Differential Effects of an Anti-Oxidant Intervention on Cardiomyocyte Expression of Adrenomedullin and Intermedin and their Receptor Components in Chronic Nitric Oxide Deficiency

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Key Words

Peptide hormones • Nitric oxide • Ventricular • Myocytes • Hypertrophy • Oxidative stress

Abstract

Background: Chronic inhibition of nitric oxide (NO) synthesis is associated with hypertension, myocardial oxidative stress and hypertrophic remodeling. Up-regulation of the cardiomyocyte adrenomedullin (AM) / intermedin (IMD) receptor signaling cascade is also apparent in NO-deficient cardiomyocytes: augmented expression of AM and receptor activity modifying proteins RAMP2 and RAMP3 is prevented by blood pressure normalization while that of RAMP1 and intermedin (IMD) is not, indicating that the latter is regulated by a pressure-independent mechanism. **Aims:** to verify the ability of an anti-oxidant intervention to normalize cardiomyocyte oxidant status and to investigate the influence of such an intervention on expression of AM, IMD and their receptor components in NO-deficient cardiomyocytes. **Methods:** NO synthesis inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 35mg/kg/day) was given to rats for 8 weeks,

with/without con-current administration of antioxidants (Vitamin C (25mg/kg/day) and Tempol (25mg/kg/day)). **Results:** In left ventricular cardiomyocytes isolated from L-NAME treated rats, increased oxidative stress was indicated by augmented (3.6 fold) membrane protein oxidation, enhanced expression of catalytic and regulatory subunits of pro-oxidant NADPH oxidases (NOX1, NOX2) and compensatory increases in expression of anti-oxidant glutathione peroxidase and Cu/Zn superoxide dismutases (SOD1, SOD3). Vitamin C plus Tempol did not reduce systolic blood pressure but normalized augmented plasma levels of IMD, but not of AM, and in cardiomyocytes: (i) abolished increased membrane protein oxidation; (ii) normalized augmented expression of prepro-IMD and RAMP1, but not prepro-AM, RAMP2 and RAMP3; (iii) attenuated (by 42%) increased width and normalized expression of hypertrophic markers, skeletal- α -actin and prepro-endothelin-1 similarly to blood pressure normalization but in contrast to blood pressure normalization did not attenuate augmented brain natriuretic peptide (BNP) expression. **Conclusion:** normalization specifically of augmented IMD/RAMP1

expression in NO-deficient cardiomyocytes by antioxidant intervention in the absence of blood pressure reduction indicates that these genes are likely to be induced directly by myocardial oxidative stress. Although oxidative stress contributed to cardiomyocyte hypertrophy, induction of IMD and RAMP1 is unlikely to be secondary to cardiomyocyte hypertrophy.

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Introduction

Excess generation of reactive oxygen species (ROS), including superoxide anion ($O_2^{\cdot -}$) catalysed by enzymes such as NADPH oxidase, can induce vascular and myocardial structural damage and result in a hypertrophic phenotype [1]. Oxidative modification of proteins represents one of the major targets for ROS [2]. The healthy myocardium is equipped with various endogenous antioxidant systems that are sufficient to metabolize ROS generated during normal cellular activity. In particular, dismutation of $O_2^{\cdot -}$ to H_2O_2 by superoxide dismutases (SOD) and degradation of H_2O_2 by glutathione peroxidase (GPx) and catalase limit the cytotoxic effects of ROS. During pathological conditions including hypertension and ischemic heart disease the balance between the generation of ROS and antioxidant enzyme activities may shift in favour of a relative increase in ROS levels resulting in increased oxidative stress [3]. Nitric oxide (NO) generated within normal myocardium and vasculature by constitutive NO synthases also plays a pivotal role in reduction of myocardial oxygen consumption and blood pressure [4], quenches ROS-induced injury [5] and exerts a regulatory influence on contractility and growth directly on cardiomyocytes [6].

NO levels are reduced in the plasma of hypertensive patients with left ventricular hypertrophy (LVH) [7]. NO-deficient hypertension induced by treatment of rats with the NOS inhibitor, N^G -nitro-L-arginine methyl ester (L-NAME) is characterised in myocardium by hypertrophy, fibrosis, ischemia and necrosis [8]. In left ventricular cardiomyocytes, general enhancement of protein synthesis and mass is observed, together with increased cell width, and phenotypic alterations, manifest as up-regulation of contractile genes such as skeletal α -actin and cardio-endocrine genes including the natriuretic peptides and endothelin-1 [9]. These phenotypic changes are completely normalized by con-current administration of blood pressure lowering agents in contrast to cell width which is only partially attenuated indicative of an additional

pressure-independent regulatory influence on cardiomyocyte hypertrophy [10].

Intermedin (IMD, adrenomedullin-2) is a recently discovered 47amino acid vasodilator peptide [11, 12] of the calcitonin gene-related peptide (CGRP) family, which also includes adrenomedullin (AM). Neutral endopeptidase (NEP), a membrane bound metallo-peptidase, has been implicated in the degradation of AM [13]. On account its close structural similarities, it is likely that IMD is also a substrate for NEP. AM has two receptors (AM_1 , AM_2) formed by the calcitonin receptor-like receptor (CRLR) combined with receptor activity modifying proteins (RAMP) 2 or 3, respectively [14]. AM also has some affinity for CGRP₁ receptors, composed of CRLR and RAMP1. Intermedin acts non-selectively at all three RAMP-CRLR complexes [11]. The (patho) physiological significance of IMD has not been extensively characterized: IMD enhances vascular NO production [15] and protects vascular endothelium from oxidative damage [16]. Furthermore, in the heart a positive inotropic effect [17], attenuation of cardiomyocyte hypertrophy [18] [Bell et al, unpublished observation] and protection against the deleterious effects of oxidative stress associated with ischemia-reperfusion injury [19, 20] are amongst effects which have recently been demonstrated. The robust increase in prepro-IMD expression in left ventricular cardiomyocytes [10] indicates an important role for the peptide in the cardiac pathology produced by NO deficiency. Up-regulation of AM and receptor components, RAMP2 and RAMP3, in cardiomyocytes of rats subjected to chronic NO-deficiency is prevented by blood pressure normalization while that of RAMP1 and prepro-IMD is not reduced significantly, indicating that these genes might be regulated concordantly by a predominantly pressure-independent mechanism [10]. Our hypothesis is that, unlike that of prepro-AM, RAMP2 and RAMP3, which are influenced by pressure-loading, expression of prepro-IMD and RAMP1 genes is induced selectively by increased oxidative stress within the myocardium and can be prevented by normalization of such oxidative stress.

Building on the previous investigation of the influence of blood pressure lowering on parameters of cardiomyocyte hypertrophy and on expression of the cardiomyocyte AM/IMD receptor system in NO-deficiency [10], the purpose of the present study therefore was to examine the effects of an anti-oxidant intervention specifically on expression of AM and IMD, their receptor components and on NEP, implicated in metabolism of these peptides. The chosen intervention was Vitamin C,

an antioxidant known to inhibit NADPH oxidase activity [21] given con-currently with Tempol, a membrane-permeable, metal-free SOD mimetic which scavenges O_2^- [22, 23]. The dual approach was designed to simultaneously reduce O_2^- generation and to scavenge any O_2^- present. This antioxidant combination was considered superior to using one agent in isolation to neutralize the influence of O_2^- . Cardiomyocyte membrane protein oxidation status was measured to confirm the efficacy of the antioxidant combination to ameliorate oxidative stress. Systolic blood pressure was assessed to confirm that the effects of the anti-oxidants were exerted directly on the heart and were independent of an attenuating influence on L-NAME induced increase in blood pressure. The effects of anti-oxidant intervention on parameters of cardiomyocyte hypertrophy – cell width, up-regulation of the hypertrophic marker, α -actin, and the neurohormones, BNP and ET-1 – were also assessed. This enabled comparison with the previously reported effects of blood pressure normalization [10] to determine the contribution of oxidative stress to cardiomyocyte hypertrophy. The possibility that any influence of oxidative stress on expression of components of the AM/IMD receptor signaling cascade might arise secondary to the influence of oxidative stress on the development of cardiomyocyte hypertrophy could therefore be excluded.

Material and 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) 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl, TPL (Tempol 25mg/kg/day, Sigma Aldrich, UK) plus Vitamin C (25mg/kg/day, Boots Group PLC, UK) in drinking water; (iii) L-NAME (35mg/kg/day) currently with Tempol (25mg/kg/day) plus Vitamin C (25mg/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. Tap water was autoclaved prior to use and Vitamin C solutions were contained in opaque drinking bottles fitted with plastic rather than metal tops to maintain stability. Systolic blood pressure (SBP) was determined by tail cuff sphygmomanometer (Harvard Instruments) [9] and the mean of four consecutive blood pressure readings was obtained for each animal weekly. Body weight was recorded weekly and water consumption daily in order to tailor drug consumption accurately to each individual animal.

Cardiomyocyte isolation

Following deep anesthesia of the rats using isoflurane (Abbott Laboratories, UK), the hearts were rapidly excised and placed in ice-cold saline. Excised hearts were cannulated through the ascending aorta, and preparations of cardiomyocytes were isolated from the left ventricles by enzymatic digestion (collagenase, 0.4mg/ml, Serva, Germany) using Langendorff perfusion [9]. 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-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), penicillin (100 IU/ml) and streptomycin (100 µg/ml).

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 as described previously [10], using the housekeeping gene, GAPDH as an internal standard.

Preparation of membrane protein, Immunodetection and Quantification

Plasma membranes were prepared from freshly isolated cardiomyocytes as described previously [10] and stored at -70°C pending analysis. 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_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; CRLR 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 against specific sequences common to human and rodent CRLR (Santa Cruz Biotechnology, sc-18007 raised in goat) or to human 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 (CRLR). Immunocomplexes were detected using secondary antibodies conjugated to horseradish peroxidase (goat anti-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 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 the specificity of the RAMP and CRLR

	Forward	Backward
AM	CATTGAACAGTCGGGCGAGTA	GGTGCGAAGCTCTCTGATTCC
BC061775	21bp [739-759]	21bp [776-796]
IMD	GGCCCAAGTTGCTGATGGT	TGCCCCGGGAGCAGGTA
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]
CRLR	CTCTACATGAAAGCTGTAAGAGCCACTC	CCTGATAGTGCATGAG AATGTGCATGAC
CAA49997	28bp [988-1015]	28bp [1102-1129]
CTa	GTCCGTGTGCTTGTGACCAA	GCCTCCAGGGAACACAACA
L14617.1	20bp [1199-1218]	20bp [1309-1328]
CTb	CATCTGCCATCAGGAACCAA	CGGATGAGTCTTGCTGGATGA
L14618.1	20bp [1689-1708]	21bp [1767-1787]
GAPDH	GAAACCCATCACCATCTTCCA	ACCCCATTTGATGTTAGCGG
AB017801	21bp [248-268]	20bp [280-299]
ET-1	TGCTCCTCCTTGATGGACAAG	TGATGTCCAGGTGGCAGAAGT
M64711	21bp [347-367]	21bp [378-398]
Skeletal α -Actin	AAGGATTCTACGTGGGCG	GGTCAGGATACCTCGCTTGCT
BC061974	19bp [300-318]	21bp [247-267]
BNP	AGGTGCTGCCCCAGATGAT	GCGGCGACAGATTAAGGAAA
M25297	19bp [71-89]	20bp [102-121]
NEP	CAA GAG GAG GAA GAC GCT GAA	CCT GAA GGC CAC AGT ATG CAT
BC085753	21bp [2719-2739]	21bp [2831-2851]
SOD1	TGT GTC CAT TGA AGA TCG TGT GA	TCT TGT TTC TCG TGG ACC ACC
NM-017050	23bp [384-406]	21bp [447-467]
SOD3	GAG TCC GGT GTC GAC TTA GCA	CCT CCC GTT GTT TTC CTA GCT
X94371	21bp [177-197]	21bp [262-282]
GPx	GGT GTT CCA GTG CGC AGA TA	CAT CAC CAA GCC CAG ATA CCA
S50336	20bp [1605-1624]	21bp [1709-1729]
NOX1	GAG CCA CTG ACG TCC TGA CA	AAA CCC CCA CCA CAG ACT TG
NM053683	20bp [1596-1615]	20bp [1690-1709]
NOX2/gp91	CTG GGA TGA ATC TCA GGC CA	TTC TCC TCA TCG TGG TGC AC
AF298656	20bp [1446-1465]	20bp [1477-1496]
p47phox	TGG TGG TTT TGC CAG ATG AA	AAT AGG ATG CAG GGA CCC AAC
AY029167	20bp [591-610]	21bp [622-642]
p22phox	TGT GAC CTT CAG TGG CTC CTG	CCT CTG GGA GCA ACA CCT TG
AJ295951	21bp [605-625]	21bp [636-655]
p67phox	GCT TCG GAA CAT GGT GTC TAA GA	AGA GTC AGG CAG TAG TTT TTC ACT
AB002664	23bp [1215-1237]	TG 26bp [1339-1364]

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*.

antibodies used to detect the RAMP monomers and CRLR protein was confirmed by comparison with immunoblots of membrane samples prepared from Cos7 cells transfected with hRAMP1, hRAMP2, hRAMP3 and hCRLR cDNAs (obtained from Dr David Poyner, Birmingham, UK) and rCRLR cDNA (obtained from Dr Walter Born, Zurich, Switzerland).

Oxidation of membrane proteins

The carbonyl groups in the membrane proteins (5 μ g protein per lane) were derivatized to 2, 4, dinitrophenylhydrazone by reaction with 2, 4, dinitrophenylhydrazine prior to separation by 12% SDS-PAGE and transfer to PVDF

membrane (0.45 μ m, Millipore, UK) as above. Non-derivatized protein samples served as negative control. The PVDF membrane was washed with phosphate buffered saline (PBS) containing 0.05% v/v Tween 20 (Sigma, UK) and blocked for 1 hour in PBS/0.05% v/v Tween 20 solution containing 1% w/v bovine serum albumin. Immunoblotting was performed using a primary antibody directed specifically against the dinitrophenyl moiety (90451 Oxyblot™ -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-Oxyblot™, Chemicon International, USA) used at a dilution

Fig. 1. (a) representative oxyblot; (b) effect on oxidation status of left ventricular cardiomyocyte membrane protein of administration of (i) L-NAME (35mg/kg/day), [n=8]; (ii) Tempol (25mg/kg/day) plus Vitamin C (25mg/kg/day), [n=4]; (iii) L-NAME (35mg/kg/day) concurrently with Tempol (25mg/kg/day) plus Vitamin C (25mg/kg/day) [n=4] for 8 weeks; (iv) age-matched untreated rats are included for comparison, [n=8]. 5 µg of membrane protein was loaded onto each lane of the gel; data are mean values + SE. Statistical analysis was performed by one way analysis of variance (ANOVA) to detect significant differences between treatment groups; Levene's test was applied to determine homogeneity of variance between groups. Since ANOVA indicated a significant outcome ($P<0.05$), the method of contrasts was applied to identify (1) difference between untreated control group and L-NAME only treatment group; * $P<0.05$ (2) difference between L-NAME only treatment group and L-NAME plus antioxidant treatment group; + $P<0.05$.

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

Peptide Radioimmunoassay

Blood was collected into chilled lavender vacutainer tubes containing EDTA and aprotinin (0.6 TIU/ml blood). Tubes were rocked gently several times immediately after collection of blood to optimize anti-coagulation and inhibition of proteinases, and centrifuged at 4°C for 20 minutes at 1600xg. The plasma was subsequently transferred to polypropylene tubes containing aprotinin (0.6 TIU / ml plasma) and stored at -70°C pending analysis. Plasma was acidified, centrifuged at 12000xg for 20 minutes at 4°C and the supernatant applied into pre-equilibrated C18 SEP columns, eluted into polypropylene tubes and evaporated to dryness using a combination of centrifugal concentration and lyophilisation. The resultant residue was reconstituted and assayed using commercially available radio-immunoassays for rat AM and rat IMD, respectively (Phoenix Pharmaceuticals Inc., California, USA).

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 and a mean value obtained.

Data analysis

Data are expressed as means ± SE where n denotes number of rats in which systolic blood pressure or plasma peptide levels were measured, or number of heart cell preparations used to analyze mRNA and protein expression levels or cell dimensions. Statistical analysis was performed using SPSS (version 14.0, SPSS Inc, Chicago, Illinois) by one way analysis of variance (ANOVA) to detect significant differences between treatment groups; Levene's test was applied to determine homogeneity of variance between groups. For significant outcomes by ANOVA ($P<0.05$), the method of contrasts was then applied to identify (1) difference between control group and L-NAME-treated group; (2) difference between L-NAME treated group and L-NAME + antioxidant treated group.

Results

Evidence for Cardiomyocyte Oxidative Stress

In left ventricular cardiomyocytes from L-NAME treated rats, increased oxidative stress was indicated by augmented (3.6 fold vs. control, Fig 1) membrane protein oxidation which was abolished by Vitamin C plus Tempol. mRNA expression of the pro-oxidant NADPH oxidases

Fig. 2. Effect on (a) prepro-AM; (b) prepro-IMD; (c) NEP; (d) CRLR; (e) RAMP1; (f) RAMP2; (g) RAMP3 mRNA expression in left ventricular cardiomyocytes of administration of (i) L-NAME (35mg/kg/day), [n=9]; (ii) Tempol (25mg/kg/day) plus Vitamin C (25mg/kg/day), [n=3]; (iii) L-NAME (35mg/kg/day) concurrently with Tempol (25mg/kg/day) plus Vitamin C (25mg/kg/day) [n=4] for 8 weeks; (iv) age-matched untreated rats are included for comparison, [n=9]. Data were expressed relative to GAPDH mRNA levels and are given as the mean values + SE. Statistical analysis was performed by one way analysis of variance (ANOVA) to detect significant differences between treatment groups; Levene's test was applied to determine homogeneity of variance between groups. Since ANOVA indicated a significant outcome ($P<0.05$) in each dataset (a-g), the method of contrasts was applied to identify (1) difference between untreated control group and L-NAME only treatment group; $*P<0.05$ (2) difference between L-NAME only treatment group and L-NAME plus antioxidant treatment group; $+P<0.05$.

(NOX1 5.1 fold and NOX2 3.7 fold vs. control, data not shown) and their associated subunits (p22phox, 3.9 fold, p47phox 1.9 fold and p67phox 3.3 fold vs. control, data not shown) was also augmented ($P<0.05$).

A compensatory increase in mRNA expression ($P<0.05$) of the counter-regulatory antioxidant enzymes, Cu/Zn SOD (SOD1 1.7 fold, SOD3 3.1 fold vs. control, data not shown) and GPx (2.6 fold vs. control, data not shown)

was also observed in cardiomyocytes in response to chronic NO-deficiency induced by L-NAME.

Adrenomedullin, Intermedin and their Receptor Component Genes

Expression of prepro-AM (1.8 fold vs. control, Fig. 2a) and prepro-IMD mRNA (8.2 fold vs. control, Fig.

2b) was increased in left ventricular cardiomyocytes following treatment with L-NAME. The L-NAME induced increase in expression of prepro-IMD mRNA was normalized by anti-oxidant intervention, while the L-NAME-induced increase of prepro-AM was not attenuated. mRNA expression of NEP, an enzyme implicated in metabolism of these peptides, was enhanced,

Fig. 4. Effect on systolic blood pressure of administration to 8 week old rats of (i) L-NAME (35mg/kg/day), [n=10], (ii) Tempol (25mg/kg/day) plus Vitamin C (25mg/kg/day), [n=3] (iii) L-NAME (35mg/kg/day) concurrently with Tempol (25mg/kg/day) plus Vitamin C (25mg/kg/day) [n=4] for 8 weeks; (iv) age-matched untreated rats are included for comparison, [n=12]. Data are given as the mean values+ SE. Statistical analysis was performed at each time point by one way analysis of variance (ANOVA) to detect significant differences between treatment groups; Levene's test was applied to determine homogeneity of variance between groups. Since ANOVA indicated a significant outcome ($P<0.05$) in each of the datasets from 9-15 weeks (but not at 8 weeks), the method of contrasts was applied to these datasets to identify (1) difference between untreated control group and L-NAME only treatment group; * $P<0.05$ (2) difference between L-NAME only treatment group and L-NAME plus antioxidant treatment group; + $P<0.05$.

although not significantly so, following treatment with L-NAME (3.2 fold vs. control, Fig 2c); this L-NAME-induced increase was normalized by anti-oxidant intervention. Expression of CRLR (1.6 fold vs. control, Fig. 2d), RAMP1 (4.1 fold vs. control, Fig. 2e), RAMP2 (2.6 fold vs. control, Fig. 2f) and RAMP3 (4.1 fold vs. control, Fig. 2g) mRNAs was also increased in left ventricular cardiomyocytes by L-NAME treatment. L-NAME-induced increase in expression of RAMP1, but not of RAMP2 and RAMP3 mRNA, was attenuated by concurrent administration of anti-oxidants. Calcitonin receptor CTa (>30 times less abundant than CRLR) and CTb mRNAs (>60 times less abundant than CRLR) were detected at very low levels in cardiomyocytes in both L-NAME treated and age-matched control rats (data not shown).

The plasma levels of the peptides were augmented in L-NAME treated rats, by 1.4 fold (Fig 3a) and 2.9 fold (Fig 3b) greater than control values for AM and IMD, respectively. Con-current administration of Vitamin C plus Tempol abolished the L-NAME-induced increase in IMD and attenuated L-NAME induced increase in AM level; however AM levels still remained greater ($P<0.05$) than

control values. At protein level treatment with L-NAME increased expression of the RAMP3 monomer (2.4 fold, Fig. 3e) and, to a lesser extent, RAMP1 monomer (1.6 fold, Fig. 3c) and RAMP2 monomer (1.6 fold, Fig 3d) within left ventricular cardiomyocytes. Enhanced expression of the RAMP1 protein was abolished by concurrent administration of anti-oxidants, while that of RAMP2 and RAMP3 was not attenuated significantly. As reported previously [10], treatment with L-NAME did not increase expression of CRLR protein, standardized to β -actin levels (L-NAME: 0.13 ± 0.02 vs. control: 0.11 ± 0.02 mean $\pm$ SE, $n=10$) significantly within left ventricular cardiomyocytes.

Hypertension, Pressure-Loading and Cardiomyocyte Hypertrophy

Systolic blood pressure (SBP) was 96 mmHg greater ($P<0.05$) in rats treated with L-NAME (35mg/kg/day) for 8 weeks relative to age-matched control values. Tempol and Vitamin C together did not influence blood pressure alone or the hypertensive effect of L-NAME (Fig.4). The widths of left ventricular cardiomyocytes were increased ($P<0.05$) by 19.9% (vs. control) following treatment with

Fig. 5. Effect on (a) cell width; (b) sk- α -actin; (c) prepro-ET-1; (d) BNP mRNA expression in left ventricular cardiomyocytes of administration to 8 week old rats of (i) L-NAME (35mg/kg/day), [n=10], (ii) Tempol (25mg/kg/day) plus Vitamin C (25mg/kg/day), [n=3] (iii) L-NAME (35mg/kg/day) concurrently with Tempol (25mg/kg/day) plus Vitamin C (25mg/kg/day) [n=4] for 8 weeks; (iv) age-matched untreated rats are included for comparison, [n=12]. Data are given as the mean values $\pm$ SE. In (a), 20 cells were measured within each heart cell preparation. In (b-d) data were expressed relative to GAPDH mRNA. Statistical analysis was performed by one way analysis of variance (ANOVA) to detect significant differences between treatment groups; Levene's test was applied to determine homogeneity of variance between groups. Since ANOVA indicated a significant outcome ($P<0.05$) in each of the datasets (a-d), the method of contrasts was applied to identify (1) difference between untreated control group and L-NAME only treatment group; * $P<0.05$ (2) difference between L-NAME only treatment group and L-NAME plus antioxidant treatment group; + $P<0.05$.

L-NAME (Fig. 5a). L-NAME induced increased width was attenuated (by 42%, $P<0.05$) by anti-oxidant therapy. The lengths of the cardiomyocytes were not altered following treatment with L-NAME (data not shown). mRNA expression levels of the hypertrophic marker gene, skeletal α -actin (3.5 fold vs. control, Fig. 5b) and of the neurohormones, ET-1 (2.1 fold vs. control, Fig. 5c) and BNP (3.3 fold vs. control, Fig. 5d) were increased ($P<0.05$) in left ventricular cardiomyocytes following treatment with L-NAME. Increased expression of sk- α -actin and prepro-ET-1 in response to L-NAME was normalized by anti-oxidant intervention while that of BNP was not attenuated.

Discussion

Amongst emerging roles for the novel peptide, IMD, is the ability to attenuate cardiac hypertrophy [18] and to protect the heart muscle against the deleterious effects of oxidative stress associated with ischemia-reperfusion injury [19, 20]. Chronic NO deficiency in rats is associated with systemic hypertension, myocardial hypertrophy, fibrosis, ischemia and necrosis [8]. The present study has indicated that the robust increase in expression of IMD and of the receptor component, RAMP1, within cardiomyocytes from rats subjected to chronic NO deficiency can be prevented by con-current admi-

nistration of exogenous anti-oxidants. These data support a specific causal association between increased oxidative stress and induction of this cardio-protective peptide, acting via CGRP₁ receptors.

Oxidative stress in NO-impaired left ventricular cardiomyocytes was evidenced in the present study firstly by the extent of oxidative modification of membrane proteins. This is in agreement with previous studies in which oxidative stress was indicated by increased protein carbonyl content, malondialdehyde content and depletion of GSH in the hearts of chronic NO-deficient rats [24, 25]. A possible source of ROS required to cause this oxidation is likely to be provided by increased generation of O₂⁻ by membrane associated NADPH oxidase since expression of pro-oxidant NOX1 and NOX2/gp91 and of the tightly associated membrane protein p22phox and regulatory subunits p47phox and p67phox was also enhanced. Secondly, expression of anti-oxidant Cu/Zn SOD (SOD1 and SOD3) and GPx was enhanced; this is likely to represent an adaptive defense mechanism to help to offset increasing oxidative stress since SOD promotes dismutation of O₂⁻ to less reactive H₂O₂ which is subsequently degraded by GPx. In agreement with these mRNA expression data, CuZn SOD activity (representing SOD1 and SOD3) has been found to be enhanced in the myocardium of L-NAME treated rats [26]. Con-current treatment with Tempol and Vitamin C reduced the oxidative stress burden upon the myocardium of the NO-deficient rats as evidenced by normalization of cardiomyocyte membrane protein oxidation status. Furthermore, this beneficial influence of Tempol and Vitamin C was attributed to a direct action locally within the heart rather than a reduction in pressure-loading since the L-NAME associated increase in systolic blood pressure was not attenuated by antioxidant therapy. A causal association between increased generation of O₂⁻ within the vasculature and development of hypertension has been proposed both in SHR_s [27] and in patients with primary hypertension [28]. Inactivation of NO by O₂⁻ and concomitant generation of ONOO⁻ may result in reduced bioavailability of NO for vasodilatation [23, 29]. These antioxidants might normally be expected therefore to quench O₂⁻ and to protect NO from interaction with O₂⁻ [23, 29, 30, 31]. NO bioavailability is unlikely to be enhanced significantly however due to the already profound inhibition of vascular NO synthesis as a consequence of chronic administration of L-NAME [8]. Indeed, chronic administration of L-NAME has been shown to abolish the ability of Tempol to lower blood pressure in SHR_s [32].

Up-regulation of prepro-AM and receptor components, RAMP2 and RAMP3, in NO-deficient left ventricular cardiomyocytes was not prevented by antioxidant therapy in agreement with previous findings that these genes are regulated concordantly by a pressure-dependent mechanism [10]. In contrast, L-NAME induced expression of prepro-IMD and of RAMP1 in LV cardiomyocytes was ameliorated by anti-oxidant therapy; these genes had previously been shown not to be influenced by blood pressure reduction [10]. Furthermore, IMD levels were increased markedly in plasma of L-NAME treated rats and these were also normalized by anti-oxidant intervention. The more robust increase in prepro-IMD expression than that of prepro-AM in ventricular cardiomyocytes indicates an important role for IMD in the cardiac pathology resulting from NO deficiency, characterised by hypertrophy, fibrosis, ischemia and necrosis [8]. IMD enhances L-arginine cellular transport, NOS activity and NO production in rat aortas [15]. A similar action is feasible in cardiomyocytes and might represent one of a number of compensatory responses recruited in response to chronic NO-deficiency. Recent reports of cardio-protective effects of IMD against ischemia-reperfusion injury, indicated by reduced myocardial leakage of lactate dehydrogenase and decreased membrane lipid peroxidation [19, 20, 33] support the hypothesis that release of this peptide represents an adaptive response to oxidative stress. Although AM has high affinity for AM₁ receptors (formed by CRLR in combination with RAMP2) and for AM₂ receptors (formed by CRLR in combination with RAMP3), AM₂ receptors are less selective for AM over other peptides of this family than AM₁ receptors [34, 35], displaying only slightly lesser affinity for CGRP and IMD [36, 37]. Furthermore, IMD (and AM) can also interact to an extent with CGRP₁ receptors formed by the interaction of CRLR with RAMP1 [14]. Neuronal release of CGRP is also enhanced in response to myocardial ischemia [38] and exerts direct cardio-protective effects against oxidative stress resulting from ischemia-reperfusion injury [39]. Enhanced levels of non-neuronally derived IMD acting on CGRP₁ receptors, may, like neuronal CGRP, serve primarily an anti-ischemic, cardio-protective function. An additional attenuating influence for IMD on cardiomyocyte hypertrophy, mediated by stimulation of the AM₂ receptor cannot be discounted, particularly since RAMP3 expression is also enhanced and IMD is known to interact at AM₂ receptors [11]. Furthermore, IMD like AM [9] can attenuate cardiomyocyte hypertrophy in vitro [18] [Bell et al,

unpublished observation] at concentrations similar to those detected in plasma of NO-deficient rats.

In addition to serving as receptors for calcitonin, the CT receptor protein can also interact with each of the RAMPs [40]. Such interaction confers a variable degree of affinity for the peptide amylin (AMY), which displays some structural similarity to the CGRP/AM/IMD peptides. IMD, like CGRP, possesses limited affinity for AMY1a receptors, formed from CTa and RAMP1, and markedly less affinity for the other AMY receptor subtypes. Although AMY influences contractility of cardiomyocytes, this effect is most probably mediated via an interaction at CGRP₁ receptors [41]. CT mRNAs were detected at very low levels in cardiomyocytes and were not increased following treatment with L-NAME (Bell et al., unpublished observation), indicating that CT and AMY receptors are unlikely to make a significant contribution to the cardiac pathology of NO-deficiency. CRLR expression was not increased as markedly as that of each of the known RAMPs. However there is currently no evidence for existence of additional IMD-specific receptor proteins with which elevated IMD levels could interact [42]. The possibility that changes in RAMP expression levels, independent of changes in CRLR protein, could reflect other roles of the RAMPs in cardiomyocytes, such as interaction with VPAC1 [43] or calcium-sensitizing receptors [44] cannot be discounted at present. It is probable however that alteration in regard to RAMP expression influence linkage specifically to regulatory events related to the CGRP/AM/IMD peptide family and CRLR-effector coupling mechanisms during the cardiac pathophysiology of NO-deficiency especially since RAMP1 and IMD were regulated in a concordant fashion.

Indeed, autocrine/paracrine factors and their receptors are often regulated in a coordinated fashion as observed for IMD and RAMP1 in the present study. Parallel changes at mRNA and protein level for RAMPs and CL within NO-deficient cardiomyocytes indicate that the expression of the AM/IMD receptor signaling cascade is predominantly regulated at transcriptional level. Attenuation of the augmented expression of RAMP1 and IMD in the NO-deficient cardiomyocyte by Tempol and Vitamin C supports the involvement of ROS such as O₂⁻, likely to be derived at least in part via the action of NADPH oxidase, in regulating the expression of these components of the signaling cascade. ROS can modify the activity of various signaling kinases implicated in hypertrophic remodeling 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) [45]. The AM and CRLR genes are known to possess binding motifs for one or more of these factors within their promoter regions [46], and the presence of similar motifs within the promoter regions of the IMD and RAMP1 genes could possibly account for induction of these genes within cardiomyocytes in response to oxidative stress. Pressure overload and oxidative stress both contribute to cardiomyocyte hypertrophy in the left ventricle of NO-deficient rats since antioxidants (this study) and blood pressure reduction [10] each partially, but incompletely, attenuated increased cardiomyocyte width. Furthermore, L-NAME induced expression of the hypertrophic marker sk- α -actin was attenuated by either antioxidants (this study) or blood pressure lowering agents [10]. In contrast, natriuretic peptide expression, an adaptive response of the left ventricle to increased stretch caused by pressure-loading [10], was regulated independently of oxidative stress, underlining the highly specific influence of the antioxidants on expression of individual genes in cardiomyocytes. Mechanical stretch is known to stimulate production of endothelin-1 [47] resulting in a hypertrophic phenotype. The endothelin-1 gene also contains an oxidative stress responsive element [48, 49]. Furthermore, endothelin-1 enhances NADPH oxidase activity in cardiomyocytes [4] and ET_A receptor antagonism attenuates cardiomyocyte hypertrophy in response to hypoxia [50]. Normalization by antioxidants (this study) and by blood pressure lowering [10] of the marked induction of preproET-1 expression in NO-deficient left ventricular cardiomyocytes underlines the complexity of the interactions between mechanical stretch, oxidative stress and cardio-endocrine signaling peptides in the elaboration of cardiomyocyte hypertrophy. Hydralazine and hydrochlorothiazide were unable to attenuate IMD and RAMP1 expression [10], despite attenuation of cardiomyocyte hypertrophic parameters (cell width, sk- α -actin mRNA) similarly to Tempol and Vitamin C. This observation strongly supports a direct influence of oxidative stress upon expression of IMD and RAMP1, independent of any indirect influence associated with the contribution of oxidative stress to development of cardiomyocyte hypertrophy. Similarly, attenuation of L-NAME induced AM and RAMP2 and RAMP3 expression by blood pressure normalization [10] but not by antioxidant therapy indicates that pressure-loading

exerts a direct influence on expression of these genes independently of any ability to prevent cardiomyocyte hypertrophy.

It should be acknowledged that the impact of such expressional changes for cell signaling within the myocardium of NO-deficient rats might be tempered in vivo by two further observations. Firstly, since RAMP2 is already the predominant RAMP present on the surface of cardiomyocytes under normal physiological conditions, and all three RAMPs are up-regulated to some extent in chronic NO-deficiency, the functional implications of changes observed in NO-deficient cardiomyocytes may be modest. Secondly, altered cell signaling would depend critically on the concentrations of the peptides in the local environment of the cardiomyocytes. NEP, which cleaves peptides endogenously at the amino side of hydrophilic residues [51], is an important enzyme implicated in the degradation of both the natriuretic peptides, ANP and BNP, and of AM [13, 52]. On account of its close structural similarities, it is likely that IMD is also a substrate for NEP. NEP mRNA expression was augmented markedly in left ventricular cardiomyocytes of L-NAME treated rats, in which increases in ANP/BNP and AM/IMD mRNA expression were observed. This finding might represent a compensatory feedback response to counter excessive production and thereby limit the actions of these peptides locally. Anti-oxidant therapy (this study) and blood pressure lowering [10] each normalized expression of NEP mRNA levels in left ventricular cardiomyocytes while

only blood pressure lowering normalized that of ANP and BNP and of AM and only antioxidant therapy normalized that of IMD. Pressure loading and oxidative stress, acting either independently or in concert would appear therefore to influence the expression of the NEP gene in left ventricular cells either directly or secondary to enhanced production of these various cardio-endocrine peptides. Enhanced expression of NEP would oppose the robust increase in IMD levels locally within the myocardium.

In conclusion, this study extends our earlier findings indicating differential regulation of components of the cardiomyocyte AM/IMD signaling cascade during chronic NO-deficiency and is compatible with emerging evidence of the cardio-protective actions of IMD [19, 20, 33] against ischemia-reperfusion injury. In contrast to AM and the receptor components, RAMP2 and RAMP3, which are strongly influenced by pressure loading [10], expression of IMD and RAMP1 is regulated by the extent of myocardial oxidative stress. Intervention with antioxidant therapy reduced the stimulus driving up-regulated expression of this endogenous cardio-protective pathway.

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