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Differential expression of components of the cardiomyocyte adrenomedullin / intermedin receptor system following blood pressure reduction in NO-deficient hypertension.

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Non-standard abbreviations:

ACE	angiotensin converting enzyme
CL	calcitonin receptor-like receptor protein
L-NAME	N^{ω}-nitro-L-arginine methyl ester
NOS	nitric oxide synthase
PBS	phosphate buffered saline
RAMP	receptor activity modifying protein

Abstract

Adrenomedullin (AM) and intermedin (IMD, adrenomedulln-2) are vasodilator peptides related to calcitonin-gene related peptide (CGRP). The actions of these peptides are mediated by the calcitonin receptor-like receptor (CLR) in association with one of three receptor activity modifying proteins. CGRP is selective for CLR/RAMP1, AM for CLR/RAMP2 and 3, and IMD acts at both CGRP and AM receptors. In a model of pressure overload induced by inhibition of nitric oxide synthase (NOS), up-regulation of AM was observed previously in cardiomyocytes demonstrating a hypertrophic phenotype. The current objective was to examine the effects of blood pressure reduction on cardiomyocyte expression of AM and IMD and their receptor components. L-NAME (35mg/kg/day) was administered to rats for 8 weeks, with or without concurrent administration of hydralazine (50mg/kg/day) and hydrochlorothiazide (7.5mg/kg/day). In left ventricular cardiomyocytes from L-NAME treated rats, increases (-fold) in mRNA expression were 1.6 (preproAM), 8.4 (preproIMD), 3.4 (CLR), 4.1 (RAMP1), 2.8 (RAMP2) and 4.4 (RAMP3). Hydralazine / hydrochlorothiazide normalised systolic BP and abolished mRNA up-regulation of hypertrophic markers, sk-α–actin and BNP and of preproAM, CLR, RAMP2 and RAMP3 but did not normalise cardiomyocyte width nor preproIMD or RAMP1 mRNA expression. The robust increase in IMD expression indicates an important role for this peptide in the cardiac pathology of this model but, unlike AM, IMD is not associated with pressure overload upon the myocardium. The concordance of IMD and RAMP1 up-regulation indicates a CGRP-type receptor action; considering also a lack of response to BP reduction, IMD may, like CGRP, have an anti-ischemic function.

Introduction

Intermedin (IMD, adrenomedullin-2) is a recently discovered 47αα vasodilator peptide (Roh *et al.*, 2004; Taylor *et al.,* 2005) of the calcitionin gene-related peptide (CGRP) family, which also includes adrenomedullin (AM). Increased plasma levels of AM have been detected in hypertension (Sumimoto *et al.,* 1997), myocardial infarction (Nagaya *et al.,* 2000) and heart failure (Willenbrock *et al.,* 1999); levels are greater in hypertensive patients with LVH than those without underlying LVH (Sumimoto *et al.,* 1997). Cardiomyocytes express the precursor peptide and secrete mature AM (Horio *et al.,* 1998). This expression is enhanced in response to mechanical stretch (Tsurada *et al.,* 2000), hypoxic stress (Nishikimi *et al.,* 1998), cytokines (Horio *et al.,* 1998) and angiotensin II (Nishikimi *et al.,* 1998). AM attenuates protein synthesis and expression of ANP in cardiomyocytes (Sato *et al.,* 1997; Tsurada *et al.,* 2000; Bell *et al.,* 2005). Intermedin is expressed, less abundantly than AM, in normal myocardium (Bell *et al.,* 2005); the (patho) physiological significance of intermedin is poorly understood at present although a cardioprotective effect against ischemia-reperfusion injury has been proposed (Taylor *et al.,* 2005).

The actions of these peptides are mediated by a calcitonin receptor- like receptor protein (CLR) in association with one of three receptor activity modifying proteins, RAMPS 1-3. AM has two specific receptors (AM_1, AM_2) formed by CLR combined with RAMP 2 or 3, respectively (Hay *et al.,* 2004). AM also has appreciable affinity for a CGRP receptor, composed of CLR and RAMP1. AM_1 receptors are highly selective for AM over CGRP and other

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peptides; AM_2 receptors show less selectivity, having considerable affinity for βCGRP (McLatchie, 1998). Intermedin acts non-selectively at all three RAMP-CLR co-receptors (Roh *et al.,* 2004). It is probable that the physiological response of a tissue to each peptide is dependent on the levels of expression of CLR and of the various RAMPs. RAMP2 predominates over RAMP1 in the heart and to a lesser extent in cardiomyocytes; RAMP3 mRNA is much less abundant (Autelitano and Ridings, 2001). A membrane-associated 'receptor component protein' (RCP) is also required for activation of signal transduction via the various RAMP-CL receptor complexes (Prado *et al.,* 2001). There is controversy as to whether the orphan receptors RDC-1 (Li *et al.,* 1996) and L1 (Chakravarty *et al.,* 2000) also interact with CGRP and AM; it is now generally considered unlikely that these proteins contribute to peptide binding in the myocardium (Autelitano, 1998; Poyner et al., 2002).

Nitric oxide (NO) generated within normal myocardium by 'endothelial type' NO synthase (eNOS) plays a pivotal role in reduction of myocardial oxygen consumption and blood pressure (Bayraktutan et al., 1998; Stauss et al., 1999). NO levels are reduced in the plasma of hypertensive patients with LVH (Hua *et al.,* 2001). Chronic administration of the NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME), to rats results in hypertension, often (Takaori *et al.,* 1997; Bernatova *et al.,* 1999) accompanied by myocardial remodeling (cardiac hypertrophy and fibrosis), vascular remodeling (medial thickening and perivascular fibrosis), cardiac ischemia and necrosis, and mechanical dysfunction. At cardiomyocyte level, enhancement of protein synthesis and mass is observed in both left and right ventricles together with increased cell

width, but phenotypic alterations, manifest as up-regulation of the contractile gene, skeletal α–actin, and cardioendocrine peptides, BNP and endothelin-1, accompanied by enhanced expression of the counter-regulatory peptide, AM, and its receptor components, particularly RAMP3, are found in LV cells only (Bell *et al.,* 2005). It is not known whether intermedin expression within the myocardium is also influenced by NO deficiency. As L-NAME does not enhance mean pulmonary arterial pressure appreciably or alter pulmonary vascular morphology, pressure overload is unlikely to account for the increased protein mass of RV cardiomyocytes (Hampl *et al,* 1993). NOS inhibition might be expected to have direct consequences for the myocardium since NO exerts an anti-growth effect on both cardiomyocytes (Matsuoka *et al.,* 1996) and non-myocytes (Sarkar *et al.,* 1995). The relative contributions of pressure-overload, caused by marked hypertension in the systemic vascular bed, and of direct inhibition of NO production locally within the myocardium, to the changes identified in the left ventricle remains unclear. The purpose of the study therefore was to investigate the contribution of pressure overload to these changes by undertaking an intervention study with blood pressure lowering agents, namely a direct smooth muscle relaxant, hydralazine (50 mg/kg/day), given concurrently with a thiazide diuretic, hydrocholorithiazide (7.5mg/kg/day); this combination is considered the best option to directly lower blood pressure compared to other anti-hypertensive drugs, which stimulate renin secretion (Goto *et al.,* 2000).

Methods

Experimental model

The study was performed in accordance with Home Office *Guidance on the operation of the Animals (Scientific Procedures) Act 1986,* published by Her Majesty's Stationary Office, London. Eight week old male Sprague-Dawley rats were assigned to receive either (i) L-NAME (35mg/kg/day, Alexis Biochemicals, Switzerland) in drinking water; (ii) hydralazine (50mg/kg/day, H-1753, Sigma Aldrich, UK) plus hydrochlorothiazide (7.5mg/kg/day, H-2910, Sigma Aldrich, UK) in drinking water; (iii) L-NAME (35mg/kg/day) currently with hydralazine (50mg/kg/day) plus hydrochlorothiazide (7.5mg/kg/day) in drinking water; (iv) drinking water only (age-matched control) for 8 weeks, and maintained at the Laboratory Service Unit, QUB, prior to sacrifice at 16 weeks of age. Systolic blood pressure (SBP) was determined by tail cuff sphygmomanometer (Harvard Instruments) (Bell *et al.,* 2004) and the mean of four consecutive blood pressure readings was obtained for each animal at weekly intervals. Body weight was recorded weekly and water consumption daily.

Cardiomyocyte isolation

Following deep anesthesia of the rats using isoflurane (Abbott Laboratories, UK), the hearts were rapidly excised, placed in ice-cold saline and weighed. Excised hearts were cannulated through the ascending aorta, and preparations of cardiomyocytes were isolated from the left and right ventricles, respectively, by enzymatic digestion (collagenase, 0.4mg/ml, Serva, Germanyl) using Langendorff perfusion (Bell *et al.,* 2005). After purification, cells were utilized immediately for extraction of RNA (RT-PCR

protocols) and preparation of membrane protein (immunoblotting protocols); for analysis of cell dimensions, a small amount of each cell preparation was retained and suspended at a concentration of approximately 50,000 viable cardiomyocytes/ml in a 'creatinine-carnitine-taurine' (CCT) medium which consisted of modified glutamine-free medium M-199 supplemented with Earle's salts (Gibco, UK), HEPES (15 mM), creatinine (5 mM), *l*-carnitine (2 mM), taurine (5 mM), ascorbic acid (100 μM), penicillin (100 IU/ml) and streptomycin (100 μg/ml).

Cardiomyocyte Dimensions

Cells were visualised using an inverted phase contrast microscope (Axiovert 10, Zeiss), and displayed on a monitor (Panasonic WV-5410) at a magnification of x988. Viable cells were selected on the basis of rod-shaped appearance without sarcolemmal blebbing and lack of spontaneous contractile activity in the absence of electrical stimulation. For each heart cell preparation, the widths and lengths (μm) were determined for 20 viable cardiomyocytes from each chamber and a mean value obtained.

Real-Time PCR:

Reported sequences for each gene (Table 1) were used to design on Primer Express software (PE Applied Biosystems), rat specific primers adapted to RT-PCR conditions, which were synthesized by Invitrogen. RT-PCR was performed using the following cycle parameters: 10 min at 95^{o}C,

followed by 40 cycles of 15 sec at 95^{o}C and 1 min at 60^{o}C using the ABI Prism Sequence Detector (PE Applied Biosystems). For each gene, RT-PCR was conducted in duplicate using ABsolute QPCR Sybr Green ROX (Abgene) in a 2:1 reaction. To ensure the quality of measurements, both negative and positive controls were included in each plate. Analysis was performed using ABI 7000 Prism software. The threshold cycle (Ct) value, at which a statistically significant increase in signal, associated with an exponential growth of PCR product was observed, was used to ascertain expression level. Analysis was performed using ABI 7000 Prism software and statistical analysis of the RT-PCR results were performed using the Ct value (Ct gene of interest – Ct reporter gene). Relative gene expression was obtained by Ct methods (Ct sample –Ct calibrator) using the control group as a calibrator for comparison of every unknown sample gene expression level. The conversion between Ct and relative gene expression level is n fold induction=2-Ct relative to GAPDH.

Preparation of membrane protein

Viable LV cardiomyocytes were suspended in a HEPES (20 mmol/L)-based homogenisation buffer (pH 7.4) containing protease inhibitors (Sigma Chemical Company, UK): aprotinin (0.8 μmol/L), bacitracin (0.1 mmol/L), benzamidizine (0.1 mmol/L), EDTA (5 mmol/L), leupeptin (2 μmol/L), PMSF (0.1 mmol/L) and sonicated on ice for 3 bursts of 10 sec at 25 watts (VIBRA-CELL sonicator, Sonics & Materials Inc., Danbury, CT) and then centrifuged at 4^{o} C for 10 min at 1000 r.p.m. (Sigma 3K18) to sediment cell nuclei and

mitochondrial fractions. The resulting supernatant, containing a crude fraction of plasma membranes, was centrifuged at 12,000 r.p.m. for 55 min at 4^{o}C and the pellet formed washed with homogenisation buffer supplemented with detergent, n-Octylglucoside (0.06mM), sonicated for 3 bursts of 10 sec at 25 Watts and incubated on ice for a further period of 15 min. Finally, the suspension was centrifuged at 12,000 r.p.m. for 55 min at 4^{o}C and the pellet formed washed with homogenisation buffer containing detergent and sonicated for 20 sec at 25 Watts prior to storage at -70^{o}C pending analysis.

Immunodetection and Quantification

Membrane protein concentration was determined by the method of Lowry. Protein samples were mixed with 4µl of 1:1 mercaptoethanol: loading buffer (0.5M Tris 25% v/v, glycerol 20%v/v, 10% sds 40% v/v, H_20 15% v/v, Bromophenol blue 0.005% w/v) prior to separation by 12% SDS-PAGE (RAMP1and RAMP2 20 µg; RAMP3 80 µg; CLR 120µg protein per lane) and transfer to PVDF membrane (0.45µm, Millipore, UK). The PVDF membrane was washed with phosphate buffered saline (PBS) containing 0.1% v/v Tween 20 (Sigma, UK) and blocked overnight in PBS/0.1% v/v Tween 20 solution containing 5% w/v *Marvel*. Immunoblotting was performed using primary antibodies directed specifically against rodent CLR (Santa Cruz Biotechnology, sc-18007 raised in goat) and rodent RAMPs 1-3 (Santa Cruz Biotechnology, sc-11379, sc-11380, sc-11381 raised in rabbit) used at a dilution of 1:500 (RAMPs) or 1:200 (CLR). Immunocomplexes were detected using secondary antibodies conjugated to horseradish peroxidase (goat anti-

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rabbit ab6721 used at a dilution of 1:20000, Abcam; donkey anti-goat sc-2020 used at a dilution of 1:40000, Santa-Cruz Biotechnology) and ECL plus (Amersham Biosciences, UK) as substrate, and quantified by densitometry (Analytical Imaging System) normalized for protein loading using β-actin (Santa Cruz Biotechnology, sc-1616 raised in goat). The specificity of the RAMP and CLR antibodies used to detect the RAMP monomers and CLR protein was confirmed by comparison with immunoblots of membrane samples prepared from Cos7 cells transfected with hRAMP1, hRAMP2, hRAMP3 and hCLR cDNAs (obtained from Dr David Poyner, Birmingham, UK) and rCLR cDNA (obtained from Dr Walter Born, Zurich, Switzerland).

Data analysis.

Data are expressed as means ± SE where n denotes number of rats in which systolic blood pressure or heart weight: body weight ratio was measured, or number of heart cell preparations used to analyze mRNA expression, protein levels or cell dimensions. Statistical analyses were performed by analysis of variance to detect significant differences for between group or within group effects and post-hoc comparisons by Bonferroni or an unpaired Student's t test as appropriate.

Results

Systolic blood pressure

Systolic blood pressure (SBP) was greater (P<0.05) in rats treated with L-NAME (35mg/kg/day) at 9 weeks of age onwards (i.e. following 1 week of treatment with drug) relative to age-matched control values. The maximum increase was attained at 14 weeks of age and was 80.6 mmHg greater than age-matched control value (Fig.1). Hydralazine (50mg/kg/day) and HCTZ (7.5mg/kg/day) together did not influence blood pressure *per se* but abolished the hypertensive effect of L-NAME.

Heart weight: body weight ratio

HW: BW ratio tended to increase (p=0.06) following treatment of 8 week old animals with 35mg/kg/day L-NAME for 8 weeks (Fig.2). This increase (10.0%) was attributed to increased cardiac weight (12.9%, P<0.05); body weight was not altered. Increases in heart weight and HW: BW ratio (P<0.05) were abolished by concurrent treatment with hydralazine (50mg/kg/day) and HCTZ (7.5mg/kg/day). Treatment with hydralazine and HCTZ also reduced body weight (P<0.05) by 14.5% in the absence of L-NAME.

Cardiomyocyte dimensions

The widths of LV and RV cardiomyocytes were increased (P<0.05) by 16.7% and 20.0%, respectively, following treatment of 8 week old animals with 35mg/kg/day L-NAME for 8 weeks (Fig.3a). These increases were only partly attenuated (p=ns) by blood pressure reduction since increases of 10.6% and 10.7% were observed following treatment of 8 week old animals with

35mg/kg/day L-NAME for 8 weeks in the presence of hydralazine (50mg/kg/day) and HCTZ (7.5mg/kg/day). The lengths of LV and RV cardiomyocytes were not increased significantly (6.1% and 5.4%, respectively) following treatment with L-NAME (Fig.3b).

Structural and cardio-endocrine genes indicative of a hypertrophic phenotype

Expression of skeletal α-actin (3.6 fold, Fig. 4a,b), β-MHC (6.6 fold, Fig. 4c,d), BNP (3.4 fold, Fig. 5a,b), ACE (1.8 fold, Fig. 5c,d) and preproET-1 (1.9 fold, Fig. 5e,f) mRNAs was increased in LV, but not RV, cardiomyocytes following treatment of 8 week old animals with 35mg/kg/day L-NAME for 8 weeks, while expression of α-MHC mRNA tended to be reduced (Fig. 4e,f); expression of c-fos and MLC-2 mRNAs was not altered by treatment in either LV or RV cells (data not shown). Increases observed in LV cardiomyocytes were completely normalized to control values by concurrent administration of blood pressure lowering agents.

Adrenomedullin, Intermedin and their Receptor Component Genes.

Expression of preproAM (1.6 fold, Fig. 6a) mRNA was increased in LV, but not RV (Fig 6b), cardiomyocytes following treatment of 8 week old animals with 35mg/kg/day L-NAME for 8 weeks; expression of preproIMD mRNA was increased in both ventricles (8.4 fold, 8.6 fold, Fig. 6c, d). Increased expression in LV cardiomyocytes of preproAM mRNA was normalized to control values, ($P<0.05$) by concurrent administration of blood pressure lowering agents; although some reduction in preproIMD mRNA expression

was evident in RV, expression was not normalized to control values in either ventricle. Expression of CL receptor, (3.4 fold, Fig 7 a, b), RAMP1 (4.1 fold, Fig 7c, d), RAMP2 (2.8 fold, Fig 7 e, f) and RAMP3 (4.4 fold, Fig 7 g, h) mRNAs was also increased ($P<0.01$) in LV, but not RV, by L-NAME treatment, while expression of RDC1, L1 and RCP mRNAs was not changed in either ventricle (data not shown). Increased expression of CL receptor and RAMP2 and RAMP3 mRNAs was completely normalized to control values by concurrent administration of blood pressure lowering agents, while that of RAMP1 mRNA was not attenuated.

At protein level (Fig 8 a-c), treatment with L-NAME increased expression of the RAMP3 monomer (2.5 fold) and to a lesser extent RAMP1 monomer (1.6 fold) and RAMP2 monomer (1.5 fold) within LV cardiomyocytes. Enhanced expression of the RAMP2 and RAMP3 proteins was abolished by concurrent administration of blood pressure lowering agents, while that of RAMP1 was not attenuated. The identity of each RAMP monomer was confirmed by use of molecular weight standards and by comparison with immunoblots of membranes prepared from Cos7 cells transfected with the respective hRAMP cDNAs and from sham-transfected Cos7 cells (Fig 9 a-c). Although additional bands were observed corresponding to proteins of larger molecular weight (>30 kDa) in both cardiomyocytes and transfected Cos7 cells, these bands were also evident in sham-transfected Cos7 cells, indicating that the antibodies might also recognize discontinuous epitopes present on other unrelated proteins of larger size. All attempts at characterising these additional bands suggested that they were artefacts and were unlikely to be

related to RAMPs or to represent CLR-RAMP complexes or RAMP 'homodimers' as previously described by other laboratories (Cueille *et al*., 2002).

At protein level (Fig 8d), treatment with L-NAME tended to increase expression of CLR (1.4 fold) within LV cardiomyocytes but increases were not statistically significant. The identity of the CLR protein (~42kDa) was confirmed by comparison with immunoblots of membranes prepared from Cos7 cells transfected with hCLR or rCLR cDNAs and from sham-transfected Cos7 cells (Fig 9d). Although an additional band was observed corresponding to a protein of larger molecular weight (~50kDa) in both transfected Cos7 cells and, less abundantly, cardiomyocytes, this band was also detected in sham-transfected Cos7 cells, indicating that the antibody used might also recognize a discontinuous epitope present on another unrelated protein (~50kDa).

Discussion

Anatomical hypertrophy was evident following treatment with L-NAME (35mg/kg/day) as indicated by elevated HW: BW. However, there were notable dose-dependent observations in relation to previous findings (Bell *et al.,* 2005) since heart weight was elevated (in contrast to the lack of increase at a lower dose of 20mg/kg/day), while body weight was maintained (in contrast to marked reduction, combined with increased morbidity and mortality, observed at 50mg/kg/day). A dose intermediate between 20 and 50mg/kg/day would appear to be well tolerated and optimal for establishing a robust and stable model of cardiac remodeling in long-term NO deficiency.

Changes at organ level reflect a combination of cardiomyocyte hypertrophy and necrosis, vascular remodeling, proliferation of non-myocytes and deposition of extracellular matrix protein. Marked attenuation of increased cardiac mass by treatment with hydralazine together with hydrochlorothiazide indicates that many of these processes are likely to be initiated in response to pressure loading of the heart. Increased width of both LV and RV cardiomyocytes is consistent with previous reports of enhanced protein synthesis and mass of cardiomyocytes from both chambers (Bell *et al.,* 2005) even though the right ventricle is not subjected to the same pressure loading as the left since long term administration of L-NAME, while causing remarkable hypertension in the systemic vascular bed does not enhance mean pulmonary arterial pressure or alter pulmonary vascular morphology (Hampl *et al.,* 1993). Incomplete attenuation of L-NAME induced increases in cell width by blood pressure reduction indicates a pressure-independent

component to the pathogenesis of cardiomyocyte hypertrophy in this model, which is likely to reflect NO deficiency within the myocardium since NO itself is known to exert direct inhibitory effects on cell growth and proliferation (Sarkar *et al.,* 1995; Matsuoka *et al.,* 1996; Bartunek *et al.,* 2000). Decreased local NO production might also compromise blood flow within the myocardium, thereby promoting ischemia and contributing indirectly to cardiomyocyte hypertrophy and necrosis (De Oliveira *et al.,* 1999).

Phenotypic alterations manifest in LV, but not RV, cardiomyocytes, namely enhanced expression of the structural proteins, β-MHC and sk-α-actin, and activation of cardio-endocrine pathways, characterized by increased expression of BNP, ACE and ET-1 mRNAs, were ameliorated by blood pressure reduction indicating that these mechanisms are recruited exclusively in response to mechanical stress upon cardiomyocytes due to pressure loading caused by profound hypertension within the systemic vasculature. Recruitment of cardio-endocrine signaling pathways might be expected to exert a modifying effect upon systemic vascular tone, affecting pre-load and after-load, in addition to influencing the coronary circulation and elaboration of cardiomyocyte hypertrophy directly.

PreproIMD mRNA was expressed under normal conditions in both LV and RV cardiomyocytes, less abundantly than preproAM mRNA. However, expression of preproIMD mRNA was increased markedly in both ventricles in response to NO deficiency following administration of L-NAME, and in contrast to preproAM mRNA, which was elevated within LV cardiomyocytes only, was not

normalized by blood pressure reduction. These data indicate divergence in regard to mechanisms of recruitment of these cardio-endocrine peptides within the cardiomyocyte. Activation of AM within the LV is initiated primarily by systemic hypertension; indeed increased plasma levels of AM have been detected in hypertension (Sumimoto *et al.,* 1997) and release of the peptide from cardiomyocytes has been demonstrated *in vitro* in response to mechanical stretch (Tsurada *et al.,* 2000). Plasma levels have been found to correlate better with LV mass than with blood pressure; augmented release of AM may therefore represent a counter-regulatory response to LV hypertrophy rather than hypertension *per se* (Sumimoto *et al.,* 1997). In contrast, synthesis of intermedin is largely attributable to a pressure-independent mechanism; demonstration of a cardioprotective effect of intermedin against ischemia-reperfusion injury (Taylor *et al.,* 2005) would support the hypothesis that this peptide is released from both ventricles in response to hypoxia. Indeed cardiac ischemia and necrosis are often reported in this model of NO deficiency (Takaori *et al.,* 1997; Bernatova *et al.,* 1999).

In agreement with previous data obtained following administration of a lower dose (20mg/kg/day) (Bell *et al.,* 2005), RAMP2, RAMP3 and CLR mRNAs were all increased in LV but not RV cardiomyocytes by L-NAME (35mg/kg/day); these further increases in expression correlated with additional increases in systolic blood pressure at the higher dose. This pattern of mRNA expression, together with the corresponding changes manifest at protein level, was completely normalized by blood pressure reduction. Since RAMP2 and RAMP3 both constitute AM receptors when in

combination with CL receptor protein (Hay *et al.,* 2004), increased abundance of these receptor complexes would be expected to act in tandem with increased expression of AM itself to augment the influence of this counter-regulatory signaling cascade in regulating myocardial contractility (Ikenounchi *et al.,* 1997; Ihara *et al.,* 2000) and opposing the pathogenesis of cardiomyocyte hypertrophy (Sato *et al.,* 1997; Tsurada *et al.,* 2000; Bell *et al.,* 2005). RAMP3, normally expressed much less abundantly than RAMP2 within the cardiomyocyte (Autelitano and Ridings, 2001), was up-regulated to the greater extent. Such changes in relative abundance of these RAMPs have implications for affinity and selectivity of mediator-receptor interactions, since AM_2 receptors are less selective for AM over other peptides of this family than AM_1 receptors (McLatchie, 1998).

In contrast, LV cardiomyocyte expression of RAMP1 mRNA, which was only slightly elevated following administration of 20mg/kg/day L-NAME despite a notable increase of systolic BP (Bell *et al.,* 2005), was markedly elevated, to a similar extent as that of RAMP3 mRNA at the higher dose employed in the present study, and this was not normalized by BP reduction. These data indicate that myocardial expression of the RAMP1 gene is largely independent of the regulatory influence of pressure loading. RAMP1, when in combination with the CL receptor protein, constitutes a CGRP-responsive receptor complex (Hay *et al.,* 2004) at which AM also has moderate affinity (McLatchie, 1998). The vasorelaxant peptide CGRP, found predominantly as a neurotransmitter localized in the heart within the sensory innervation comprising capsaicin sensitive A(δ) and C fiber afferent nerves (Mulderry *et*

al., 1985), often in association with the coronary vasculature, from which nerve fibers extend into the myocardium, forming a nerve plexus which is particularly well developed within papillary muscles, exerts multiple effects on the cardiomyocyte *in vitro* including positive inotropy (Bell and McDermott, 1994), initiation of a hypertrophic phenotype (Bell *et al.,* 1995; Bell *et al.,* 1997) and direct cardioprotective effects against ischemic injury (Li *et al.,* 1996). Neuronal release of CGRP is enhanced in response to myocardial ischemia (Franco-Cereceda *et al.,* 1987). Enhanced expression of the RAMP1 protein would increase the probability of interaction of this neuronally released CGRP with its receptor, thereby augmenting cellular responsiveness. In addition to possible beneficial effects on coronary haemodynamics, localized release of CGRP could also serve to counter the cardiac ischemia and necrosis associated with NO deficiency, and also to promote compensatory hypertrophy and improve contractility of surviving myocardium. It is interesting to note in this regard that cardiomyocyte hypertrophy is also initiated *in vitro* in response to oxidative stress (Sabri *et al.,* 1998; Siwik *et al.,* 1999).

Expression of L1, which is normally found at very low levels within cardiomyocytes (Autelitano, 1998), of RDC-1 (Li *et al.,* 1996), expressed abundantly in the myocardium, and of the RCP protein, which moderates the efficacy of coupling of the RAMP/CLR complex to G-protein activation (Prado *et al.,* 2001) was not influenced by L-NAME administration, indicating that altered abundance of these proteins within the cardiomyocyte are unlikely to participate in the pathophysiological changes induced by NO deficiency. This

supports the conclusion of Poyner and colleagues (2002) that LI and RDC-1 do not represent receptors for the CGRP/AM family, but rather are orphan GPCR for which the true ligands remain to be identified.

Intermedin interacts non-selectively both at AM receptors, formed by the association of RAMP2 or 3 with CLR, and at CGRP receptors formed by the association of RAMP1 with CL (Poyner *et al.,* 2002; Roh *et al.,* 2004). Although enhanced expression of intermedin mRNA is not accompanied by enhanced expression of receptor component genes in RV, in contrast to LV, nonetheless some increase in activity of intermedin signaling pathways might also be anticipated as a consequence of increased peptide levels. While the functional significance of this peptide is poorly understood at present, the robust increase in intermedin expression in indicates an important role in the cardiac pathology resulting from NO deficiency but, unlike AM, intermedin is probably not associated primarily with pressure overload upon the myocardium. The concordance of intermedin and RAMP1 up-regulation in LV supports a CGRP-type receptor action; considering also the relative unresponsiveness to BP reduction, intermedin may, like CGRP, serve primarily an anti-ischemic, cardio-protective function although an additional attenuating influence for this peptide on cardiomyocyte hypertrophy, mediated by stimulation of the AM_2 receptor, cannot be discounted, particularly since RAMP3 expression is also enhanced. Expression of intermedin and RAMP1 mRNAs is likely to be a dynamic process, tightly regulated in response to prevailing levels of myocardial ischemia, and extent of ongoing cell necrosis

and apoptosis, and requirement for remodelling of adjacent healthy myocardium.

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FIGURE LEGENDS

Fig. 1: Temporal changes in systolic blood pressure following chronic administration to 8 week old rats of (i) L-NAME (35mg/kg/day) (•), (ii) hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day) (▼), (iii) L-NAME (35mg/kg/day) concurrently with hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day) (■); (iv) age-matched untreated rats are included for comparison (▲). Data are the mean values± SE of 5-7 rats. *denotes significant variation from age-matched control response ($P<0.05$).

Fig. 2:

Effect on (a) heart weight; (b) body weight; (c) HW:BW ratio of administration to 8 week old rats of (i) L-NAME (35mg/kg/day), (ii) hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day), (iii) L-NAME (35mg/kg/day) concurrently with hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day) for 8 weeks ; (iv) age-matched untreated rats are included for comparison. Data are the mean values+ SE of 6-9 rats. *denotes significant variation from age-matched control response ($0.01<P\leq0.05$). $^{+}$denotes significant difference between responses in the absence and presence of blood pressure lowering agents (+ $0.01<P\leq0.05$; ++ $0.005<P\leq0.01$; +++ $P\leq0.005$)

Fig. 3:

Effect on (a) width; (b) length of LV and RV cardiomyocytes following administration to 8 week old rats of (i) L-NAME (35mg/kg/day) (N), (ii) hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day) (HH), (iii) L-NAME

(35mg/kg/day) concurrently with hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day) for 8 weeks (NHH); (iv) age-matched untreated rats are included for comparison (C). Data are the mean values+ SE of 3-7 heart cell preparations, within which 20 cells were measured from each chamber. *denotes significant variation from age-matched control response
(* $0.01<P\leq0.05$; ** $0.005<P\leq0.01$; *** $P\leq0.005$)

Fig. 4: Effect on (a, b) skeletal-α-actin; (c, d) β-myosin heavy chain; (e, f) α-myosin heavy chain mRNA expression in left and right ventricular cardiomyocytes of administration of (i) L-NAME (35mg/kg/day), (ii) hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day), (iii) L-NAME (35mg/kg/day) concurrently with hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day) for 8 weeks; (iv) age-matched untreated rats are included for comparison. Data are expressed relative to GAPDH mRNA levels and are the mean values + SE of 4-6 heart cell preparations. *denotes significant variation from control response (*$0.01<P<0.05$; **$0.005<P<0.01$;***$P<0.005$). $^{+}$denotes significant difference between responses in the absence and presence of blood pressure lowering agents (+ $0.01<P\leq0.05$; ++ $0.005<P\leq0.01$; +++ $P\leq0.005$)

Fig. 5: Effect on (a, b) BNP; (c, d) ACE; (e, f) ET-1 mRNA expression in left and right ventricular cardiomyocytes of administration of (i) L-NAME (35mg/kg/day), (ii) hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day), (iii) L-NAME (35mg/kg/day) concurrently with hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day) for 8 weeks; (iv) age-matched untreated rats are

included for comparison. Data are expressed relative to GAPDH mRNA levels and are the mean values + SE of 4-6 heart cell preparations. *denotes significant variation from control response (*0.01<P<0.05; **0.005<P<0.01;***P<0.005). $^{+}$denotes significant difference between responses in the absence and presence of blood pressure lowering agents (+ 0.01<P≤0.05; ++ 0.005<P≤0.01; +++ P≤0.005)

Fig. 6: Effect on (a, b) preproAM; (c, d) preproIMD mRNA expression in left and right ventricular cardiomyocytes of administration of (i) L-NAME (35mg/kg/day), (ii) hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day), (iii) L-NAME (35mg/kg/day) concurrently with hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day) for 8 weeks; (iv) age-matched untreated rats are included for comparison. Data are expressed relative to GAPDH mRNA levels and are the mean values + SE of 4-6 heart cell preparations. *denotes significant variation from control response (*0.01<P<0.05; **0.005<P<0.01;***P<0.005). $^{+}$denotes significant difference between responses in the absence and presence of blood pressure lowering agents (+ 0.01<P≤0.05; ++ 0.005<P≤0.01; +++ P≤0.005).

Fig. 7: Effect on (a, b) CL receptor; (c, d) RAMP1; (e, f) RAMP2; (g, h) RAMP3 mRNA expression in left and right ventricular cardiomyocytes of administration of (i) L-NAME (35mg/kg/day), (ii) hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day), (iii) L-NAME (35mg/kg/day) concurrently with

hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day) for 8 weeks; (iv) age-matched untreated rats are included for comparison. Data are expressed relative to GAPDH mRNA levels and are the mean values + SE of 4-6 heart cell preparations. *denotes significant variation from control response (*0.01<P<0.05; **0.005<P<0.01;***P<0.005). $^{+}$denotes significant difference between responses in the absence and presence of blood pressure lowering agents (+ 0.01<P$\leq$0.05; ++ 0.005<P$\leq$0.01; +++ P$\leq$0.005).

Fig. 8: Effect on (a) RAMP1; (b) RAMP2; (c) RAMP3 monomer; (d) CL receptor protein expression in left ventricular cardiomyocytes of administration of (i) L-NAME (35mg/kg/day), (ii) hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day), (iii) L-NAME (35mg/kg/day) concurrently with hydralazine (50mg/kg/day) plus HCTZ (7.5mg/kg/day) for 8 weeks; (iv) age-matched untreated rats are included for comparison. Data are expressed relative to β-actin levels and are the mean values + SE of 4-6 heart cell preparations. *denotes significant variation from control response (*0.01<P<0.05; **0.005<P<0.01;***P<0.005). $^{+}$denotes significant difference between responses in the absence and presence of blood pressure lowering agents (+ 0.01<P$\leq$0.05; ++ 0.005<P$\leq$0.01; +++ P$\leq$0.005).

Fig 9: Representative immunoblots to confirm the presence of (a) RAMP1; (b) RAMP2; (c) RAMP3 monomers and (d) CLR protein in membranes prepared from LV cardiomyocytes and from Cos7 cells transfected with hRAMPs cDNAs (a-c) and rCLR or hCLR cDNAs (d) but not in membranes prepared from sham-transfected Cos7 cells.

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Table 1: Primers Sequences

	Forward	Backward
AM BC061775	CATTGAACAGTCGGGCGAGTA 21bp [739-759]	GGTGCGAAGCTCTCTGATTCC 21bp [776-796]
IMD NM201426	GGCCCAGTTGCTGATGGT 18bp [175-192]	TGCCCGGGAGCAGGTA 16bp [224-239]
RAMP-1 AB042887	AGCATCCTCTGCCCTTTCATT 21bp [268-288]	GACCACCAGGGCAGTCATG 19bp [315-333]
RAMP-2 AB042888	TGTCAAGGACTGGTGCAACTG 21bp [272-292]	AAGCAATACCGCAGGTTGCT 20bp [315-334]
RAMP-3 AB042889	CAACCTGTCGGAGTTCATCGT 21bp [198-218]	TGTCTCCATCTCCGTGCAGTT 21bp [238-258]
CL CAA49997	CTCTACATGAAAGCTGTAAGAGCCACTC 28bp [988-1015]	CCTGATAGTGCATGAGAATGTGCATGAC 28bp [1102-1129]
LI S79811	TGCCAACATGTACAGCAGCAT 21bp [623-643]	GAGGTATTGGTGAGGGTCACGTA 23bp [753-775]
RDC1 AJ010828	CCGAGCACAGCATCAAGGA 19bp [685-703]	GGACAGCAAAACCCAAGATGA 21bp [753-773]
RCP BC059117	GCAGCCCTCTGGGTAGGA 19bp [811-829]	TGAATAGCTCCCCACTGTGTACA 21bp [876-896]
GAPDH AB017801	GAAACCCATCACCATCTTCCA 21bp [248-268]	ACCCCATTTGATGTTAGCGG 20bp [280-299]
ET-1 M64711	TGCTCCTCCTTGATGGACAAG 21bp [347-367]	TGATGTCCAGGTGGCAGAAGT 21bp [378-398]
Skeletal α-Actin BC061974	AAGGATTCCTACGTGGGCG 19bp [300-318]	GGTCAGGATACCTCGCTTGCT 21bp [247-267]
MLC-2 M11851	CGAAAGCTCCAAGGTGTTCT 20bp [77-96]	TCCTTCTCTTCTCCGTGGGT 20bp [516-534]
ACE BC085760	GAGCCATCCTTCCCTTTTTC 20bp [2428-2447]	GGCTGCAGCTCCTGGTATAG 20bp [2562-2581]

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BNP M25297	AGGTGCTGCCCCAGATGAT 19bp [71-89]	GCGGCGACAGATTAAGGAAA 20bp [102-121]
α-MHC K01464	TGTGAAAAGATTAACCGGAGTTTAAG 26bp [1210-1235]	TCTGACTTGCGGAGGTATCG 20bp [1630-1649]
β–MHC X15939	AAGTCCTCCCTCAAGCTCCTAAGT 24bp [1841-1864]	TTGCTTTGCCTTTGCCC 17bp [1909-1925]
c-fos X06769	AATAAGATGGCTGCAGCCAA 20bp [573-592]	TTGGCAATCTCGGTCTGCAA 20bp [669-688]

Accession Numbers are taken from the European Molecular Biology Laboratory (EMBL) database which is part of the *International Nucleotide Sequence Database Collaboration*

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Fig 1:

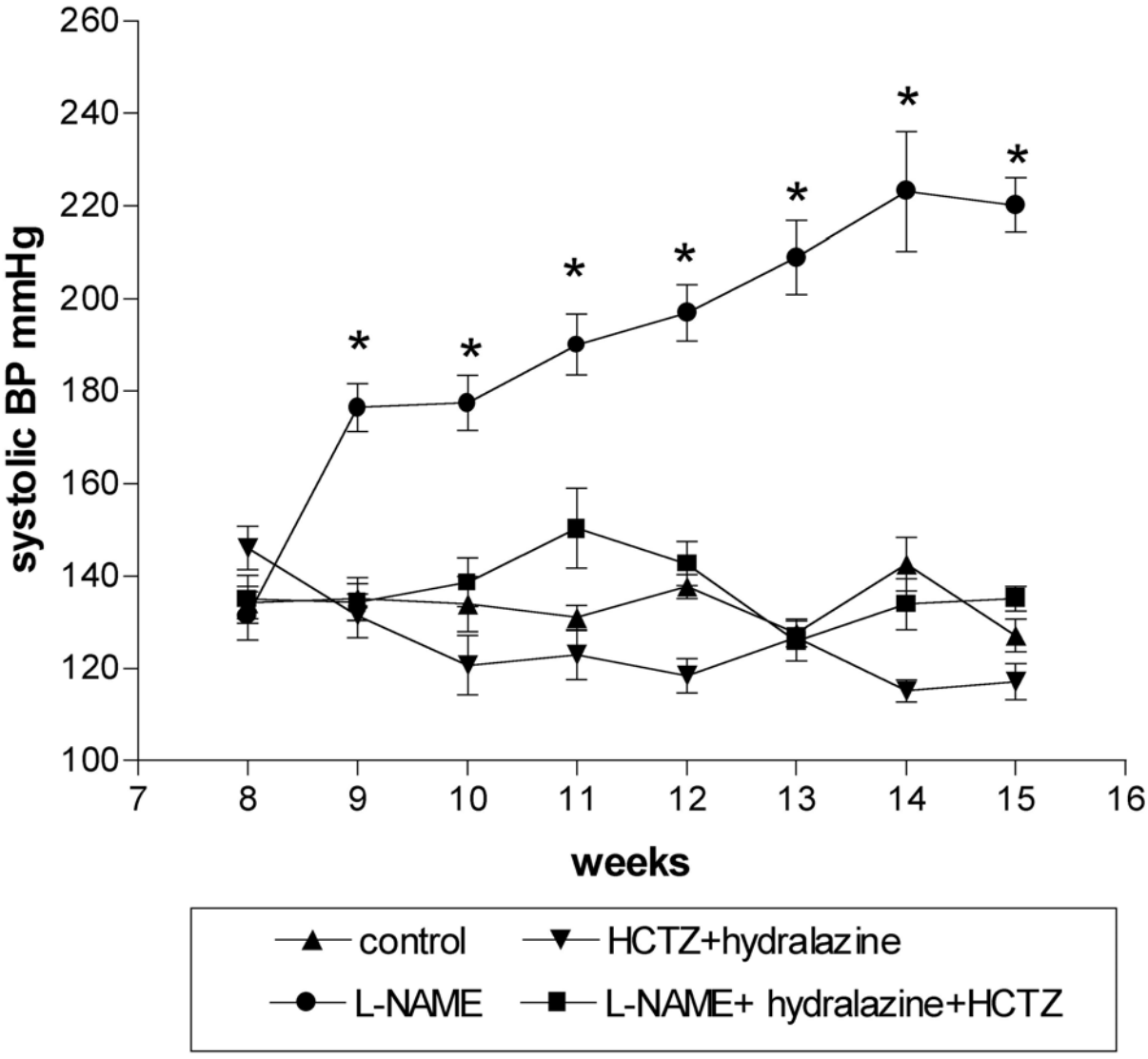

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Fig. 2

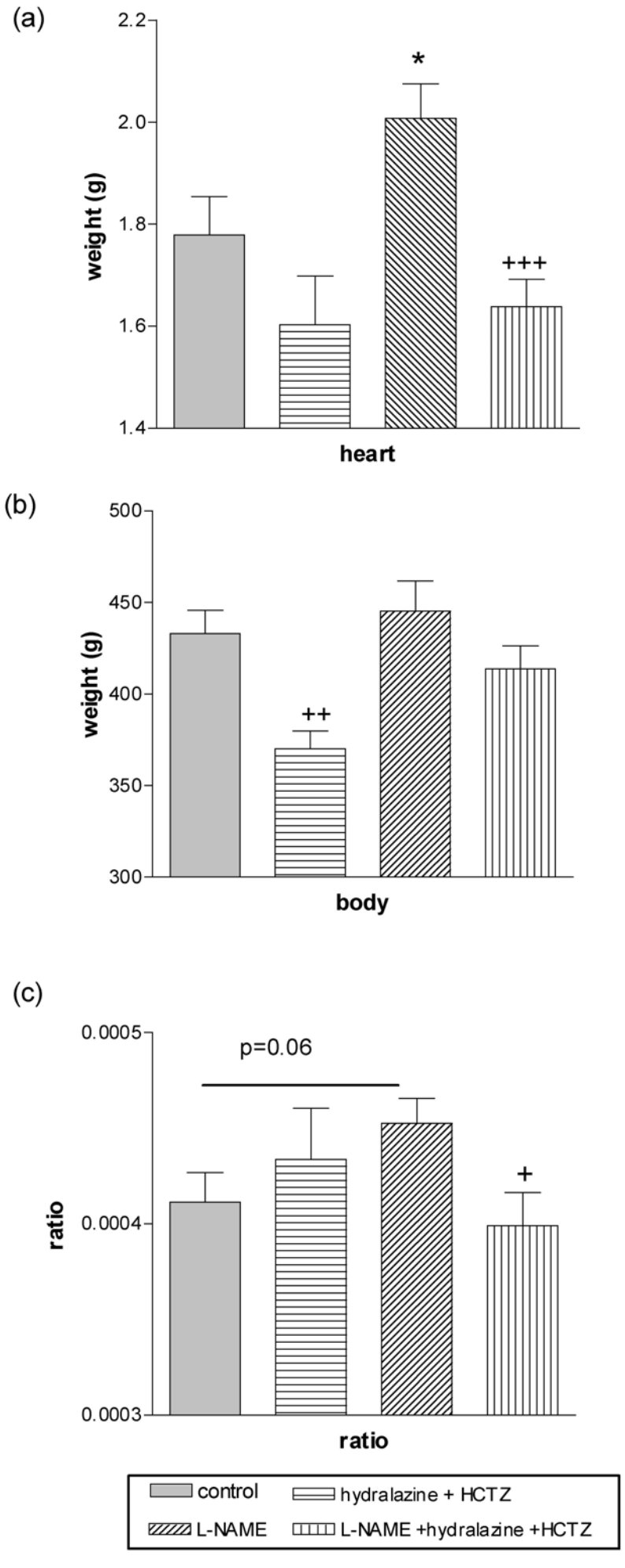

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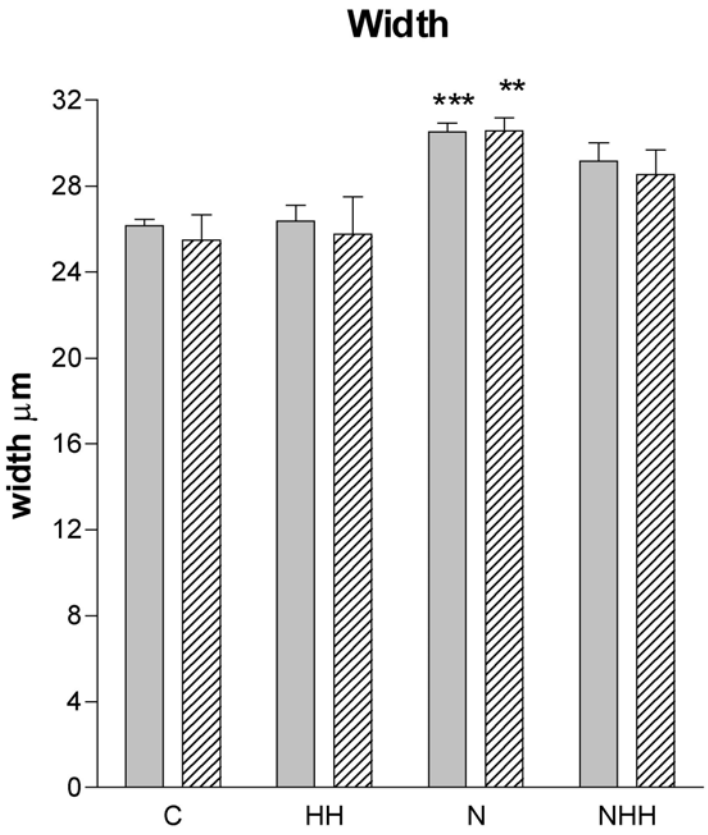
Figure 3

(a)



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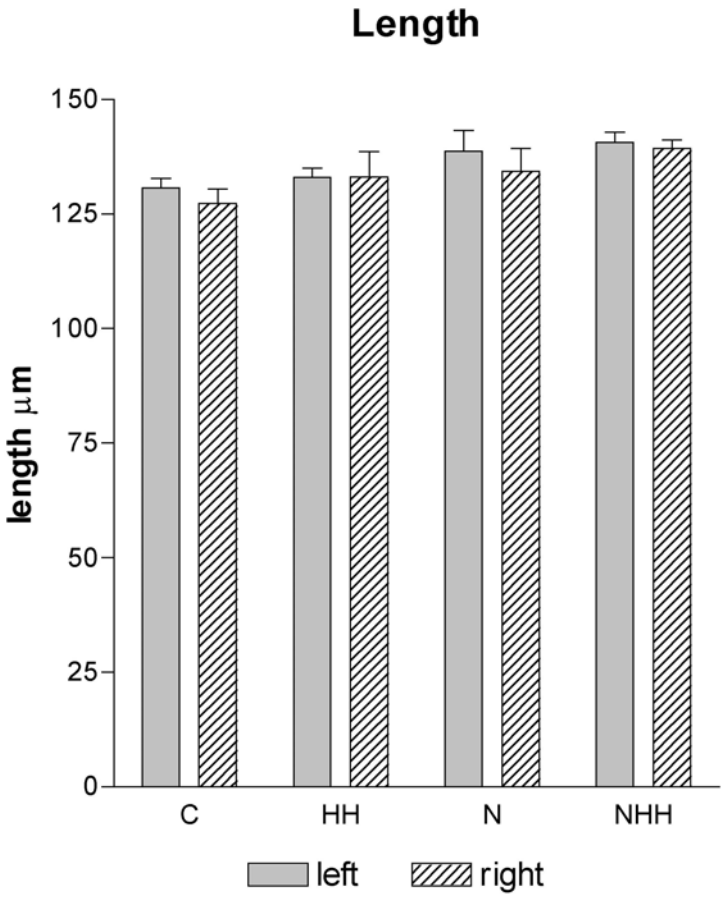

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Fig, 4

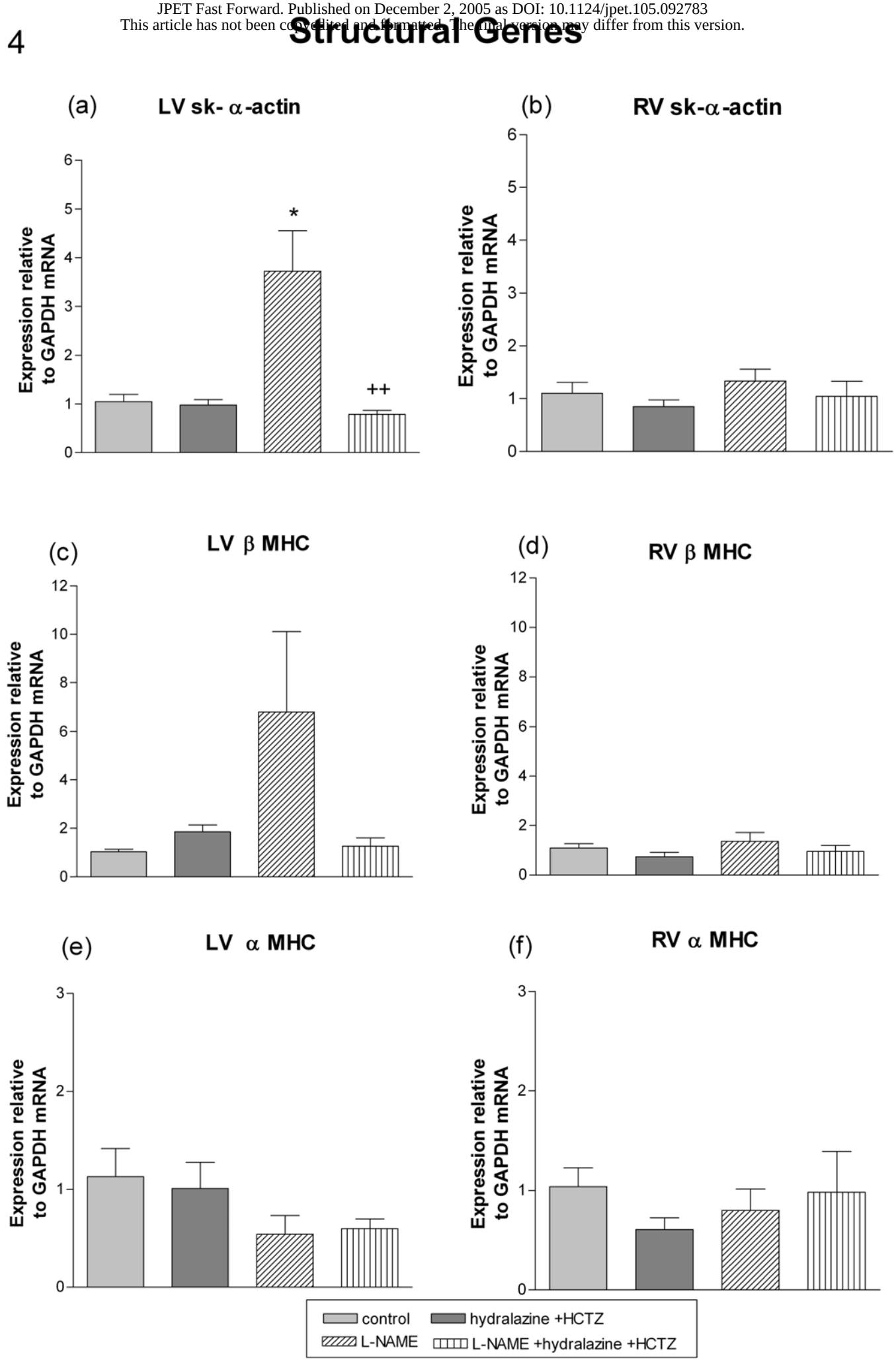

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Fig. 5

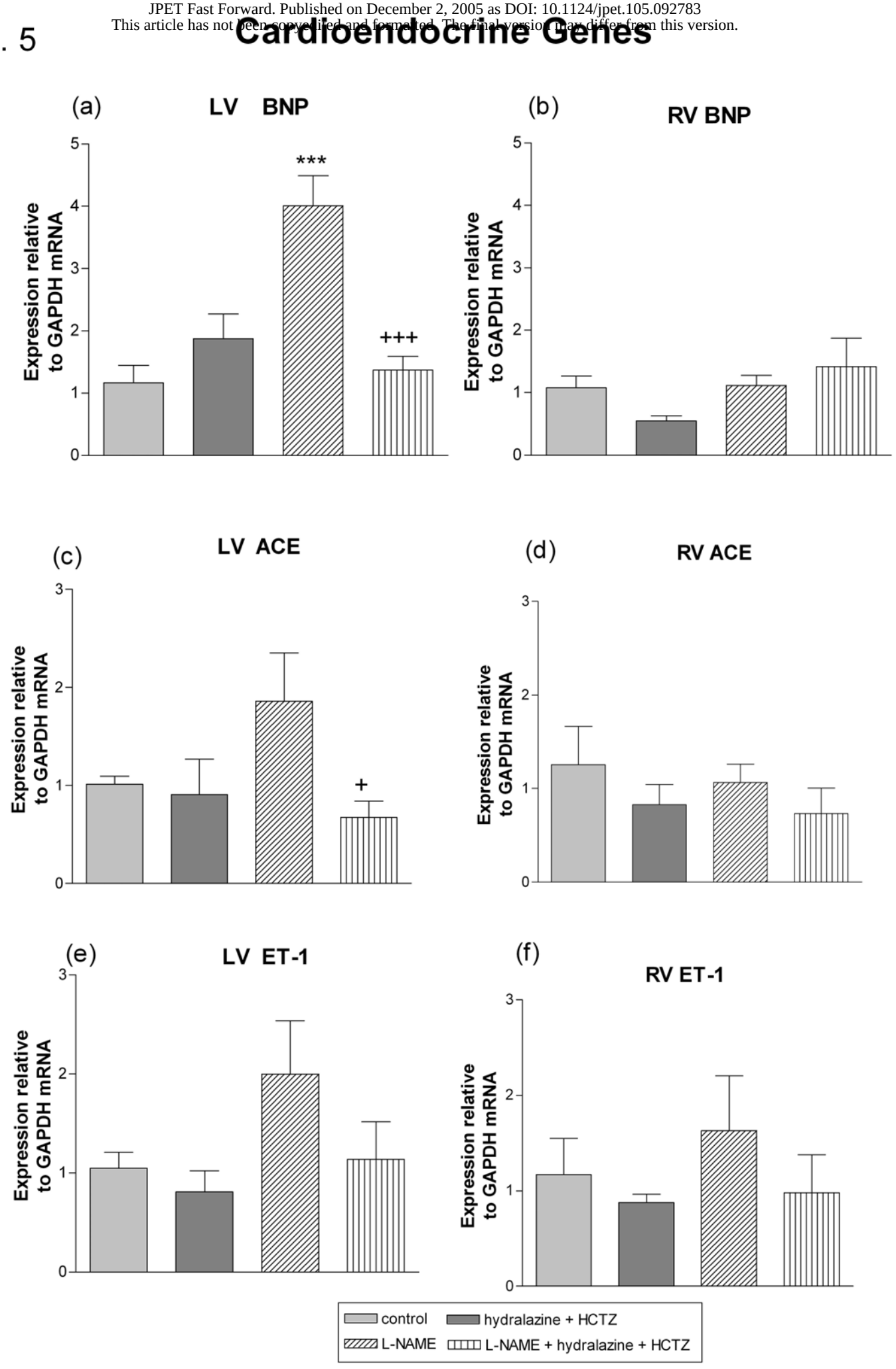

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Fig. 6

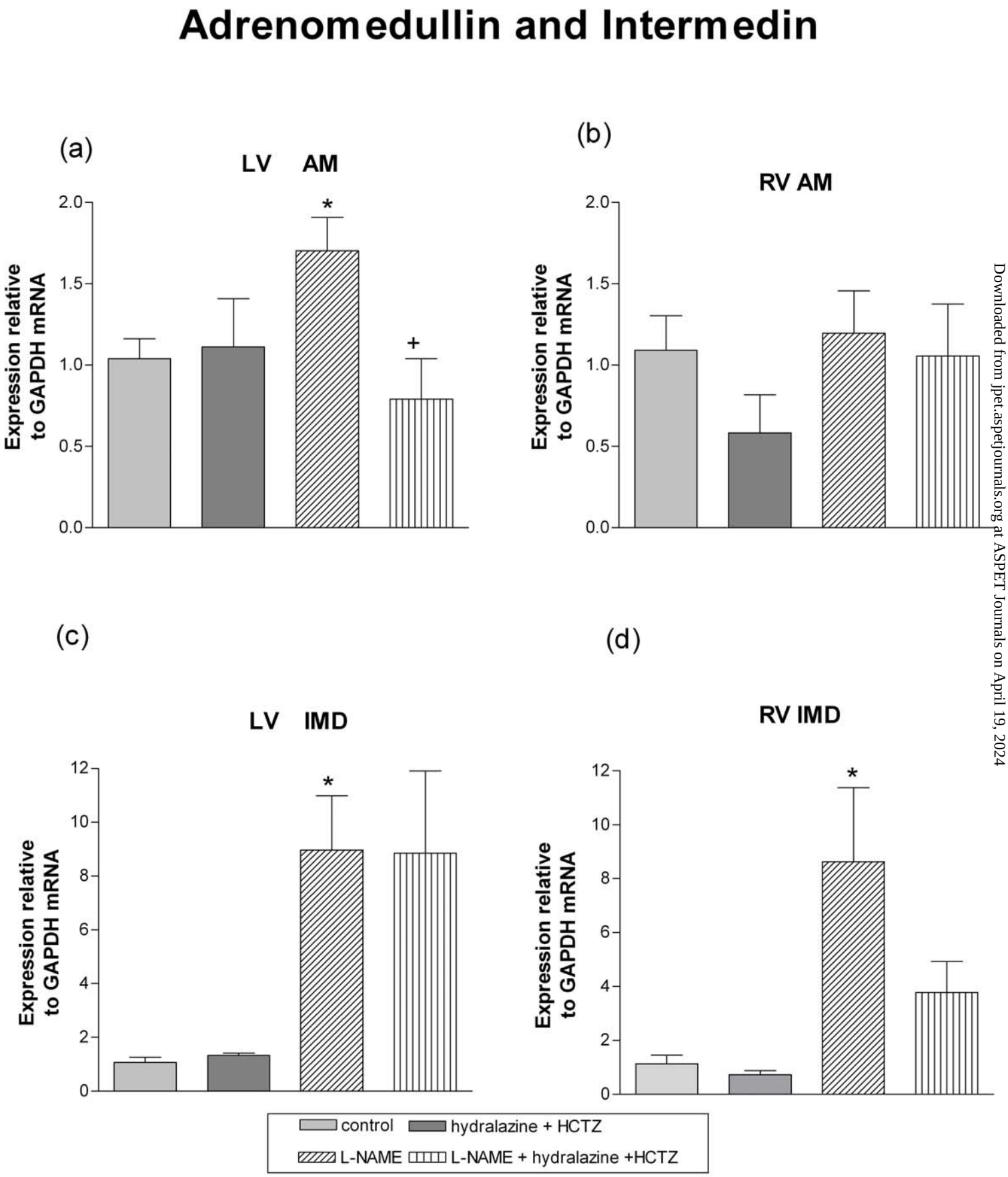

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Fig. 7

AM Receptor Component Genes

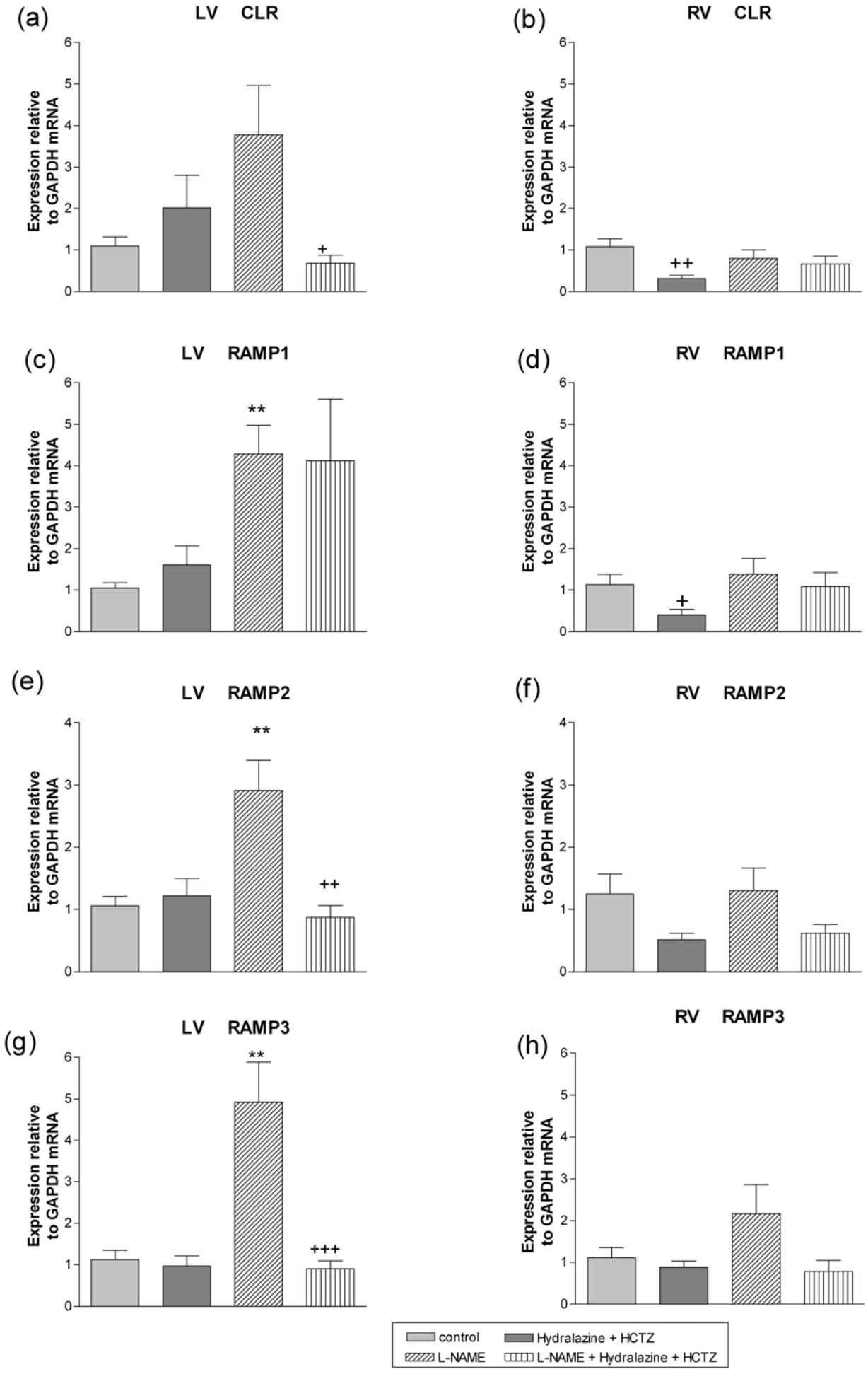
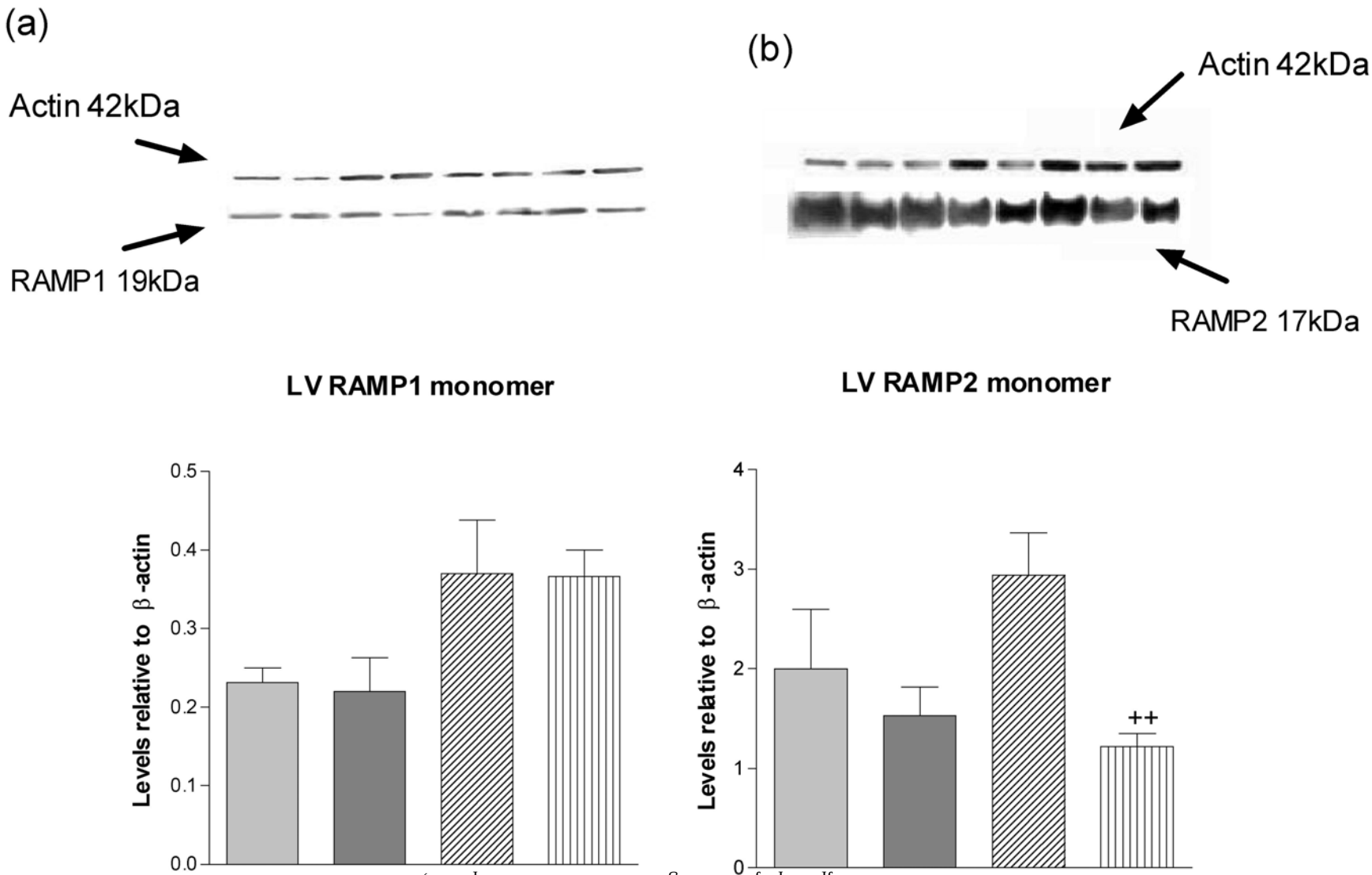


Figure 8 a,b



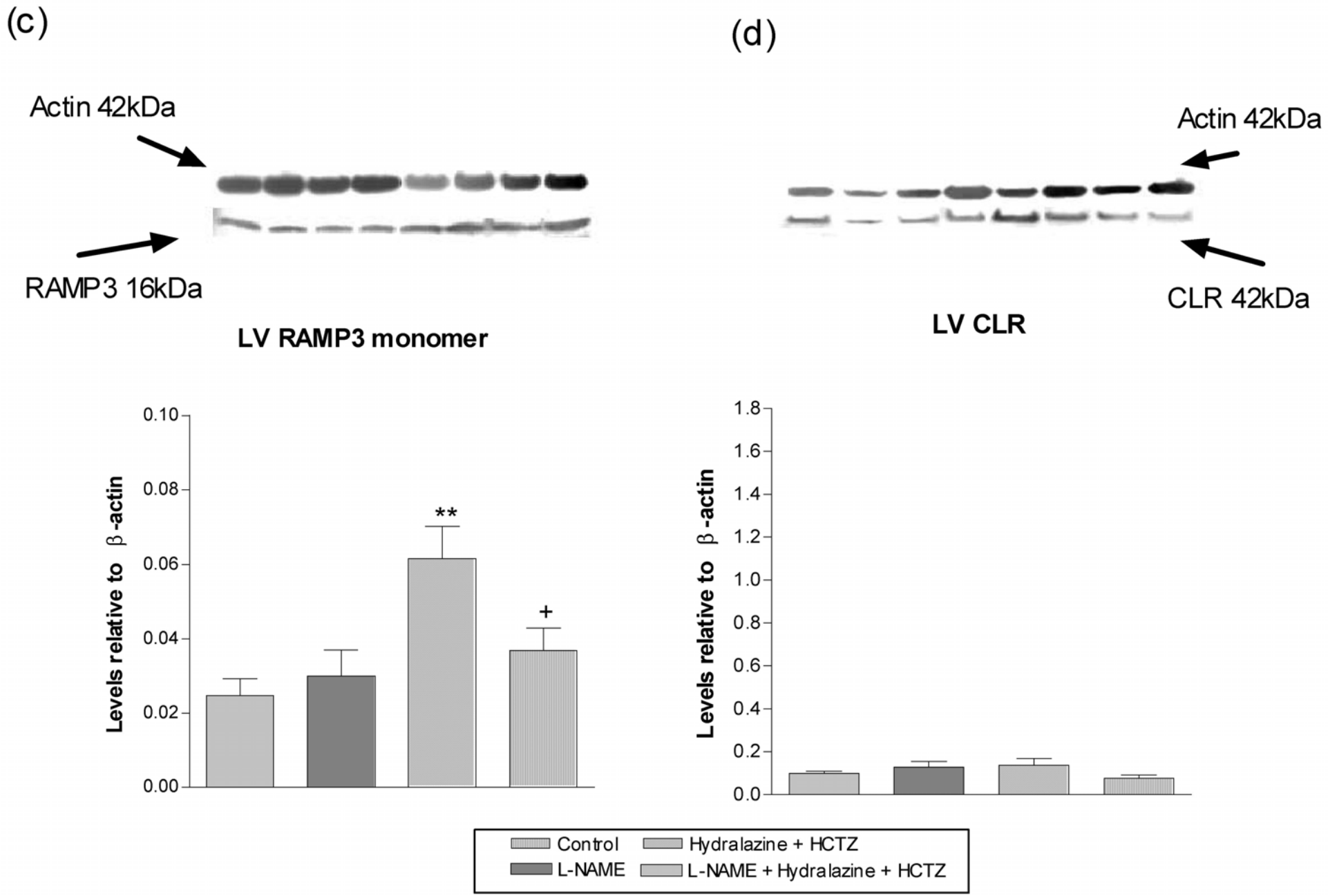
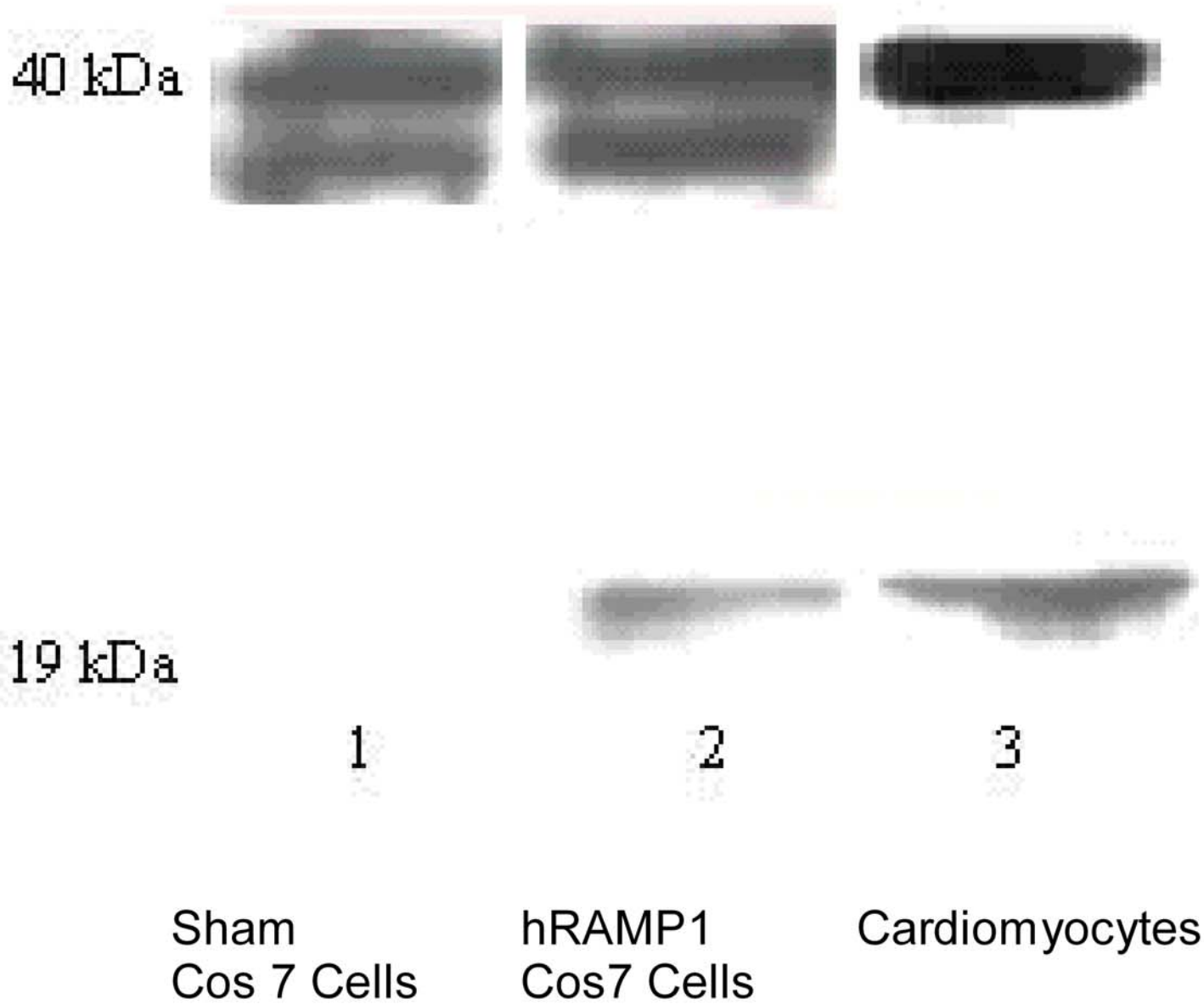


Figure 9 (a)



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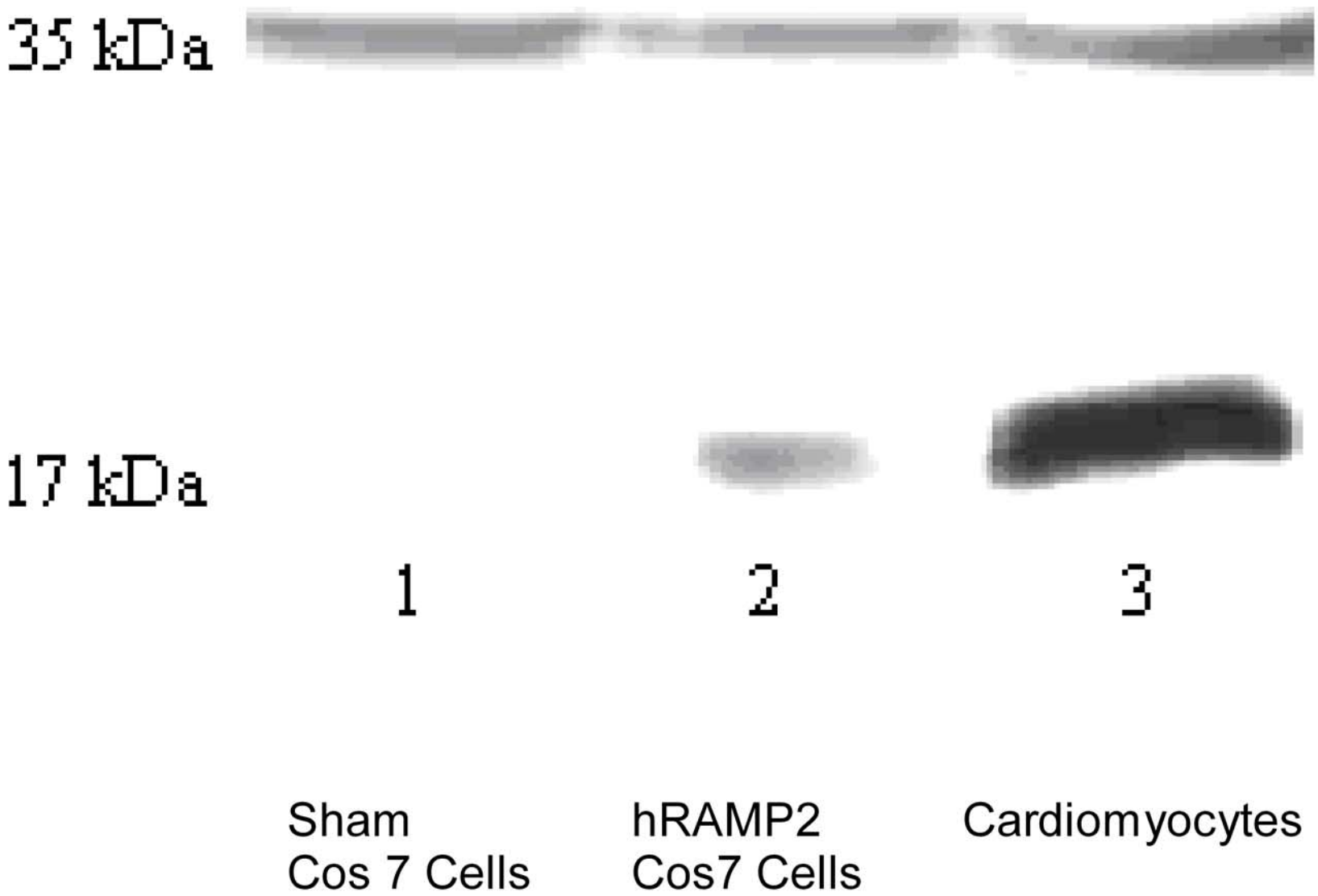

Fig. 9 (c)

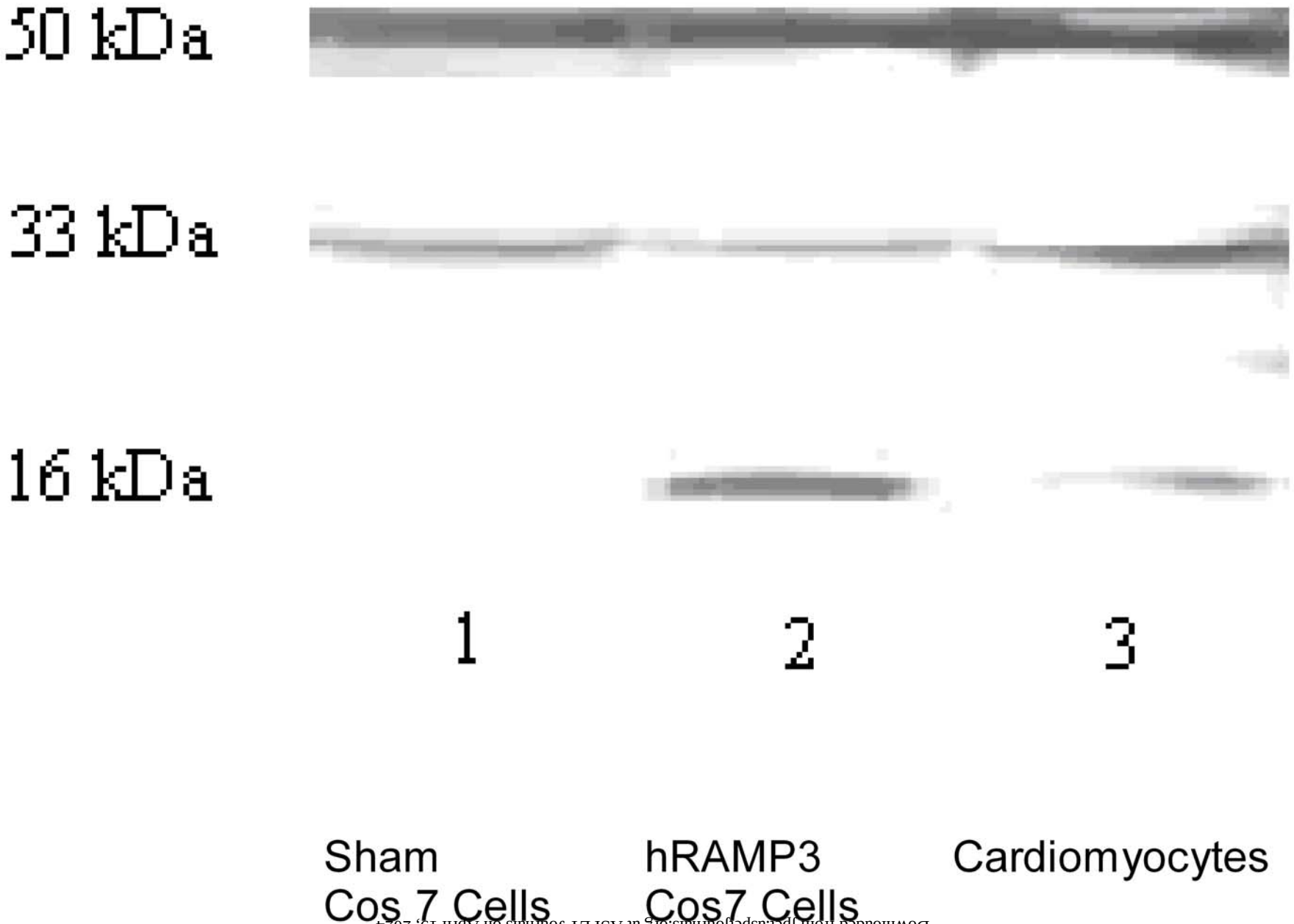

Fig. 9 (d)