Influence of Atenolol and Nifedipine on Nitric-Oxide Deficient Cardiomyocyte Hypertrophy and Expression of the Cardio-Endocrine Peptide Intermedin and its Receptor Components

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Key Words

Ventricular • Oxidative stress • Cardiac peptides • RAMPs • Hypertension • Intermedin • Adrenomedullin • Myocardial ischemia • Cardiomyocytes • Hypertrophy

Abstract

Background /aims: Chronic inhibition of nitric oxide (NO) synthesis is associated with hypertension, myocardial ischemia, oxidative stress and hypertrophy; expression of adrenomedullin (AM) and intermedin (IMD) and their receptor activity modifying proteins (RAMPs 1-3) is augmented in cardiomyocytes, indicating that the myocardial AM/IMD system may be activated in response to pressure loading and ischemic insult. The aim was to examine effects on (i) parameters of cardiomyocyte hypertrophy and on (ii) expression of AM and IMD and their receptor components in NO-deficient cardiomyocytes of an intervention chosen specifically for ability to alleviate pressure loading and ischemic injury concurrently. **Methods:** The NO synthesis inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 35mg.kg⁻¹.day⁻¹) was given to rats for 8 weeks, with/

without concurrent administration of β -adrenoceptor antagonist, atenolol (25mg.kg⁻¹.day⁻¹) / calcium channel blocker, nifedipine (20mg.kg⁻¹.day⁻¹). **Results:** In L-NAME treated rats, atenolol / nifedipine abolished increases in systolic blood pressure and plasma AM and IMD levels and in left ventricular cardiomyocytes: (i) normalized increased cell width and mRNA expression of hypertrophic (sk- α -actin) and cardio-endocrine (ANP, BNP, ET) genes; (ii) normalized augmented membrane protein oxidation; (iii) normalized mRNA expression of AM, IMD, RAMP1, RAMP2 and RAMP3. **Conclusions:** normalization of blood pressure and membrane oxidant status together with prevention of hypertrophy and normalization of the augmented expression of AM, IMD and their receptor components in NO-deficient cardiomyocytes by atenolol / nifedipine supports involvement of both pressure loading and ischemic insult in stimulating cardiomyocyte hypertrophy and induction of these counter-regulatory peptides and their receptor components. Attenuation of augmented expression of IMD in this model cannot however be explained simply by prevention of cardiomyocyte hypertrophy.

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Introduction

Intermedin (IMD) is a novel vasodilator peptide [1] of the calcitonin gene-related peptide (CGRP) family which includes adrenomedullin (AM). Neutral endopeptidase (NEP) degrades AM [2]; it is likely that IMD is also a substrate for NEP. 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]. IMD acts non-selectively at all three RAMP-CRLR complexes [1]. IMD enhances vascular nitric oxide (NO) production [4] and protects endothelium from oxidative damage [5]. In the heart, administration of IMD exerts a positive inotropic effect [6], attenuates cardiomyocyte hypertrophy [7] and protects against deleterious effects of oxidative stress associated with ischemia-reperfusion injury [8].

NO-deficient hypertension induced by chronic treatment with the NOS inhibitor, N^G-nitro-L-arginine-methyl-ester (L-NAME) is characterised in myocardium by hypertrophy and ischemia [9]. Previous studies in our laboratory indicate two important findings relating to cardiomyocytes subjected to chronic NO deficiency. Firstly, pressure loading and oxidative stress, exerted directly on the myocardium, both contribute to cardiomyocyte hypertrophy since antihypertensive therapy (smooth muscle relaxant hydralazine given concurrently with the diuretic hydrochlorothiazide, [10]) and antioxidant therapy in the absence of blood pressure reduction (Vitamin C in combination with Tempol, [11]) each partially, but incompletely, attenuated increased cardiomyocyte width despite normalizing blood pressure and cardiomyocyte membrane oxidant status, respectively. Furthermore, L-NAME induced expression of the hypertrophic markers, sk- α -actin and prepro-ET1, was abolished either by blood pressure lowering agents [10] or by antioxidants in the absence of blood pressure reduction [11]; in contrast, however, augmented expression of the natriuretic peptides, ANP and BNP, was normalized by blood pressure lowering [10] but not by antioxidants in the absence of blood pressure reduction [11].

Secondly, IMD is normally expressed at extremely low levels in healthy adult myocardium [7]. A much more robust increase in IMD expression relative to AM in cardiomyocytes [10] of rats treated chronically with

L-NAME indicates an important counter-regulatory role for IMD in the cardiac pathology of NO deficiency. Expression of all three RAMPs is also augmented in cardiomyocytes. L-NAME-induced expression of AM and RAMP2 and RAMP3 was normalized by blood pressure normalization by hydralazine / hydrochlorothiazide [10] but not by antioxidant therapy with Tempol/ Vitamin C in the absence of blood pressure reduction [11], despite a similar reduction in the extent of cardiomyocyte hypertrophy indicating that pressure-loading exerts a direct influence on expression of these genes independently of any ability to prevent cardiomyocyte hypertrophy. Unlike Tempol and Vitamin C [11], hydralazine and hydrochlorothiazide were unable to attenuate augmented IMD and RAMP1 expression [10] despite attenuation of cardiomyocyte hypertrophic parameters to a similar extent. 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.

The purpose of the present study was to investigate the effects on parameters of cardiomyocyte hypertrophy and expression of the counter-regulatory peptides, AM and IMD and their various receptor components of an intervention chosen specifically for ability to target both hypertension and ischemic insult upon the myocardium simultaneously. The combination of a vascular-selective calcium channel blocker, nifedipine, and β adrenoceptor antagonist, atenolol, which act by different mechanisms and neutralize some of each other's unwanted effects, is often used in management of angina with hypertension due to effectiveness in lowering blood pressure, reducing cardiac work and correcting intermittent mismatch between myocardial oxygen supply and demand [12]. Cardiomyocyte membrane protein oxidation status was measured to demonstrate efficacy of atenolol/nifedipine to ameliorate L-NAME induced oxidative stress. Systolic blood pressure was assessed to confirm that this combination normalized L-NAME induced hypertension. The results relating specifically to the atenolol/nifedipine arm of a larger study are presented here and these have been compared and contrasted in discussion with those of the hydrochlorothiazide / hydralazine [10] and Tempol / Vitamin C [11] arms of the study which have been reported recently elsewhere.

	Forward	Backward
AM	CATTGAACAGTCGGGCGAGTA	GGTGCGAAGCTCTCTGATTCC
BC061775	21bp [739-759]	21bp [776-796]
IMD	GGCCCAGTTGCTGATGGT	TGCCCCGGAGCAGGTA
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	CCTGATAGTGCATGAGAATGTGCATGAC
CAA49997	28bp [988-1015]	28bp [1102-1129]
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]
ANP	CTGGGACCCCTCCGATAGA	GCAGAGCCCTCAGTTTGCTT
M27498	19bp [300-318]	20bp [333-352]
BNP	AGGTGCTGCCCCAGATGAT	GCGGCGACAGATTAAGGAAA
M25297	19bp [71-89]	20bp [102-121]
NEP	CAAGAGGAGGAAGACGCTGAA	CCTGAAGGCCACAGTATGCAT
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.

Materials and Methods

Experimental design: intervention study

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 (35 mg.kg⁻¹.day⁻¹, Alexis Biochemicals, Switzerland) in drinking water; (ii) atenolol (25 mg.kg⁻¹.day⁻¹, Sigma Aldrich, UK) plus nifedipine (20 mg.kg⁻¹.day⁻¹, Sigma Aldrich, UK) in drinking water; (iii) L-NAME (35 mg.kg⁻¹.day⁻¹) concurrently with atenolol (25 mg.kg⁻¹.day⁻¹) plus nifedipine (20 mg.kg⁻¹.day⁻¹) in drinking water; (iv) drinking water only (age-matched control) for 8 weeks, and maintained at the Laboratory Service Unit, QUB (under 12 hour light/dark cycles and receiving standard rat chow) prior to sacrifice at 16 weeks of age. Atenolol and nifedipine were first dissolved in absolute ethanol prior to dilution in drinking water (final alcohol concentration 1.2%vol.vol⁻¹); fresh solutions of these drugs were prepared daily. To discount any confounding influence of alcohol alone, alcohol (1.2% vol.vol⁻¹) was also added to drinking water given to rats assigned to groups (i) and (iv). Systolic blood pressure (SBP) was determined by tail cuff sphygmomanometer (Harvard Instruments) [10] 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 by inhalation of 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⁻¹, 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 cell dimensions, a small amount of each cell preparation was retained and suspended at a concentration of approximately 50,000 viable cardiomyocytes.ml⁻¹ in a 'creatinine-carnitine-taurine' (CCT) medium [10].

Measurements made

A) 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 SYBR Green detection (Abgene Biotechnologies Ltd, Surrey, UK) and the housekeeping gene, GAPDH as an internal standard.

B) 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

Fig. 1. 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 (35 mg.kg⁻¹.day⁻¹), [n=10]; (ii) atenolol (25 mg.kg⁻¹.day⁻¹) plus nifedipine (20 mg.kg⁻¹.day⁻¹), [n=3]; (iii) L-NAME (35 mg.kg⁻¹.day⁻¹) concurrently with atenolol (25 mg.kg⁻¹.day⁻¹) plus nifedipine (20 mg.kg⁻¹.day⁻¹) [n=4] for 8 weeks; (iv) age-matched untreated rats are included for comparison, [n=12]. 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 of the datasets (a)-(c) and (e)-(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 atenolol / nifedipine treatment group; $+P < 0.05$. [For control and L-NAME only treatment groups, data have been pooled from those values published previously (Fig.2 [11]) together with values from samples not available for analysis at the time of publication of that paper)].

concentration was determined by the method of Lowry. Protein samples were mixed with 4μl of 1:1 mercaptoethanol: loading buffer (0.5M Tris 25% vol.vol⁻¹, glycerol 20% vol.vol⁻¹, 10% SDS 40% vol.vol⁻¹, H₂O 15% vol.vol⁻¹, Bromophenol blue 0.005% wt.vol⁻¹) 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% vol.vol⁻¹ Tween 20 (Sigma, UK) and blocked overnight in PBS/0.1% vol.vol⁻¹ Tween 20 solution containing 5% wt.vol⁻¹ 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 report from the same laboratory [10]. In that study the specificity of the RAMP and CRLR antibodies used to detect the RAMP monomers and CRLR

Fig. 2. Effect on (a) AM; (b) IMD plasma level; (c) RAMP1; (d) RAMP2; (e) RAMP3 monomer expression in left ventricular cardiomyocytes of administration of (i) L-NAME (35 mg.kg⁻¹.day⁻¹), [n=10]; (ii) atenolol (25 mg.kg⁻¹.day⁻¹) plus nifedipine (20 mg.kg⁻¹.day⁻¹), [n=3]; (iii) L-NAME (35 mg.kg⁻¹.day⁻¹) concurrently with atenolol (25 mg.kg⁻¹.day⁻¹) plus nifedipine (20 mg.kg⁻¹.day⁻¹) [n=4] for 8 weeks; (iv) age-matched untreated rats are included for comparison, [n=12]. In (c)-(e), representative immunoblots are [from left-right] duplicate samples from a representative animal taken from each of the four treatment groups (i)-(iv). Protein expression data are expressed relative to β -actin levels and are given as the mean values $\pm$ SE of n animals. 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-e), 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 atenolol / nifedipine treatment treatment group; $+P < 0.05$.

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).

C) Oxidation of membrane proteins: detection of protein carbonylation. The carbonyl groups in the membrane proteins (5 μ g protein per lane) were denatured by addition of 12% SDS and derivatized to 2, 4, dinitrophenylhydrazones by reaction with 2, 4, dinitrophenylhydrazine (DNPH) for 15 min prior to

separation by 12% SDS-PAGE and transfer to PVDF membrane (0.45 μ m, Millipore, UK) as above [13]. A series of oxidized molecular weight standards were included as positive controls. 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

Fig. 3. Effect on systolic blood pressure of administration to 8 week old rats of (i) L-NAME (35 mg.kg⁻¹.day⁻¹), [n=10], (ii) atenolol (25 mg.kg⁻¹.day⁻¹) plus nifedipine (20 mg.kg⁻¹.day⁻¹), [n=3] (iii) L-NAME (35mg.kg⁻¹.day⁻¹) concurrently with atenolol (25 mg.kg⁻¹.day⁻¹) plus nifedipine (20 mg.kg⁻¹.day⁻¹) [n=4] for 8 weeks; (iv) age-matched untreated rats are included for comparison, [n=12]. Data are given as the mean values± SE. At each time point, 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 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 atenolol / nifedipine treatment group; + $P<0.05$.

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

D) Peptide Radioimmunoassay. Blood was collected into chilled lavender vacutainer tubes containing EDTA and aprotinin (0.6 TIU.ml⁻¹ 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⁻¹ 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).

E) 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.

F) Data analysis and statistical procedures. 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 untreated control group and L-NAME only treatment group; (2) difference between L-NAME only treatment group and L-NAME plus atenolol / nifedipine treatment group.

Results

Adrenomedullin, intermedin and their receptor component genes

mRNA expression of prepro-AM (1.8 fold vs. control, Fig. 1a), prepro-IMD (8.1 fold vs. control, Fig. 1b) and of NEP (3.2 fold vs. control, Fig. 1c), an enzyme implicated in metabolism of these peptides, was increased in left ventricular cardiomyocytes following treatment with L-NAME. These L-NAME induced increases were prevented by concurrent intervention with atenolol and nifedipine. Expression of RAMP1 (3.8 fold vs. control, Fig. 1e), RAMP2 (2.6 fold vs. control, Fig. 1f) and

Fig. 4. (a) representative oxyblot (using cardiomyocyte membranes prepared from one animal taken from each of four treatment groups respectively) depicting non-derivatised protein samples (negative control) and protein samples derivatised by DNPH; oxidized molecular weight standards are included as a positive control; A/N atenolol/nifedipine (b) effect on oxidation status of left ventricular cardiomyocyte membrane protein of administration of (i) L-NAME (35 mg.kg⁻¹.day⁻¹), [n=8]; (ii) atenolol (25 mg.kg⁻¹.day⁻¹) plus nifedipine (20 mg.kg⁻¹.day⁻¹), [n=4]; (iii) L-NAME (35 mg.kg⁻¹.day⁻¹) concurrently with atenolol (25 mg.kg⁻¹.day⁻¹) plus nifedipine (20 mg.kg⁻¹.day⁻¹) [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. Immuno-complexes formed within the entire lane for each derivatised 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-derivatised protein samples was also quantified as an estimate of background and the value obtained subtracted from those of the matched derivatised samples. 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 the dataset, 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 atenolol / nifedipine treatment treatment group; +P<0.05.

RAMP3 (4.4 fold vs. control, Fig. 1g) mRNAs was also increased in left ventricular cardiomyocytes by L-NAME treatment. In each case, L-NAME-induced increase in mRNA expression was normalized by con-current administration of atenolol and nifedipine. Expression of CRLR mRNA was not increased significantly by L-NAME treatment (1.6 fold vs. control, Fig. 1d).

The plasma levels of the peptides were augmented in L-NAME treated rats, by 1.4 fold (Fig 2a) and 2.9 fold (Fig 2b) greater than control values for AM and IMD, respectively. Con-current administration of atenolol and nifedipine abolished the L-NAME-induced increase in AM. Atenolol and nifedipine did not influence baseline IMD values in the absence of L-NAME but reduced L-NAME induced IMD levels to less than baseline values. At protein level treatment with L-NAME increased expression of the RAMP3 monomer (2.4 fold, Fig. 2e) and, to a lesser extent, RAMP1 monomer (1.6 fold,

Fig. 2c) and RAMP2 monomer (1.6 fold, Fig 2d) within left ventricular cardiomyocytes. L-NAME induced expression of each RAMP monomer was attenuated by con-current administration of atenolol and nifedipine. 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.

Systolic blood pressure

Systolic blood pressure (SBP) was 96 mmHg greater (P<0.05) in rats treated with L-NAME for 8 weeks relative to age-matched control values (Fig.3). Atenolol and nifedipine together did not influence blood pressure in normotensive rats but abolished the hypertensive effect of L-NAME.

Fig. 5. Effect on (a) cell width; (b) sk- α -actin; (c) ANP; (d) BNP; (e) prepro-ET-1 mRNA expression in left ventricular cardiomyocytes of administration to 8 week old rats of (i) L-NAME (35 mg.kg⁻¹.day⁻¹), [n=10], (ii) atenolol (25 mg.kg⁻¹.day⁻¹) plus nifedipine (20 mg.kg⁻¹.day⁻¹), [n=3] (iii) L-NAME (35 mg.kg⁻¹.day⁻¹) concurrently with atenolol (25 mg.kg⁻¹.day⁻¹) plus nifedipine (20 mg.kg⁻¹.day⁻¹) [n=4] for 8 weeks; (iv) age-matched untreated rats are included for comparison, [n=12]. In (a), 20 cells were measured within each heart cell preparation and a mean value obtained; mean+SE in the figure are based on the mean values for n individual heart cell preparations. In (b-e) 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-e), 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 atenolol / nifedipine treatment group; + $P<0.05$.

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 4) membrane protein oxidation which was abolished by concurrent administration of atenolol and nifedipine.

Cardiomyocyte Hypertrophy

The width of left ventricular cardiomyocytes was increased ($P<0.05$) by 19.8% following treatment with L-NAME (Fig.5a); this increase was completely normalized by atenolol and nifedipine. Cardiomyocyte lengths were not altered following treatment with L-NAME (data not shown). Expression of sk α -actin (3.4 fold, Fig. 5b), ANP (7.3 fold, Fig 5c), BNP (3.2 fold, Fig. 5d) and preproET-1 (2.3 fold, Fig. 5e) mRNAs was increased in left ventricular cardiomyocytes following treatment with L-NAME; these increases were abolished by concurrent therapy with atenolol and nifedipine. mRNA

expression of AM, IMD, RAMP1, RAMP2 and RAMP3 in left ventricular cardiomyocytes (Fig.1) was correlated positively ($P<0.05$) with that of the cardioendocrine genes, ANP, BNP and prepro-ET-1 (Fig.5). Pearson r values and P values are given in Table 2.

Discussion

In the present study, atenolol/nifedipine completely normalized L-NAME-induced increased left ventricular cardiomyocyte width in contrast to the finding, reported previously [10], that hydralazine / hydrochlorothiazide caused only partial attenuation despite similar ability to normalize systolic blood pressure and abolish augmented cardiomyocyte expression of ANP and BNP in response to pressure-loading upon the myocardium. This confirms that normalization of cell width by atenolol/nifedipine cannot be entirely accounted for by abolition of L-NAME-

X	Y	Pearson r	P value
BNP	AM	0.5084	0.0068
	IMD	0.6132	0.0014
	RAMP1	0.6635	0.0002
	RAMP2	0.6716	0.0001
ANP	RAMP3	0.6486	0.0003
	AM	0.5262	0.0143
	IMD	0.6334	0.0021
	RAMP1	0.6114	0.0032
ET-1	RAMP2	0.7055	0.0004
	RAMP3	0.7337	0.0003
	AM	0.5899	0.0015
	IMD	0.6295	0.0010
	RAMP1	0.6620	0.0002
	RAMP2	0.4766	0.0138
	RAMP3	0.4949	0.0102

Table 2. Correlation between mRNA expression of cardioendocrine genes and AM, IMD and receptor components in left ventricular cardiomyocytes.

induced hypertension and that additional mechanisms contribute. There are a number of possible explanations. Firstly, NO deficiency is associated with increased systolic BP variability, due to loss of NO to exert negative feedback regulation in response to increased sheer stress upon resistance vessels, which in turn causes more severe myocardial structural damage at a given level of BP [14] together with narrowing of coronary arteries and greater fluctuation in coronary perfusion leading to intermittent ischemia and re-oxygenation [15]. The combination of a calcium channel blocker and β -adrenoceptor antagonist can reduce systolic BP variability (and LVH) in spontaneously hypertensive rats [16]; indeed the combination has a markedly greater and longer-lasting action than either drug alone. Although thiazides also reduce systolic BP variability to a lesser extent [17], hydralazine does not [18]; this could account for the lesser ability of this combination to attenuate increased cardiomyocyte width reported previously [10] compared to that of atenolol/nifedipine in the present study.

Secondly, the vascular selective calcium channel blocker nifedipine was chosen for ability to increase myocardial oxygen supply as a consequence of coronary vasodilatation, and to reduce myocardial oxygen consumption by reducing preload and after-load as a consequence of systemic vasodilatation [19]; both effects are due to direct relaxation of vascular smooth muscle independent of NO release, in contrast to the action of

hydralazine which is primarily an arteriolar dilator, whose mechanism is dependent in part on NO-related cyclic GMP accumulation [20] and likely to be influenced by NO-deficiency. Dihydropyridine calcium channel modulators, including nifedipine, can also scavenge ROS [21]. The β -adrenoceptor antagonist, atenolol was selected to reduce oxygen demand by reducing cardiac work and improve oxygen supply by reducing heart rate, thereby improving perfusion time [22]. Atenolol prevents reflex activation of the sympathetic nervous system and tachycardia by calcium channel blockade [12] and prevents exacerbation of ischemia; conversely, calcium channel blockade prevents reflex increase in vascular resistance due to β -adrenoceptor blockade [23]. Atenolol / nifedipine abolished L-NAME induced cardiomyocyte membrane protein oxidation. Generation of O_2^- by pro-oxidant enzymes including NADPH oxidases results in a hypertrophic phenotype [24] [25]. SOD mimetics such as Tempol lower blood pressure by scavenging O_2^- and improving NO bioavailability in experimental hypertension [26]. We have reported previously that, despite inability to reduce systolic BP in L-NAME treated rats due to the profound inhibition of vascular NO synthesis prevailing as a consequence of chronic administration of L-NAME [9], antioxidant therapy with Tempol /Vitamin C normalized cardiomyocyte oxidant status and partly attenuated L-NAME induced increase in cardiomyocyte width [11] implying a contribution of oxidative stress directly to cardiomyocyte hypertrophy. Reduced myocardial oxygen requirement, together with less fluctuation in coronary perfusion and frequency and duration of periods of ischemia-reoxygenation associated with oxidative stress may also have contributed therefore to the ability of atenolol/ nifedipine to normalize cardiomyocyte cell width.

Thirdly, a direct action of atenolol to antagonize β_1 adrenoceptors present on cardiomyocytes might have contributed to attenuation of L-NAME induced increase in cardiomyocyte width. However the influence of β_1 adrenoceptor stimulation on cardiomyocyte hypertrophy is unclear since both positive and negative effects have been reported [27, 28].

L-NAME-induced cardiomyocyte expression of each of the cardio-endocrine peptides, IMD and AM, and all three receptor activity modifying proteins was prevented in the present study by concurrent administration of atenolol / nifedipine. This finding is in contrast to the differential influence of hydralazine/ hydrochlorothiazide [10] and Vitamin C/Tempol [11] treatment reported previously. The combination of

β -adrenoceptor antagonist and vascular-selective calcium channel blocker, a commonly used therapy for angina pectoris in hypertensive patients, would be expected to reduce both mechanical stress associated with myocardial pressure-loading and oxidative stress caused by intermittent periods of ischemia-reoxygenation resulting from chronic administration of L-NAME in rats; this was confirmed in the present study by demonstration of the amelioration of augmented ANP and BNP expression and membrane protein oxidation, respectively. The ability of atenolol/nifedipine in the present study, but not of hydralazine / hydrochlorothiazide [10], to normalize prepro-IMD and RAMP1 expression, despite eliciting similar decreases in systolic blood pressure and ameliorating expression of prepro-AM and RAMP2 and RAMP3, and also of ANP and BNP, is consistent with the conclusion that prepro-IMD and RAMP1 are regulated by a pressure-independent mechanism. The ability of atenolol/nifedipine to attenuate expression of IMD, AM and their RAMPs is unlikely to occur secondary to prevention of cardiomyocyte hypertrophy by indirect or direct means (for example blockade of the β_1 adrenoceptor), since Tempol/Vitamin C [11] and hydralazine/hydrochlorothiazide [10] exerted differential effects on expression of these peptides and their RAMPs, despite comparable partial decreases in the extent of the change in cardiomyocyte width in response to L-NAME. Reports of cardio-protective effects of IMD against ischemia-reperfusion injury, indicated by reduced myocardial leakage of lactate dehydrogenase and decreased membrane lipid peroxidation [8] would support the hypothesis that release of this peptide represents an adaptive response to oxidative stress. RAMP1, when in combination with the CRLR receptor protein, constitutes a CGRP₁-receptor [3]. Neuronal release of CGRP is also enhanced in response to myocardial ischemia [29] and exerts direct cardio-protective effects against ischemic injury [30]. Non-neuronal IMD may, like neuronal CGRP, serve primarily an anti-ischemic, cardio-protective function although an additional influence for IMD as a negative regulator of cardiomyocyte hypertrophy, mediated by stimulation of the AM₂ receptor, cannot be discounted, particularly since RAMP3 expression is also markedly enhanced and IMD is known to interact both at AM₂ receptors, formed by the association of RAMP3 with CRLR, and at CGRP₁ receptors formed by the association of RAMP1 with CRLR [1]. However in contrast to other negative regulators of cardiomyocyte hypertrophy such as ANP, BNP and AM, induction of IMD is influenced to a much greater extent by oxidative

stress than by pressure loading.

Reactive oxygen species (ROS) modify the activity of various signaling kinases implicated in hypertrophic remodeling including protein kinase C, src tyrosine kinase and MAP kinase all of which influence gene expression [31]. Oxidative stress also regulates expression of transcription factors such as NF- κ B, AP-1, hypoxia-inducible factor HIF-1 α and endothelial PAS domain protein 1 [32]. The presence of binding motifs for such factors within the promoter regions of the IMD and RAMP1 genes could explain induction of these genes within cardiomyocytes in response to oxidative stress. Daiber et al (2005) [33] reported that hydralazine scavenged ROS in vitro. However, hydralazine/hydrochlorothiazide caused only partial, non-significant attenuation of augmented cardiomyocyte membrane protein oxidation (Bell and Zhao, unpublished observation) in NO-deficient rats in contrast to atenolol/nifedipine which caused completed attenuation. Inability of hydralazine / hydrochlorothiazide to ameliorate augmented cardiomyocyte IMD and RAMP1 expression in NO-deficient rats in contrast to Tempol/Vitamin C and atenolol/nifedipine could reflect the lesser ability of hydralazine to scavenge ROS or divergence in mechanisms by which each of the drug combinations offset oxidative stress and therefore influence gene expression. Unexpectedly, hydralazine has recently been found to induce, rather than attenuate, expression of HIF-1 α [33] albeit in vascular tissues.

In conclusion, simultaneous targeting of hypertension and myocardial ischemia by atenolol/nifedipine ameliorated cardiomyocyte hypertrophy and normalized augmented cardiomyocyte expression of both AM and IMD and all three RAMPs in NO-deficient rats. These data are consistent with involvement of both pressure loading and ischemic insult in stimulating cardiomyocyte hypertrophy and in induction of these counter-regulatory peptides and their receptor components by mechanisms independent of the extent of cardiomyocyte hypertrophy. This study has revealed important differences between antihypertensive regimens in regard to attenuation of cardiomyocyte hypertrophy and recruitment of cardiac counter-regulatory mechanisms. The superiority of atenolol/nifedipine over hydralazine/ hydrochlorothiazide for attenuation of L-NAME induced cardiomyocyte hypertrophy probably reflects properties of the β -blocker/ calcium channel modulator combination in addition to normalization of mean blood pressure. Attenuation of augmented cardiomyocyte expression of IMD and RAMP1 by atenolol / nifedipine is likely to reflect, at least

in part, the ability of this drug combination to ameliorate myocardial ischemic insult and re-oxygenation injury, while abolition of the augmented expression of RAMP2, RAMP3 and AM may reflect primarily the influence of this drug combination on myocardial pre-load and after-load and hence, mechanical stress.

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