Adrenomedullin is a regulated modulator of neonatal cardiomyocyte hypertrophy in vitro

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Abstract

Objective: Adrenomedullin is a potent hypotensive, natriuretic and diuretic peptide that is coexpressed in the heart with its receptor, suggesting that it may have localized actions as a modulator of cardiac function. Although expression of adrenomedullin is upregulated in the pathological heart, its cardiac function has not been clearly elucidated and it is not known whether this represents a common feature of cardiac hypertrophy, nor whether this is restricted to cardiac myocytes. We have determined the direct effects of hypertrophic agents on cardiomyocyte adrenomedullin gene expression and peptide secretion and have examined the effects of adrenomedullin on biochemical markers of cardiomyocyte hypertrophy. Methods: Regulation of adrenomedullin expression and its effects on the hypertrophic response were studied in cultured rat neonatal ventricular cardiomyocytes. Results: Incubation with phenylephrine or endothelin for 48 h led to a hypertrophic response with an associated fivefold stimulation of ANP gene expression. In contrast, adrenomedullin mRNA was inhibited by 30–50% in response to phenylephrine or endothelin-mediated hypertrophy, and this was associated with a 35–45% reduction in secretion of immunoreactive adrenomedullin. Phorbol ester mediated activation of protein kinase C and increasing intracellular Ca with ionomycin led to significant downregulation of adrenomedullin gene expression in cardiomyocytes. Co-incubation with 100 nM adrenomedullin for 48 h inhibited phenylephrine-induced cardiomyocyte hypertrophy as determined by protein:DNA ratio. Adrenomedullin partially blocked phenylephrine-mediated transcriptional activation of ANP and MLC-2 reporter gene expression in cardiomyocytes and this effect was mimicked by 2 \textmu M forskolin, suggesting that this response was mediated via the activation of adenylate cyclase. Conclusion: These data demonstrate that the cardiomyocyte adrenomedullin gene is repressed by phenylephrine or endothelin-mediated hypertrophy. The inhibitory effects of adrenomedullin on the cardiomyocyte hypertrophic response suggests that this peptide acts as a regulated autocrine or paracrine modulator of cardiomyocyte function and that downregulation of adrenomedullin expression may play a role in induction and maintenance of cardiomyocyte hypertrophy. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Adrenomedullin (AM) is a vasoactive peptide hormone that has been shown to have potent hypotensive actions in a variety of species [1]. In addition to its vascular effects, administration of AM has natriuretic and diuretic actions [1], as well as direct actions on the heart [2], suggesting that AM represents an important hormone involved in regulation of cardiovascular physiology. Immunoreactive (ir)-AM has been found in human plasma, with significantly elevated levels that increase in proportion to clinical severity in hypertensive [3] and congestive heart failure patients [4,5]. Immunocytochemical staining of human heart tissue showed that ir-AM is expressed in cardiomyocytes [5] and in addition, ir-AM has been shown to be released into the coronary sinus in response to myocardial ischemia, suggesting that such damage results in release of AM from the heart [6]. High expression of AM mRNA and ir-AM has also been demonstrated in vascular endothelium and smooth muscle cells [1], indicating that

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cells other than cardiomyocytes may produce AM in the heart, and raising the possibility that these non-myocardial cells may additionally contribute to the overall cardiac expression of AM. Animal models have demonstrated that expression of AM can be upregulated in the heart in some models of hypertension, cardiac hypertrophy and cardiac ischemia. Aorto caval shunt-induced volume overload caused sustained elevation of AM mRNA and ir-AM peptide levels in rat LV [7], while pressure overload hypertrophy induced by monocrotaline [7] or aortic banding [8] had no effect on LV AM mRNA. Experimental ischemic heart failure in rats also causes a marked and prolonged upregulation of AM expression, both within and outside the infarcted region of the myocardium [9].

A number of studies have indicated that factors such as angiotensin II, endothelin, and insulin-like growth factor can be activated within the heart and are likely to play an autocrine/paracrine role in the establishment of cardiomyocyte hypertrophy and progression to heart failure [10–12]. Adrenomedullin binding sites are abundant in heart [13], and we have previously shown that adrenomedullin receptor genes are expressed in cardiac myocytes [14]. Taken together, the localised expression of adrenomedullin and its receptor strongly suggest that this peptide may function as an intracardiac regulator of myocyte function. This notion is supported by studies demonstrating that exogenously administered AM inhibits angiotensin II-stimulated [14]phenylalanine incorporation in neonatal cardiomyocyte cultures, and in addition, blocking endogenous AM action with a monoclonal anti-AM antibody enhances the effects of angiotensin II [15]. Despite in vivo evidence that AM can be upregulated during cardiac hypertrophy and the progression to heart failure, whether or not these changes are specific to the cardiomyocyte and the mechanisms involved in regulating its expression are not known. In the present study, we have used cultured neonatal cardiomyocytes to directly determine whether AM expression is regulated by agents known to induce a hypertrophic phenotype and have also determined the effects of exogenous AM on the cardiomyocyte hypertrophic growth response and on expression of hypertrophy-induced genes. The current data demonstrate that AM has anti-hypertrophic effects and that cardiomyocyte AM gene expression is downregulated in response to phenylephrine or endothelin, suggesting that repression of local AM production may play a role in the maintenance of the hypertrophic phenotype in the cardiac myocyte.

2. Methods

2.1. Neonatal cardiomyocyte cultures

Primary cultures of cardiac myocytes (MC) were prepared from 1–2-day-old Sprague–Dawley rat pups in accordance with the Australian Code of Practise for the Care and Use of Animals for Scientific Purposes as described [16]. Following initial digestion with 0.1% collagenase for 15 min at 37°C, ventricles were washed in dissociation buffer (calcium-free Dulbecco’s phosphate buffered saline containing 1 mM MgSO4) and single cell preparations were then prepared by multiple 5-min incubations with 0.08% trypsin and 0.01% DNase. Cells were then pre-plated in 100-mm culture dishes at 37°C for 30 min to remove the majority of fibroblasts. MC were removed, counted and plated at an initial density of 750 cells/mm² for 18 h in DMEM containing 10% FCS+5% horse serum, 0.1 mM bromodeoxyuridine (BdU), and penicillin-streptomycin-fungizone solution (1:100 dilution, CSL, Parkville, VIC, Australia). MC were then washed in serum-free DMEM containing 0.1 mM BdU, 10 μg/ml bovine transferrin (Sigma) and 2.5 U/ml human insulin (Novo Nordisk Pharmaceuticals, Australia), incubated in this medium for a further 48 h, and experiments were performed by incubating cells in the same medium without BdU. Phenylephrine-hydrochloride (PE) and propandiololol-hydrochloride (PRO) were purchased from Sigma Chemicals and were used at final concentrations of 25 μM and 1 μM, respectively. In all experiments using PE, PRO was included to block β-adrenergic receptors. Human endothelin-1 (ET) was obtained from Auspep (Parkville, VIC, Australia) and rat adrenomedullin was purchased from Phoenix Pharmaceuticals (Belmont, CA, USA) and were used in culture experiments at the concentrations indicated. Phorbol-12-myristate-13-acetate (PMA) and ionomycin were both purchased from Calbiochem and were dissolved in DMSO.

2.2. RNA preparation and ribonuclease protection analysis

Following incubation with test substances, medium was aspirated from MC cultures, the cells washed with phosphate buffered saline and total RNA isolated as previously described [16]. A 613-nt RT-PCR cloned rat AM cDNA fragment encompassing the entire coding region, a 755-nt rat ANP cDNA and a 177-nt rat GAPDH cDNA clone (GAPDH-177) were used as templates to generate 32P-labeled cRNA probes for use in solution hybridization/RNase protection analysis as previously described [16,17]. AM and ANP probes were labelled to a specific activity of ~5×10⁸ cpm/μg RNA and GAPDH probes to 2×10⁸ cpm/μg RNA. Solution hybridization and RNase digestion was carried out as described previously [16,17] and protected RNA hybrids were analyzed on non-denaturing polyacrylamide gels and quantitated on a Fuji BAS-1000 phosphorimaging system. Quantitation of ANP and AM mRNA transcripts was achieved by comparison to standard curves generated following RNase protection of known amounts of in vitro synthesized sense RNA transcripts as
described [16]. Levels of GAPDH mRNA, simultaneously hybridized in each sample, were used to correct for minor differences in RNA loading.

2.3. Transient transfection and reporter gene assays

For transient transfection, MC in 12-well plates were washed 24 h post-plating with DMEM with no additives and then transfected with 0.5 μg DNA and 1.5 μl Tfx-50 (Promega) in 400 μl DMEM for 1 h. Transfection medium was overlaid with 1 ml DMEM containing 0.1 mM BdU, 10 μg/ml bovine transferrin and 2.5 U/ml human insulin and MC were incubated for a further 24 h. Medium was then replaced with DMEM containing 10 μg/ml bovine transferrin, 2.5 U/ml human insulin and the appropriate test substances as indicated. Transcriptional activity of the ANP and myosin light chain 2 (MLC-2) promoters were measured using a plasmid containing a 700 base-pair fragment of the rat ANP promoter linked to firefly luciferase (ANP328 [18], courtesy of Dr. M. Nema) or the MLC-2 promoter linked to firefly luciferase ([19]; courtesy of Dr. J. Heller Brown). Luciferase activity was measured in 20 μl aliquots after 24–48 h treatment following lysis in 200 μl reporter lysis buffer (Promega).

2.4. Radioimmunoassay of AM

Media samples were extracted on Sep-Pak C18 columns (Waters, MA, USA) with 80% methanol+0.1% trifluoro-acetic acid as eluting solvent and peptide extracts were lyophilized and reconstituted in radioimmunoassay buffer for determination of immunoreactive (ir)-AM concentration as described previously [17]. The DNA content of each well was determined [20] as an indicator of MC number in each experiment and was used to normalize ir-AM secretion data.

2.5. Statistical analysis

For all multi-group comparisons, data were analyzed by one-way ANOVA followed by Fisher’s PLSD for post hoc comparisons. Student’s t-test was used for comparisons between two groups. All data are presented as mean±S.E.M. and P<0.05 was considered to be significantly different.

3. Results

3.1. Cardiomyocyte hypertrophy in vitro inhibits AM gene expression and peptide release

To directly determine the influence of hypertrophy on cardiomyocyte AM expression, MC cultures were treated for 48 h with the alpha adrenergic (α-AR) receptor agonist phenylephrine (PE) or endothelin-1 (ET), which are well recognised stimulators of cardiomyocyte hypertrophy [21]. MC maintained in defined serum free did not hypertrophy, but were readily responsive to various hypertrophic agents as previously shown [16]. After 48 h incubation with 25 μM PE or 50 nM ET, cardiomyocytes displayed an increase in cell size, had made extensive cell–cell contacts, and were beating synchronously. Associated with these changes in cellular morphology was a significant increase in cellular protein and total RNA, indicative of a hypertrophic response (data not shown).

In addition to changes in cellular morphology and increased biosynthesis of both RNA and protein, levels of ANP mRNA were also assessed in MC cultures as an indicator of cardiomyocyte hypertrophy (Fig. 1B). In ventricular MC cultures incubated in the presence of PE or ET for 48 h, ANP mRNA was elevated 4–5-fold above control levels (Con, 26±8; PE, 113±6; ET, 130±5 amol/μg RNA).

In sharp contrast to ANP, RNase protection analysis of AM mRNA using a probe that spans part of exon 1 and the entire AM prohormone coding region (all of exons 2, 3 and part of exon 4) demonstrated that AM mRNA expression is downregulated as part of the hypertrophic phenotype in the cardiomyocyte (Fig. 1A). Both PE and ET caused significant inhibition of AM mRNA expression (Con, 1.3±0.14; PE, 0.87±0.06; ET, 0.43±0.05 amol/μg RNA, mean±S.E.M. of 7–8 cultures) in MC cultures treated for 48 h (Fig. 1A). Using a different AM mRNA probe complimentary to 225 nucleotides of exon 4 only, it was similarly shown that AM mRNA was downregulated ~5-fold in hypertrophied MC following 48 h stimulation with PE (Fig. 1C). Furthermore, RT-PCR using primers spanning exon 1–exon 4 of the rat AM sequence confirmed that PE led to downregulation of full length AM mRNA transcripts, while ANP primers demonstrated upregulated ANP mRNA in the same samples (Fig. 1D).

Release of ir-AM from cardiomyocyte cultures was measured by specific radioimmunoassay following 48 h incubation with or without hypertrophic agents. Control cardiomyocytes actively secreted ir-AM into the medium (116±22 pg/μg DNA over a 48 h period), and peptide release was significantly inhibited in MC that had been incubated in the presence of either PE or ET (Table 1). Accumulated ir-AM secreted over a 48 h period was 35–45% lower (64±2 and 74±6 pg/μg DNA) in PE or ET treated cells, respectively, in comparison to control MC.

3.2. Decay of AM mRNA in control versus hypertrophied cardiomyocytes

To test whether the downregulation of AM mRNA in hypertrophied MC may be due to alterations in transcript stability potentially related to AU rich motifs in the 3′ tail of the AM mRNA, MC were treated for 48 h with PE after which actinomycin D (2 μg/ml) was added to the cultures...
Fig. 1. Solution hybridization/RNase protection of AM, ANP and GAPDH mRNA following in vitro induction of hypertrophy in neonatal ventricular cardiomyocytes. Cardiomyocyte cultures were treated for 48 h with 25 μM PE+1 μM PRO or 50 nM ET and total RNA was used for simultaneous hybridization with either (A) GAPDH and a probe spanning the entire AM prohormone coding region (2 μg RNA) or (B) ANP/GAPDH probes (0.2 μg RNA). Phosphorimages shown represent RNase protected bands from individual cultures. Quantitative data represents mean±S.E.M. of 7±8 cultures. *P<0.01 versus control. (C) RNase protection of a 225-nt fragment of exon 4 of rat AM mRNA following 48 h PE-induced hypertrophy. (D) RT-PCR of a 613-nt fragment of AM mRNA spanning exons 1–4 or a 402-nt fragment of ANP mRNA from control or 48 h PE-treated cardiomyocyte RNA.

to arrest gene transcription. Prior to the addition of actinomycin D, AM mRNA expression was downregulated to ~15% of control levels in MC treated with PE for 48 h (Fig. 2). Although the level of GAPDH mRNA expression
however, PE failed to cause a significant hypertrophic response in cardiomyocyte cultures as measured by protein:DNA ratio (Con, 5.0 ± 0.2; PE, 7.90 ± 1.1; AM, 4.3 ± 0.25; AM+PE, 5.7 ± 0.35; mean ± S.E.M., n = 4).

To determine whether AM influences the transcriptional activity of genes known to be activated as part of the hypertrophic phenotype, we examined the effects of AM on the PE-stimulated activity of luciferase reporters under the control of either the MLC-2 or ANP promoters. Basal transcriptional activity of the MLC-2 or the ANP promoter constructs was not significantly altered after 48 h incubation in the presence of 100 nM AM (Fig. 4B and C). PE alone led to significant transcriptional stimulation of the MLC-2 (7.4-fold above control, P < 0.0001) and ANP (2.9-fold above control, P < 0.0001) promoters. In the presence of 100 nM AM, the stimulatory effect of PE on both MLC-2 and ANP reporter gene transcription was partially blocked relative to the effects of PE alone. AM inhibited PE-induced transcription of the MLC-2 and ANP gene promoters by ~40% and 25%, respectively (Fig. 4B and C).

We further determined whether the inhibitory effects of AM on PE-stimulated ANP gene transcription could be mimicked by the adenylate cyclase activator forskolin. Incubation with PE (24 h) led to an eightfold increase in ANP-Luc activity which was inhibited by ~40% in the presence of 100 nM AM (Fig. 4D). Coincubation of cardiomyocyte cultures with low dose forskolin (0.1 μM) had no effect, however, 2 μM forskolin blocked PE-stimulated ANP-Luc activity to a similar extent as 100 nM AM suggesting that the inhibitory effects of AM may be mediated via the activation of cAMP.

4. Discussion

Since its discovery in 1993, AM has been shown to have a range of biological effects including vasodilation, natriuresis and diuresis [1], suggesting that AM may play a similar role to ANP in regulating cardiovascular responses. The sustained upregulation of ANP gene expression in ventricular cardiomyocytes is a consistent marker of hypertrophy in response to a wide variety of agents both in vivo and in vitro [21]. In the present study, we have shown that AM mRNA expression is downregulated in hypertrophied cardiomyocytes, suggesting that these two genes are differentially expressed and regulated in cardiomyocytes. The relatively low level of cardiac expression of AM and the colocalization of AM receptors in cardiomyocytes [14] is consistent with this peptide having autocrine/paracrine effects.

Both α-AR and ET-1 receptors mediate hypertrophic signalling in cardiomyocytes via coupling to Gq which in turn leads to PKC activation, alterations in Ca2+ signalling...
Fig. 2. Decay of AM mRNA in control and hypertrophied neonatal cardiomyocyte cultures. MC cultures were treated in the presence or absence of 25 μM PE+1 μM PRO for 48 h and actinomycin D (2 μg/ml) was then added for the indicated times to arrest gene transcription. Total RNA was harvested from each well and the entire amount (one well equivalent) was used for simultaneous solution hybridization/RNase protection of AM and GAPDH mRNA. (A) Representative RNase protected bands showing changes in AM and GAPDH mRNA, and (B) quantitative changes in AM and GAPDH mRNA transcripts. Data represent mean±S.E.M. of three individual cultures.

and activation of mitogen activated protein kinase (MAPK) pathways [22]. Direct activation of PKC with PMA leads to hypertrophic growth of cardiomyocytes [23] associated with transcriptional activation of ANP and MLC-2 gene promoters [19], suggesting that PKC is a key mediator of the cardiomyocyte hypertrophic response. In our studies, activation of α-AR or ET-1 receptors led to an obvious hypertrophic response in cardiomyocytes that was characterised by induction of total cellular RNA and protein and MLC-2 and ANP gene transcription. In marked contrast to MLC-2 and ANP, however, the hypertrophic response to ET or PE in cultured MC caused significant downregulation of AM mRNA and ir-AM secretion. The phorbol ester PMA and the Ca²⁺ ionophore ionomycin similarly had opposite effects on ANP and AM gene expression in cardiomyocyte cultures, raising the possibility that PKC and intracellular Ca²⁺ are key mediators of the hypertrophy-mediated repression of AM gene expression. A diverse range of genes have been shown to be upregulated in the hypertrophied heart [24], however,
relatively few downregulated genes have been described. Like AM, the cardiomyocyte sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) gene has been shown to be downregulated by hypertrophic agents such as endothelin in a PKC-dependent manner [25,26], suggesting that the AM and SERCA2 genes may be repressed during cardiomyocyte hypertrophy in a similar manner. Although the precise molecular mechanisms have not been fully elucidated, there is evidence to suggest that PMA-induced mRNA destabilization may in part be responsible for SERCA2 mRNA downregulation [26]. AM mRNA transcripts contain multiple AU-rich sequences that may be involved in mRNA destabilization, however, AM mRNA half-life was unchanged following PE-induced hypertrophy, suggesting that transcriptional repression may be the major mechanism involved in downregulating AM mRNA levels. In mouse C3H10T1/2 fibroblasts, transcription of the AM gene is downregulated by Myc-mediated repression via an initiator element (INR) in the AM promoter [27]. Since c-myc gene expression is rapidly induced in cardiomyocytes in response to \(\alpha\)-AR activation [28], it is possible that this might represent a mechanism for transcriptional repression of the AM gene. Despite the clear inhibitory effects of hypertrophy on neonatal cardiomyocyte AM expression in vitro, in vivo studies in adult rats show differential effects on cardiac AM expression. Whether this reflects differences between neonatal and adult cardiomyocytes or merely the cumulative measurement of AM expression in multiple cell types in whole heart is not clear. Volume overload induced by aortocaval shunt causes sustained elevation of AM mRNA and ir-AM peptide levels in rat LV [7], while monocrotaline-induced pressure overload hypertrophy [7] or aortic banding [8] had no effect on LV AM mRNA. Taken together, these data suggest that the cardiac AM gene is not upregulated as part of the hypertrophic phenotype per se and that its induction is dependent upon the etiology of the hypertrophy. Consistent with this, it was shown that acute pressure overload produced in rats by infusion of arginine vasopressin for 2 h caused increased ventricular expression of AM mRNA that was independent of myocyte hypertrophy [29]. On the other hand, ventricular AM gene expression is consistently induced in the failing heart [5,8,9,30], suggesting AM gene activation as ventricular dysfunction progresses. Conflicting data have been presented regarding the precise cellular localization of these changes however, with increased immunocytochemical staining of ir-AM shown in cardiac myocytes [5] and
microvascular endothelial cells [9] of the failing heart. Thus it is likely that AM expression may be regulated in multiple cell populations in the pathological heart, and that overall cardiac AM levels do not necessarily reflect expression in cardiac myocytes.

The heart has a high density of AM binding sites [13] and putative AM receptor genes are expressed in cardiomyocytes [14], implying that these cells are direct targets of AM action. Consistent with a previous study demonstrating that AM inhibits angiotensin II-stimulated \(^{14}C\)phenylalanine incorporation in neonatal cardiomyocytes [15], we have shown that AM can block PE-mediated cardiomyocyte hypertrophy as determined by inhibition of increases in cellular protein:DNA ratio. In addition to its effects on cellular hypertrophy, AM partially blocked the PE-stimulated transcription of two genes (ANP and MLC-2) that are well-established markers of the cardiomyocyte hypertrophic phenotype [21].

The dose-dependent accumulation of cAMP in cardiomyocytes in response to AM has been described [31,32], suggesting that elevated levels of cAMP may play a role in the antihypertrophic effects of AM. Consistent with this, receptor-independent activation of adenylyl cyclase with 2 μM forskolin led to similar inhibitory effects on PE-stimulated ANP gene transcription. Although the present data provide evidence for AM-mediated inhibitory effects on several parameters of the cardiomyocyte hypertrophic response that may be dependent upon increased cAMP accumulation, the molecular mechanisms involved are not known. Hypertrophic signalling in cardiomyocytes appears to involve Ras-dependent activation of Raf-1 which in turn leads to activation of MAPK pathways [21]. An inhibitory role for cAMP-dependent protein kinase A has been demonstrated in several cell types that effectively blocks Ras-dependent activation of Raf-1 and subsequent activation of downstream MAPK [33]. Though not established in the cardiomyocyte, inhibitory PKA-dependent phosphorylation of Raf-1 could account for some of the antihypertrophic effects of increased intracellular cAMP. In addition, it has been proposed that cAMP may have either positive or negative effects on cardiomyocyte ANP secretion that are related to its effects on Ca\(^{2+}\) transients and cellular contractile state [34]. While low doses of forskolin (0.1 μM) were shown to increase cardiomyocyte Ca\(^{2+}\) transients, contractile frequency and ANP secretion, higher doses of forskolin inhibited these parameters [34], implying that the absolute level of cAMP may be a crucial determinant of the cellular response. Since cellular contraction is a potent stimulus for cardiomyocyte hypertrophy [23], it is possible that the antihypertrophic effects of AM and 2 μM forskolin seen in the current study may be due to subtle alterations in the contractile state of the cardiomyocyte cultures. The differences in previously demonstrated direct cAMP-dependent [35] and cAMP-independent [36] positive inotropic actions, as well as negative inotropic effects of AM [37] may also relate to the concentration of AM used.
In summary, the present study demonstrates that in marked contrast to the ANP gene, induction of cardiomyocyte AM expression is not a general feature of hypertrophy, and that in vitro, hypertrophy induced by the activation of Gq-gcoupled receptors (α1-AR and ET-1) leads to significant repression of cardiomyocyte AM gene expression and peptide secretion. The inhibitory actions of AM on the cardiomyocyte hypertrophic response suggest that AM acts as an intracardiac modulator of cardiomyocyte function and that repression of AM gene expression may act in part to maintain hypertrophic signalling.

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References


