Expression of the Counter-Regulatory Peptide Intermedin is Augmented in the Presence of Oxidative Stress in Hypertrophied Cardiomyocytes

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Key Words

Ventricular • Oxidative stress • Cardiac peptides • RAMPs • Hypertension • Intermedin • Adrenomedullin • Myocardial ischemia • Cardiomyocyte hypertrophy

Abstract

Background: Intermedin (IMD), a novel cardiac peptide related to adrenomedullin (AM), protects against myocardial ischemia-reperfusion injury and attenuates ventricular remodelling. IMD's actions are mediated by a calcitonin receptor-like receptor in association with receptor activity modifying proteins (RAMPs 1-3). **Aim/method:** using the spontaneously hypertensive rat (SHR) and normotensive Wistar Kyoto (WKY) rat at 20 weeks of age, to examine (i) the presence of myocardial oxidative stress and concentric hypertrophy; (ii) expression of IMD, AM and receptor components. **Results:** In left and right ventricular cardiomyocytes from SHR vs. WKY cell width (26% left, 15% right) and mRNA expression of hypertrophic markers ANP (2.7 fold left, 2.7 fold right) and BNP (2.2 fold left, 2.0 fold right) were enhanced. In left ventricular cardiomyocytes only (i) oxidative stress was indicated by increased membrane protein carbonyl content (71%) and augmented production

of O₂⁻ anion (64%); (ii) IMD (6.8 fold), RAMP1 (2.5 fold) and RAMP3 (2.0 fold) mRNA was increased while AM and RAMP2 mRNA was not altered; (iii) abundance of RAMP1 (by 48%), RAMP2 (by 41%) and RAMP3 (by 90%) monomers in cell membranes was decreased. **Conclusion:** robust augmentation of IMD expression in hypertrophied left ventricular cardiomyocytes indicates a prominent role for this counter-regulatory peptide in the adaptation of the SHR myocardium to the stresses imposed by chronic hypertension. The local concentration and action of IMD may be further enhanced by down-regulation of NEP within the left ventricle.

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Introduction

Intermedin (IMD) is a novel member of the calcitonin gene-related peptide (CGRP) family which also includes adrenomedullin (AM) [1, 2]. AM has two receptors (AM₁, AM₂) formed by the calcitonin receptor-like receptor (CRLR) combined with receptor activity modifying proteins (RAMP) 2 or 3 respectively. AM also

has some affinity for CGRP₁ receptors, composed of CRLR and RAMP1 [3]. A membrane-associated receptor component protein (RCP) is required for optimum activation of signal transduction [4]. IMD acts non-selectively at all three RAMP-CRLR complexes [2]. Neutral endopeptidase (NEP), a membrane-bound metalloproteinase [5], is implicated in degradation of these peptides [6]. In the heart, a positive inotropic effect [7], attenuation of cardiomyocyte hypertrophy [8] and protection against ischemia-reperfusion injury are observed in response to administration of IMD [9].

The IMD transcript is expressed abundantly in rat neonatal cardiomyocytes [8] and is detected, but present at substantially lower levels than that of AM, in adult cardiomyocytes [10] consistent with greater prominence of IMD during embryonic development of the myocardium. In human tissue obtained at autopsy from subjects without known cardiac disease, IMD is localised to myocardial cells but not endocardium, pericardial adipose tissue or vasculature of heart [11]. Expression of IMD is augmented in myocardial tissue in a model of sub-acute myocardial ischemic injury and hypertrophy induced by chronic administration of isoprenaline in rat [8]. Expression of IMD in cardiomyocytes is increased robustly [12] in nitric oxide-deficient hypertension induced by administration of the nitric oxide synthase (NOS) inhibitor, *N*^G-nitro-L-arginine-methyl-ester (L-NAME), which is characterised in myocardium by hypertrophy, fibrosis, ischemia and necrosis [13]. IMD binding capacity in myocardial sarcolemmal membranes [14] and cardiomyocyte expression of RAMPs [10] is also enhanced in ischemic and hypertrophied myocardium.

The spontaneously hypertensive rat (SHR) is a useful model of chronic pressure loading [15, 16] in which hypertension occurs from 7 weeks of age and is followed initially by development of compensated left ventricular hypertrophy (LVH); the influence of pressure loading on cardiomyocytes is manifest as increased cell width, indicative of concentric hypertrophy, and altered phenotype [17] at 20 weeks relative to that of Wistar Kyoto (WKY) rat, employed as a normotensive control. Augmented expression in SHR hearts of the natriuretic peptides, ANP and BNP, which offset increased cardiac preload and after-load by promoting natriuresis, diuresis and vasodilatation [18] is normalised by blood pressure lowering [19]. Modest pulmonary hypertension is also present in SHR at 20 weeks [20] but right ventricular hypertrophy (RVH) is not evident in non-failing SHR hearts [21]. Hearts of SHR also experience more oxidative stress, evidenced by greater carbonylation of

myocardial proteins relative to age-matched WKY at 16 weeks [22].

The hypothesis that IMD is an endogenous cardio-protective peptide which is up-regulated specifically in the presence of myocardial oxidative stress and hypertrophic remodeling in response to chronic pressure loading was examined using the SHR model. The presence of oxidative stress and concentric hypertrophy was verified in left ventricular cardiomyocytes obtained from SHR at 20 weeks; expression of IMD was determined and contrasted with that of AM and their receptor components and also with expression of ANP, BNP and the enzyme, NEP. Right ventricular cardiomyocytes from SHR and left and right ventricular cardiomyocytes from age-matched WKY rat were used as a source of tissue for comparison studies. The findings of the present study indicate a much more prominent role for IMD than AM in the cardiac pathology of the SHR during early compensation in response to pressure-loading and suggest discordance in regard to induction of this novel counter-regulatory peptide and that of the natriuretic peptides.

Materials and Methods

Experimental model

The study was performed in accordance with NIH guidelines 'Principle of laboratory animal care' and UK Home Office 'Guidance on the operation of the Animals (Scientific Procedures) Act 1986', published by Her Majesty's Stationary Office, London. Male SHR and age/sex-matched WKY were obtained from Harlan (Blackthorn, Oxon, UK) at 4 weeks of age and maintained at the Laboratory Service Unit, QUB, prior to sacrifice at 20 weeks of age. Systolic blood pressure was determined by tail cuff sphygmomanometer (Harvard Instruments) [17] and the mean of four consecutive blood pressure readings was obtained for each animal at weekly intervals (8 animals per group). Body weight was recorded weekly.

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, Germany) using Langendorff perfusion [10]. After purification, cells were utilized immediately for extraction of RNA (RT-PCR protocols) and preparation of membrane protein (immunoblotting protocols). For analysis of superoxide production, cells were suspended at a concentration of

	Forward	Backward
AM	CATTGAACAGTCGGGCGAGTA	GGTGCGAAGCTCTCTGATTCC
BC061775	21bp [739-759]	21bp [776-796]
IMD	GGCCAGTTGCTGATGGT	TGCCCGGAGCAGGTA
NM201426	18bp [175-192]	16bp [224-239]
RAMP-1	AGCATCCTCTGCCCTTTCATT	GACCACCAGGGCAGTCATG
AB042887	21bp [268-288]	19bp [315-333]
RAMP-2	TGTCAAGGACTGGTGCAACTG	AAGCAATACCGCAGGTTGCT
AB042888	21bp [272-292]	20bp [315-334]
RAMP-3	CAACCTGTCGGAGTTCATCGT	TGTCTCCATCTCCGTGCAGTT
AB042889	21bp [198-218]	21bp [238-258]
RCP	GCAGCCCTCTGGGTAGGA	TGAATAGCTCCCCACTGTGTACA
BC059117	19bp [811-829]	21bp [876-896]
CRLR	CTCTACATGAAAGCTGTAAGAGCCACTC	CCTGATAGTGCATGAGAATGTGCATGAC
CAA49997	28bp [988-1015]	28bp [1102-1129]
GAPDH	GAAACCCATCACCATCTTCCA	ACCCCATTTGATGTTAGCGG
AB017801	21bp [248-268]	20bp [280-299]
BNP	AGGTGCTGCCCCAGATGAT	GCGGCGACAGATTAAGGAAA
M25297	19bp [71-89]	20bp [102-121]
ANP	CTGGGACCCCTCCGATAGA	GCAGAGCCCTCAGTTTGCTT
M27498	19bp [300-318]	20bp [333-352]
NEP	CAA GAG GAG GAA GAC GCT GAA	CCT GAA GGC CAC AGT ATG CAT
BC085753	21bp [2719-2739]	21bp [2831-2851]

Table 1. Primers Sequences: Accession Numbers are taken from the European Molecular Biology Laboratory (EMBL) database which is part of the International Nucleotide Sequence Database Collaboration.

approximately 1×10^6 viable cardiomyocytes/ml in Krebs-Ringer solution supplemented with 1mM Ca^{2+} ion and bovine serum albumin (4% w.v⁻¹). For analysis of cell dimensions, cells were suspended at a concentration of 5×10^4 viable cardiomyocytes/ml in a 'creatinine-carnitine-aurine' (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°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°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. 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 $n \text{ fold induction} = 2^{-\text{Ct relative to GAPDH}}$.

Preparation of membrane protein

Viable 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 $\mu\text{mol/L}$), bacitracin (0.1 mmol/L), benzamidazine (0.1 mmol/L), EDTA (5 mmol/L), leupeptin (2 $\mu\text{mol/L}$), 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°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°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°C and the pellet formed washed with

Fig. 1. (a) systolic blood pressure; (b) heart weight: body weight ratio; (c) ventricular cardiomyocyte width; (d) ventricular cardiomyocyte length of 20 week old SHR and WKY rat. Data are the mean values $\pm$ SE, n=8. In (c) and (d), 20 cells were measured within each heart cell preparation. *variation between strains (P<0.05).

homogenisation buffer containing detergent and sonicated for 20 sec at 25 Watts prior to storage at -70°C pending analysis.

Immunodetection and quantification of RAMPs

Membrane protein concentration was determined by the method of Lowry. Protein samples were mixed with 4 μl of 1:1 mercaptoethanol: loading buffer (0.5M Tris 25% v/v, glycerol 20% v/v, 10% SDS 40% v/v, H_2O 15% v/v, Bromophenol blue 0.005% w/v) prior to separation by 12% SDS-PAGE (RAMP1 and RAMP2 20 μg ; RAMP3 80 μg protein per lane) and transfer to PVDF membrane (0.45 μm , 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 RAMPs 1-3 (Santa Cruz Biotechnology, sc-11379, sc-11380, sc-11381 raised in rabbit) used at a dilution of 1:500. Immunocomplexes were detected using secondary antibodies conjugated to horseradish peroxidase (goat anti-rabbit ab6721 used at a dilution of 1:20000, Abcam; 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 particular batch of each antibody used was identical in each case to that used in a recent study from the same laboratory

[10]. In that study, specificity of the antibodies used to detect the RAMP monomers was confirmed by comparison with immunoblots of membrane samples prepared from Cos7 cells transfected with hRAMP1, hRAMP2 and hRAMP3 cDNAs (provided by Dr David Poyner, Birmingham, UK).

Oxidation of membrane proteins: detection of protein carbonylation

The carbonyl groups in the membrane proteins (5 μg protein per lane) were denatured by addition of 12% SDS and derivatized to 2, 4, dinitrophenylhydrazone by reaction with 2, 4, dinitrophenylhydrazine (DNPH) for 15 min [12, 23] prior to separation by 12% SDS-PAGE and transfer to PVDF membrane (0.45 μm , Millipore, UK). A series of oxidized molecular weight standards was included as positive control. Non-derivatized protein samples served as negative control. The PVDF membrane was washed with phosphate buffered saline (PBS) containing 0.05% vol.vol $^{-1}$ Tween 20 (Sigma, UK) and blocked for 1 hour in PBS/0.05% vol.vol $^{-1}$ Tween 20 solution containing 1% wt.vol $^{-1}$ bovine serum albumin. Immunoblotting was performed using a primary antibody directed specifically against the dinitrophenyl moiety (90451 OxyblotTM -anti-DNP, raised in rabbit, Chemicon International, CA, USA) used at a dilution of 1:150. Immunocomplexes were detected using secondary antibodies conjugated to horseradish peroxidase (goat anti-rabbit 90452- OxyblotTM, Chemicon International, USA)

Fig. 2. Oxidation status of membrane protein from left and right ventricular cardiomyocytes of 20 week old SHR and WKY rat: (a) *oxyblot* of protein carbonyl content of membrane proteins obtained from a representative SHR and WKY; 5 μ g of membrane protein was loaded onto each lane of the gel; non-derivatized protein samples (negative control) and protein samples derivatized by DNPH are indicated; oxidized molecular weight standards are included as a positive control; (b) mean values $\pm$ SE, $n=4$. Immunocomplexes formed within the entire lane for each derivatized protein sample using a primary antibody specific to the DNP moiety were quantified by densitometric analysis (Analytical Imaging System); a similar area within the adjacent lanes containing the non-derivatized protein samples was also quantified as an estimate of background and the value obtained subtracted from those of the matched derivatized samples.

used at a dilution of 1:300, and ECL plus (Amersham Biosciences, UK) as substrate, and quantified by densitometry (Analytical Imaging System).

Quantification of the production of superoxide anion

Release of superoxide anion from intact cardiomyocytes maintained in suspension at 37° C was determined in response to the activator of protein kinase C, phorbol 12 myristoyl 13 acetate (10^{-8} M); the total volume of reaction was 200 μ l. Superoxide release (expressed as relative light units. sec^{-1}) was quantified by enhanced chemiluminescence detection using lucigenin (Sigma-Aldrich, UK) as a luminescent substrate as described previously by Gyllenhammar et al [24] and modified by Skatchkov et al [25]. Superoxide release was measured in duplicate samples and a mean value obtained for each heart cell population.

Data analysis and statistical procedures

Data are expressed as means $\pm$ 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, production of superoxide anion or cell dimensions. Statistical analyses were performed by unpaired Student's t test to detect inter-strain differences.

Results

Confirmation of hypertension and ventricular hypertrophy in SHR

Systolic blood pressure (SBP) was 114 mmHg greater ($P<0.05$) in SHR at 20 weeks of age relative to age-matched WKY rat (Fig.1a). Heart weight: body weight ratio was increased 16.7% ($P<0.05$) in SHR versus WKY rat (Fig.1b). The widths of left and right ventricular cardiomyocytes were increased ($P<0.05$) by 26% and 15%, respectively (Fig.1c) in SHR, while the lengths of left and right ventricular cardiomyocytes were not altered (Fig.1d) relative to those of WKY rat.

Evidence for enhanced myocardial oxidative stress in left ventricle of SHR

Increased myocardial oxidative stress in the left ventricle of SHR was indicated by the greater carbonyl content of membrane proteins in left (71%, Fig 2), but not in right, ventricular cardiomyocytes relative to that of WKY rat. PMA-stimulated production of superoxide anion was enhanced in left ventricular cardiomyocytes

Fig. 3. PMA (10^{-8} M) stimulated production of superoxide anion in suspensions of intact cardiomyocytes from left (a, c) and right (b, d) ventricles of 20 week old SHR and WKY rat: (a, b) representative experiments; (c, d) mean values $\pm$ SE, $n=3$. AUC= area under curve. *variation between strains ($P<0.05$).

(64%, $P<0.05$, Fig 3) but was not altered in right ventricular cardiomyocytes of SHR relative to WKY rat.

Cardio-endocrine gene expression

mRNA expression of ANP (2.7 fold increase, $P<0.05$, Fig.4a), BNP (2.2 fold increase, $P<0.05$, Fig. 4b) and IMD (6.8 fold, $P<0.05$, Fig 4c) was augmented while AM was unchanged (Fig 4d) and NEP was reduced (by 64%, $P<0.05$, Fig.4e) in left ventricular cardiomyocytes of SHRs relative to WKY rats. Expression of ANP (2.7 fold increase, $P<0.05$) and BNP (2.0 fold increase, $P<0.05$) was also increased in right ventricular cardiomyocytes of SHRs relative to WKY rats, but IMD, AM and NEP were unchanged.

Receptor component gene expression

In left ventricular cardiomyocytes, mRNA expression of RAMP1 (2.5 fold, $P<0.05$, Fig. 5a), RAMP3 (2.0 fold, $P<0.05$, Fig.5c) and RCP (1.7 fold, $P<0.05$, Fig. 5e) was greater while that of RAMP2 (Fig. 5b) and CRLR (Fig 5.d) was unchanged in SHR relative to WKY rat. In right ventricular cardiomyocytes, mRNA expression of

RCP was reduced (66% decrease, $P<0.05$) while that of RAMP1, RAMP2, RAMP3 and CRLR was unchanged in SHR relative to WKY rat. The abundance of the RAMP monomers in sarcolemmal membranes obtained from left ventricular cardiomyocytes of SHR was reduced (RAMP1 by 48%, RAMP2 by 41%, RAMP3 by 90%) relative to that of WKY rat (Fig 6).

Discussion

Concentric hypertrophy in response to pressure loading was confirmed in left ventricular cardiomyocytes by increased cardiomyocyte width in SHR at 20 weeks relative to age-matched normotensive WKY. Modest pressure loading is also exerted on the right ventricle of SHR [20]. Although proliferation of non-myocytes contributes to increased ventricular mass this does not occur to a significant extent during the early compensatory response to pressure loading [26]. This could explain why right ventricular hypertrophy is not evident at chamber level in non-failing hearts [21] despite the observation in

Fig. 4. Expression of (a) ANP; (b) BNP; (c) IMD; (d) AM; (e) NEP mRNA in left and right ventricular cardiomyocytes of 20 week old SHR and WKY rat. Data are expressed relative to GAPDH mRNA levels and are the mean values $\pm$ SE, $n=8$. *variation between strains ($P<0.05$).

the present study that right ventricular cardiomyocytes are hypertrophied, albeit to a lesser extent than those in the left ventricle, during early compensation to pressure-loading.

Left (but not right) ventricular cardiomyocytes from SHR hearts experienced greater oxidative stress than their WKY counterparts. This was indicated by the greater extent of modification of membrane proteins by oxygen free radicals, manifest as introduction of carbonyl groups into protein side chains. This finding is in agreement with a previous study that hearts of SHR exhibit greater protein carbonylation than age-matched WKY at 16 weeks [22] and consistent with the observation in the present study that PMA-simulated production of superoxide anion is enhanced in suspensions of left but

not right ventricular cardiomyocytes from SHR relative to those of WKY. Excess generation of reactive oxygen species, in particular superoxide anion, can induce contractile dysfunction and structural damage [27-29] and contribute to cardiomyocyte hypertrophy [30-33].

IMD is expressed at very low levels in healthy adult heart [11]. The robust induction of IMD expression in hypertrophied left ventricular cardiomyocytes of SHR, taken together with the observation that IMD attenuates cardiomyocyte hypertrophy in vitro [8], [Bell and Zhao, unpublished observation] is consistent with a role for the peptide as an endogenous negative feedback regulator of cardiomyocyte hypertrophy. Such counter-regulatory mechanisms are inactive at baseline but are recruited in response to hypertrophic stimuli [reviewed in 34].

Fig. 5. Expression of (a) RAMP1; (b) RAMP2; (c) RAMP3 (d) CRLR; (e) RCP mRNA in left and right ventricular cardiomyocytes of 20 week old SHR and WKY rat. Data are expressed relative to GAPDH mRNA levels and are the mean values + SE, n=8 *variation between strains ($P<0.05$).

AM also attenuates cardiomyocyte hypertrophy *in vitro* [35] and AM gene therapy lowers blood pressure and reduces the extent of cardiac hypertrophy in SHR *in vivo* [36]. However the absence of induction of AM expression in hypertrophied left ventricular cardiomyocytes of SHR is consistent with previous reports that AM expression is not induced in left ventricular tissue in hypertensive SHR prior to or during early onset of cardiac hypertrophy [37-39]. These findings suggest a much more prominent role for IMD than AM locally in the myocardium as an endogenous negative feedback regulator of cardiomyocyte hypertrophy.

In contrast to the selective induction of IMD in the left ventricle, the natriuretic peptides, ANP and BNP, were up-regulated in both left and right ventricular

cardiomyocytes of SHR. Increased ANP and BNP expression in left ventricular cardiomyocytes is considered to be a compensatory response to pressure-loading since these peptides serve to reduce both cardiac preload and after-load by their natriuretic, diuretic and vasodilator action [18]. Augmented expression of these peptides in both chambers of SHR may therefore reflect sensitivity of these genes to the regulatory influence of pressure loading since modest pressure loading is also evident in the right ventricle of SHR [20]. Normalisation of systemic blood pressure in SHR attenuates augmented expression of ANP and BNP in the heart [19]. In contrast, blood pressure normalisation in rats subjected to chronic nitric oxide deficiency does not attenuate the augmented expression of IMD observed in both ventricles in that

Fig. 6. Abundance of (a) RAMP1; (b) RAMP2; (c) RAMP3 monomers in membranes prepared from left ventricular cardiomyocytes of 20 week old SHR and WKY rat. Representative blots are samples obtained from 4 WKY (lanes 1-4) and 4 SHR (lanes 5-8). Data are expressed relative to β -actin levels and are the mean values $\pm$ SE, $n=8$. *variation between strains ($P<0.05$).

model [10]. Furthermore, the robust increase in IMD expression in right ventricular cardiomyocytes of rats subjected to chronic nitric oxide deficiency is likely to have occurred against a background of oxidative stress but little, if any, pulmonary hypertension [40] and natriuretic peptide expression is not up-regulated in right ventricular cardiomyocytes of nitric oxide deficient rats. These findings argue against the involvement of pressure-loading directly in induction of IMD expression in the cardiomyocyte and indicate that this peptide is likely to be recruited in response to different regulatory influences than the natriuretic peptides. Neither IMD nor AM was up-regulated in right ventricular cardiomyocytes. This observation could reflect the less marked hypertrophy of the right ventricle, insufficient to activate negative-feedback regulators. However, it is probable that oxidative stress makes an important contribution to induction of IMD expression since oxidative stress was evident in left, but not right, ventricular cardiomyocytes of SHR. Furthermore, administration of IMD protects the isolated rat heart against the deleterious effects of oxidative stress associated with ischemia-reperfusion injury [9]. Consideration was initially given to undertaking an intervention study with an antioxidant regimen (Vitamin C in combination with Tempol) in the SHR model to test the hypothesis that prevention of augmentation of oxidative stress in SHR hearts would prevent augmented expression of IMD by left ventricular cardiomyocytes; indeed a similar pharmacological approach was recently applied to the model of cardiomyocyte hypertrophy induced by chronic nitric oxide deficiency (administration of L-NAME 35mg/kg/day in normal Sprague-Dawley rats [12]). In that study, augmented cardiomyocyte expression of IMD was normalised by Tempol/Vitamin C in the absence of blood pressure reduction indicating that augmented expression of IMD is likely to be induced directly by myocardial oxidative stress. This approach was not considered suitable however in regard to the SHR model since intervention with antioxidants including Tempol and Vitamin C is associated with blood pressure lowering in SHR [41]; the results of such an intervention would therefore be much more difficult to interpret and the outcome inconclusive in regard to mechanistic insight due to the confounding influence of the antioxidants on blood pressure.

Reactive oxygen species can modify the activity of various signalling kinases implicated in hypertrophic remodelling including protein kinase C, src tyrosine kinase and MAP kinase all of which can exert an influence on gene expression; oxidative stress has also been shown

specifically to regulate expression of various other transcription factors such as NF- κ B, AP-1, hypoxia-inducible factor HIF-1 α and endothelial PAS domain protein 1 (EPAS-1, HIF-2 α) [42]. The AM and CRLR genes are known to possess binding motifs for such factors within their promoter regions [43] and the presence of similar motifs within the promoter region of the IMD gene might account for induction of the latter in cardiomyocytes in response to oxidative stress.

NEP expression was attenuated in left but not right ventricular cardiomyocytes of SHR relative to WKY in agreement with the observation [44] that expression of this enzyme was reduced in left ventricular tissue from SHR at 10 weeks of age. NEP metabolises the natriuretic peptides [5] and has also been implicated in the metabolism of AM (and IMD) [6] although it is possible that more specific enzymatic pathways remain to be identified for AM and IMD. Down regulation of this enzyme would work together with augmented cardiomyocyte expression of ANP, BNP and IMD to increase levels of these counter-regulatory peptides locally in hypertrophied myocardium of SHR.

RAMP1 and RAMP3 mRNA levels were also elevated in left, but not right ventricular cardiomyocytes of SHR relative to WKY. Like that of IMD, induction of the RAMP1 gene is predominantly influenced, as is that of the RAMP3 gene to a lesser extent, by the level of oxidative stress encountered by cardiomyocytes of rats subjected to chronic nitric oxide deficiency [12]. RAMP1 confers CGRP selectivity on CRLR while CGRP also reacts at AM₂ receptors formed by RAMP3/CRLR, albeit with reduced potency [2]. Neuronal release of CGRP is enhanced in response to myocardial ischemia [45] and, like non-neuronal IMD, CGRP exerts a cardio-protective effect against oxidative stress resulting from ischemia-reperfusion injury [46]. Enhanced oxidative stress in left, but not right, cardiomyocytes of SHR could explain the inter-chamber difference in up-regulation of RAMP1 (and RAMP3) mRNA.

Paradoxically, RAMP proteins, particularly the RAMP1 and RAMP3 monomers, were less abundant in cell membranes of left ventricular cardiomyocytes of SHR than WKY. Furthermore, the contractile response to IMD (10⁻⁷ M) is also blunted in hypertrophied left ventricular cardiomyocytes of SHR relative to that of

normotensive WKY rats at 20 weeks consistent with down regulation of IMD receptor number from the cell surface (Bell and Zhao, unpublished observation). Reduced membrane RAMP levels may contribute to, or alternatively arise as a compensatory response to augmented IMD levels. IMD acts non-selectively at all three RAMP-CRLR complexes [2] so it is feasible that persistent elevation of IMD (and neuronal CGRP) in the vicinity of the cardiomyocyte might cause internalisation of cell surface receptors. However, it is worth noting that the tissue distribution of RAMPs extends further than that of CRLR indicating a much broader influence of RAMPs extending beyond alteration of the binding affinity and selectivity of the CRLR for various members of the CGRP/AM peptide family. In membranes of ventricular cardiomyocytes of rats, all three RAMP proteins are present in much greater numbers than CRLR with RAMP2 being most abundant [10, 12]. Amongst novel binding partners for RAMPs are calcium-sensitising receptors [47] and the coupling of VPAC receptors for the secretin/VIP/PACAP family of peptides to G-protein dependant signalling cascades [48]. Indeed secretin and VIP-stimulated adenylate cyclase activity is reduced in cardiac membranes of SHR [49] so it is possible that changes in RAMP levels observed in membranes of cardiomyocytes from SHR are associated with altered regulation of signalling processes in SHR myocardium which are unrelated, or not exclusively related, to the CGRP/AM/IMD peptide family.

In conclusion, the robust induction of IMD, but not AM, in left ventricular cardiomyocytes of SHR indicates that this novel cardio-endocrine peptide may provide an important endogenous mechanism to attenuate hypertrophic remodelling and counter the detrimental consequences of oxidative stress during the early compensatory changes occurring in the myocardium subjected to pressure loading. It is likely that induction of IMD is under different regulatory control to that of the natriuretic peptides.

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