

Estrogen protects against the development of salt-induced cardiac hypertrophy in heterozygous *proANP* gene-disrupted mice

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Abstract

Cardiovascular disease is the leading cause of morbidity and mortality in both men and women, but the incidence for women rises sharply after menopause. This has been mainly attributed in the reduction of the female sex hormone estrogen during menopause, suggesting that estrogen may have cardioprotective effects, although how estrogen exerts its cardioprotective effects is not fully understood. Moreover, the beneficial effect of estrogen on end-organ damage such as cardiac hypertrophy (CH) remains unclear. The aim of the present study was to examine the interaction between estrogen and the natriuretic peptide system (NPS) and their possible roles during the development of CH by using the *proANP* heterozygous atrial natriuretic peptide (ANP +/–) mouse as a model of salt-sensitive CH. Male, female ANP +/– mice,

and also ovariectomized (Ovx) female ANP +/– mice treated with oil or estrogen were fed either a normal or high salt (HS) diet. After a 5-week treatment period, marked CH was noted in the male and oil-injected Ovx female ANP +/– mice treated with HS. The cardiac NPS, i.e. ANP, B-type natriuretic peptide, and natriuretic peptide receptor-A, was activated in these ANP +/– mice. Interestingly, the female and estrogen-injected Ovx female ANP +/– mice did not exhibit CH, and the cardiac NPS remained unchanged. Collectively, we provide direct evidence that estrogen has the ability to resist the induction of salt-induced CH in ANP +/– mice. Furthermore, the development of hypertrophy may be activating the cardiac NPS in an attempt to blunt these structural changes.

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Introduction

Cardiac hypertrophy (CH) is an important predictor of cardiovascular morbidity and mortality, independent of other cardiovascular risk factors. The heart adapts in response to an array of mechanical, hemodynamic, hormonal, and pathological stimuli due to the increased demand for cardiac activity by increasing myocardial mass through the induction of a hypertrophic response (Hunter & Chien 1999). At the cellular level, cardiomyocytes respond by initiating several different mechanisms that lead to hypertrophy. Further, in most forms of CH, there is an increase in the expression of natriuretic peptides (NP) and contractile protein genes, both of which belong to the fetal gene program and are induced by a complex series of signaling events (Chien *et al.* 1993). The initiation of expression of the NP genes such as atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) is one of the most reliable markers for the activation of the hypertrophic program in both clinical and experimental models associated with CH (Hunter & Chien 1999). Furthermore, these genes are also a valuable prognostic indicator of clinical severity of CH in all mammalian species (Hunter & Chien 1999). ANP is mainly produced in the atrium of the heart and initiates natriuresis, diuresis, and vasodilatation via binding to the natriuretic peptide receptor-A (NPR-A), all of which contribute to the

maintenance of cardiovascular homeostasis (Munagala *et al.* 2004). A complete absence of the *NPR-A* gene causes hypertension in mice and leads to CH, particularly more so in males, resulting in sudden death in these 'knockout' mice before 6 months of age (Oliver *et al.* 1997). Furthermore, mice lacking a functional *proANP* gene, and therefore not expressing ANP, have elevated blood pressure and exhibit marked CH (John *et al.* 1995). However, the role of ANP leading to cardiac pathophysiology in the *proANP* gene-disrupted mouse is poorly understood, particularly as it pertains to gender differences.

Studies have shown that the rate of cardiovascular morbidity and mortality in women of childbearing age is significantly lower than in men, while in post-menopausal women it is higher (Baker *et al.* 2003). This observation has led to much speculation as to what physiological changes are associated with menopause that is responsible for the significant increased incidence of CH. 17 β -Estradiol (E2), an important hormone, whose production is significantly decreased in post-menopausal women, is believed to have CH-reducing effects, which are thought to be mediated via increased ANP expression (Grohe *et al.* 1998, van Eickels *et al.* 2001, Babiker *et al.* 2004). E2 also regulates cardiac endothelial and inducible nitric oxide synthase, modulates the activity of the mitogen-activated protein kinase pathways,

and down-regulates the activity of the renin–angiotensin system (Nickenig *et al.* 1998, Nuedling *et al.* 1999a,b). Presently, the underlying mechanism of how E2 and ANP interact and their possible cardioprotective actions are poorly understood.

In the present study, we utilized the *proANP* heterozygous (ANP +/–) mouse as a model of salt-sensitive CH to examine the interaction between E2 and the natriuretic peptide system (NPS) and their possible roles during the development of CH. Male ANP +/– mice, which express approximately half of the ANP content as wild-type mice (ANP +/+), are normotensive and have normal cardiac geometry when maintained on a normal salt (NS) diet (John *et al.* 1995, Sangaralingham *et al.* 2004). However, these mice develop marked CH and become hypertensive when fed a high-salt (HS) diet (John *et al.* 1995, Sangaralingham *et al.* 2004). The ANP +/– mouse is an excellent model to study the progression of salt-induced CH since it can be extrapolated to the human population, as humans are more likely to have a partial rather than absolute deficiency in ANP expression (Rutledge *et al.* 1995, Gruchala *et al.* 2003, Franco *et al.* 2004). Thus, we fed male, female ANP +/– mice, and Ovx female ANP +/– mice treated with oil or E2, with normal (0.8% NaCl) or high (8.0% NaCl) dietary salt for 5 weeks in order to induce CH and examine the expression of components of the NPS. Our data indicates that the cardiac NPS is activated in the salt-loaded mice that have less E2 and the presence of E2 seems to protect against the development of salt-induced CH.

Materials and Methods

Animals

Experimental protocols pertaining to the use of mice in this study have been approved by the Animal Care Committee of Queen's University in accordance with the guidelines of the Canadian Council on Animal Care. ANP gene-disrupted mice initially established by John *et al.* (1995) were bred and maintained in the Animal Care Facility at Queen's University. Male and female ANP +/– mice (9–11 weeks of age) were housed in plastic cages (one to three animals per cage) and kept at a room temperature of 21 ± 1 °C with a 12 h light:12 h darkness schedule.

Ovariectomy surgical procedure

Female ANP +/– mice were anesthetized with 20 mg/kg Xylazine and 100 mg/kg Ketamine intraperitoneally. The abdomen was then shaved and decontaminated with Hibitane, Betadine and rinsed with 70% alcohol. A laparotomy was performed and the small intestines were temporarily placed onto sterile gauze soaked with sterile saline solution. Using forceps, the periovarian fat was gently teased away to exteriorize the ovary. Mosquito forceps were used to grasp

the cranial most part of the uterine horn distal to the ovary and the ovary was removed by a High-temperature cauterizer (World Precision Instruments Inc., Sarasota, FL, USA) above the clamped area. The uterine horn was returned into the abdomen and the process was repeated on the opposite side. Once the ovaries were removed, the small intestines were placed back within the abdominal cavity and the abdominal wall was closed with a continuous locking suture pattern using 5–0 silk. The skin was then closed using wound clips. Mice were placed in a bedded cage heated by a temperature-controlled heating pad and carefully monitored for 24 h. An s.c. injection of buprenorphine (0.1 mg/kg) was administered every 12 h for 3 days for post-operative pain. Normal chow and water were made available *ad libitum*. The mice were allowed to recover for 3 days before the dietary/treatment regimen began.

Dietary/treatment regimen

Age-matched male and female ANP +/– mice were fed either a NS chow containing 0.8% NaCl or a HS chow containing 8.0% NaCl (Lab Diet 5001, Purina Feed, Brentwood, MO, USA) for a total of 5 weeks. A total of 20 mice, 10 of each sex and 5 of each dietary group, were used. Tap water was made available *ad libitum* to the animals. To determine whether estrogen was involved in attenuating CH in ANP +/– mice, ovariectomized (Ovx) female ANP +/– mice were injected daily with 100 µl sterile corn oil (O; Sigma–Aldrich), which acted as a vehicle or 100 µg 17-β-estradiol (E2)/100 µl sterile corn oil (Sigma–Aldrich) for 5 weeks. O–Ovx and E2–Ovx ANP +/– mice were also fed either NS or HS during the same 5-week period. A total of 20 mice, 10 of each treatment group and 5 of each dietary group, were used. Tap water was made available *ad libitum* to all the mice.

Plasma and tissue collection

Blood was collected by cardiac puncture from mice that were anesthetized with sodium pentobarbital (45–50 mg/kg). Blood samples were added immediately to pre-chilled tubes containing Aprotinin (1000 KIU/ml) and EDTA, disodium salt (1 mg/ml) and centrifuged for 10 min at 4000 g to isolate the plasma. Plasma samples were stored at –70 °C until assayed.

The heart was dissected, blotted, and weighed, after which the left and right atria were removed and the right ventricle was carefully separated from the left ventricle and inter-ventricular septum (LV). Ventricular samples were trimmed of any remaining atrial tissue and the apical portions were divided into quarters. All tissues were rapidly snap-frozen in liquid nitrogen and stored at –70 °C until use.

Plasma estrogen measurement

E2 in plasma samples was determined by an enzyme immunoassay as per manufacturer's instructions (Diagnostic Automation Inc., Calabasas, CA, USA).

RNA isolation

Total RNA was extracted from frozen tissue samples by a modification of the acid guanidinium–phenol–chloroform method (Tri Reagent, Sigma) as per the manufacturer's instructions. Precipitated RNA was resuspended in RNase-free (DEPC-treated) water and stored in -70°C until use for northern blot analyses.

Production of RNA probes

ANP and BNP probes were generated from cardiac atria and ventricle of ANP +/+ mice respectively, by RT-PCR using specific primers designed according to published sequence of the mouse ANP and BNP genes (GenBank, <http://www.ncbi.nlm.nih.gov/>). The sequences of primers and their annealing temperatures were as follows:

ANP (sense (S)): 5'-AACCCACCTCTGAGAGAC-3'
 ANP (antisense (AS)): 5'-GGAAGCTGTTGCAGC-CTA-3', 55°C
 BNP (S): 5'-GACAGCTCTTGAAGGACC-3'
 BNP (AS): 5'-GTACCGATCCGGTCTAT-3', 55°C

Resultant PCR products were ligated directly into a plasmid vector (pCRII vector, Invitrogen). Positive clones were identified by sequencing. Nucleotide sequence of cloned PCR fragments were determined by the dideoxynucleotide sequencing method by Cortec DNA Service Laboratories Inc. (Kingston, ON, USA). cDNA sequences were compared with existing sequences in GenBank using the basic local alignment search tool (BLAST) algorithm (<http://www.ncbi.nlm.nih.gov/BLAST>). The above vectors were linearized and antisense digoxigenin (DIG)-labeled riboprobes were produced by *in vitro* transcription as previously described (Tse *et al.* 2001).

Northern blot analysis

Detection of specific mRNA using DIG-ANP and BNP probes (10 ng/ml) were conducted as previously described (Tse *et al.* 2001), and performed as per manufacturer's protocol (Roche Molecular Biochemicals). Quantification of the expression of the specific mRNA bands was normalized with those of the ribosomal 18S rRNA.

Real-time quantitative RT-PCR to measure NPR-A, NPR-C & neutral endopeptidase (NEP) expression

Levels of expression of the NPR-A, NPR-C, and NEP mRNAs were determined in the LV of ANP +/- mice by real-time quantitative RT-PCR. A two-step RT-PCR method was employed. Complimentary first strand DNA was synthesized from oligo dT₁₈ primed total RNA using the Qiagen OmniScript RT kit (Qiagen Inc.). Relative

quantitation by real-time PCR was performed using SYBR[®]-Green-I detection of PCR products using the Rotor-Gene 3000 Thermal Cycler (Corbett Research, Montreal Biotech Inc., Montreal, QU, Canada) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Reactions were prepared in duplicate and were heated to 95°C for 2 min followed by 40 cycles at 95°C for 15 s, 62°C for 20 s, and 72°C for 25 s. To detect the amount of amplification of product, the fluorescence quantification of product was determined after the extension step of each cycle. Melting curves were also measured to ensure that only a single product was amplified. The oligonucleotide primers were designed according to published GenBank (www.ncbi.nlm.nih.gov/Genbank) sequences. Relative quantification was performed with the manufacturer's software using a standard curve for each primer set. Sequences and annealing temperatures of the primers were as follows:

NPR-A (S): 5'-GGTTCGTTCTTATTGGCTC-3'
 NPR-A (AS): 5'-CCACCATCTCCATCCTCTC-3', 62°C
 NPR-C (S): 5'-CAGCAGACTTGGAACAGGA-3'
 NPR-C (AS): 5'-CCATTAGCAAGCCAGCAC-3', 62°C
 NEP (S): 5'-GATAGCAGAGGCGGACAA-3'
 NEP (AS): 5'-CAAGTCGAGGCTGGTCAA-3', 62°C
 GAPDH (S): 5'-GCATCGAAGGTGGAAGAG-3'
 GAPDH (AS): 5'-ACCACAGTCCATGCCATC-3', 62°C

ANP RIA

Atrial and LV samples were prepared for extraction by Sep-Pak C₁₈ cartridges (Waters Ltd, Mississauga, ON, USA), as previously described (Tse *et al.* 2001). The processed samples were lyophilized and the resultant powder samples were stored at -70°C until RIA. The RIA method employed in this study has been previously described (Tse *et al.* 2001). The ANP antiserum (Peninsula Laboratories Inc., Belmont, CA, USA) was used at a dilution of 1:100 000. A detection limit of 0.8 pg/tube and the 50% binding value of 12.5 pg/tube were achieved for the assay.

Statistical analysis

Northern blots and cDNA bands were scanned using a Hewlett Packard Scan Jet II cx/T equipped with a transilluminator. Densitometric analysis was performed using AlphaEaseFC Software, Version 3.1.2. (Alpha Innotech Corporation, San Leandro, CA, USA 1993–2002). Results were expressed as means \pm S.E.M. Differences between experimental groups were determined by performing one-way ANOVA followed by Tukey's *post hoc* test using the GraphPad PRISM program (Version 4.03, GraphPad, San Diego, CA, USA). $P < 0.05$ were considered statistically significant.

Results

Salt-induced alterations on cardiac mass of male and female ANP +/− mice

The effects of a 5-week HS diet on cardiac weight between male and female ANP +/− mice are shown in Table 1. On a NS diet, the male ANP +/− mice showed a significant increase in heart-to-body weight (HW:BW) ratio when compared with female ANP +/− mice. On HS diet, male ANP +/− mice exhibited a marked CH with a 14% increase in cardiac weight compared with those fed NS diet. Female ANP +/− mice, however, did not develop significant CH and had only a 5% increase in heart-to-body WH:BW when fed a HS diet compared with those fed NS diet.

Salt-induced changes in the cardiac NPS between male and female ANP +/− mice

Representative northern blot analysis of atrial ANP and their quantified expression levels are displayed in Fig. 1A and B. Both male and female ANP +/− mice showed significant increases (67 and 35% respectively) in atrial ANP mRNA expression under HS conditions. Neither male nor female atrial ANP protein expression differed after salt treatment (Fig. 1C). LV ANP mRNA and protein expression significantly increased only in male ANP +/− mice fed HS diet and remained unchanged in the female group (Fig. 2A–C). LV BNP mRNA expression increased 204% in the male ANP +/− mice fed HS diet and remained unchanged in the female ANP +/− mice (Fig. 2D and E). Under HS conditions, the mRNA expression of NPR-A was significantly increased in male ANP +/− mice and remained unchanged in female ANP +/− mice (Fig. 3A). NPR-C mRNA expression was unaffected by the HS diet in both male and female ANP +/− mice (Fig. 3B). The expression of NEP mRNA was not altered by diet, even though a tendency towards a down-regulation could be seen under HS conditions in both male and female ANP +/− mice; however, this did not reach statistical significance (Fig. 3C).

Salt-induced changes in heart size of O-injected and E2-injected Ovx female ANP +/− mice

Since the female ANP +/− mice had a lower HW:BW ratio when compared with their male counterparts under normal conditions and were able to resist significant changes in cardiac weight when fed a HS diet, we sought to test the hypothesis that E2 plays a role in preventing this change in the heart by using the following paradigm: Ovx female ANP +/− mice received an O-injection or E2-injection while maintained on a NS and HS diet for 5 weeks. Mice receiving E2-injections had a circulating E2 level of 34 ± 8 pg/ml whereas the circulating E2 level for those mice receiving O-injections fell below the detection limit (10 pg/ml) of the assay. Also, the uteri of mice receiving E2-injections showed typical fluid retention at the time of sampling following the treatment. Nonetheless, the effects of the HS treatment on cardiac weight between O-Ovx and E2-Ovx ANP +/− mice are shown in Table 2. O-Ovx ANP +/− mice on a HS diet exhibited a significant (13%) increase in WH:BW compared with O-Ovx ANP +/− mice under NS conditions. In contrast, the E2-Ovx ANP +/− mice did not develop CH and had only a 5% increase in WH:BW under the influence of a HS diet when compared with NS diet fed E2-Ovx ANP +/− mice.

Salt-induced changes in the cardiac NPS between O-injected and E2-injected Ovx female ANP +/− mice

No significant increases were seen in either O-Ovx or E2-Ovx ANP +/− mice with regards to atrial ANP mRNA and protein expression under HS conditions (Fig. 4). An approximate twofold increase in atrial ANP mRNA was detected between the E2-Ovx and O-Ovx ANP +/− mice. However, a significant increase in LV ANP mRNA and protein expression was observed only in the O-Ovx ANP +/− mice treated with HS (Fig. 5A and B). LV ANP mRNA and protein levels remained unchanged in E2-Ovx ANP +/− mice with HS treatment (Fig. 5A and B). LV BNP mRNA expression in the O-Ovx ANP +/− mice under a HS diet followed a similar trend to that of the HS diet fed male ANP +/− mice, in that its expression was elevated (78%), while the LV BNP mRNA level for the E2-Ovx ANP +/− mice remained unchanged (Fig. 5C). Under HS conditions, the mRNA expression of NPR-A was

Table 1 Salt-induced physical changes in male and female atrial natriuretic peptide (ANP) +/− mice. Values are means \pm S.E.M.

	DIET	n	BW (g)	WHW (mg)	WH:BW (mg/g)
ANP +/− Male	0.8% NaCl	5	31.43 \pm 0.73	154 \pm 4	4.90 \pm 0.05
	8.0% NaCl	5	30.96 \pm 1.50	173 \pm 10*	5.58 \pm 0.07*
Female	0.8% NaCl	5	31.93 \pm 0.74	132 \pm 5	4.13 \pm 0.09
	8.0% NaCl	5	28.19 \pm 0.26*	123 \pm 3	4.35 \pm 0.08

* $P < 0.05$ compared to 0.8% NaCl diet of the same sex. BW, body weight; WH, whole heart weight; WH:BW, whole heart: body weight.

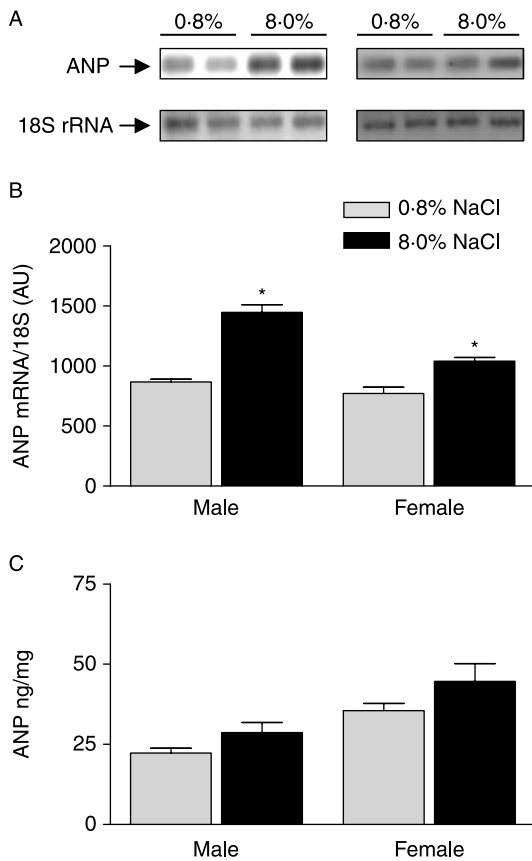


Figure 1 (A) Representative northern blot of atrial ANP mRNA expression of male and female ANP +/– mice treated with 0.8% NaCl diet compared with 8.0% NaCl diet. (B) Graph summarizing atrial ANP mRNA expression as assessed by northern blot normalized to the 18S rRNA. (C) Atrial ANP protein expression as determined by RIA. (*n* = 4 for each group). **P* < 0.05 vs 0.8% NaCl of the same sex. AU, arbitrary units.

significantly increased in the O-Ovx ANP +/– mice and remained unchanged in the E2-Ovx ANP +/– mice (Fig. 6A). NPR-C mRNA expression was unaffected by the HS diet in both O-Ovx and E2-Ovx ANP +/– mice (Fig. 6B). The expression of NEP mRNA was not altered, even though a tendency towards a down-regulation could be seen under HS salt conditions in both O-Ovx and E2-Ovx ANP +/– mice; however, this did not reach statistical significance (Fig. 6C).

Discussion

Gender-specific differences with regards to the development of CH have been well-documented in the human and animal population (de Simone *et al.* 1995, Oliver *et al.* 1997). In recent years, a growing body of evidence has suggested that E2 may contribute to the cardioprotective effects prior to the

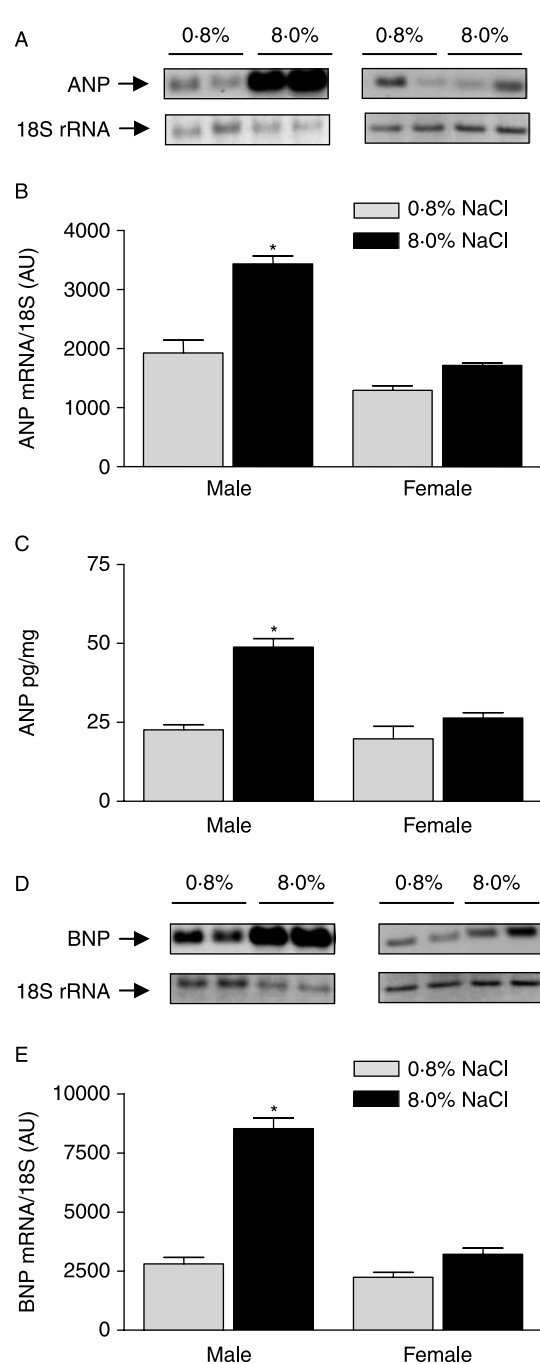


Figure 2 (A) Representative northern blot of LV ANP mRNA expression of male and female ANP +/– mice treated with 0.8% NaCl diet compared with 8.0% NaCl diet. (B) Graph summarizing LV ANP mRNA expression as assessed by northern blot normalized to the 18S rRNA. (C) LV ANP protein expression as determined by RIA. (D) Representative northern blot of LV BNP mRNA expression of male and female ANP +/– mice treated with 0.8% NaCl diet compared with 8.0% NaCl diet. (E) Graph summarizing LV BNP mRNA expression as assessed by northern blot normalized to the 18S rRNA. (*n* = 4 for each group). **P* < 0.05 vs 0.8% NaCl of the same sex. AU, arbitrary units.

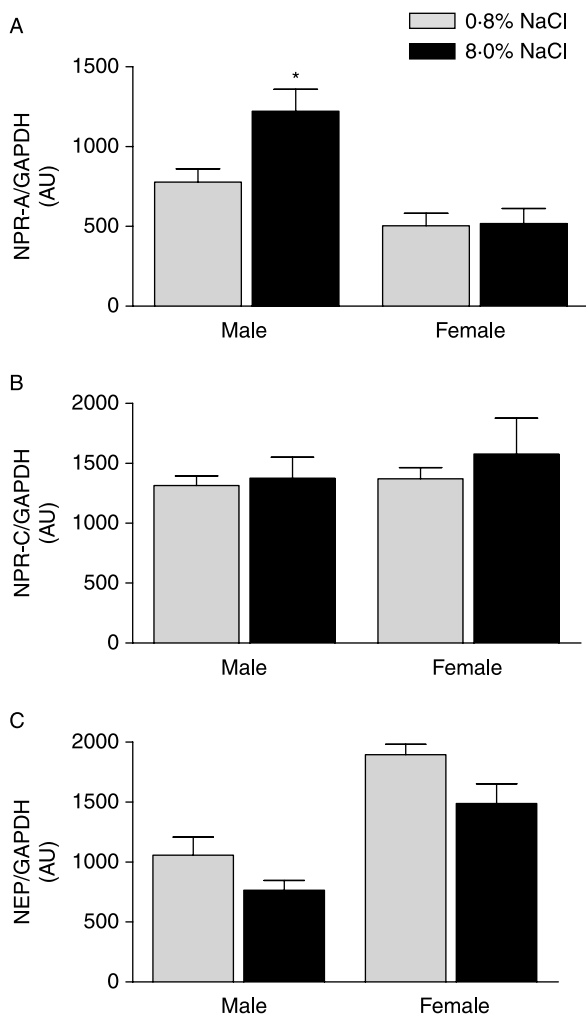


Figure 3 Graphs summarizing (A) LV NPR-A, (B) NPR-C, and (C) NEP mRNA expression of male and female ANP +/− mice treated with 0.8% NaCl diet compared with 8.0% NaCl diet as assessed by real-time PCR analysis normalized to GAPDH. ($n=4$ for each group). * $P<0.05$ vs 0.8% NaCl of the same sex. AU, arbitrary units.

onset of menopause. This is based on studies that show that premenopausal women have a significantly lower prevalence of cardiac disease compared with their age-matched male counterparts (Gorodeski 1994). However, the underlying

mechanism(s) responsible for this lower incidence of cardiac disease, such as CH, in females is poorly understood. Previous investigations have shown that male mice completely lacking ANP gene developed salt-sensitive hypertension and CH with extensive remodeling (John *et al.* 1995, Sangaralingham *et al.* 2004). Although male ANP +/− mice, which are partially deficient in ANP expression, do not exhibit hypertension or CH on an NS diet, the changes in cardiac structure during the development of salt-induced CH between genders have not been extensively studied (John *et al.* 1995, Sangaralingham *et al.* 2004). More importantly, the ANP +/− mouse model provides us with a unique opportunity to study the progression of salt-induced CH, which is clinically relevant due to the fact that genetic variations in the expression of the ANP gene have been described in the human population, particularly in African-American salt-sensitive hypertensives (Rutledge *et al.* 1995, Campese *et al.* 1996). Since dietary salt intake has been shown to modulate CH independently of blood pressure (Feng *et al.* 2003), our primary focus for this study was to assess E2's role in salt-induced end-organ damage such as CH in the ANP +/− mice. Thus, in the present study, we show, for the first time, that gender differences exist in the development of salt-induced CH and that E2 had antihypertrophic effects in hearts of ANP +/− mice under HS conditions. This is quite evident when we challenged the ANP +/− mice with a prolonged HS intake, where only the male and O-Ovx ANP +/− mice developed marked CH and had an activated cardiac NPS. Conversely, the female and E2-Ovx ANP +/− mice did not develop salt-induced CH and their cardiac NPS remained unchanged.

To further elucidate the mechanism involved in the ability of E2 to retard the progression of salt-induced CH, we studied the expression of the cardiac NPS. This system defends against excess salt and water retention, promotes vasodilatation, inhibits the production and action of vasoconstrictor peptides, and inhibits sympathetic outflow (Levin *et al.* 1998). ANP also has been shown to play an important role in the development and progression of CH and is E2 responsive (Deng & Kaufman 1993, Masciotra *et al.* 1999). Furthermore, recent studies have shown the antihypertrophic properties of ANP and its interaction with E2; for instance, there is an increase in the expression of ANP during pressure overload hypertrophy (Horio *et al.* 2000, van Eickels *et al.* 2001). In the

Table 2 Salt-induced physical changes in O-Ovx and E2-Ovx atrial natriuretic peptide (ANP) +/− mice. Values are means ± S.E.M.

	DIET	<i>n</i>	BW (g)	WHW (mg)	WH:BW (mg/g)
O-Ovx female	0.8% NaCl	5	27.02 ± 0.78	113 ± 3	4.20 ± 0.08
	8.0% NaCl	5	25.81 ± 0.61	123 ± 3*	4.75 ± 0.04*
E2-Ovx female	0.8% NaCl	5	27.87 ± 0.73	112 ± 3	4.02 ± 0.05
	8.0% NaCl	5	29.76 ± 0.67	126 ± 2*	4.23 ± 0.06

* $P<0.05$ compared to 0.8% NaCl diet of the same treatment group. BW, body weight; WH, whole heart weight; WH:BW, whole heart: body weight; O, oil-injected; E2, estrogen-injected; Ovx, Ovariectomized.

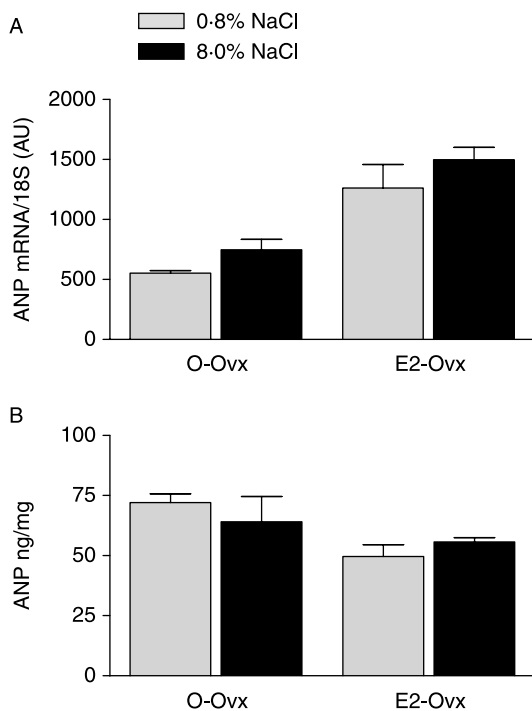


Figure 4 (A) Graph summarizing atrial ANP mRNA expression of oil-injected (O) and estrogen-injected (E2) Ovx female ANP +/– mice treated with 0.8% NaCl diet compared with 8.0% NaCl diet as assessed by northern blot normalized to the 18S rRNA. (B) Atrial ANP protein expression as determined by RIA. ($n=4$ for each group). No significant differences were observed between the HS versus NS of the same treatment group. AU, arbitrary units.

present study, both the male and female ANP +/– mice had an increased atrial ANP mRNA and protein expression under HS conditions; however, only the mRNA expression reached significant levels. Moreover, atrial mRNA expression in the HS male ANP +/– mice rose 32% more than that in its female HS counterparts. Two of atrial ANP's main functions are natriuresis and diuresis. Under HS conditions, in order to maintain normal cardiovascular homeostasis, we speculate that the male ANP +/– mice secreted more of its ANP as evident by the lower amounts of the peptide in the atrium, which in turn correlates with the greater increase in ANP mRNA expression than the female ANP +/– mice. When fed a HS diet, O-Ovx and E2-Ovx ANP +/– mice showed an increase in atrial ANP mRNA expression, although these levels did not reach statistical significance. Interestingly, E2-Ovx ANP +/– mice had an approximately twofold increase in atrial ANP mRNA than O-Ovx ANP +/– mice. Hence, the E2-Ovx ANP +/– mice appeared to have secreted more of its stored ANP that, in turn, resulted in an increase in ANP mRNA expression to replenish the ANP granule stores. Since the E2-Ovx ANP +/– mice exhibited fluid retention as noticed by the fluid-filled uteri of these mice during autopsy, we speculate that the increase in ANP expression

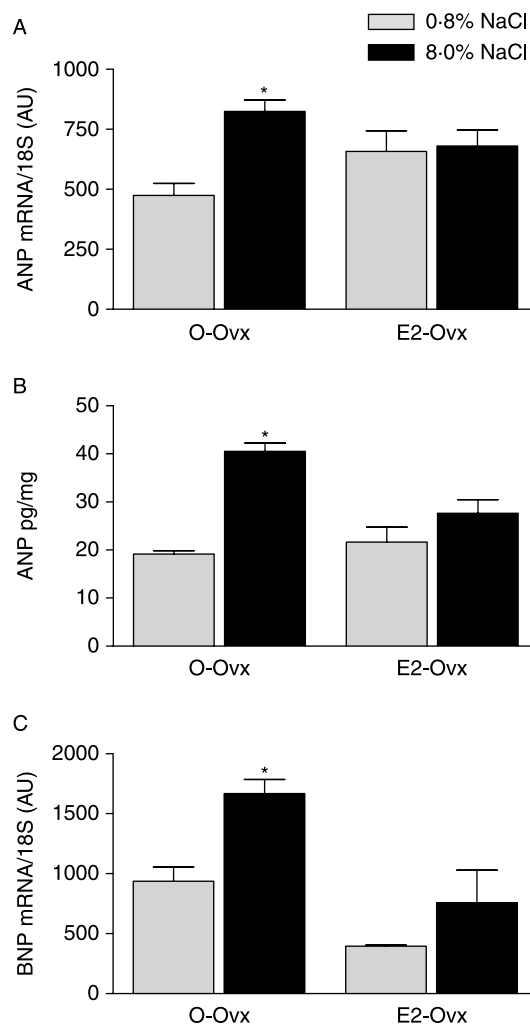


Figure 5 (A) Graph summarizing LV ANP mRNA expression of oil-injected (O) and estrogen-injected (E2) Ovx female ANP +/– mice treated with 0.8% NaCl diet compared with 8.0% NaCl diet as assessed by northern blot normalized to the 18S rRNA. (B) LV ANP protein expression as determined by RIA. (C) Graph summarizing LV BNP mRNA expression as assessed by northern blot normalized to the 18S rRNA. ($n=4$ for each group). * $P<0.05$ vs 0.8% NaCl of the same treatment group. AU, arbitrary units.

seen in the E2-Ovx ANP +/– mice is in response to this fluid accumulation.

In the current study, male and O-Ovx ANP +/– mice clearly resulted in significant CH in response to stress caused by a HS diet when compared with female and E2-Ovx ANP +/– mice. It is known that ANP is produced in minimal quantities in normal adult ventricular tissue; however, it is present in greater amounts in hypertrophied ventricles (Levin *et al.* 1998). Here, we observed a significant increase in LV ANP expression in male and O-Ovx ANP +/– mice fed HS versus the respective NS ANP +/– mice compared with no change in LV ANP expression at both the mRNA

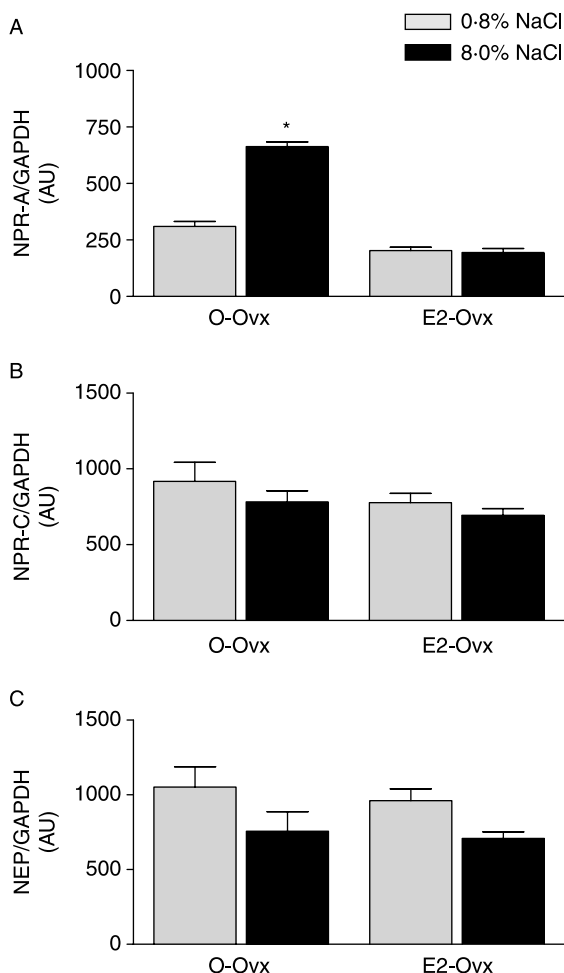


Figure 6 Graphs summarizing (A) LV NPR-A, (B) NPR-C, and (C) NEP mRNA expression of oil-injected (O) and estrogen-injected (E2) ovariectomized female (Ovx) ANP $+/-$ mice treated with 0.8% NaCl diet compared with 8.0% NaCl diet as assessed by real-time PCR analysis normalized to GAPDH. ($n=4$ for each group). * $P<0.05$ vs 0.8% NaCl of the same treatment group. AU, arbitrary units.

and protein level in female and E2-Ovx ANP $+/-$ mice fed a HS diet. In addition, we show that only the male and O-Ovx ANP $+/-$ mice exhibited a significant increase (204 and 78% respectively) in LV BNP expression under HS conditions. As BNP is an important marker of cardiac dysfunction, higher BNP expression is often related to more severe cardiac damage (Gardner 2003, Uusimaa *et al.* 2004). Nevertheless, what remains unclear is whether the expression of ANP and BNP, which are markedly increased in patients and animals with CH or failing hearts, are induced to normalize cardiac structure or their expression is simply a consequence of the CH itself (Chien *et al.* 1991). Our data support the notion that the over-expression of both ANP and BNP in the male and O-Ovx ANP $+/-$ mice likely occurred in response to the salt-induced CH in an

attempt to normalize cardiac size and to possibly protect the heart from additional undesirable effects. This was further supported by the fact that the expression of the NPR-A receptor, which binds both ANP and BNP to exert its beneficial effects, was significantly elevated only in the male and O-Ovx ANP $+/-$ mice under HS conditions. Studies involving the NPR-A receptor knockout mice have shown that the male mice develop salt-sensitive hypertension and marked CH, and in some instances resulting in sudden death before the age of 6 months (Oliver *et al.* 1997, Holtwick *et al.* 2003). In addition, the stable expression of the NPR-C receptor and the decrease in NEP expression may be necessary to maintain the high levels of ANP and BNP in the LV because both NPR-C and NEP are responsible for degrading the NP. However, caution must be exercised while interpreting the NEP data because, unlike the NPR-C receptor, NEP not only regulates the NP but also many other peptides such as angiotensin II, kinin peptides, and endothelin (Campbell 2003).

Our results seem to differ in relation to other *in vitro* studies because they have shown that E2 stimulates ANP gene expression in response to hypertrophic stimuli in the heart and prevents hypertrophy by inhibiting protein synthesis in cardiomyocytes via a cGMP-dependent process (Horio *et al.* 2000, Babiker *et al.* 2004). However, these previous data were generated using neonatal cardiomyocytes as a model that cannot be readily generalized to adult cardiomyocytes. In fact, it has been shown that during post-natal life, the cardiomyocytes undergo dramatic changes in phenotype, structure, and cellular composition (Kuroski de Bold 1999). Furthermore, an *in vivo* study also showed an E2-induced increase in ventricular ANP expression; however, this study employed a different hemodynamic stress to induce CH than our study (transverse aortic constriction versus HS diet; van Eickels *et al.* 2001). This suggests that the cardioprotective effects of E2 may very well be achieved by completely different mechanisms depending on the hemodynamic stress involved. Overall, the data in the literature suggest that there are two drives that increase ventricular ANP expression, one being estrogen and the other CH. In our study, the increased CH seen in the male and O-Ovx ANP $+/-$ mice was a stronger stimulus to ANP than E2. Hence, our findings suggest one of two possibilities that: (1) E2 protects the heart against CH by a mechanism other than an increase in cardiac ANP, or (2) E2 protects the heart against CH by reducing the hemodynamic stress to the heart.

Cardiovascular disease, the major cause of morbidity and mortality for both men and women, occur more commonly in post-menopausal women than in premenopausal women. This difference has been attributed to the loss of E2 at the time of menopause, and studies investigating the relationship between E2 and cardiovascular health have resulted in much debate particularly in defining E2's role in cardiovascular health. Recent advances in research have favored the idea that E2 is cardioprotective, but the mechanism as to

why this occurs remains largely unknown (Grodstein *et al.* 2006, Hsia *et al.* 2006). The development of CH is a complex process and the antihypertrophic effects of E2 may possibly be mediated by the modulation of not only the cardiac NPS, but also by several different pathways. Here, we provide novel data which reveal that E2 has the ability to protect against the induction of salt-induced CH in ANP +/– mice, and we also show that the hypertrophy itself may be activating the cardiac NPS. These findings may eventually form the basis for new therapeutic strategies in controlling CH, particularly for those individuals who have a genetic variation of the ANP gene.

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