Adrenomedullin Gene Delivery is Cardio-protective in a Model of Chronic Nitric Oxide Deficiency Combining Pressure Overload, Oxidative Stress and Cardiomyocyte Hypertrophy

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Key Words

Ventricular • Oxidative stress • Adenoviral vectors • Adrenomedullin • Myocardial ischemia • Cardiomyocytes • Hypertrophy • Nitric oxide

Abstract

Background/aims: Chronic inhibition of nitric oxide (NO) synthesis is associated with hypertension, myocardial ischemia, oxidative stress and hypertrophy; expression of the vasodilator peptide, adrenomedullin (AM) and its receptors is augmented in cardiomyocytes, indicating that the myocardial AM system may be activated in response to pressure loading and ischemic insult to serve a counter-regulatory, cardio-protective role. The study examined the hypothesis that oxidative stress and hypertrophic remodeling in NO-deficient cardiomyocytes are attenuated by adenoviral vector-mediated delivery of the human adrenomedullin (hAM) gene *in vivo*. **Methods:** The NO synthesis inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 15mg.kg⁻¹.day⁻¹) was given to rats for 4 weeks following systemic administration via the tail vein of a single injection of either adenovirus harbouring hAM cDNA under the

control of the cytomegalovirus promoter-enhancer (Ad.CMV-hAM-4F2), or for comparison, adenovirus alone (Ad.Null) or saline. Cardiomyocytes were subsequently isolated for assessment of the influence of each intervention on parameters of oxidative stress and hypertrophic remodelling. **Results:** Cardiomyocyte expression of the transgene persisted for ≥4 weeks following systemic administration of adenoviral vector. In L-NAME treated rats, relative to Ad.Null or saline administration, Ad.CMV-hAM-4F2 (i) reduced augmented cardiomyocyte membrane protein oxidation and mRNA expression of pro-oxidant (p22phox) and anti-oxidant (SOD-3, GPx) genes; (ii) attenuated increased cardiomyocyte width and mRNA expression of hypertrophic (sk- α -actin) and cardio-endocrine (ANP) genes; (iii) did not attenuate hypertension. **Conclusions:** Adenoviral vector mediated delivery of hAM resulted in attenuation of myocardial oxidative stress and hypertrophic remodelling in the absence of blood pressure reduction in this model of chronic NO-deficiency. These findings are consistent with a direct cardio-protective action in the myocardium of locally-derived hAM which is not dependant on NO generation.

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Introduction

Left ventricular hypertrophy (LVH), characterized by increased mass [1, 2] and altered phenotype [3, 4] in cardiomyocytes, is a compensatory response to increased cardiac workload progressing from an initial physiological to a pathophysiological response. It is a common complication of hypertension and ischemic heart disease. Excess generation of reactive oxygen species (ROS), including superoxide anion (O_2^-) catalysed by enzymes such as NADPH oxidase, induce vascular and myocardial structural damage and result in a hypertrophic phenotype [5]. Oxidative modification of proteins represents a major target for ROS [6]. 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^- 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 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 [7].

Nitric oxide (NO) generated within normal myocardium and vasculature by the enzyme NO synthase (NOS) also plays a pivotal role in reduction of myocardial oxygen consumption and blood pressure [8], quenches ROS-induced injury [9] and exerts a regulatory influence directly on cardiomyocyte contractility and growth [10]. NO levels are reduced in the plasma of hypertensive patients with LVH [11]. Three isoforms of NOS are recognised: eNOS, nNOS and iNOS; all three are expressed in cardiomyocytes and contribute to the regulation of various physiological and pathophysiological processes [12]. NO-deficient hypertension induced by treatment of rats with the isoform non-selective NOS inhibitor, *N* ω -nitro-L-arginine methyl ester (L-NAME), is characterised in myocardium by hypertrophy, fibrosis, ischemia and necrosis [12]. In cardiomyocytes, general enhancement of protein synthesis and mass is observed, together with increased cell width, and phenotypic alterations, manifest as up-regulation of contractile and cardio-endocrine genes [13]; oxidative stress is evidenced by cardiomyocyte membrane protein oxidation and augmented expression of pro-oxidant and anti-oxidant enzymes [14]. Pressure-dependant and oxidative stress-dependent mechanisms contribute to development of cardiomyocyte hypertrophy in this model [13-16].

Adrenomedullin (AM) possesses vasodilator [17] and

natriuretic [18] effects. Although AM was first isolated from adrenal medulla [18], the peptide is also found in plasma and in the heart [19-22]. Increased plasma levels of AM have been detected in hypertension [19], myocardial infarction [22] and heart failure [20, 21] and are greater in hypertensive patients with evidence of LVH than hypertensive patients without evidence of underlying LVH [20]. Increased ventricular levels of AM also correlate with increased left ventricular: body mass (LV:BM) ratio in pressure-overload hypertrophy induced by aortic banding in rats; infusion of AM reduced LV:BM ratio while infusion of the direct acting vasodilator, hydralazine, given at a dose designed to lower blood pressure to a similar extent, did not [23]. Furthermore, AM attenuates cardiomyocyte hypertrophy *in vitro* [14, 24, 25]. Cardiomyocytes express the precursor peptide mRNA and secrete AM into culture medium [22, 26]. This expression is enhanced in response to hypertrophic stimuli including mechanical stretch [27] and hypoxic stress [28]. These data imply a possible direct role for AM, produced locally within the heart, in attenuation of cardiac remodelling. AM also attenuates myocardial ischaemia-reperfusion injury, cardiomyocyte membrane lipid peroxidation and leakage of myoglobin from Langendorff-perfused hearts *in vitro* [29].

Cardiomyocyte expression of AM and its receptors is augmented in the chronic NO-deficient rat [13], providing further evidence that the myocardial AM system may be activated in response to pressure loading and ischemic insult and may serve a counter-regulatory, cardio-protective role. Administration of adenovirus harbouring human AM cDNA under the control of the cytomegalovirus promoter/enhancer lowers blood pressure and attenuates cardiac hypertrophy and fibrosis in hypertensive animal models [30-34] and oxidative stress and ischemia-reperfusion injury following occlusion of the rat coronary artery [35]. Cyclic AMP and/or NO/cGMP-dependant signalling pathways have been implicated in the various actions of the peptide, depending on model and end-point assessed [28, 30-37].

The purpose of the present study therefore was firstly to determine whether systemic delivery of an adenoviral vector harbouring the hAM gene attenuated manifestation of parameters of cardiomyocyte oxidative stress and hypertrophy in the chronic NO-deficient rat. L-NAME was preferentially selected as the means of inducing chronic NO deficiency because this compound is more soluble in drinking water than other isoform non-selective inhibitors such as N^G -nitro-L-Arginine; L- N^G -Nitroarginine (L-NNA). L-NAME is however metabo-

lised to L-NNA by the action of cellular esterases *in vivo*. Secondly, the dependence of any effects of the peptide on attenuation of cardiomyocyte oxidative stress and hypertrophy in this model on the extent of blood pressure reduction were examined. Finally, since NO/cGMP-dependant signalling is suppressed by chronic NOS inhibition, this model was chosen to provide novel mechanistic insight into the extent of involvement of this signalling pathway in the cardio-protective actions of AM.

Materials and Methods

Preparation of replication-deficient adenovirus vectors

Recombination-deficient adenovirus is an excellent vector for gene delivery because of its high transfection efficiency in infecting cells and its ability to target non-dividing cells. Vectors encoding green fluorescent protein (GFP) cDNA facilitate microscopic identification of transgene expression. Adenoviruses harbouring human AM (hAM) or GFP cDNAs under the control of the cytomegalovirus (CMV) enhancer/promoter were initially prepared in the laboratory of Chao (Medical University of South Carolina) using the AdEasy system as described previously [38]; additional elements incorporated into these recombinant vectors [30] were the Adenoviral E3 region (to prevent antiviral and cellular immune responses) and the 4F2 transcription enhancer (to bind multiple nuclear proteins and augment and prolong transgene expression resulting in prolonged exposure to elevated hAM levels *in vivo* during cardiac remodelling). To make these constructs, a 500bp 4F2 transcription enhancer element was amplified by PCR from human genomic DNA and inserted between the human AM or GFP cDNAs and bovine growth hormone polyA signal [39]. A CMV promoter-driven E3 region was incorporated into the adenovirus vectors as described [40, 41]. After transferring hAM and E3 expression cassettes into the shuttle vector pHM3 with appropriate restriction and ligation manipulation, adenoviral vectors were constructed as previously described [39] [42]. Briefly, hAM and GFP cDNAs were cloned into the adenoviral shuttle vector, pAdTrack-CMV and linearised using PmeI. Adenoviral shuttle vector alone (Ad.Null) or shuttle vectors carrying hAM or GFP cDNAs were co-transformed into *E. coli* BJ5183 cells along with an adenoviral backbone plasmid, pAdEasy-1; the resulting plasmids, pAd.CMV-hAM-4F2 and pAd.CMV-GFP-4F2, linearised by PacI, were transfected into human embryonic kidney (HEK) 293 packaging cells and purified by modified caesium chloride banding as described previously [34, 38, 43] to produce high-titre adenoviral vectors. Sufficient stocks of these various adenovirus vectors for the planned experiments were generated, purified and stored in the laboratory of Cosby in Belfast.

The following studies were performed under license in accordance with Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationary Office, London.

Preliminary experiments to confirm ability of cardiomyocytes to take up the vector and express the transgene (i) in vitro and (ii) in vivo

(i) ventricular cardiomyocytes were isolated from healthy male Sprague-Dawley rats (12-13 weeks of age) as described below. These cells were suspended at a concentration of approximately 50,000 viable cardiomyocytes.ml⁻¹ 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). Aliquots of cell suspension (1 ml) were pipetted gently onto Petri dishes, pre-incubated for 2 h with foetal calf serum (4% vol.vol⁻¹) in CCT Medium. After 1 h viable cardiomyocytes became attached to the surface of the dish. The dishes were then washed with fresh CCT medium to remove non-attached cells and cell debris and the attached cells were exposed for 24 h to Ad.CMV-GFP-4F2 (10¹¹, 10¹² pfu.ml⁻¹) in CCT medium before visualization by fluorescence imaging. (ii) Healthy male Sprague-Dawley rats aged 12-13 weeks received a single injection of Ad.CMV-GFP-4F2 at 2x10¹¹ pfu.ml⁻¹ in 100 µl saline via the tail vein; rats were sacrificed at weekly intervals and cardiomyocytes isolated as described below. Cells were suspended in CCT medium and GFP expression determined by fluorescence microscopy.

Experimental protocol for administration of adenoviral vectors to the nitric oxide-deficient rat

Male Sprague-Dawley rats (aged 12-13 weeks) were assigned to receive either (i) L-NAME (15 mg.kg⁻¹.day⁻¹, Alexis Biochemicals, Switzerland) in drinking water plus saline injection; (ii) L-NAME (15 mg.kg⁻¹.day⁻¹) in drinking water plus Ad.CMV-hAM-4F2 injection in saline; (iii) L-NAME (15 mg.kg⁻¹.day⁻¹) in drinking water plus Ad.Null injection in saline; (iv) drinking water only plus saline injection (age-matched normotensive control); for 4 weeks, and maintained at the category 2 containment facility at the Laboratory Service Unit, QUB (under 12 h light/dark cycles and receiving standard rat chow) prior to sacrifice at 16-17 weeks of age. Solutions of L-NAME were replenished daily during the experimental period. Adenoviral vectors were administered at the beginning of the experimental period at 2x10¹¹ pfu.ml⁻¹ in a single injection of 100µl via the tail vein. Systolic blood pressure was determined by tail cuff sphygmomanometer (Harvard Instruments) [14] 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 L-NAME consumption accurately to each individual animal.

Cardiomyocyte isolation

Following deep anaesthesia 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 ventricular cardiomyocytes were isolated by enzymatic digestion (collagenase, 0.4mg.ml⁻¹, Serva, Germany) using Langendorff perfusion [14]. After purification, cells were utilized immediately for extraction of RNA (RT-PCR protocols) and

	Forward	Backward
hAM	AAA CTG ATT TCT CAC GGC GTG 21bp [1046-1067]	AAT AGT GAG GCT TGC GCC C 19bp [1078-1097]
GAPDH	GAA ACC CAT CAC CAT CTT CCA 21bp [248-268]	ACC CCA TTT GAT GTT AGC GG 20bp [280-299]
AB017801	AAG GAT TCC TAC GTG GGC G 19bp [300-318]	GGT CAG GAT ACC TCG CTT GCT 21bp [247-267]
Skeletal α -Actin	CTG GGA CCC CTC CGA TAG A 19bp [300-318]	GCA GAG CCC TCA GTT TGC TT 20bp [333-352]
BC061974	GAG TCC GGT GTC GAC TTA GCA 21bp [177-197]	CCT CCC GTT GTT TTC CTA GCT 21bp [262-282]
ANP	GGT GTT CCA GTG CGC AGA TA 20bp [1605-1624]	CAT CAC CAA GCC CAG ATA CCA 21bp [1709-1729]
M27498	TGT GAC CTT CAG TGG CTC CTG 21bp [605-625]	CCT CTG GGA GCA ACA CCT TG 20bp [636-655]
SOD3		
X94371		
GPx		
S50336		
p22phox		
AJ295951		

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.

preparation of membrane protein (immunoblotting protocols); for analysis of cell dimensions, a small amount of each cell preparation was suspended at a concentration of approximately 50,000 viable cardiomyocytes.ml⁻¹ in CCT medium.

Real-Time PCR

Total RNA was extracted from cardiomyocytes using TRIzol reagent. Reported sequences for each gene (Table 1) were used to design on Primer Express software (PE Applied Biosystems) rat specific primers for hypertrophic and oxidative stress marker genes and human specific primers for AM, adapted to RT-PCR conditions, which were synthesized by Invitrogen. RT-PCR was performed as described previously [14], using SYBR Green detection (Abgene Biotechnologies Ltd, Surrey, UK) and the housekeeping gene, GAPDH, as an internal standard. Southern blot analysis of human AM expression was performed as described previously [31]; briefly RT-PCR was stopped after 40 cycles (prior to the dissociation stage) and 5 μ L removed from the sample wells. Subsequently the samples underwent electrophoresis on an agarose gel and were stained with ethidium bromide before viewing using a UVP autochemi imager.

Oxidation of membrane proteins/detection of protein carbonylation

Plasma membranes were prepared from freshly isolated cardiomyocytes as described previously [14, 15] and stored at -80°C pending analysis. Membrane protein concentration was determined by the method of Lowry. 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). 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 h 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 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 of 1:300, and ECL plus (Amersham Biosciences, UK) as substrate, and quantified by densitometry (Analytical Imaging System).

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 (μ m) were determined for 50 viable cardiomyocytes and a mean value obtained.

Data analysis and statistical procedures

Data are expressed as means $\pm$ SE where *n* denotes number of rats in which systolic blood pressure was 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 normotensive control group and L-NAME only treatment group; (2) difference between L-NAME only treatment group and L-NAME plus adenoviral vector treatment groups.

Fig. 1. Expression of GFP in rat ventricular heart muscle cells; *in vitro* 24 h after administration of the adenoviral vector Ad.CMV-GFP-4F2 (a; 10^{11} pfu.ml $^{-1}$; b 10^{12} pfu.ml $^{-1}$); 4 weeks after delivery of the Ad.CMV-GFP vector *in vivo* by tail vein injection (c; 2×10^{11} pfu ml $^{-1}$ in 100 µl saline injection); all cells are clearly expressing the protein.

Results

Evidence for uptake of adenoviral vectors into cardiomyocytes

Uptake of replication-deficient adenoviral vectors into heart muscle cells was first confirmed *in vitro* by incubating healthy rat ventricular heart muscle cells in culture with Ad.CMV-GFP-4F2 vector (10^{11} , 10^{12} pfu.ml $^{-1}$); the presence of GFP expression within cells was evident within 24 h (Fig. 1 a, b). Administration of Ad.CMV-GFP-4F2 (2×10^{11} pfu.ml $^{-1}$ in 100 µl saline injection) to healthy normotensive control rats *in vivo* via the tail vein resulted in successful delivery of the adenoviral vector to the ventricular myocardium; cardiomyocytes uniformly expressed GFP and expression persisted for at

Fig. 2. Representative Southern blot demonstrating expression of the hAM gene in ventricular cardiomyocytes isolated after administration to 12-13 week old rats for 4 weeks of L-NAME (15 mg.kg $^{-1}$.day $^{-1}$) plus saline injection (100 µl) containing Ad.CMV-hAM-4F2 2×10^{11} pfu.ml $^{-1}$; additional treatment groups are shown for comparison (i) L-NAME (15 mg.kg $^{-1}$.day $^{-1}$) plus saline injection (100 µl) (ii) L-NAME (15 mg.kg $^{-1}$.day $^{-1}$) plus saline injection (100 µl) containing Ad.Null 2×10^{11} pfu.ml $^{-1}$ (iii) age-matched rats receiving saline injection (100 µl) only. Ad./saline was administered on Day 1 of the experimental schedule. RT-PCR was stopped after 40 cycles (but prior to the dissociation stage) and 5 µl removed from the sample wells. Subsequently the samples underwent electrophoresis on an agarose gel and were stained with ethidium bromide before viewing using a UVP autochemi imager.

least 4 weeks post injection (Fig. 1 c). Similarly, expression of hAM mRNA was still evident (Fig. 2) in cardiomyocytes 4 weeks after administration of Ad.CMV-hAM-4F2 (2×10^{11} pfu.ml $^{-1}$ in 100 µl saline injection) via the tail vein in rats receiving L-NAME (15 mg.kg $^{-1}$.day $^{-1}$).

Hypertension, Pressure-Loading and Cardiomyocyte Hypertrophy

Systolic blood pressure was 72 mmHg greater ($P < 0.05$) in rats treated with L-NAME (15 mg.kg $^{-1}$.day $^{-1}$) for 4 weeks relative to age-matched control values. Neither Ad.Null nor Ad.CMV-hAM-4F2 administration (2×10^{11} pfu.ml $^{-1}$ in 100 µl saline injection) attenuated the development of hypertension in response to L-NAME

Fig. 3. Effect on systolic blood pressure of administration to 12-13 week old rats of (i) L-NAME (15 mg.kg⁻¹.day⁻¹) plus saline injection (100 µl), [n=3], (ii) L-NAME (15 mg.kg⁻¹.day⁻¹) plus saline injection (100 µl) containing Ad.CMV-hAM-4F2 2x10¹¹pfu.ml⁻¹, [n=4], (iii) L-NAME (15 mg.kg⁻¹.day⁻¹) plus saline injection (100 µl) containing Ad.Null 2x10¹¹pfu.ml⁻¹, [n=4], for 4 weeks; (iv) age-matched rats receiving saline injection (100 µl) only are included for comparison, [n=4]. Ad./saline was administered on Day 1 of the experimental schedule. Data are given as the mean values ± SE. Difference between untreated control group and L-NAME only treatment group *P<0.05.

(Fig. 3). The widths of ventricular cardiomyocytes were increased (P<0.05) by 43.4% (vs. control) following treatment with L-NAME (Fig. 4a). L-NAME-induced increased width was attenuated by Ad.CMV-hAM-4F2 (by 84%, P<0.05) but not by Ad.Null administration. 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, (1.75 fold vs. control, Fig. 4b) and of the cardio-endocrine hormone, ANP (2.51 fold vs. control, Fig. 4c), were increased (P<0.05) in ventricular cardiomyocytes following treatment with L-NAME. Increased mRNA expression of skeletal-α-actin and ANP in response to L-NAME was attenuated by 73% and 61%, respectively, (P<0.05) by Ad.CMV-hAM-4F2 but not by Ad.Null administration.

Fig. 4. Effect on (a) cell width; (b) sk-α-actin; (c) ANP mRNA expression in ventricular cardiomyocytes of administration to 12-13 week old rats of (i) L-NAME (15 mg.kg⁻¹.day⁻¹) plus saline injection (100 µl), [n=3], (ii) L-NAME (15 mg.kg⁻¹.day⁻¹) plus saline injection (100 µl) containing Ad.CMV-hAM-4F2 2x10¹¹pfu.ml⁻¹, [n=4], (iii) L-NAME (15 mg.kg⁻¹.day⁻¹) plus saline injection (100 µl) containing Ad.Null 2x10¹¹pfu.ml⁻¹, [n=4], for 4 weeks; (iv) age-matched rats receiving saline injection (100µl) only are included for comparison, [n=4]. In (a), 50 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-c) data were expressed relative to GAPDH mRNA. Difference between untreated control group and L-NAME only treatment group *P<0.05; difference between L-NAME only treatment group and L-NAME plus adenoviral vector treatment group +P<0.05.

Fig. 5. (a) representative oxyblot (using cardiomyocyte membranes prepared from one animal taken from each of four treatment groups respectively) depicting protein samples derivatised by 2-4 Dinitrophenylhydrazine (DNPH, left-hand lanes) and non-derivatised protein samples (negative control, right-hand lanes); (b) effect on oxidation status of left ventricular cardiomyocyte membrane protein of administration of (i) L-NAME (15 mg.kg⁻¹.day⁻¹) plus saline injection (100 µl), [n=3], (ii) L-NAME (15 mg.kg⁻¹.day⁻¹) plus saline injection (100 µl) containing Ad.CMV-hAM-4F2 2x10¹¹pfu.ml⁻¹, [n=3], (iii) L-NAME (15 mg.kg⁻¹.day⁻¹) plus saline injection (100 µl) containing Ad.Null 2x10¹¹pfu.ml⁻¹, [n=3], for 4 weeks; (iv) age-matched rats receiving saline injection (100 µl) only [n=3]; 5 µg of membrane protein was loaded onto each lane of the gel; data are mean values + SE. Immunocomplexes 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. Difference between untreated control group and L-NAME only treatment group *P<0.05; difference between L-NAME only treatment group and L-NAME plus adenoviral vector treatment group *P<0.05.

Fig. 6. Effect on (a) p22phox; (b) SOD3; (c) GPx mRNA expression in ventricular cardiomyocytes of administration to 12-13 week old rats of (i) L-NAME (15 mg.kg⁻¹.day⁻¹) plus saline injection (100 µl), [n=3], (ii) L-NAME (15 mg.kg⁻¹.day⁻¹) plus saline injection (100 µl) containing Ad.CMV-hAM-4F2 2x10¹¹pfu.ml⁻¹, [n=4], (iii) L-NAME (15 mg.kg⁻¹.day⁻¹) plus saline injection (100 µl) containing Ad.Null 2x10¹¹pfu.ml⁻¹, [n=4], for 4 weeks; (iv) age-matched rats receiving saline injection (100 µl) only are included for comparison, [n=4]. Ad./saline was administered on Day 1 of the experimental schedule. Data were expressed relative to GAPDH mRNA. Difference between untreated control group and L-NAME only treatment group *P<0.05; difference between L-NAME only treatment group and L-NAME plus adenoviral vector treatment group *P<0.05.

Cardiomyocyte Oxidative Stress

In ventricular cardiomyocytes from L-NAME treated rats, increased oxidative stress was indicated by

augmented (2.20 fold vs. control, $P < 0.05$, Fig. 5) membrane protein oxidation which was attenuated by Ad.CMV-hAM-4F2 (by 47%, $P < 0.05$) but not by Ad.Null administration. Cardiomyocyte mRNA expression of the NADPH oxidase associated subunit, p22phox, was augmented in L-NAME-treated rats (2.12 fold vs. control, $P < 0.05$, Fig. 6a); expression of this pro-oxidant gene was attenuated by Ad.CMV-hAM-4F2 (by 53%, $P < 0.05$) but not by Ad.Null administration. Similarly, L-NAME treatment augmented expression of SOD3 and GPx mRNAs in cardiomyocytes 2.13 and 1.68-fold, respectively, (Fig. 6b, c); expression of these anti-oxidant genes was attenuated by Ad.CMV-hAM-4F2 (by 78% and 51%, respectively, $P < 0.05$) but not by Ad.Null administration.

Discussion

Inadequate control of systemic blood pressure results in progressive left ventricular remodelling and dysfunction. Most classes of antihypertensive drugs have at best modest ability to attenuate LVH. Concomitant delivery of an exogenous supply of AM could potentially prevent increases in cellular mass and phenotypic changes associated with development of cardiomyocyte hypertrophy [23]. AM cannot be given in tablet form as the peptide would be readily metabolized in the gut and plasma before it reached the heart; the necessity for frequent intravenous infusion may limit the therapeutic potential of this peptide. One way to overcome this difficulty is to harness modern molecular biology techniques to deliver a source of the AM gene packaged in an adenoviral vector. Following a single intravenous injection of a suspension containing the virus and subsequent cellular uptake, the gene encoding AM is switched on, causing the body to sustain the manufacture of large amounts of the peptide [30-35]. This study confirms the feasibility of adenoviral vector mediated gene delivery to cardiomyocytes via the systemic route *in vivo*. Transgene protein expression would be expected to occur within 24 h of uptake of the adenoviral vectors by cardiomyocytes as shown by the *in vitro* experiments. Incorporation of the 4F2 transcription enhancer into the adenoviral vectors [30] prolonged transgene expression *in vivo* since expression of GFP and hAM persisted in cardiomyocytes for at least four weeks after adenovirus administration. In contrast, transgene expression has been found previously to peak after ~5-9 days and then decline markedly between 17-25 days when adenoviral vectors

lacking the 4F2 transcription enhancer were used [32-34]. Uniform and abundant expression of GFP within the populations of viable cardiomyocytes isolated (combined with the demonstration of functional consequences of hAM gene delivery discussed below) confirmed that systemic delivery via the tail vein enabled the adenoviral vectors to be taken up by the myocardium in sufficient quantities despite the probability of significant uptake of adenovirus by other organs, notably the liver [30, 33]. The presence of the 4F2 transcription enhancer has been shown previously to increase transgene expression by 10-15 fold compared to vectors lacking this modification [30]. Localized and abundant expression of hAM would therefore have occurred in the myocardium throughout the duration of the experimental protocol.

Adenoviral mediated delivery of hAM markedly attenuated development of cardiomyocyte hypertrophy *in vivo* relative to saline or Ad.Null administration as evidenced by reduction of the extent of increase in cell width and mRNA expression of ANP and sk- α -actin, genes associated with acquisition of the hypertrophic phenotype, providing evidence of a clinically relevant biological response to the peptide *in vivo*. This observation is consistent with previous reports in which adenoviral vector mediated delivery of hAM attenuated hypertensive cardiac hypertrophy in spontaneously hypertensive [31], two kidney one clip (2K1C) [33], Dahl-salt sensitive [34] and DOCA-salt [32] rats. However while attenuation of cardiomyocyte hypertrophy by hAM in these previous studies could reflect a composite of direct and indirect (pressure-dependent) effects of the peptide, in the NO-deficient rat this observation can be attributed to a direct action of the peptide locally upon the myocardium for several reasons. Firstly, AM is known to be a negative regulator of cardiomyocyte hypertrophy which is up-regulated in response to a hypertrophic stimulus [44]: receptors for AM are expressed on cardiomyocytes and the peptide has been shown to attenuate protein synthesis in response to hypertrophic stimuli such as foetal bovine serum or the protein kinase C activator, phorbol ester, in neonatal [25, 27] and adult cardiomyocytes [13] *in vitro*. Secondly, and perhaps more importantly, adenoviral mediated delivery of hAM did not attenuate the rise in blood pressure observed in response to administration of L-NAME; at all time points during this 4 week intervention study, systolic blood pressure levels were similar in L-NAME-treated rats which received Ad.CMV-hAM-4F2 as in those receiving Ad.Null or saline injection. AM is a potent vasodilator (reviewed in [45]); infusion of the peptide lowers blood pressure in rat models of

hypertension [46, 47]. Similarly, delivery of the hAM gene has been shown to evoke a hypotensive effect in other models of cardiac hypertrophy in response to pressure loading, including the spontaneously hypertensive [31], 2KIC [33], DOCA-salt [32] and Dahl-salt sensitive rat [34]. In some of those early studies, vectors lacking the 4F2 transcription enhancer were employed, and as a consequence the hypotensive effect peaked at 8-10 days and waned after 2-3 weeks. In the current study, the use of vectors incorporating the 4F2 transcription enhancer ensured prolonged, augmented expression of the transgene *in vivo*, as evidenced in cardiomyocytes, so an even greater hypotensive action might have been anticipated. While there is some variation with respect to species and vessel, the vasorelaxant action of AM has been attributed predominantly to an endothelium-dependent mechanism involving release of NO [36, 45] although endothelium-independent effects, associated with activation of outward potassium currents and hyperpolarisation of vascular smooth muscle have also been reported [45, 48]. Furthermore, blood pressure is elevated in heterozygote AM knockout mice, accompanied by suppression of NO production [49] and in transgenic mice over-expressing the AM gene, NOS inhibition normalizes the observed reduction in blood pressure [50] indicating a role for NO/cGMP in mediating AM's hypotensive effect. AM stimulates constitutive NOS leading to enhanced NO release from aortic endothelial cells [51]. We [15] and others [52] have reported that the ability of the antioxidant Tempol to lower blood pressure, which has also been attributed to a NO-dependent mechanism, is abolished following chronic administration of L-NAME. Taken together, these results indicate that the absence of a hypotensive effect of hAM following adenoviral vector-mediated delivery of the transgene in the presence of chronic administration of L-NAME is a consequence of the inhibition of vascular NO production; therefore an indirect contribution to attenuation of cardiomyocyte hypertrophy, mediated by blood pressure reduction, can be discounted.

Adenoviral vector-mediated delivery of hAM was also shown to attenuate cardiomyocyte oxidative stress in the absence of blood pressure lowering, as evidenced by reduction in the extent of membrane protein oxidation and expression levels of pro-oxidant and anti-oxidant enzymes. A possible source of ROS required to cause membrane protein oxidation is likely to be provided by increased generation of O_2^- by membrane-associated NADPH oxidase [5-7]. The present findings are consistent with previous reports that cardiomyocyte

expression of pro-oxidant NOX1 and NOX2/gp91 and of the tightly associated membrane protein p22phox and regulatory subunits p47phox and p67phox are enhanced in response to chronic NO-deficiency [15, 16]. Augmented expression of anti-oxidant Cu/Zn SOD (including SOD3) and GPx in response to chronic NO-deficiency is also in agreement with previous reports [15]; this is likely to represent an adaptive defence mechanism to help to offset increasing oxidative stress since SOD promotes dismutation of O_2^- to less reactive H_2O_2 which is subsequently degraded by GPx. In agreement with such mRNA expression data, CuZn SOD activity has been found to be enhanced in the myocardium of L-NAME treated rats [53]. The present findings are also consistent with previous reports that administration of AM to the Langendorff-perfused heart attenuated the extent of ischaemia-reperfusion injury, cardiomyocyte membrane lipid peroxidation and leakage of myoglobin *in vitro* [29] and that adenoviral vector-mediated delivery of hAM [35] reduced the ratio of infarct size to ischaemic area at risk and blunted NADPH oxidase activity and O_2^- production in an *in vivo* model of myocardial ischaemia-reperfusion injury. Generation of O_2^- by pro-oxidant enzymes including NADPH oxidases results in a hypertrophic phenotype [5]. We have reported previously that, despite inability to reduce systolic blood pressure in L-NAME treated rats due to the profound inhibition of vascular NO synthesis prevailing as a consequence of chronic administration of L-NAME [12], antioxidant therapy with Tempol/Vitamin C normalized cardiomyocyte oxidant status and partly attenuated L-NAME induced increase in cardiomyocyte width [15] implying a contribution of myocardial oxidative stress directly to development of cardiomyocyte hypertrophy. Attenuation of cardiomyocyte hypertrophy by hAM, directly, in the absence of blood pressure lowering, may also therefore reflect in part the contribution of attenuation of oxidative stress.

G_s -mediated activation of adenylate cyclase represents a major signalling pathway coupled with AM receptors [37] although $G_{q/11}$ -mediated coupling to phospholipase C- βG_i and/or G_o -mediated regulation of potassium channels, activation of phosphatidylinositol 3-kinase (reviewed in [54]), and NO-dependent activation of guanylate cyclase [36] resulting in attenuation of the calcineurin-NFAT hypertrophic signalling pathway [44] and inhibition of O_2^- production [55], have been reported. The cardio-protective effects, anti-proliferative effects on fibroblasts, and anti-apoptotic effects on cardiomyocytes of AM are mediated in part by accumulation of cAMP [26, 35, 56]. Adenoviral vector-

mediated delivery of hAM to the DOCA-salt rat [32] was associated with increased cardiac content of cAMP. In contrast, cardiac cGMP levels were elevated in response to adenoviral vector-mediated delivery of hAM in a model of myocardial ischaemia-reperfusion injury while those of cyclic AMP were not [35]. In the present study conducted in a model of chronic NO deficiency, attenuation by hAM of cardiomyocyte oxidative stress and hypertrophic remodelling cannot be attributed to an NO/cyclic GMP dependent mechanism. Although isoform-selective inhibitors of NOS have been developed, established rat models of chronic nitric oxide deficiency have generally employed isoform non-selective inhibitors of NOS such as L-NAME. This strategy was considered appropriate for our experiments since we had extensively characterised this model and wished to investigate evidence specifically for NO-independent actions of AM. However, NOS isoform involvement in those actions of AM which are likely to be mediated via NO-cGMP dependant signalling (for example blood pressure reduction) could be explored in subsequent studies either in transgenic mice models in which individual NOS isoforms are specifically over-expressed or knocked-down respectively, or through establishment of rat models of chronic inhibition of specific NOS isoforms using appropriate pharmacological inhibitors.

Rather than an NO/cyclic GMP dependent mechanism, the actions of hAM in the present study are most likely to be accounted for by alternative, probably cAMP dependent, signalling pathways. This conclusion is in agreement with the observation of Pan and co-workers [57] who reported that AM stimulated cAMP accumulation in myocardial tissue sections *in vitro* and that accumulation of this second messenger substance was augmented in tissue from rats subjected to chronic NO deficiency, consistent with an up-regulation in receptor number, which they [57] and we have [13] observed in myocardial tissue and cardiomyocytes,

respectively. Further studies are warranted to address receptor-subtype involvement and coupling to signal transduction pathways in protection against cardiomyocyte oxidative stress and hypertrophy. Due to the relative non-selectivity of antagonists such as CGRP₈₋₃₇ and AM₂₂₋₅₂ [45], such questions might best be answered in transgenic mice models targeting knock-down of specific receptor activity modifying proteins.

In conclusion, adenoviral vector mediated delivery of hAM resulted in attenuation of myocardial oxidative stress and hypertrophic remodelling in the absence of blood pressure normalization in a model of chronic NO-deficiency. This finding provides novel mechanistic insight, consistent with a direct cardio-protective action in the myocardium of locally-derived hAM which is not dependant on generation of NO and subsequent accumulation of cGMP. Since the cardio-protective action of AM was independent of blood pressure reduction, delivery of a source of the AM gene packaged in an adenoviral vector might ultimately provide a useful addition to standard blood pressure lowering therapy in managing the cardiac complications of hypertension, such as LVH, and reducing overall cardiovascular risk.

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