Immunoblotting was performed using antibodies against APJ (rabbit anti-rat APJ; Neuromics, Minneapolis, MN) and apelin (rabbit anti-human apelin; Phoenix Pharmaceuticals, Belmont, CA). The Supersignal West Pico Chemillumenescent Substrate (Pierce, Rockford, IL) was used for detection and expression levels were quantified with Kodak1D image analysis software v3.6. To confirm equal loading of total protein into each lane,

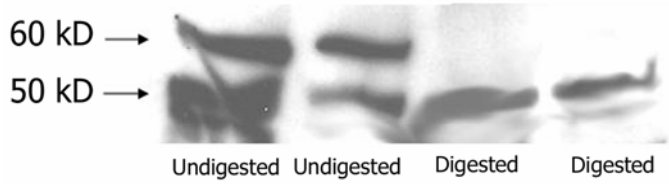

Fig. 1. Western blot analysis of myocardial protein with and without N-linked digestion demonstrating staining of the 50 kD native and 60 kD glycosylated APJ receptors without digestion. The 60 kD glycosylated receptor is not present following N-linked digestion.

the SDS-PAGE gels were stained with coomassie blue and the levels of actin were quantified with Kodak1D image analysis software v3.6. Total concentration of myocardial apelin and APJ protein was expressed as intensity thereby providing a relative comparison of protein concentration between sham and heart failure animals.

APJ was identified at 50 kD for the deglycosylated and 60 kD for the glycosylated forms [5]. In order to confirm the presence of 50 kD deglycosylated and 60 kD glycosylated forms of APJ an N-linked digestion was performed on myocardial protein (Enzymatic Deglycosylation Kit, Prozyme Inc, San Leandro, CA). Analysis demonstrates the disappearance of the 60 kD band following N-linked enzymatic digestion, Fig. 1.

An additional western blot with standard apelin-36, 16, 13, and 12 isotypes was performed to determine the molecular weight of apelin isotypes. Western blot analysis demonstrated similar weights for the various subtypes of apelin standards. It is feasible to determine total myocardial apelin concentration, but not individual apelin isotypes concentrations.

Enzyme immunoassay (EIA) measurement of total apelin protein expression in ischemic heart failure

Endogenous total myocardial apelin concentration in heart failure and sham animals was quantified in duplicate utilizing the commercially available Apelin-36 EIA Kit (Phoenix Pharmaceuticals, Belmont, CA). This kit detects apelin-36 as well as smaller biologically active isoforms (i.e. Apelin-19, 16, 15, 13, and 12).

Both, standard peptide solutions and myocardial protein samples were loaded onto antibody treated 96 well plates containing primary antiserum and biotinylated peptide and allowed to incubate for 2 hours at room temperature. Following the initial reaction, streptavadin-horseradish peroxidase solution was added to the samples and allowed to react for 1 hour at room temperature. The samples were thoroughly rinsed and substrate was added to each sample well, yielding a color reaction. Absorbance was recorded at 450 nm. A standard logarithmic curve was plotted and used to calculate protein concentration in the myocardial samples using apelin-36 as a standard.

Confirmation of inotropic activity of the apelin-13 ligand

We have previously demonstrated enhanced inotropic effects of apelin-16 in vivo in the normal and ischemic cardiomyopathic heart [15]. The effects of infusion of the shorter apelin-13 ligand on myocardial function in both sham and ischemic cardiomyopathic hearts was studied to confirm acute inotropic activity of this isotype. All animals underwent induction and maintenance of anesthesia, as described above, six weeks after the original left thoracotomy.

After adequate anesthesia was determined, a midline sternotomy was performed to expose the mediastinum. The aortico-pulmonary window was dissected free and a 2.5 mm perivascular ascending aortic flow probe (Transonic Systems, Ithaca, NY) was placed around the ascending aorta to provide a continuous, dynamic measure of the mean cardiac output (ml/min). A 2-French pressure-

volume catheter was inserted retrograde via the apex of the heart into the left ventricle as previously described [21]. Hemodynamic pressure (mm Hg) and volume (relative volume units) measurements were analyzed utilizing the ARIA 1 Pressure Volume Analysis software (PVAN, Millar Instruments, Houston, TX). Open chest hemodynamic measurements were recorded while the animal was fully anesthetized. A 22-gauge angiocatheter was inserted into the right femoral vein under direct visualization for apelin infusion.

Initial hemodynamic measurements from the ascending aortic flow probe and the intraventricular pressure-volume catheter were recorded to establish baseline hemodynamic function. Subsequently, the animals received a continuous infusion of apelin-13 (Phoenix Pharmaceuticals, Belmont, CA) reconstituted in 0.45% normal saline (200 μg/l) at a rate of 0.05 ml/min (0.01 μg apelin-13/min) with a peristaltic pump (Variable Flow Mini-Pump, Low Flow, model 3385, Control Company) via the femoral venous catheter. Hemodynamic measurements were recorded at 5-minute intervals for a total of 15 minutes after the infusion was started.

Statistical analysis

Statistical analysis was performed utilizing the unpaired Student's *t* test to calculate the statistical significance between two groups for protein analysis or between data at baseline (0 minutes) and 15 minutes following infusion. All results are presented as mean ± standard error of the mean. Differences were considered statistically significant at a p-value <0.05.

RESULTS AND DISCUSSION

Myocardial APJ receptor protein concentration is increased in ischemic heart failure

Western blot analysis was utilized to measure total myocardial APJ receptor levels in heart failure animals and non-infarcted, sham animals (n=8), Fig. 2.

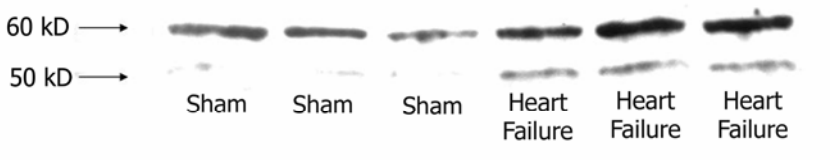

Fig. 2. Western blot analysis demonstrating staining of the 50 kD native and 60 kD glycosylated APJ receptors within the left ventricle of heart failure and sham hearts.

Antibody binding at the 50 and 60 kD band widths provided biologic evidence for the presence of the G-protein coupled APJ receptor within the normal left ventricle. The 50 kD band corresponds to the naked APJ receptor whereas the 60 kD band represents the glycosylated receptor. The concentration of APJ

receptors within the failing left ventricle increased compared to sham animals, possibly indicating a compensatory increase in the inotropic receptor as a means to enhance myocardial function. Quantitative analysis of left ventricular APJ receptor concentrations exhibited a statistically significant increase in both the native and glycosylated forms of the receptor in the setting of heart failure as compared to sham control, Fig. 3 (850875 ± 122125 vs. 229200 ± 89440 intensity units; 1039875 ± 75187 vs. 712166 ± 127777; 1890750 ± 133500 vs. 901600 ± 143120, for the 50 kD, 60kD and total APJ levels, respectively).

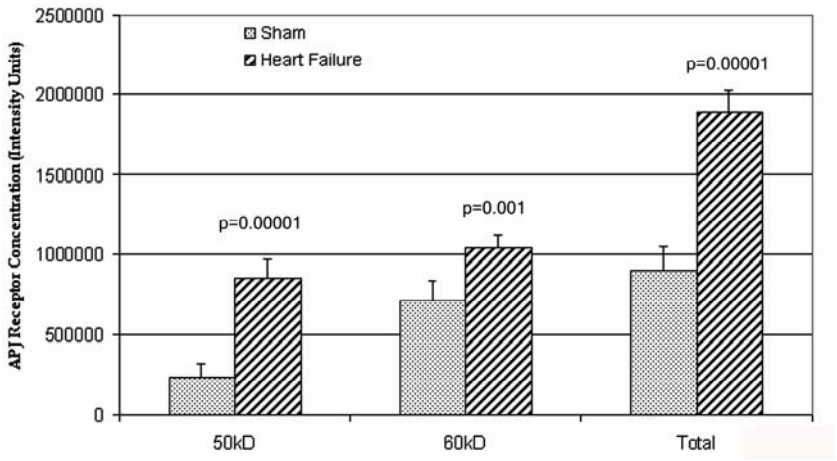

Fig. 3. Increased left ventricular expression of 50 kD nonglycosylated, 60 kD glycosylated, and total APJ receptor concentrations in the presence of ischemic heart failure as measured by western blot analysis.

Endogenous expression of the apelin is increased in ischemic heart failure

Enzyme immunoassay (EIA) was used to quantitate the total endogenous myocardial concentration of apelin. EIA analysis revealed low basal levels of enzymatically processed apelin within the sham myocardium. Total apelin concentration was dramatically increased within the failing left ventricle, with a nearly twelve-fold increase in endogenous total left ventricular apelin concentration in the setting of ischemic cardiomyopathy, Fig. 4 (12.0 ± 4.6 ng/ml vs. 1.0 ± 1.2 ng/ml, n=5).

Western blot analysis was used to evaluate the left ventricular concentration of apelin. A representative western blot demonstrates the presence of staining for apelin, Fig. 5. Left ventricular apelin concentrations were increased in the presence of ischemic heart failure as compared to sham control (1579400 ± 477733 vs. 943000 ± 157600 intensity units, n=10, p=0.008). The increase in myocardial apelin ligand concentration correlates with the development of heart failure. The enhanced expression of this endogenous isotype may be a compensatory mechanism for heart failure that is not activated in the sham hearts.

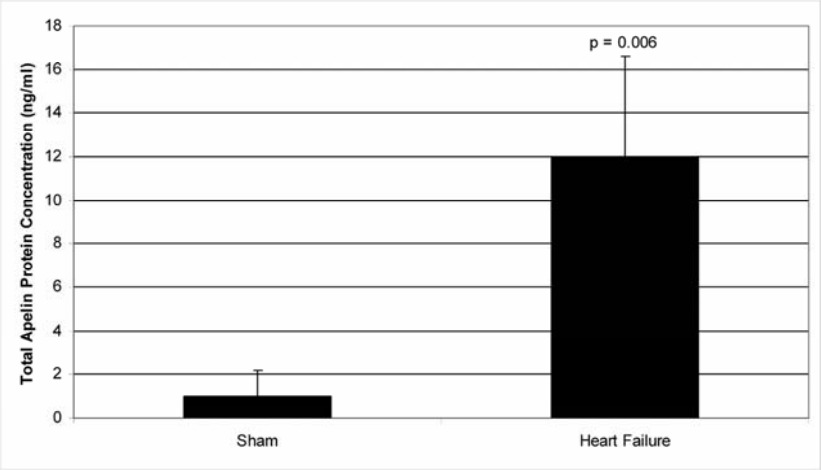

Fig. 4. Increased total left ventricular apelin levels in the presence of ischemic heart failure as measured by enzyme immunoassay.

Fig. 5. Western blot analysis demonstrating staining for apelin within the left ventricle of heart failure and sham hearts.

Exogenous administration of apelin-13 enhances myocardial function

Apelin-13 infusion increased inotropic function in both sham and ischemic cardiomyopathic hearts (n=5), Tabs 1 and 2. Fifteen minutes of apelin-13 infusion did not result in statistically significant changes in heart rate, maximum volume, or minimum volume. There was also no difference in tau (the coefficient of relaxation), indicating that apelin-13 does not play a noticeable role in isovolumetric relaxation during diastole. However, apelin-13 infusion did induce a statistically significant increase in maximum pressure, stroke volume, cardiac output, stroke work, and maximum dP/dt. As we have previously published, these improvements in myocardial function are not due to volume infusion but rather a result of the apelin [15]. These results follow the same pattern as our previously published exhaustive study that demonstrated a pronounced inotropic effect of apelin-16 infusion in vivo in the sham and ischemic cardiomyopathic heart [15] indicating that apelin-13 also has a potent inotropic effect on normal and failing myocardium. As with apelin-16 infusion, significant differences in cardiac output, maximum dp/dt and maximum ventricular pressure were evidenced with apelin-13 infusion. A roughly 22.3%,42.3% and 26.0% increases in cardiac output, maximum dp/dt, and

Tab. 1. Hemodynamic profile at baseline and during continuous infusion of Apelin-13 in normal rat hearts, statistical comparison between baseline (0 minutes) and 15 minutes following apelin-13 infusion are displayed (n=5/group).

	Baseline (0 minutes)	5 minutes infusion	10 minutes infusion	15 minutes infusion	P =
Heart rate (bpm)	192±29	189±20	173±32	179±23	NS
Maximum volume (µl)	322±73	328±52	367±61	372±62	NS
Minimum volume (µl)	221±46	220±26	241±35	246±38	NS
Maximum ventricular pressure (mm Hg)	77.7±4.2	92.5±8.8	93.9±12.5	97.9±8.4	0.008
Stroke volume (µl)	193±24	215±31	249±22	251±26	0.03
Stroke work (mm Hg*µl)	4886±1207	5761±2305	7385±1853	7612±1786	0.005
Maximum dP/dt (mm Hg/sec)	3136±267	3398±558	3926±309	4464±900	0.04
Cardiac output (ml/min)	36.8±6.6	40.4±7.1	43.0±8.0	45.0±7.6	0.006

Tab. 2. Hemodynamic profile at baseline and during continuous infusion of Apelin-13 in ischemic cardiomyopathic rat hearts, statistical comparison between baseline (0 minutes) and 15 minutes following apelin-13 infusion are displayed (n=5/group).

	Baseline (0 minutes)	5 minutes infusion	10 minutes infusion	15 minutes infusion	P =
Heart rate (bpm)	176 ±19	187 ± 5	188 ± 3	186 ± 4	NS
Maximum volume (µl)	359 ± 103	257 ± 70	261 ± 65	249 ± 72	NS
Minimum volume (µl)	292 ± 101	154 ± 61	158 ± 58	148 ± 62	NS
Maximum ventricular pressure (mm Hg)	71.5 ± 1.9	75.9 ± 1.9	75.7 ± 1.6	74.5 ± 2.6	0.02
Stroke volume (µl)	136±27	153±28	158±29	163±35	0.04
Ejection fraction (%)	19.4 ± 7.2	45.8 ± 11.6	44.6 ± 11.3	45.7 ± 11.4	0.006
Stroke work (mm Hg*µl)	2684 ± 1297	5579 ± 1187	5576 ± 1028	5386 ± 1187	0.04
Maximum dP/dT (mm Hg/sec)	2611 ± 306	3533 ± 639	3556 ± 582	3430 ± 655	0.03
Preload adjusted max. power (mWatts/µl^2)	1.4 ± 0.3	7.7 ± 3.6	8.0 ± 4.8	9.8 ± 5.4	0.05
Cardiac output (ml/min)	25.2 ±4.2	28.4 ± 4.9	30.0 ± 5.4	30.8 ± 5.9	0.01

maximum ventricular pressure were evidenced with apelin-13 infusion in normal hearts as compared to 32.0%, 60.2%, 28.6% for apelin-16 infusion respectively. In our experience apelin-13 appears to demonstrate decreased potency at peak inotropic activity when compared to apelin-16.

The APJ receptor has been identified in several tissues throughout the body including the central nervous system, spinal cord, lung, spleen, gastrointestinal tract, mammary gland, and heart [5, 7]. Regulatory functions of apelin and the APJ receptor are multifold, involving the modulation of thirst and water intake, food intake, immunological modulation, receptor mediated human immunodeficiency virus entry, gastrointestinal hormone release, nitric oxide mediated arterial vasodilatation, and enhanced inotropic activity [8-12]. Szokodi and colleagues have demonstrated potent inotropic activity of apelin-16 on the isolated rat ventricle [13]. We have recently demonstrated acute in vivo activity of apelin-16 on both ischemic heart failure and normal hearts [15]. Similarly, a recent report demonstrated that a chronic 2-week infusion of the apelin-13 glutamylated isoform enhanced contractility without unwanted myocardial hypertrophy [14]. In this study we have illustrated the acute, potent inotropic activity of the apelin-13 ligand.

Though the physiologic effects of the apelin-APJ receptor interaction have been characterized, there is little information on the biologic properties of the ligand, receptor and their interaction. Apelin is initially translated into a pre-proprotein. The 41 amino acids at the amino terminus provide for secretory activity and are cleaved by peptidases, yielding the physiologically active 36 amino acid carboxy terminal residues that interact with the APJ receptor to provide for homeostatic activity [2, 9]. As discussed previously, further processing of apelin-36 results in even shorter and more biologically potent molecules [2-6]. This finding has prompted our group to focus our study on the apelin-13 ligand.

Reports in the literature that have attempted to investigate the alterations in both APJ receptor and apelin concentrations in heart failure have been limited. Foldës et al demonstrated decreased atrial and plasma apelin protein concentrations in the explanted hearts of end-stage heart failure patients [17]. Chen et al noted significantly increased plasma apelin levels in heart failure patients that eventually decreased with progression of disease to end-stage heart failure [16]. We sought to evaluate ventricular apelin and APJ concentrations in the setting of ischemic cardiomyopathy.

In this study, we demonstrate an increase in myocardial APJ receptor and total apelin concentrations in the setting of ischemic cardiomyopathy. Based upon our findings and the findings of previous groups, it appears that there is a compensatory increase in APJ receptor and apelin levels in heart failure. However, with progression of heart failure to end stage, there appears to be an ultimate decrease in this inotropic APJ-apelin interaction [16].

Apelin immunoreactivity has been localized to the endothelium and, to a lesser extent, cardiomyocytes[13, 16]. Measurements of plasma and tissue levels of apelin demonstrate more concentrated levels of apelin within the tissue,

suggesting tissue level production and activity [4, 8, 9]. Based upon these findings, it appears that apelin manifests paracrine activity. The increased left ventricular levels of apelin that we report are therefore likely a local, myocardial tissue level increase in protein expression.

While peripheral vascular studies have demonstrated nitric oxide as the mediator for vasodilatation following apelin-APJ interaction [9], this is not the mechanism for enhancing inotropy. Apelin acts independently of the adrenergic system. Apelin interaction with APJ results in an increase in intracellular calcium as demonstrated by enhanced calcium transits and patch clamp electrophysiology. It appears that this interaction results in an increase in phospholipase C with subsequent activation of protein kinase C. Ultimately, there may be an increase in Na^+/H^+ exchange and reverse mode Na^+/Ca^{2+} exchange, leading to elevated intracellular calcium levels. Intracellular calcium binds to troponin C, leading to removal of tropomyosin and allowing for actin-myosin coupling. On a global, organ level this results in enhanced, more efficient myocardial contractions. [5, 13].

The increase in concentration of myocardial APJ receptors provides a very attractive treatment option for both acute and chronic heart failure. Though there is also an increase in apelin levels during heart failure, the ability to enhance inotropic activity with exogenous apelin administration suggests that the endogenously produced apelin is not sufficient to saturate the APJ receptors and maximize APJ receptor mediated signal transduction for enhancing contractility. Apelin may provide a novel adjunctive inotropic therapy for heart failure in addition to β_1-agonists and phosphodiesterase inhibitors. The ability of apelin to enhance vasodilatation and decrease systemic vascular resistance offers the potential to decrease afterload, cardiac work and myocardial oxygen demand as well as enhance contractility and cardiac output with a single agent.

Apelin-13 manifests potent inotropic activity in both normal and failing hearts. In the setting of ischemic cardiomyopathy there is a significant increase in endogenous myocardial apelin and APJ receptor protein concentrations. This compensatory increase in protein expression provides a paracrine mechanism to augment myocardial inotropy in the setting of ischemic cardiomyopathy. Exogenous administration of apelin may provide clinical therapeutic benefit in both acute and chronic heart failure.

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