Antioxidant actions contribute to the antihypertrophic effects of atrial natriuretic peptide in neonatal rat cardiomyocytes

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Abstract

Objective: Reactive oxygen species (ROS) such as superoxide have been linked to the hypertrophic response of the heart to stimuli including angiotensin II (AngII), mechanical stretch, and pressure overload. We have previously demonstrated that cGMP and protein kinase G mediate the antihypertrophic actions of the natriuretic peptides in rat cardiomyocytes and isolated whole hearts. The impact of natriuretic peptides on cardiac ROS generation, however, has not been investigated. We tested the hypothesis that reduced superoxide accumulation contributes to the antihypertrophic action of atrial natriuretic peptide (ANP).

Methods: Neonatal rat cardiomyocytes were cultured in serum-free medium with and without AngII (1 μmol/L) or endothelin-1 (ET1, 60nmol/L) in the presence and absence of ANP (1 μmol/L) or tempol (100 μmol/L). Hypertrophic responses, cardiomyocyte superoxide generation, and cardiomyocyte expression of NADPH oxidase were determined.

Results: AngII induced increases in cardiomyocyte size (to 176±9% n=8 p<0.001, at 48h), β-myosin heavy chain expression (to 4.0±1.6-fold n=6 p<0.01, at 6h), c-fos expression (to 1.9±0.5-fold n=7 p<0.01, at 48h), superoxide generation (to 181±21% n=8 p<0.005, at 24h), and expression of the gp91phox subunit of NADPH oxidase (to 2.4±0.5-fold n=7 p<0.05, at 48h). These effects were all significantly inhibited by ANP: cardiomyocyte size, β-myosin heavy chain expression, c-fos expression, superoxide generation and gp91phox expression were reduced to 107 ±5% (n=5 p<0.05), 1.2±0.2-fold (n=6 p<0.05), 0.9±0.2-fold (n=7 p<0.05), 141±21% (n=8 p<0.05), and to 1.0±0.5-fold (n=7 p<0.05), respectively. These effects were mimicked by tempol. ANP and tempol also significantly inhibited ET1-induced increases in cardiomyocyte size and superoxide generation, but had no effect on markers of hypertrophy when studied alone.

Conclusion: This data indicates that the antihypertrophic actions of ANP are accompanied by reduced levels of superoxide, suggesting an antioxidant action contributes to the antihypertrophic actions of ANP.

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Keywords: ANP; Antioxidant; Cardiomyocytes; NADPH oxidase; Oxygen radicals; Peptide hormones

1. Introduction

Cardiac hypertrophy, the enlargement of the heart, is a common complication of many cardiovascular disorders. It initially develops in vivo as an adaptive response to maintain myocardial function, for example in hypertension when cardiac workload is chronically elevated or in response to neurohumoral activation [1–3]. At the level of the cardiomyocyte, hypertrophy is evident as an increase in cell volume, largely in the absence of cell division [4]. A complex process of changes in gene and protein expression is one of the first detectable signs of hypertrophy in myocytes. Cardiomyocyte hypertrophy encompasses the rapid expression of immediate-early genes and transcription factors, re-
expression of a number of embryonic genes such as atrial natriuretic peptide (ANP), and a switch in prevalence of contractile protein expression from α-myosin heavy chain to β-myosin heavy chain with resultant effects on cardiomyocyte sarcomeric organization [3–6]. In the longer term, cardiac hypertrophy progresses to a maladaptive state, with progressive decline in both ventricular contractility and diastolic function [2,6], in addition to increased cardiac sympathetic activity [7]. Ultimately, cardiac hypertrophy becomes an important blood pressure-independent predictor of heart failure and cardiovascular mortality [1,2,8].

The natriuretic peptide family, comprising ANP, B-type (BNP) and C-type (CNP) natriuretic peptides are secreted cardiac hormones that negatively regulate cardiac hypertrophy, cellular growth and proliferation [9–12]. We have previously shown that both ANP and BNP elicit antihypertrophic actions in isolated cardiomyocytes and isolated hearts by activating the natriuretic peptide receptor NPRA, and have previously shown that both ANP and BNP elicit antihypertrophic actions in isolated cardiomyocytes and isolated hearts by activating the natriuretic peptide receptor NPRA, which is coupled to guanylate cyclase, and hence activates cGMP production [9,10]. However, little is known about the exact mechanism of the protective action of ANP beyond cGMP production [9,10]. However, little is known about the exact mechanism of the protective action of ANP beyond cGMP production. Reactive oxygen species (ROS, such as superoxide) have emerged as important triggers of the hypertrophic response, both in the intact heart in vivo [13,14], and in cardiomyocytes in vitro, whether in response to stretch [15] or other hypertrophic stimuli such as angiotensin II [16]. Production of ROS also triggers stimulation of c-fos gene expression [17,18] and activity of ERK1/2 [19], both of which are among the first indicators of hypertrophy. ANP prevents oxidative cell damage in the liver, and inhibits superoxide generation in neutrophils [20,21], but similar studies in cardiomyocytes are lacking. The aim of this study therefore was to test the hypothesis that ANP mediates its antihypertrophic actions via inhibition of superoxide signalling in neonatal rat cardiomyocytes.

2. Methods

This investigation conforms with both the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996) and the National Health and Medical Research Council of Australia guidelines, and was approved by the Animal Ethics Committee of the Baker Heart Research Institute.

2.1. Materials

Angiotensin II, N,N′-dimethyl-9-9′-biacridianion dinitrate (lucigenin), β-nicotinamide adenine dinucleotide phosphate (NADPH) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol) were purchased from Sigma Chemical Company (St. Louis, United States). DNA-free kits were obtained from Ambion (Austin, United States). Rat ANP-28 was from Bachem Feinchemikalien AG (Bubendorf, Switzerland). All reagents for cell culture were of tissue culture grade. Real Time PCR (fluorogenic probes, TaqMan® Universal PCR master mix and TaqMan® RT reagents) reagents were all purchased from Applied Biosystems (Scoresby, Australia). All other materials were purchased from Sigma except where indicated, and were of analytical grade or higher.

2.2. Cell isolation

Cardiomyocytes were isolated from neonatal (1–3 day old) Sprague-Dawley rats using serial enzymatic digestion as previously described [22]. Cardiomyocytes were suspended in sterile Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with penicillin 100 U/mL, streptomycin 100 μg/mL and amphotericin B 0.25 μg/mL and containing 10% foetal bovine serum (FBS). The cells were pre-plated twice (45 min at 37°C) to reduce fibroblast contamination, prior to plating at a density of 1 × 10^5 cells/cm² for determination of cardiomyocyte superoxide generation (using 96-well Optiplate, Perkin Elmer, Rowville Australia) and changes in gene expression (60 mm dishes). Cardiomyocytes were plated at a density of 2 × 10^4 cells/cm² for measurement of cell size (13 mm round coverslips). Following removal of cardiomyocyte-enriched culture medium at the end of the two pre-plating steps, the remaining attached cells (cardiac fibroblasts, assessed by microscopic evaluation) were cultured in DMEM supplemented with 10% FBS and grown to confluence. Cardiac fibroblasts were then plated to confluence in 96-well Optitray plates (Perkin Elmer) for determination of cardiac fibroblast superoxide generation. Fibroblasts up to passage level 3 were used for experiments (with 24 h serum starvation prior to study). The cardiomyocyte culture medium was changed to serum-free DMEM after 48 h and cells were then incubated at 37°C for 48 h until the time of study.

2.3. Quantitation of superoxide levels

Superoxide generation in neonatal rat cardiomyocytes and cardiac fibroblasts was determined using NADPH (100 μmol/L)-driven lucigenin-enhanced chemiluminescence, as previously described [23]. Cells were incubated for 24 h with angiotensin II (1 μmol/L), in the presence or absence of ANP (0.3–3.0 μmol/L, as indicated), tempol (100 μmol/L) and/or KT5823 (250 nmol/L, Calbiochem–Novabiochem, La Jolla, CA). ANP, tempol and KT5823 were present for 24 h prior to, and for the duration of, angiotensin II incubation. Media was replaced with a Krebs buffer containing NADPH and lucigenin (5 μmol/L) for chemiluminescence measurements. Each measurement was expressed as relative light units per second (RLU/sec). Background luminescence (in the absence of cells) was subtracted from the average of 8 readings. The results for cardiomyocyte superoxide generation were confirmed using 2′,7′-dichlorodihydro-fluorescein diacetate fluorescence (H2DCF-DA), which detects a range of ROS including...
superoxide, hydrogen peroxide and peroxynitrite. Cardiomyocytes were incubated as above, prior to replacement of medium with phosphate-buffered saline supplemented with H$_2$DCF-DA (10μmol/L). Fluorescence measurements (excitation 485nm, emission 520nm) were expressed as relative fluorescence units/second (RFU/sec), with background fluorescence subtracted from the average of 8 readings. The average level of ROS generation was then calculated and expressed relative to paired control cardiomyocytes.

### 2.4. Measurement of cardiomyocyte size

Cardiomyocytes were plated onto sterile coverslips (13mm diameter) at low density, specifically to permit delineation of single (not overlapping) cells. Following 48h of incubation with study drugs (angiotensin II±ANP or tempol), coverslips were inverted onto microscope slides. Using a phase contrast microscope at 1034× 1300 resolution, 6–8 fields of live myocytes were randomly chosen and photographed at 10× magnification. Images were analysed using Optimas software (Media Cybernetics, Silver Springs, USA), with a photographed scale of the 10× objective lens for calibration. The 2D area (μm$^2$) of cardiomyocytes was calculated by tracing edges of live cells. For each treatment, 30–40 individual myocytes were measured. Data were represented as mean area± SEM, % of paired control.

### 2.5. Changes in hypertrophic gene expression

We have previously determined that maximal induction by angiotensin II of the immediate-early gene c-fos and the β-isoflorm of the contractile protein myosin heavy chain is evident after 6h and 48h of incubation, respectively using real-time Polymerase Chain Reaction (PCR, Ritchie unpublished). In the present study, cardiomyocytes were incubated for either 6h or 48h with angiotensin II (1μmol/L), for determination of c-fos and β-myosin heavy chain gene expression, respectively. The effect of ANP (1μmol/L) on angiotensin II-induced changes in gene expression was determined. In both cases, ANP was present for 48h, i.e. added 24h prior to angiotensin II for c-fos measurement and concomitantly with angiotensin II for β-myosin heavy chain measurement. At the end of the incubation, total RNA was extracted from cardiomyocytes using the single-step acid guanidine-thiocyanate method, as previously described [9,10,24]. In brief, DNase-treated RNA was reverse-transcribed using Taqman® reagents and the GeneAmp PCR system 9700 (Applied Biosystems). Primer and probes for real time PCR are shown in Table 1. Reactions for the test gene (either c-fos or β-myosin heavy chain) and 18S were amplified in the same tube to determine the increase in their number of transcripts relative to 18S [9,10,24]. In a parallel series of experiments, cardiomyocyte de novo protein synthesis was also determined, via [$^3$H]phenylalanine incorporation, following 24h of incubation with angiotensin II±ANP, as previously described [9].

#### 2.6. Changes in NADPH oxidase expression

Cardiomyocytes were incubated for 48h with angiotensin II (1μmol/L), for determination of gene expression of gp91$^{phox}$, a sarcolemmal subunit of NADPH oxidase (also known as Nox2). The effects of both ANP (1μmol/L) and tempol (100μmol/L) on angiotensin II-induced changes in gene expression were determined. At the end of the incubation, total RNA was extracted for real time PCR analysis as above (see Table 1). In a parallel series of experiments, cardiomyocyte protein expression of gp91$^{phox}$ was also determined, via Western analysis. After electrophoresis and transfer, we used an affinity purified goat

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Optimal concentration</th>
<th>cDNA template amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-fos forward primer</td>
<td>CAACGAGCCCTCTCCTCTGACT</td>
<td>10nmol/L</td>
<td>5ng</td>
</tr>
<tr>
<td>c-fos reverse primer</td>
<td>TGCCTCTCTGACTGCTACA</td>
<td>10nmol/L</td>
<td></td>
</tr>
<tr>
<td>c-fos probe</td>
<td>6FAM-CTGACGCTGACCAACTGCTAGC-TAMRA</td>
<td>10nmol/L</td>
<td></td>
</tr>
<tr>
<td>β-MHC forward primer</td>
<td>GTCAAGCTCTAGTAATCTGTT</td>
<td>10nmol/L</td>
<td>4ng</td>
</tr>
<tr>
<td>β-MHC reverse primer</td>
<td>GAAAGGTAGCATCTCTTGGC</td>
<td>10nmol/L</td>
<td></td>
</tr>
<tr>
<td>β-MHC probe</td>
<td>6FAM-CTACAGGTGGCATGACT-ACT-MGBNFQ</td>
<td>10nmol/L</td>
<td></td>
</tr>
<tr>
<td>gp91$^{phox}$ forward primer</td>
<td>TCAAAGTGCTGCCAGGTATCACA</td>
<td>250nmol/L</td>
<td>25ng</td>
</tr>
<tr>
<td>gp91$^{phox}$ reverse primer</td>
<td>TTCTCTACGAGCAAGGCTAGT</td>
<td>250nmol/L</td>
<td></td>
</tr>
<tr>
<td>gp91$^{phox}$ probe</td>
<td>6FAM-CTAGGGTGGCACCAT-T-MGBNFQ</td>
<td>250nmol/L</td>
<td></td>
</tr>
<tr>
<td>18S forward primer</td>
<td>TGTTCCCATGAGGCTAGATC</td>
<td>120nmol/L</td>
<td>N/A</td>
</tr>
<tr>
<td>18S reverse primer</td>
<td>TGGTGCGGTGAAAAATCC</td>
<td>120nmol/L</td>
<td></td>
</tr>
<tr>
<td>18S probe</td>
<td>VIC-TGCTGTGGCACCAGACTTGCCCT-A TAMRA</td>
<td>125nmol/L</td>
<td></td>
</tr>
</tbody>
</table>

Taqman® chemistry was used, as previously described [9,10,25]. Fluorogenic probes were labelled at the 5′ end with the reporter dye of FAM (c-fos, β-MHC or gp91$^{phox}$) or VIC (18S) and at the 3′ end with a quencher molecule, either TAMRA or MGBNFQ. Reactions for the test gene (either c-fos, β-MHC or gp91$^{phox}$) and 18S were amplified in the same tube to determine the increase in their number of transcripts relative to 18S. Thermocycler conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15s at 95°C and 60°C for 1 min. β-MHC, β-myosin heavy chain; N/A, not applicable (18S amplified in the same tube as test gene).
polyclonal antibody raised against a peptide mapping at the C-terminus of human gp91phox (1:1000, Santa Cruz Biotechnology, Inc, Santa Cruz CA), followed by donkey anti-goat secondary IgG (1:1000, Sigma-Aldrich). Proteins were visualized by enhanced chemiluminescence (kit from Amersham; Buckinghamshire, UK) prior to densitometric quantitation.

2.7. Statistical analysis

All results are presented as mean ± standard error. The “n” value for each set of results represents the number of myocyte preparations studied. For changes in superoxide generation and cardiomyocyte size, results were expressed as a percent of results obtained with paired control cardiomyocytes for each experiment. Changes in gene expression (c-fos, β-myosin heavy chain and gp91phox normalised to 18S) were expressed as fold of paired control. Comparative statistical analyses were all performed using one-way analysis of variance (ANOVA) to compare the effect of angiotensin II with paired controls, or of antihypertrophic interventions (e.g. ANP or tempol) with angiotensin II alone, as indicated. The Newman-Keuls Method for pairwise multiple comparisons was applied where appropriate. Values of $P<0.05$ were accepted as significant.

3. Results

3.1. Antihypertrophic actions of ANP in neonatal cardiomyocytes

We firstly demonstrated that two different hypertrophic stimuli, angiotensin II and endothelin-1, induced hypertrophic responses in neonatal rat cardiomyocytes. Responses to either stimulus were abolished by the natriuretic peptide ANP. As shown in Fig. 1 (representative

![Fig. 1](https://example.com/fig1.jpg)  
*Fig. 1. Representative results for cardiomyocyte size for each treatment studied (following 48h of incubation), at 1034×1300 resolution and photographed at 10×magnification. (a) Control, (b) Angiotensin II (Ang II, 1μmol/L), (c) endothelin-1 (ET1, 60nmol/L), (d) atrial natriuretic peptide (ANP, 1μmol/L), (e) Ang II+ANP, (f) ET1+ANP, (g) tempol (100μmol/L), (h) Ang II+tempol, and (i) ET1+tempol.*
results in individual cardiomyocytes) and Fig. 2 (pooled data from all cardiomyocyte preparations studied), incubation with angiotensin II (1 μmol/L) for 48h increased cardiomyocyte size to 176±9% of control, using computer-aided analysis of two-dimensional area (n=8, P<0.001). A similar hypertrophic response was seen with endothelin-1 treatment (60nmol/L); cardiomyocyte size was increased to 183±6% of control (n=8, P<0.001) after 48h. ANP completely abolished both the angiotensin II-induced increase in cardiomyocyte size, to 107±5% of control (n=5, P<0.05 versus angiotensin II, Fig. 2a), and that induced by endothelin-1 (n=5, P<0.001 versus endothelin-1, Fig. 2b). Cardiomyocyte size was similarly protected by tempol, whether in the presence of angiotensin II (n=4, P<0.05 versus angiotensin II, Fig. 2c) or endothelin-1 (n=4, P<0.001 versus endothelin-1, Fig. 2d). Neither ANP nor tempol alone had any effect on neonatal cardiomyocyte size compared to paired controls.

Real-time PCR was used to measure changes in gene expression of both the immediate-early gene c-fos and the fetal isoform of the contractile protein β-myosin heavy chain, as additional markers of cardiomyocyte hypertrophy. Angiotensin II increased c-fos expression to 1.9±0.5-fold of control neonatal rat cardiomyocytes after 6h of incubation (P<0.01, n=7, Fig. 3a). After 48h of incubation, angiotensin II also increased β-myosin heavy chain expression to 4.0±1.6-fold of control (P<0.05, n=6, Fig. 3b). ANP completely prevented these angiotensin II-stimulated increases; c-fos and β-myosin heavy chain expression were restricted to 0.9±0.2-fold and 1.2±0.2-fold respectively. ANP alone had no effect on either c-fos or β-myosin heavy chain expression in neonatal cardiomyocytes. These results were confirmed using cardiomyocyte protein...
Angiotensin II increased $[3H]$phenylalanine incorporation by 22 ± 4% ($n = 3$, $P < 0.05$); this was completely prevented by ANP, which had no effect alone (results not shown).

3.2. Antioxidant actions of ANP in neonatal cardiomyocytes

Angiotensin II (1 μmol/L) significantly increased NADPH-driven superoxide generation in neonatal rat cardiomyocytes, to 177 ± 24% of control at 24h ($n = 9$, $P < 0.005$ versus control). This was inhibited by ANP in a concentration-dependent fashion (0.03–3.0 μmol/L, $P < 0.05$ versus angiotensin II, Fig. 4a). At a concentration of 1 μmol/L, ANP diminished cardiomyocyte superoxide generation in the presence of angiotensin II to only 127 ± 15% of control ($n = 17$, $P < 0.001$ versus angiotensin II alone, Fig. 4b). The superoxide spin trap tempol (100 μmol/L) mimicked the ANP effect, reducing cardiomyocyte superoxide generation induced by angiotensin II to 134 ± 27% of control ($P < 0.05$ versus angiotensin II, Fig. 4e). Addition of tempol only in the final 30 min of the 24-h incubation with angiotensin II also inhibited the increase in superoxide levels to a similar extent ($n = 5$, $P < 0.05$, data not shown). Moreover, the protein kinase G inhibitor KT5823 (250 nmol/L) significantly attenuated ANP suppression of cardiomyocyte superoxide ($n = 6$, $P < 0.001$ versus angiotensin II + ANP, Fig. 4d). ANP also abolished superoxide generation induced by a second hypertrophic stimulus, endothelin-1. As shown in Fig. 5, cardiomyocyte superoxide generation was significantly increased by endothelin-1 (60 nmol/L), to 287 ± 68% of control at 24h ($n = 6$, $P < 0.05$). ANP (1 μmol/L) inhibited the endothelin-1 response ($P < 0.05$ versus endothelin-1, Fig. 5a), an effect that was again mimicked by tempol (100 μmol/L, $P < 0.05$ versus endothelin-1, Fig. 5b). Addition of tempol only in the final 30 min of the 24-h incubation with angiotensin II also inhibited the increase in superoxide levels to a similar extent ($n = 5$, $P < 0.05$, data not shown). Neither ANP, tempol nor KT5823 alone had any effect on superoxide generation in control cardiomyocytes.

The results obtained with lucigenin detection of cardiomyocyte superoxide generation were confirmed using H$_2$DCF-DA fluorescence, to detect a range of ROS in cardiomyocytes. Angiotensin II (1 μmol/L) significantly increased cardiomyocyte ROS generation 2.6 ± 0.7-fold of control at 24h ($n = 8$, $P < 0.05$ versus control, results not shown). Angiotensin II-induced cardiomyocyte ROS generation was inhibited by ANP (1 μmol/L) and tempol (100 μmol/L), by 27 ± 17% and 35 ± 19% respectively ($P < 0.05$ versus angiotensin II on one-way ANOVA, results not shown).

3.3. Antioxidant actions of ANP in neonatal cardiac fibroblasts

In neonatal rat cardiac fibroblasts, angiotensin II (1 μmol/L) also significantly increased cardiomyocyte superoxide generation to 214 ± 71% of control at 24h ($n = 5$, $P < 0.05$). Similar to the effect in cardiomyocytes, this was inhibited by the natriuretic peptide ANP in cultured fibroblasts, to 118 ± 12% of control ($P < 0.05$ versus angiotensin II, Fig. 6a). The ANP effect was mimicked by tempol (100 μmol/L), which reduced fibroblast superoxide generation to 78 ± 15% of control ($P < 0.05$ versus angiotensin II, Fig. 6b). Neither ANP nor tempol alone had any effect on superoxide generation in control (non-angiotensin II stimulated) fibroblasts.
3.4. ANP inhibits cardiomyocyte expression of NADPH oxidase

Real-time PCR was used to measure changes in cardiomyocyte gene expression of the gp91phox subunit of NADPH oxidase (one of the major enzyme sources of superoxide in cardiomyocytes). Angiotensin II (1μmol/L) increased gp91phox expression to 2.5±0.5-fold of control neonatal rat cardiomyocytes after 48h of incubation (P<0.05, n=8, Fig. 6c). ANP (1μmol/L) completely prevented this angiotensin II-stimulated increases; gp91phox expression were restricted to 1.0±0.5-fold. ANP alone had no effect on gp91phox expression in neonatal cardiomyocytes. These results were confirmed using Western analysis of cardiomyocyte gp91phox protein expression. Angiotensin II increased gp91phox expression to 145±21% of control neonatal rat cardiomyocytes after 48h of incubation (P<0.01, n=8, Fig. 6d); this was completely prevented by ANP, which had no effect alone.

4. Discussion

The key finding to emerge from this study was that the antihypertrophic action of ANP is accompanied by inhibition of superoxide production in neonatal cardiomyocytes. Moreover, ANP-dependent inhibition of the oxidant and hypertrophic responses to angiotensin II were mimicked by the antioxidant tempol. The antioxidant and antihypertrophic actions of both agents were comparable whether determined in response to angiotensin II or endothelin-1, suggesting the cardioprotective actions of ANP are independent of the hypertrophic stimulus used. We also demonstrated that important contributing mechanisms of antioxidant ANP actions were activation of cGMP-dependent signalling.
which is consistent with previous studies [25]. The angiotensin II-induced increase in two-dimensional cell size was also accompanied by changes in hypertrophic gene expression (c-fos and β-myosin heavy chain) and de novo protein synthesis. Moreover, a second, distinct hypertrophic stimulus endothelin-I elicited a similar increase in cardiomyocyte size as angiotensin II. Taken together, agreement between four different markers of hypertrophy and two different hypertrophic stimuli, validates the use of two-dimensional area as an index of cardiomyocyte hypertrophy.

ROS are emerging as growth-signalling factors in cardiomyocytes and many other cell types. Superoxide is one of the most abundant ROS, and many of the others, including H$_2$O$_2$, peroxynitrite and the hydroxyl radical, are derived from superoxide [26]. In vascular smooth muscle cells, the hypertrophic effect of angiotensin II depends on an increased production of superoxide via NADPH oxidase [27]. A similar finding has been described in fibroblasts, in which increases in superoxide have been implicated in mediating cellular growth by acting through Ras [28]. On this basis, we chose to specifically measure accumulation of superoxide in our neonatal cardiomcyocytes, using the superoxide-selective probe, lucigenin. Using this technique, we observed that angiotensin II almost doubled cardiomyocyte superoxide accumulation, i.e. to an extent comparable with its effects on both cardiomyocyte size and expression of the immediate-early gene c-fos. Furthermore, using the same methodology to detect superoxide accumulation in neonatal rat cardiac fibroblasts, we observed that angiotensin II again almost doubled superoxide accumulation in these cells, in line with previous studies [38].

Tempol is a hydrophilic, low molecular weight nitroxide that functions as a superoxide spintrap [29]. In experimental models of hypertension, tempol reduces levels of superoxide and other markers of oxidative stress, in conjunction with lowering blood pressure [30,31]. In the present study, tempol potently blocked the superoxide responses to angiotensin II. Moreover, when tempol was present only for the final 30 min of the 24-h incubation period, angiotensin II-induced superoxide levels were similarly decreased, indicating that tempol was acting to specifically remove superoxide rather than affecting superoxide-stimulated signalling pathways. Taken together, our findings that angiotensin II stimulates superoxide accumulation and ROS generation (using H$_2$DCF-DA), in parallel to hypertrophic responses in cardiomyocytes, and that both responses are blocked by tempol, are compelling evidence that the hypertrophic response to angiotensin II in neonatal cardiomyocytes is mediated specifically via superoxide production. This is in agreement with previous studies [16]. Unlike lucigenin however, H$_2$DCF-DA is unable to distinguish between superoxide and other radical species such as H$_2$O$_2$, other peroxides, and peroxynitrite [26].

There is substantial evidence supporting a protective role for natriuretic peptides in cardiovascular pathophysiology.

![Fig. 5. Superoxide generation by cardiomyocytes (% of paired control). ET$_1$ (60nmol/L)-stimulated increases in cardiomyocyte superoxide generation were significantly attenuated by both (a) ANP (1μmol/L, n=6 P<0.05 on one-way ANOVA) and (b) Tempol (100μmol/L, n=6 P<0.05 on one-way ANOVA). Neither ANP nor tempol alone affected superoxide generation in cardiomyocytes. RLU, relative light units. *P<0.05 vs control, #P<0.05 vs ET$_1$.](https://academic.oup.com/cardiovascres/article-abstract/72/1/112/296196)
We have previously shown that natriuretic peptides prevent hypertrophy in cardiomyocytes, via activation of guanylate cyclase-coupled NPR\_A natriuretic peptide receptors and cGMP-dependent signalling [9], with similar effects in isolated hearts [10]. Absence of either ANP or its receptor NPRA results in a marked hypertrophy evident both in the intact heart and individual cardiomyocytes. Furthermore, this hypertrophy is greater than could be attributed to the concomitant hypertension observed [32–34]; moreover mice with cardiomyocyte-restricted deletion of NPRA exhibit blood pressure-independent cardiac (and cardiomyocyte) hypertrophy [35,36]. Conversely, overexpression of either ANP or NPR\_A significantly decreases heart and cardiomyocyte size [32,37]. Accordingly, both we and others have proposed that ANP is an endogenous antihypertrophic mediator in the heart, serving as a brake to curb a more exaggerated cardiac growth response [9–11,38]. In the present study, we have demonstrated that the antihypertrophic effects of ANP in neonatal rat cardiomyocytes are evident across cell size as well as hypertrophic gene

Fig. 6. Superoxide generation by neonatal rat cardiac fibroblasts (% of paired control). Ang II (1 \(\mu\)mol/L)-stimulated increases in cardiac fibroblast superoxide generation were significantly attenuated by (a) ANP (1 \(\mu\)mol/L) and (b) tempol (100 \(\mu\)mol/L). Neither ANP nor tempol alone affected fibroblast superoxide generation. RLU, relative light units. Both \(n=5\), \(P<0.05\) on one-way ANOVA. ANP also completely prevents Ang II-stimulated increases in gp91\_phox expression in neonatal rat cardiomyocytes, at both the (c) gene (\(n=8\), \(P<0.01\) on one-way ANOVA) and (d) protein levels (\(n=8\), \(P<0.01\) on one-way ANOVA). A representative image of gp91\_phox protein expression from two experiments is shown in the inset of (d). *\(P<0.05\) vs control, #\(P<0.05\) vs Ang II.
expression (c-fos, β-myosin heavy chain). This antihypertrophic action of ANP (and indeed of tempol) was cardiomyocyte-specific and not secondary to changes in load as might be a factor in vivo. Of particular interest, these antihypertrophic actions were accompanied by reduced generation of superoxide, by both cardiomyocytes and cardiac fibroblasts. Given that cardiomyocytes interact with fibroblasts in vivo and that both cell types are a source of superoxide, taken together with the paracrine nature of their interaction [28,39], it is particularly relevant that we have demonstrated that the antioxidant actions of ANP are observed in both cardiomyocytes and fibroblasts. ROS generated by either cell type in the heart in vivo will thus likely be suppressed by ANP.

Furthermore, we have now demonstrated that angiotensin II-induced upregulation of the gp91phox subunit of NADPH oxidase is completely prevented by ANP. Thus, at least one mechanism by which ANP suppresses generation of ROS is by decreasing the levels of their major source in the heart, NADPH oxidase. Based on both the findings presented here and our previous work [9,10], we propose that the mechanism of antihypertrophic action of ANP on cardiomyocyte hypertrophy encompasses activation of NPR_A receptors to initiate cGMP-dependent signalling. Following NPR_A activation, ANP prevents both induction of NADPH oxidase expression and NADPH-driven generation of ROS such as superoxide. Given the emerging role of ROS in the development of cardiomyopathy [13–16], this antioxidant action of ANP likely prevented the superoxide-triggered stimulation of c-fos [17,18] in addition to downstream components of the cardiomyocyte hypertrophy phenotype, including upregulation of both β-myosin heavy chain expression and cardiomyocyte size. Transient induction of c-fos is a key trigger in the initial stages of hypertrophy [40] and its induction may be directly stimulated by superoxide in cardiomyocytes [17]. Although not specifically tested in the present study, ANP would presumably also block superoxide-triggered activation of ERK1/2 [19], an early trigger of the cardiomyocyte hypertrophic response. Evidence in support of this comes from both mice with cardiomyocyte-restricted deletion of NPR-A, in which upregulation of ERK1/2 activity accompanies cardiomyocyte hypertrophy [35], and BNP-transgenic mice, in which angiotensin II-induced hypertrophy is diminished due to inhibition of the MEK-ERK transduction pathway [41]. From the present study, we now propose that ANP antioxidant actions are however clearly downstream of cGMP-dependent signalling (Fig. 4d). The antioxidant effect of ANP, illustrated in Fig. 7, is a novel mechanism of action for this endogenous antihypertrophic mediator, not previously described. Whether ANP-induced decreases are also attributable to inhibition of other enzymatic sources of superoxide (e.g. mitochondria, xanthine oxidase, nitric oxide synthase, etc), upregulation of endogenous antioxidant mechanisms (e.g., superoxide dismutase), or superoxide scavenging action, remains to be elucidated. Although natriuretic peptides inhibit superoxide release from neutrophils [21] and probably also from vascular cells [42], ours is the first report to address this in cardiomyocytes. Whatever the mechanism, the ANP-induced reduction in cardiomyocyte superoxide levels is likely responsible, at least in part, for its antihypertrophic actions. In conclusion, the present study demonstrates that the inhibitory effect of ANP on cardiomyocyte hypertrophy is accompanied by inhibition of superoxide and is mimicked by an antioxidant. This suggests that the antihypertrophic actions of ANP are associated, at least in part, with an

Fig. 7. Proposed mechanism of antihypertrophic action of ANP on cardiomyocyte hypertrophy. Both Ang II (via activation of the AT_1 angiotensin receptor) and ET_1 (via activation of the ET_2 endothelin receptor) stimulate expression of NADPH oxidase, a major source of reactive oxygen species such as superoxide (•O_2^-) in cardiomyocytes. gp91phox is a subunit of NADPH oxidase. The resultant increase in superoxide generation triggers the hypertrophic response in cardiomyocytes (including upregulated expression of c-fos and β-myosin heavy chain in addition to cardiomyocyte size). ANP activates cardiomyocyte particulate guanylate cyclase (pGC)-coupled natriuretic peptide NPR_A receptors; the subsequent cGMP stimulation of protein kinase G (PKG) then suppresses the induction of NADPH oxidase, and hence decreases the amount of superoxide generated by this enzyme. This antioxidant action of ANP mediates a significant component of its antihypertrophic response in cardiomyocytes.
antioxidant effects and are evident at the level of cardiomyocyte size and hypertrophic gene expression. Inhibition of superoxide, such as with an NPR$_A$ agonist, may be a potential target for pharmaceutical prevention of hypertrophy.

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References


