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# Estrogen Attenuates Left Ventricular and Cardiomyocyte Hypertrophy by an Estrogen Receptor-Dependent Pathway that Increases Calcineurin Degradation

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## Abstract

Left ventricular (LV) hypertrophy commonly develops in response to chronic hypertension and is a significant risk factor for heart failure and death. The serine-threonine phosphatase, calcineurin (CnA), plays a critical role in the development of pathologic hypertrophy. Previous experimental studies in murine models show that estrogen limits pressure overload-induced hypertrophy; our purpose was to explore further the mechanisms underlying this estrogen effect. Wild type, ovariectomized female mice were treated with placebo or 17β-estradiol (E2), followed by transverse aortic constriction (TAC) to induce pressure overload. At two weeks, mice underwent physiologic evaluation, immediate tissue harvest, or dispersion of cardiomyocytes. E2 replacement limited TAC-induced LV and cardiomyocyte hypertrophy while attenuating deterioration in LV systolic function and contractility. These E2 effects were associated with reduced abundance of CnA. The primary downstream targets of CnA are the nuclear factor of activated T-cell (NFAT) family of transcription factors. In transgenic mice expressing a NFAT-activated promoter-luciferase reporter gene, E2 limited TAC-induced activation of NFAT. Moreover, the inhibitory effects of E2 on LV hypertrophy were absent in CnA knockout mice supporting that CnA is an important target of E2-mediated inhibition. In cultured rat cardiac myocytes, E2 inhibited agonist-induced hypertrophy while also decreasing CnA abundance and NFAT activation. Agonist stimulation also reduced CnA ubiquitination and degradation that was prevented by E2; all in vitro effects of estrogen were reversed by an ER antagonist. These data support that E2 reduces pressure overload induced hypertrophy by an ER-dependent mechanism that increases CnA degradation, unveiling a novel mechanism by which E2 and ERs regulate pathologic LV and cardiomyocyte growth.

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### Keywords

Estrogen; LV Remodeling; Pressure Overload; Myocyte Hypertrophy; Calcineurin; Proteasome; Ubiquitin

Chronic pressure overload such as hypertension commonly leads to left ventricular (LV) hypertrophy that is a major risk factor for the development of heart failure and death.[1,2] LV hypertrophy is reflective of changes in the extracellular matrix and cardiomyocyte hypertrophy, both of which result from activation of signaling pathways and a reprogramming of gene expression.[3,4] The calcium-calmodulin dependent, serine-threonine phosphatase, calcineurin, plays an important role in pathologic cardiomyocyte hypertrophy.[5,6] The primary targets of calcineurin are the nuclear factor of activated T cell (NFAT)-family of transcription factors. NFAT intracellular localization and function is regulated by its phosphorylation state. Under basal conditions, NFAT proteins are hyper-phosphorylated and localize to the cytoplasm.[7] Upon hypertrophic stimulation, specific N-terminal serine residues in NFAT proteins are dephosphorylated by the catalytic "A" subunit of calcineurin (CnA), allowing nuclear localization of NFAT that in cooperation with other transcription factors, activates the hypertrophic gene program.

Clinical and experimental studies have established that gender influences the patterns of LV hypertrophy.[8] For example, in response to pressure overload such as hypertension or aortic stenosis, men's hearts exhibit LV dilatation or eccentric hypertrophy, while women's hearts tend to maintain normal chamber size but develop increased wall thickness, consistent more with concentric hypertrophy.[9] Though gender differences provide no direct evidence that sex hormones influence the patterns of hypertrophy, we and others have demonstrated that physiologic replacement of 17β-estradiol (E2), the main circulating form of estrogen in premenopausal women, to ovariectomized female mice limits pressure overload-induced LV hypertrophy.[10,11] In this study, we characterize further the molecular mechanisms that contribute to the effects of estrogen on LV hypertrophy in response to pressure overload, focusing on components of the calcineurin signaling pathway.

## Methods

### Materials

All chemical agents were obtained from Sigma. Polymerase chain reaction reagents were obtained from Invitrogen. Please see Supplemental Methods for details regarding antibodies, plasmid and adenoviral vectors.

### Animals

Eight to 10 week old, female C57BL/6 mice were cared for as described.[10,12] This protocol was approved by the Institutional Animal Care and Use Committee at the Tufts-New England Medical Center. Transgenic mice that ubiquitously express a NFAT-activated promoter/luciferase reporter gene and mice with a disrupted CnA beta gene (CnAβ knockout mice) have been previously reported.[13,14]

### Surgical Procedures

Procedures were performed under general anesthesia using 2.0–2.5% isoflurane. Ovariectomy, subcutaneous pellet placement, and TAC were performed as described previously.[10,12] Estrogen or placebo was administered via 60-day release pellets placed subcutaneously (0.25mg/60 day release pellet; Innovative Research of America, Sarasota FL).

### Echocardiography

Transthoracic echocardiography was performed under light sedation with 1.0% isoflurane as described.[12,15]

### Closed Chest Hemodynamic Evaluation

Closed chest hemodynamics were measured as described.[10,15] Pressure-volume (PV) loop analyses were performed from the right carotid artery using a fully calibrated, 1.0Fr pressure-volume (PV) conductance catheter (PVR-1045, Millar Instruments, Houston Texas). TAC pressure gradients were quantified by also cannulating the left carotid with a 1.0Fr microtip pressure transducer (Model # SPR-1000, Millar instruments) and measuring aortic pressure beyond the stricture. Hemodynamics were recorded and analyzed with IOX version 1.8.11 software (EMKA Instruments, Falls Church, Virginia). Preload recruitable stroke work (PRSW) was derived from the PV loops recorded during IVC occlusion as this measure is derived independent of loading conditions, chamber size or calibration.[16] All hemodynamic derivations were based on previous work of Georgakopoulos et al.[17] Parallel conductance corrections were performed in duplicate for each animal by injecting 10ul of 15% NaCl solution into the external jugular vein.

### Dispersion of Adult Mouse Myocytes for Morphometric Analysis

Myocytes from adult mice were enzymatically dispersed as described.[18] The final buffer was supplemented with an excess of KCl (30mM) to suppress contraction. Cells were cover slipped and imaged at 100× magnification; cell size measurements were performed in a blinded fashion.

### Primary Neonatal Rat Ventricular Myocyte (NRVM) Culture and Transfection

NRVMs were cultured as described.[19] For plasmid-DNA transfection, the calcium phosphate method was used as described.[20] For replication deficient adenoviruses, a MOI of 5 was added upon plating and allowed to incubate overnight.

### Adult Rat Ventricular Myocyte (ARVM) Cultures

ARVMs were isolated from male rats weighing 200–250 grams (approximately 8–10 weeks old) as described.[21] Cells were allowed to settle by gravity through 6% BSA in media (low glucose DMEM supplemented with 0.1% BSA, 5mM taurine, 5mM carnitine, and 2mM creatine (ACCT media)) and the final pellet was resuspended in ACCT media and plated on to laminin coated plates.

### Semi-quantitative Real Time Reverse Transcription Polymerase Chain Reaction (rtPCR)

From frozen LV segments or cells in culture, total cellular RNA was isolated as described.[15] cDNA was made using the Quantifect cDNA synthesis kit (Qiagen) according to the manufacturer's instructions. Real time PCR was performed on all samples in triplicate using SYBR Green (Applied Biosystems).

### Immunoprecipitation (IP)

IPs were performed in lysis buffer supplemented with 10mM N-ethylmaleimide to inhibit ubiquitin-degrading enzymes. Five ug of anti-CnA (BD Biosciences, Clone G182-1847) or anti-myc antibody (mouse monoclonal, Tufts University Core Facility)) was added to lysates (300ug), and rocked overnight at 4°C followed by rocking with Protein A or G coated sepharose beads (GE-Amersham) for 1 hour at 4°C. Negative controls consisted of non-immune, mouse IgG. Beads were pelleted by centrifugation, washed X4 in 750ul of ice cold PBS, and suspended in 2x Sample buffer. Pellets were analyzed by SDS-PAGE.

### Luciferase Assays

Twenty four hours after stimulation, cells were lysed and assayed for luciferase (Luciferase Assay System (Promega)) and ß-galactosidase activity (Applied Biosystems) to normalize for transfection efficiency. All assays were performed with a 96 well plate luminometer (Ascent, ThermoElectron, Waltham, MA).

### Statistical Analysis

All data are shown as mean ± standard error of the mean (sem). When comparing multiple groups, one-way ANOVA was performed and when significant ($p<0.05$), comparisons between each group were conducted using the Student Newman-Keuls test and a p-value of $<0.05$ was considered statistically significant. No significant differences between placebo-Sham and E2-Sham groups were observed in any of the variables studied. These data were therefore pooled.

## Results

The experimental design for most *in vivo* studies is shown in Online Figure I. In studies utilizing wild type mice, there were 4 groups: placebo-Sham, E2-Sham, placebo-TAC, and E2-TAC. A total of 88 female C57/BL6 mice were used: 26 animals were divided equally into placebo-sham and E2-sham groups; 62 mice underwent TAC, 29 were randomized to placebo and 33, E2. One death occurred in the shams (E2), 3 deaths in the placebo-TAC and 3 deaths in the E2-TAC groups giving a total of 81 survivors.

E2 replacement increased uterine weight (79.8±7.8mg) compared to placebo-treated mice (sham and TAC groups (9.7±1.1mg, $p<0.001$ vs. E2-treated). Plasma estradiol levels were not measured but the protocol utilized in these studies is identical to that from prior studies in which E2 levels were in the high physiologic range.[10,12]

TAC significantly increased LV mass in the placebo group that was limited by E2 replacement (See Table 1). TAC led to increases in LV end diastolic diameter (LVEDD) and

end systolic diameter (LVESD), and decreased fractional shortening (FS) in placebo-treated mice; E2 replacement normalized these parameters. A subgroup of mice underwent echocardiography while awake (N=27) demonstrating similar findings Online Figure II). TAC produced similar LV systolic pressures and gradients in the E2- and placebo-treated mice, both being statistically greater than shams. In the placebo group, TAC significantly increased LVEDP and decreased maximal LV dP/dt, both of which were improved by E2-replacement.

Data from PV loop analyses (N=25) are shown in Table 1 and Figure 1A. TAC led to reductions in stroke volume and cardiac output both of which were improved by E2 replacement. TAC markedly decreased two measures of contractility derived from PV loop analyses: the slope of the ESPVR and PRSW. Both of these measures were normalized by E2 replacement. As displayed in Figure 1A, TAC caused significant LV dilatation in the placebo group as indicated by a rightward shift in the steady state PV loop. E2 replacement attenuated TAC-induced LV dilatation, and partially restored LV ejection fraction.

Morphometric measurements were performed on freshly dispersed cardiomyocytes obtained from 23 mice (Figure 1B). TAC significantly increased myocyte area, length and width in placebo-treated mice compared to shams; E2 replacement attenuated the increases in myocyte area and length while no significant effect was observed on myocyte width post-TAC. These data support that E2 replacement limits pressure overload-induced hypertrophy by preventing myocyte elongation, LV chamber dilation, and dysfunction, thereby promoting a more concentric pattern of hypertrophy. TAC also increased atrial natriuretic peptide (ANP) and β-myosin heavy chain (β-MHC) gene expression, both of which were limited by E2 replacement (Online Figure III A).

We next examined the effects of E2 on TAC-induced changes in the cardiac expression of the catalytic “A” subunit of calcineurin (CnA). Of the three known isoforms, CnAβ has been implicated in pathologic cardiac hypertrophy.[14] Neither TAC nor E2 treatment significantly altered the abundance of CnAβ mRNA (See Online Figure III B). However, CnA protein abundance rose significantly in placebo-treated mice following TAC (Figure 2A), and this was reversed by E2-replacement. We measured the expression of a splice variant of modulatory calcineurin interacting protein 1 (MCIP1.4) that harbors multiple NFAT binding sites in the introgenic region upstream to exon 4. MCIP1.4 expression is therefore a reflection of calcineurin-NFAT activation.[22] MCIP1.4 mRNA was increased in placebo-TAC hearts compared to shams and this was attenuated by E2 replacement (Figure 2A). Taken together these data support that E2 attenuates the rise CnA protein expression and activity following TAC.

To examine further the effect of E2-replacement on calcineurin-NFAT activation, we utilized transgenic mice (N=18) that express a NFAT-activated promoter/luciferase reporter transgene, applying the same paradigm (Online Figure I). Luciferase assays performed on myocardial lysates demonstrated significantly increased luciferase activity in the placebo-TAC group compared to shams that was attenuated by E2 replacement (Figure 2B) supporting further that E2 limits TAC-induced calcineurin-NFAT activation. We next examined the effects of E2 replacement on TAC-induced hypertrophy in female

homozygous CnAβ knockout (KO) mice (N=20) using their wild type littermates for controls (N=27). The same experimental paradigm was followed (Online Figure I). However, as CnAβ mice have been previously shown to have a marked blunting of the hypertrophic response 14 days following abdominal aortic banding,[14] mice were analyzed 28 days following TAC to allow for the development of a greater degree of hypertrophy. E2 replacement attenuated LV hypertrophy in wild types following TAC (Figure 2C). The percent increase in LV mass compared to shams in placebo-treated CnAβ KO mice was considerably less than corresponding wild types (28.7±7.3% vs. 70.0±4.6%, $p<0.01$); E2 had no effect on LV hypertrophy in CnAβ KO mice indicating that the attenuation of pressure overload-induced hypertrophy by E2 requires CnAβ, supporting further that calcineurin is a critical target of E2-mediated inhibition.

To explore the mechanisms of E2 effects on cardiomyocyte hypertrophy and calcineurin signaling, we utilized primary cultures of NRVMs stimulated with the $alpha_1$-adrenergic receptor agonist, phenylephrine (PE). Physiologic concentrations of E2 (10nM) inhibited PE-induced increase in cardiomyocyte cell area (Figure 3A) and ANP expression (Figure 3B), both of which were reversed by the estrogen receptor (ER) antagonist, ICI 182,780 (ICI) supporting an ER-dependent mechanism. Similar findings were observed in ARVMs (Online Figure 4A and C).

E2 also attenuated the PE-induced increase in CnA protein abundance that was reversed by ICI (Figure 4A) supporting an ER-dependent mechanism. Moreover, E2 limited the PE-induced increase in CnAβ protein levels while no effect on CnAβ mRNA was observed at 6 and 24 hours following PE stimulation (Online Figure 4B). In NRVMs transfected with an NFAT-activated promoter/luciferase-reporter plasmid, E2 attenuated the PE-induced increase in NFAT activation (Figure 4A). To ascertain whether the E2-effects on CnA were required for inhibition of cell growth, NRVMs were infected with an adenovirus encoding a constitutively active mutant of CnA (caCnA), or GFP as a control. E2 had no effect on the increase in cell area in NRVMs expressing caCnA, though E2 limited the PE-induced increase in cell size in AdvGFP infected NRVMs (Figure 4B). E2 also had no effect on NFAT activation in the caCnA infected cells, but attenuated NFAT activation in GFP expressing cells stimulated with PE. NRVMs were next pretreated with E2, the calcineurin inhibitor, cyclosporine (500ng/ml), or their combination (Figure 4C) followed by stimulation with PE. Importantly, E2 exhibited no additional inhibition of cell growth when combined with cyclosporine. Taken together, these findings support that calcineurin is an important target of E2-ER mediated inhibition during agonist-induced hypertrophy.

Because E2 limits the PE-induced increase in CnA protein abundance without significantly altering mRNA levels, we explored whether E2 influences CnA degradation. NRVMs were pretreated with the protein synthesis inhibitor, cycloheximide (40ug/ml) before stimulation with PE. As shown in Figure 4D, the half-life of CnA in control cells was 15.1 hrs and this was significantly prolonged by PE supporting that the PE-induced increase in CnA abundance is due, in part, to reduced CnA degradation. In the presence of cycloheximide, E2 attenuated the PE-induced stabilization of CnA that was reversed by ICI, supporting that E2 limits the increase in CnA abundance by increasing CnA degradation through an ER-dependent mechanism.

Calcineurin degradation occurs via the 26S proteasome.[23] We therefore pretreated cardiomyocytes with the 26S proteasome inhibitor, lactacystin (10uM). Lactacystin increased CnA levels (Figure 5A) compared to untreated cells, and this was not altered further by PE treatment and/or E2, supporting that a functional 26S proteasome is important for the regulation of CnA expression both by PE and E2. Lactacystin also abolished the E2-mediated attenuation of PE-induced increase in cardiomyocyte cell size (Figure 5B). A critical component of protein degradation is the addition of poly-ubiquitin chains to specific lysine residues on targeted proteins. We thus examined whether E2 pretreatment influenced CnA ubiquitination during agonist-induced hypertrophy. Figure 5C demonstrates that CnA ubiquitination was decreased by PE stimulation, while E2 pre-treatment increased CnA ubiquitination following PE stimulation. Taken together, these data support that E2 attenuates the hypertrophic agonist-induced increase in CnA protein abundance by an ER dependent mechanism that increases CnA ubiquitination and proteasomal degradation.

CnA degradation has previously been shown to depend on the specific E3 ubiquitin ligase, atrogin1, which associates with calcineurin and catalyzes CnA ubiquitination.[23] We therefore quantified the mRNA expression of Atrogin1 and two other E3 ubiquitin ligases, mouse double minute 2 (MDM2) and muscle-specific ring finger protein 1 (MuRF1) using myocardial RNA obtained from our TAC study in wild type mice (N=25 samples, Figure 6). TAC induced a significant decline in the expression of atrogin1, MDM2, and MuRF1 in placebo-treated mice. E2 replacement had no effect on the TAC-induced decrease in atrogin1 expression but restored the expression of both MDM2 and MuRF1 to levels similar to shams.

## Discussion

### Summary of Current Findings

We investigated the molecular mechanisms underlying the inhibitory effects of estrogen replacement on pressure overload-induced LV hypertrophy in the mouse TAC model. Consistent with prior studies, including our own,[10,11] estrogen replacement limits pressure overload induced LV hypertrophy. Perhaps more importantly, estrogen favorably influenced the remodeling phenotype by reversing TAC-induced LV dilatation and systolic dysfunction, while also preventing the decline in contractility following TAC. One striking and novel finding of our study was that E2 replacement limits TAC-induced myocyte hypertrophy largely by inhibiting myocyte elongation without affecting myocyte width. Taken together, our *in vivo* data support that E2 replacement promotes a more concentric pattern of hypertrophy, and preserves LV systolic function and contractility.

The favorable effects of E2 replacement on LV and myocyte hypertrophy were accompanied by a reduction in the TAC-induced increase in calcineurin protein abundance and activity. Furthermore, absence of an effect of E2 in CnAβ knockout mice following TAC further strengthens our hypothesis that calcineurin is likely a critical target of estrogen-mediated inhibition.

Our *in vitro* experiments provide further insight into a novel mechanism by which E2 influences hypertrophy and calcineurin signaling. First, the inhibitory effects of E2 on

hypertrophic agonist-induced increase in calcineurin protein expression and cardiomyocyte hypertrophy are reversed by an ER antagonist supporting an ER-dependent pathway. Additionally, we observed no additive effect of E2 when combined with pharmacologic inhibition of calcineurin. The absence of E2 effects in NRVMs and ARVMs overexpressing a constitutively active mutant of CnA support further that the calcineurin pathway is an important E2-regulated target. We also observed the novel finding that estrogen limits the hypertrophic stimulus-induced increase in CnA abundance by increasing its ubiquitination and proteasomal degradation, thereby limiting CnA-dependent activation of the hypertrophic gene program. Taken together, these data support the overall conclusion that estrogen replacement limits LV and cardiomyocyte hypertrophy, in part, by an ER-dependent pathway that increases proteasome-mediated degradation of calcineurin.

## Prior Studies

The mechanisms by which estrogen and its receptors influence pathologic cardiomyocyte growth are complex and multifaceted. Van Eickels et al demonstrated *in vivo* that E2-mediated reduction in TAC-induced hypertrophy in ovariectomized female mice was associated with an unexpected rise in ANP expression.[11] In our studies, the effects of E2 on ANP mRNA expression mirrored its effects on cardiomyocyte growth. Differences between our findings and that of van Eickels may be related to the time period after TAC (2 vs. 4 weeks) or that we measured mRNA while van Eickels quantified ANP protein by western blotting.

Pedram et al[24] demonstrated that E2 increased the expression of the calcineurin regulatory protein, MCIP1, and that siRNA knockdown of MCIP1 abolished the inhibitory effects of E2 on agonist-induced hypertrophy. Our results appear at face value to differ from these in several respects though the study by Pedram utilized cultured neonatal cardiac myocytes while our results on MICP1 expression were obtained from *in vivo* myocardial samples. Moreover, we quantified the MCIP1 isoform whose expression is driven by NFAT activation (MCIP1.4). MCIP1.4 mRNA levels corroborated with LV mass and calcineurin protein expression data, increasing in the placebo-TAC group and being attenuated by E2 replacement. We also quantified the expression of exon 7, common to all MCIP1 splice variants, and observed no significant differences following TAC. Studies using overexpression strategies *in vitro*[25,26] and cardiac-specific, transgenic approaches *in vivo*[27] have shown that MCIP1 inhibits calcineurin activation and pathologic hypertrophy. However, MCIP1 knockout mice surprisingly developed paradoxically *less* TAC-induced hypertrophy and calcineurin activation,[28] suggesting that at physiologic expression levels, MCIPs may *facilitate*, rather than inhibit calcineurin activation and LV hypertrophy. It is apparent that regulation of calcineurin activation and cardiomyocyte growth by MCIPs is complex, and the manner in which these calcineurin regulatory proteins participate in the E2 effects on hypertrophy deserves further investigation.

We demonstrate here that E2 limits the hypertrophic stimulus-induced rise in CnA protein expression by increasing its degradation, an effect which is ER dependent. Calcineurin was shown by Li et al to associate with the E3 ubiquitin ligase, atrogin 1.[23] Overexpression of atrogin 1 inhibited agonist- induced NRVM hypertrophy *in vitro* and pressure overload-

induced LV hypertrophy *in vivo*, while concomitantly limiting the rise in calcineurin protein abundance and activity. We observed *in vivo* that TAC decreases the expression of atrogin 1 with no effect of E2 replacement suggesting that the E2 effects on calcineurin degradation are not the result of an effect on atrogin 1 transcription. Interestingly, E2 replacement normalized the TAC-induced decrease in the expression of two other E3 ubiquitin ligases that may have potentially important effects on myocyte hypertrophy.[30] Further work will be required to elucidate the mechanisms by which E2 and ERs increase calcineurin degradation and the contribution of MDM2 and MuRF1 to the estrogen-mediated attenuation of pressure-overload hypertrophy. Based on our data, we propose a model shown in Figure 7.

The current findings may have important clinical implications as the development of cardiac-specific, selective estrogen receptor modulators (SERM) that produce favorable estrogenic effects on the myocardium without inducing unfavorable effects in other tissues (e.g. activation of coagulation factors) may hold therapeutic promise for reducing left ventricular hypertrophy.

In summary, we demonstrate that estrogen replacement limits pressure overload-induced LV dilation and cardiomyocyte hypertrophy *in vivo* and *in vitro*, promoting a more concentric pattern of LV and myocyte remodeling, resulting in preservation of LV function and contractility. Furthermore, we show for the first time, that the inhibition of hypertrophy by estrogen is dependent on estrogen-mediated regulation of calcineurin protein abundance through an ER-dependent pathway that increases calcineurin ubiquitination and degradation. These data represent a novel and previously unrecognized mechanism by which estrogen and its receptors regulate pathologic cardiomyocyte growth.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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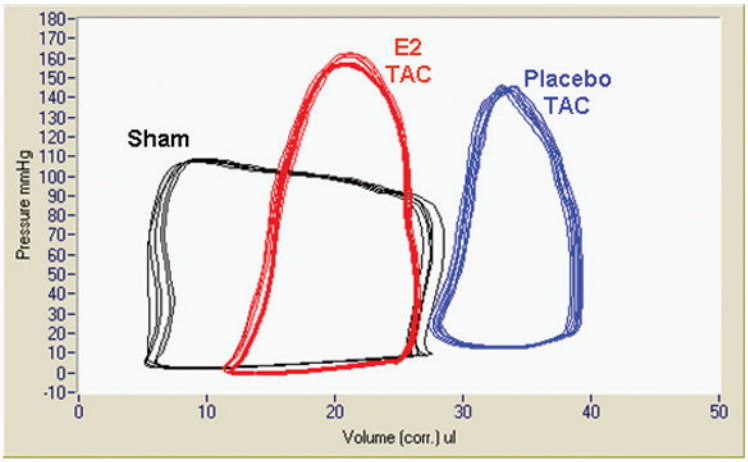
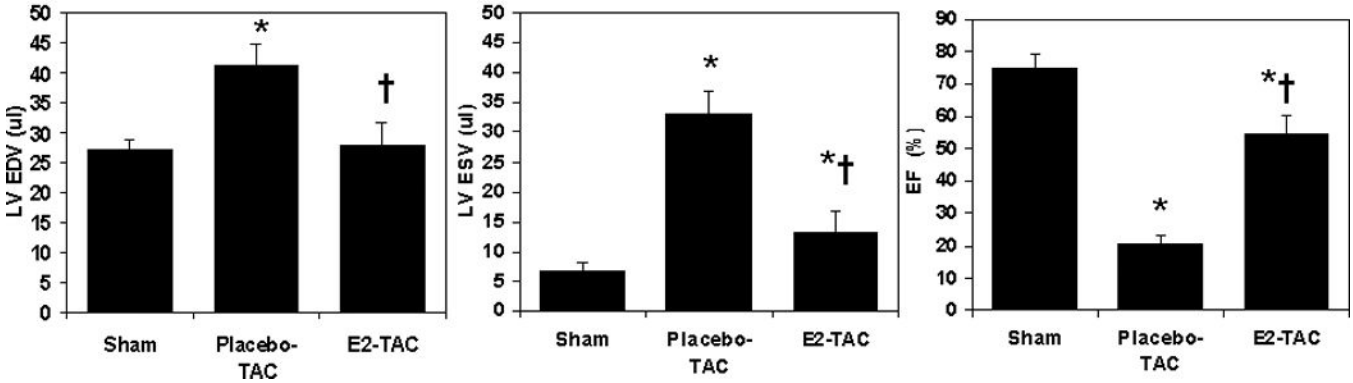


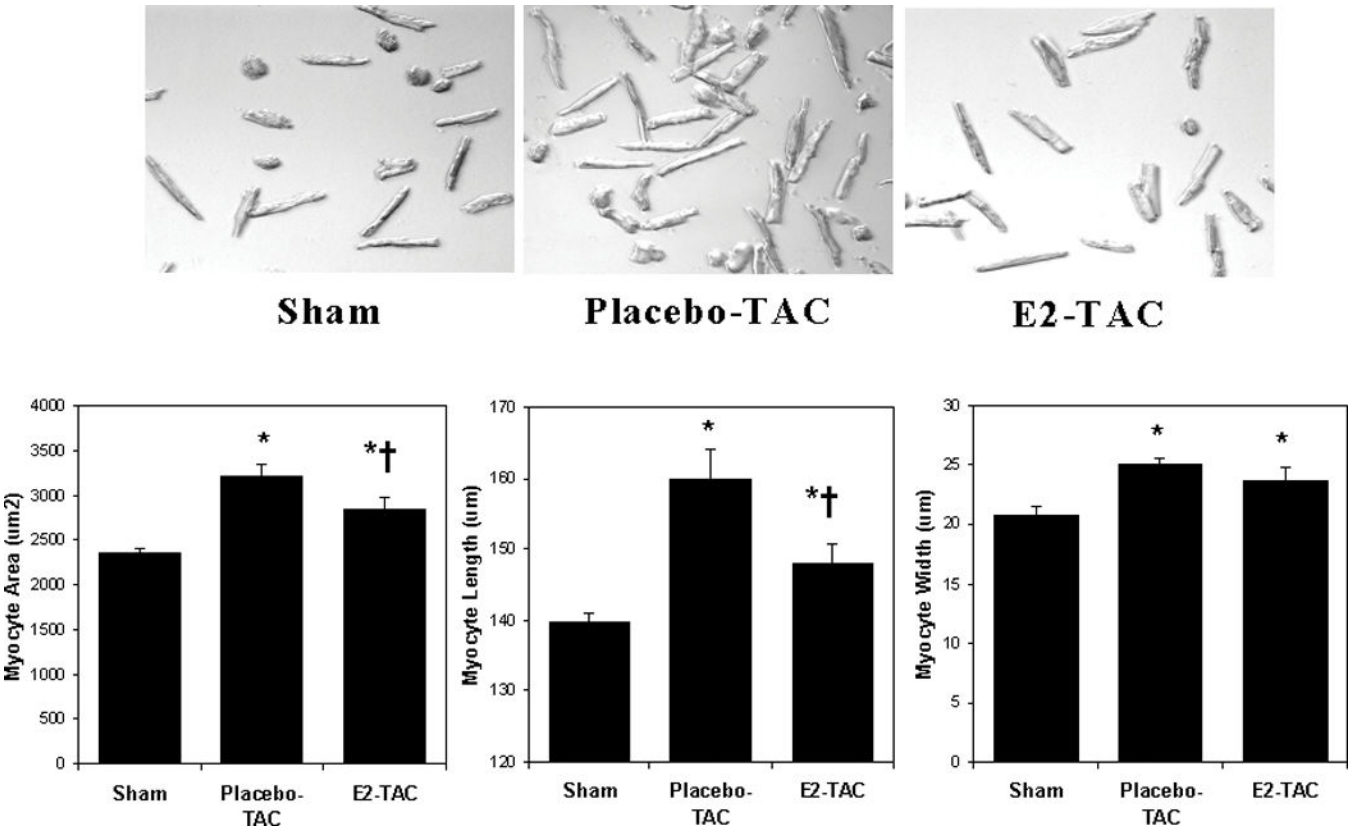
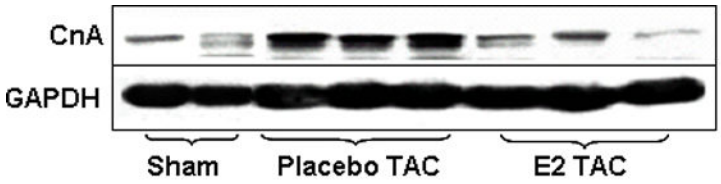
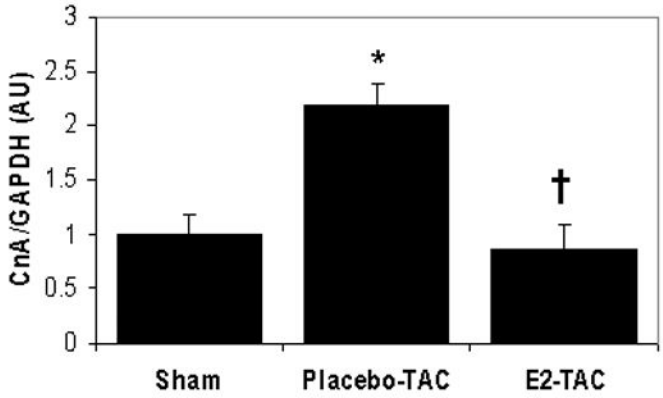
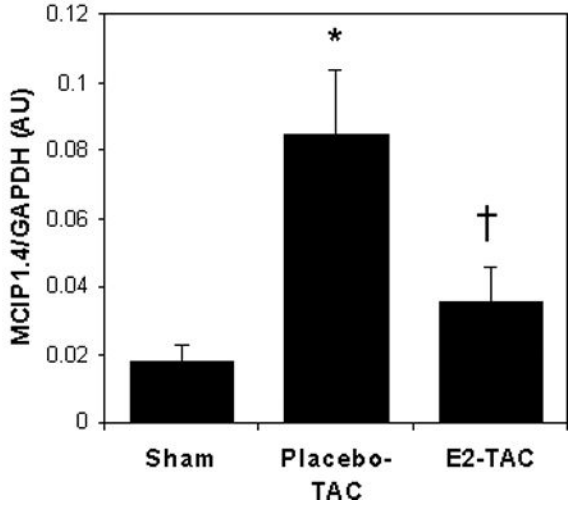


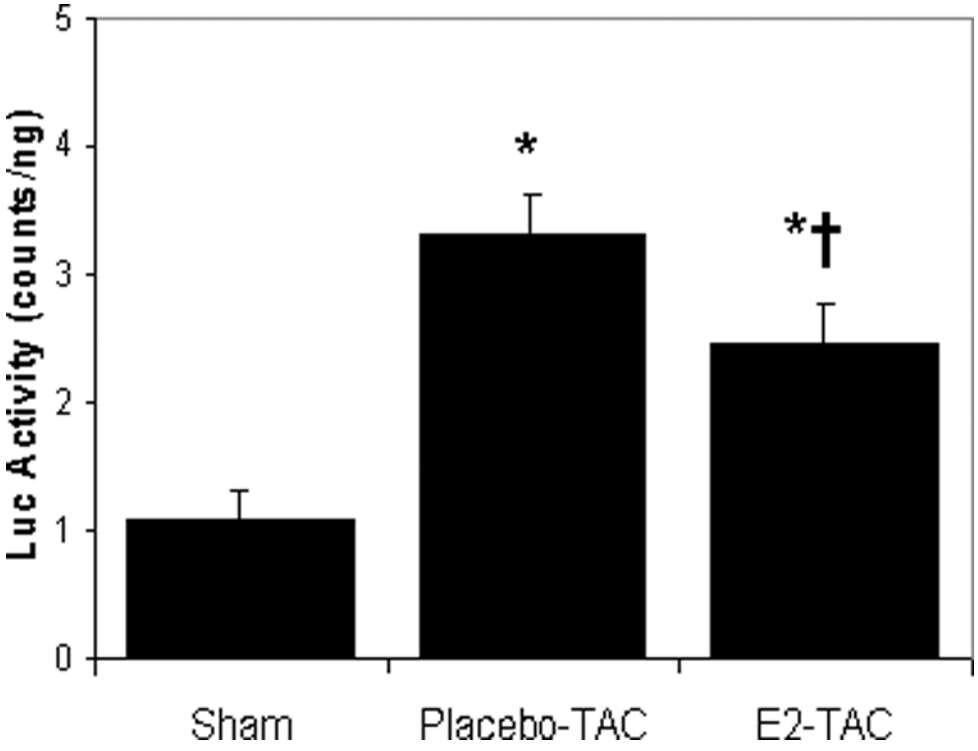

**Figure 1. Estrogen Promotes Concentric Remodeling Following TAC**

**A. PV Loop Analyses:** (Also see Table 1). **Top:** Representative steady state PV loops from sham, placebo-TAC and E2-TAC mice. The placebo-TAC heart exhibits a rightward shift in the PV loop consistent with LV dilation which is reversed by E2 replacement. **Bottom Left:** LV end diastolic volume (EDV); **Middle:** LV end systolic volume (ESV); **Right:** LV Ejection Fraction (EF). *p<0.01 vs. Shams; † p<0.05 vs. Placebo-TAC. **B. Myocyte Morphometry Results: Top:** Representative images of dispersed myocytes from a Sham, Placebo-TAC and E2-TAC heart. **Bottom Left:** Myocyte 2-dimensional area; **Middle:** Myocyte Length; **Right:** Myocyte Width. TAC significantly increased myocyte area, length, and width in the placebo-treated mice. E2 replacement limited myocyte enlargement by attenuating the increase in myocyte length with no effect on myocyte width. N=9 Shams, 6 Placebo-TAC and 8 E2-TAC mice. Equivalent TAC gradients were confirmed by pulse wave Doppler measurements during echocardiography. * p<0.05 vs. Shams; † p<0.05 vs. Placebo-TAC.



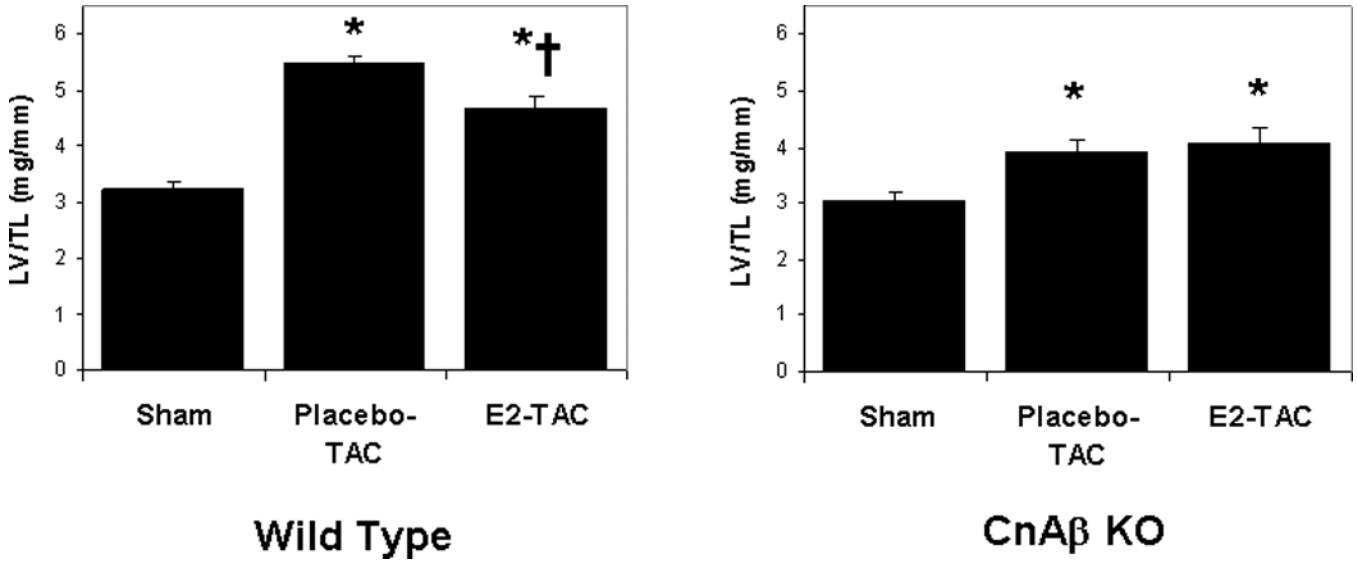



**Figure 2. Influence of E2 Replacement on Calcineurin Signaling Following TAC**

**A. Left: Calcineurin A protein expression** increased following TAC in placebo-treated mice but not those treated with E2. **Right: MCIP1.4 gene expression.** TAC increased the mRNA level of MCIP1.4 that is prevented by E2 replacement. $p<0.05$ vs. Shams; † $p<0.05$ vs. Placebo-TAC. **B. Myocardial Luciferase activity from NFAT-Luc Transgenic Mice** 2 weeks following TAC. TAC led to a significant 3.2 fold increase in myocardial luciferase activity that is attenuated by E2 replacement. * $p<0.05$ vs. Shams; † $p=0.05$ vs. Placebo-TAC. **C. E2 Reduces LV hypertrophy in Wild Type but not in CnAβ knockout mice.** LV mass indexed to tibial length 4 weeks post-TAC in ovariectomized, female CnA beta KO mice (Right) and wild type littermates (Left). * $p<0.01$ vs. Sham; † $p<0.05$ vs. placebo-TAC.

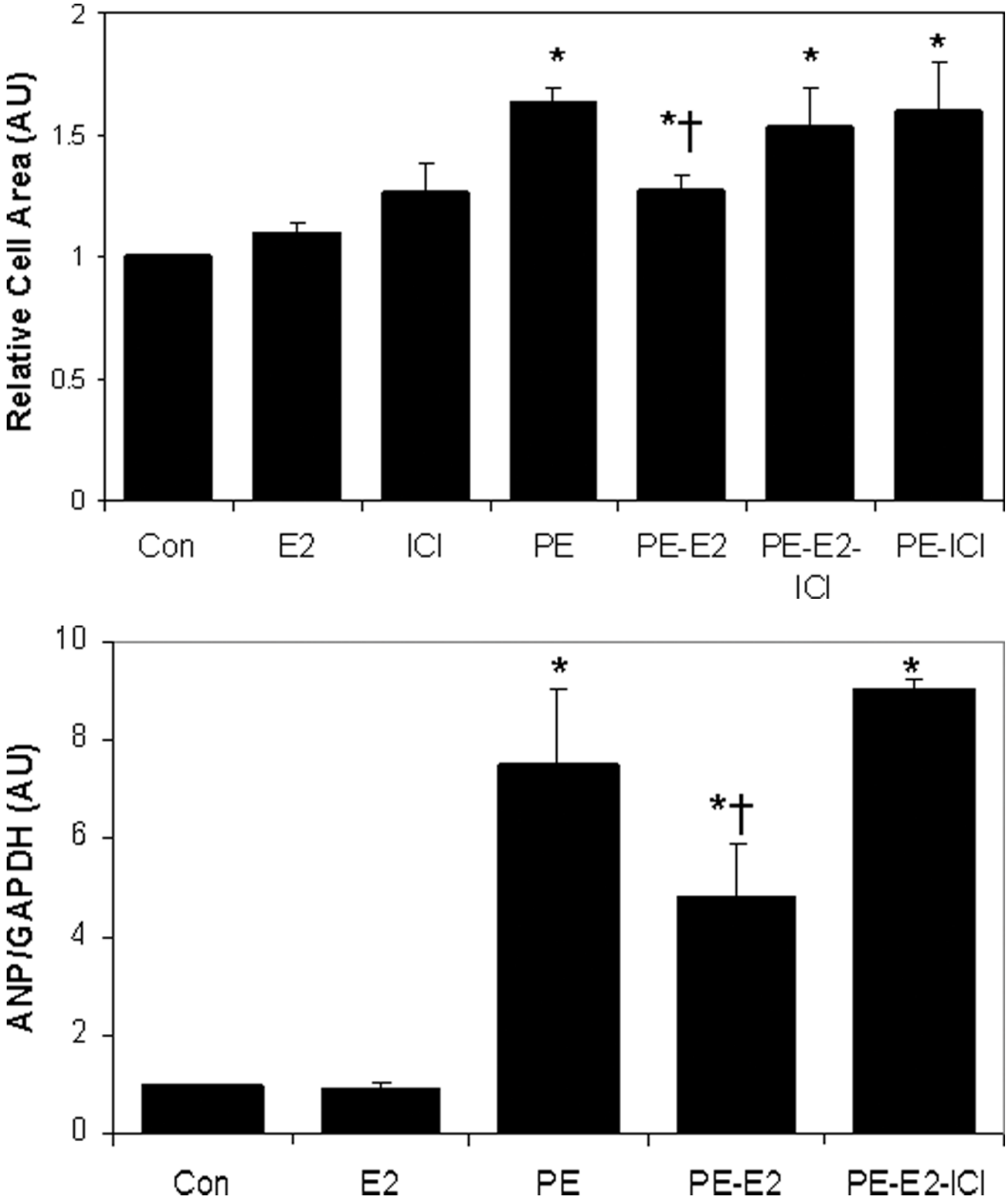
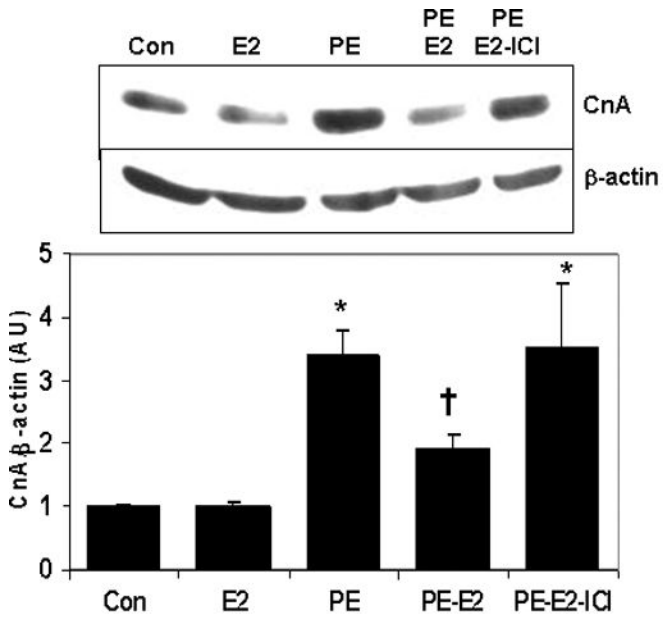
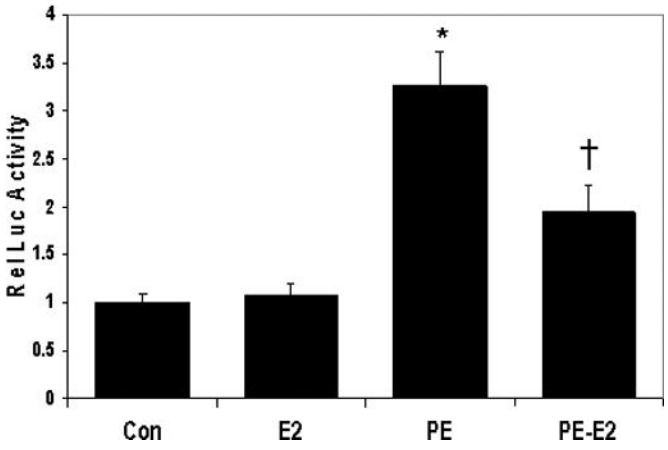
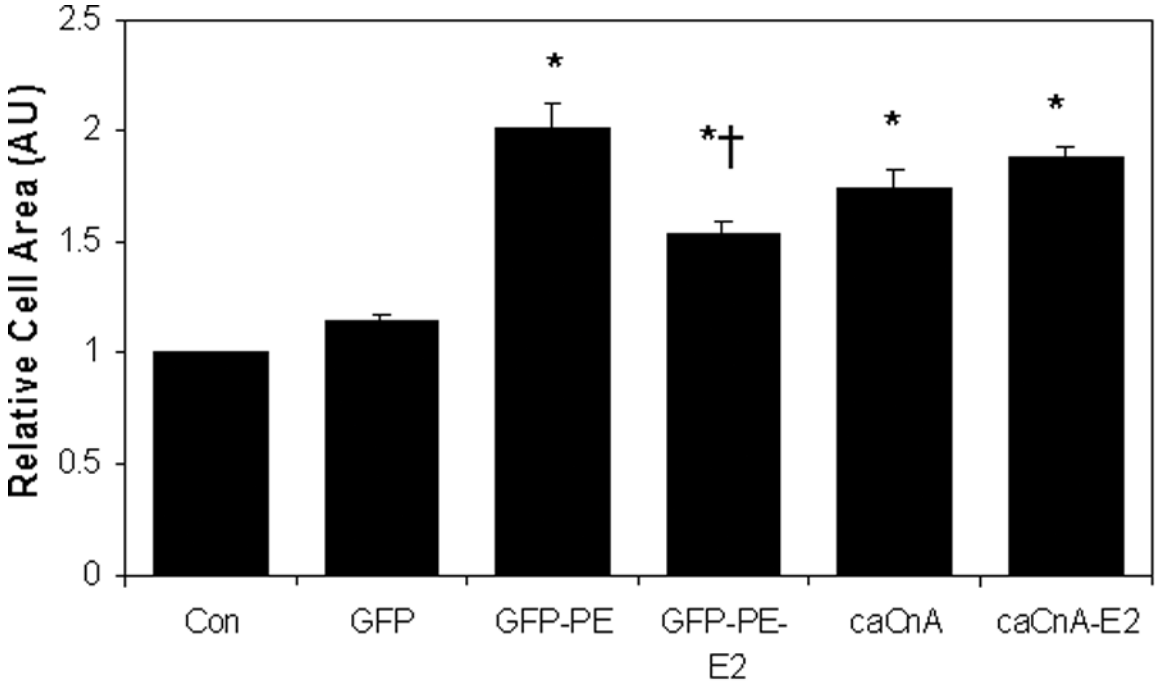


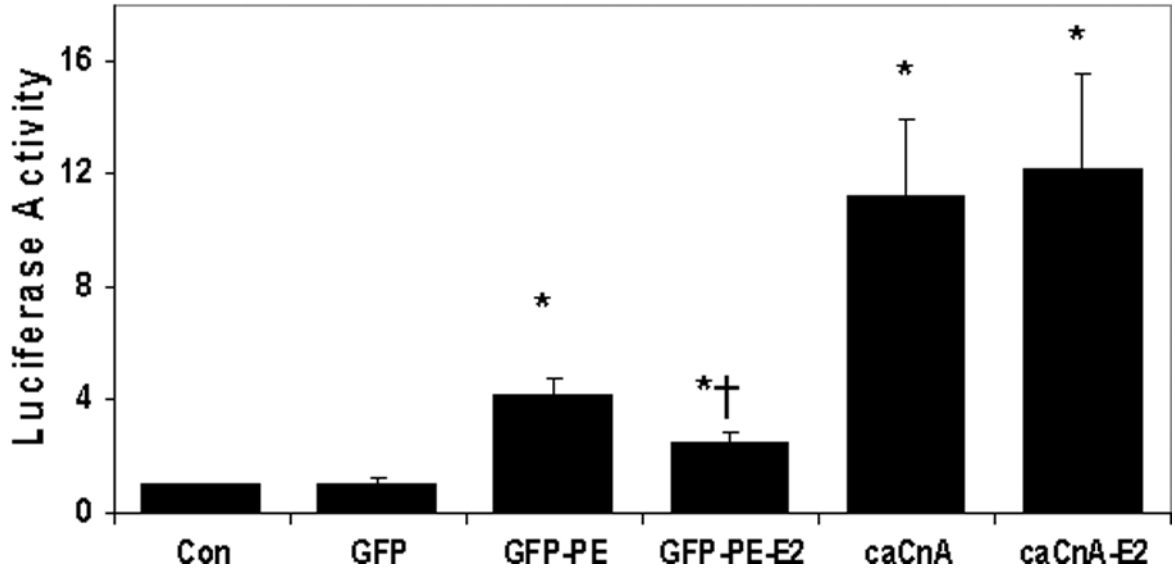

**Figure 3. Estrogen Inhibits Agonist Induced Cardiomyocyte Hypertrophy *in vitro***

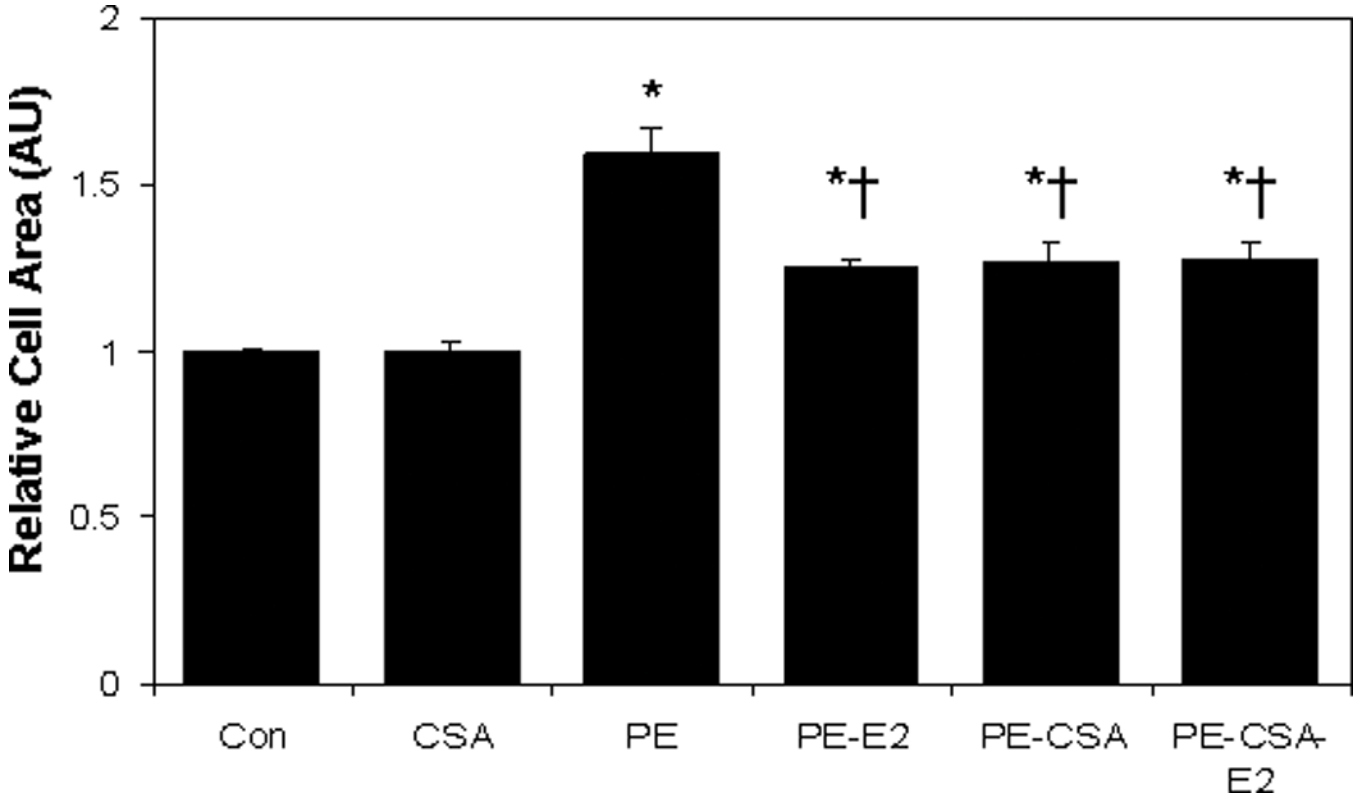
**A. Myocyte Cell Size:** Neonatal rat ventricular myocytes (NRVMs) were plated in serum containing media for 24 hours. 17 β-estradiol (E2, 10nM), ICI 182,780 (ICI, 1.0uM), both, or vehicle control were added in serum free media overnight. Cells were then stimulated with phenylephrine (50uM) for 48 hours. Pretreatment with E2 limited PE-induced cardiomyocyte enlargement that was reversed by the ER antagonist, ICI. These data are representative of 6 independent experiments. * $p<0.01$ vs. control cells; † $p<0.05$ vs. PE. **B. ANP Gene Expression.** Total cellular RNA was harvested from $2.5\times10^6$ cells plated on 10cm dishes and real time rtPCR performed to quantify the level of ANP expression. E2 (10nM) had no effect on basal levels of ANP expression but significantly limited the PE-induced increase in ANP mRNA that was reversed by ICI (0.5uM). * $p<0.01$ vs. control cells; † $p<0.05$ vs. PE.







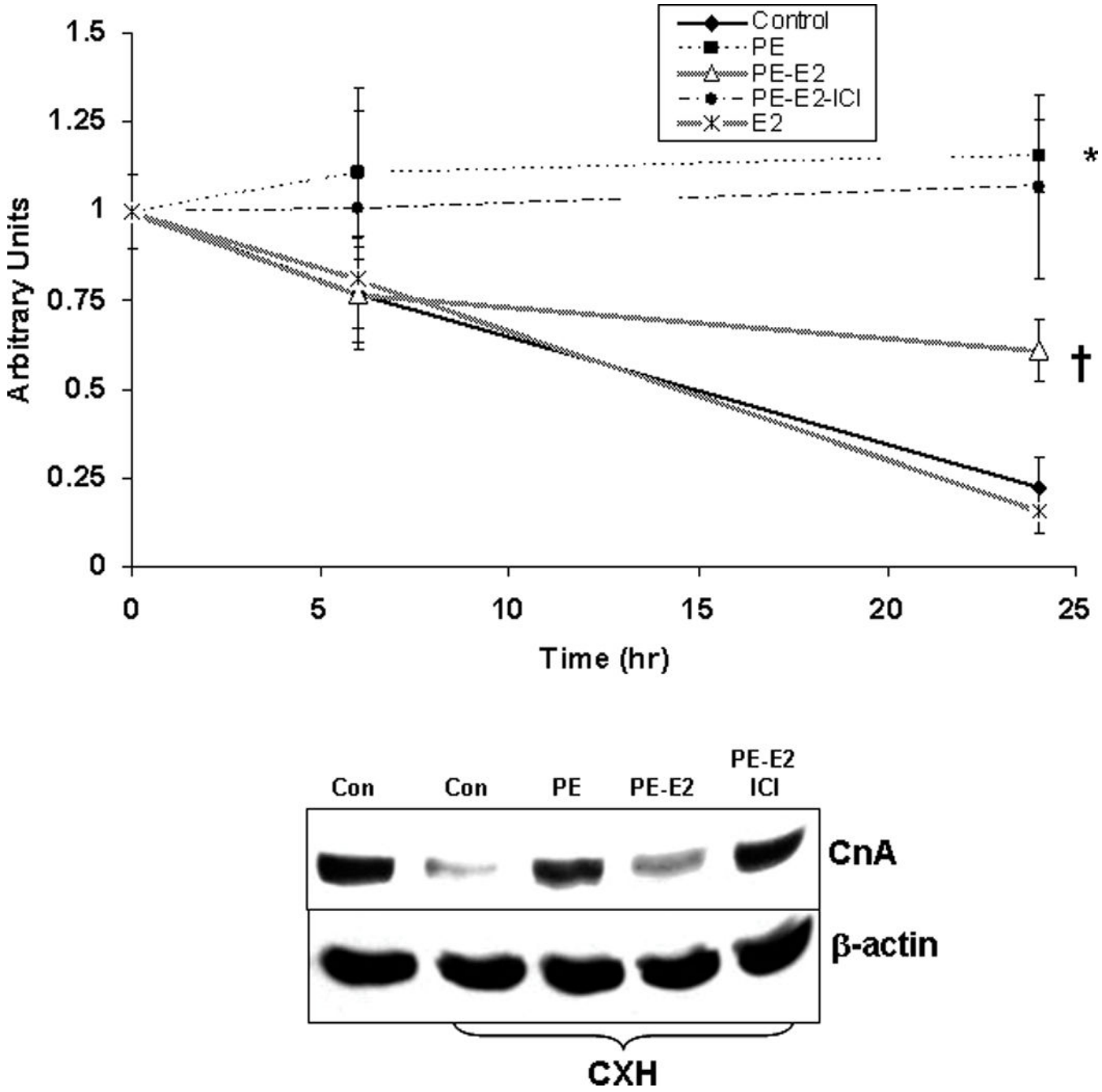
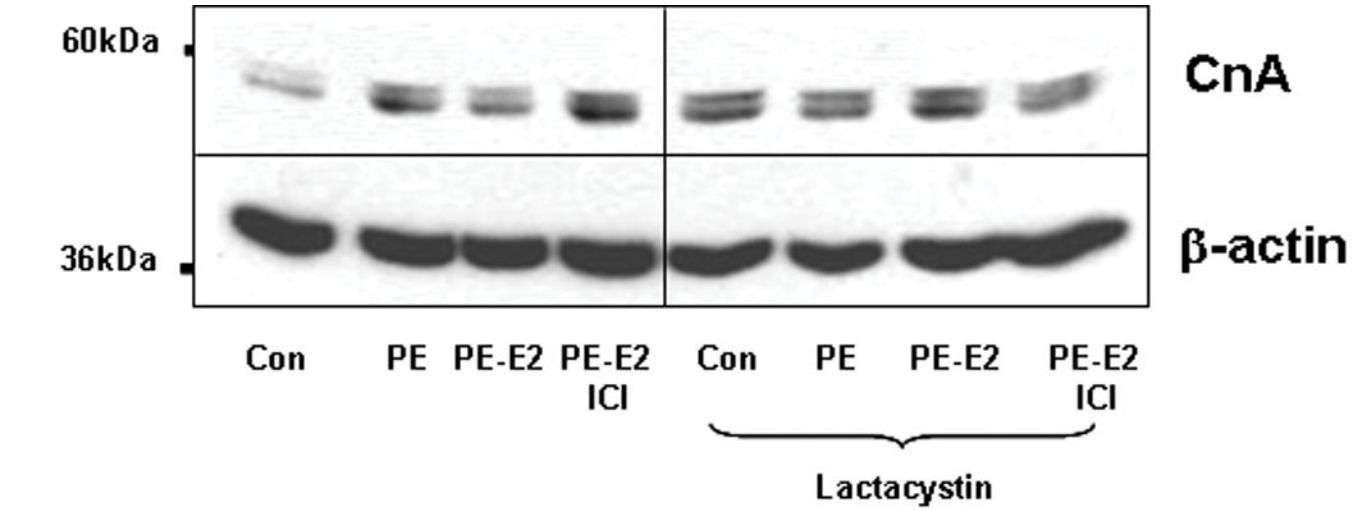
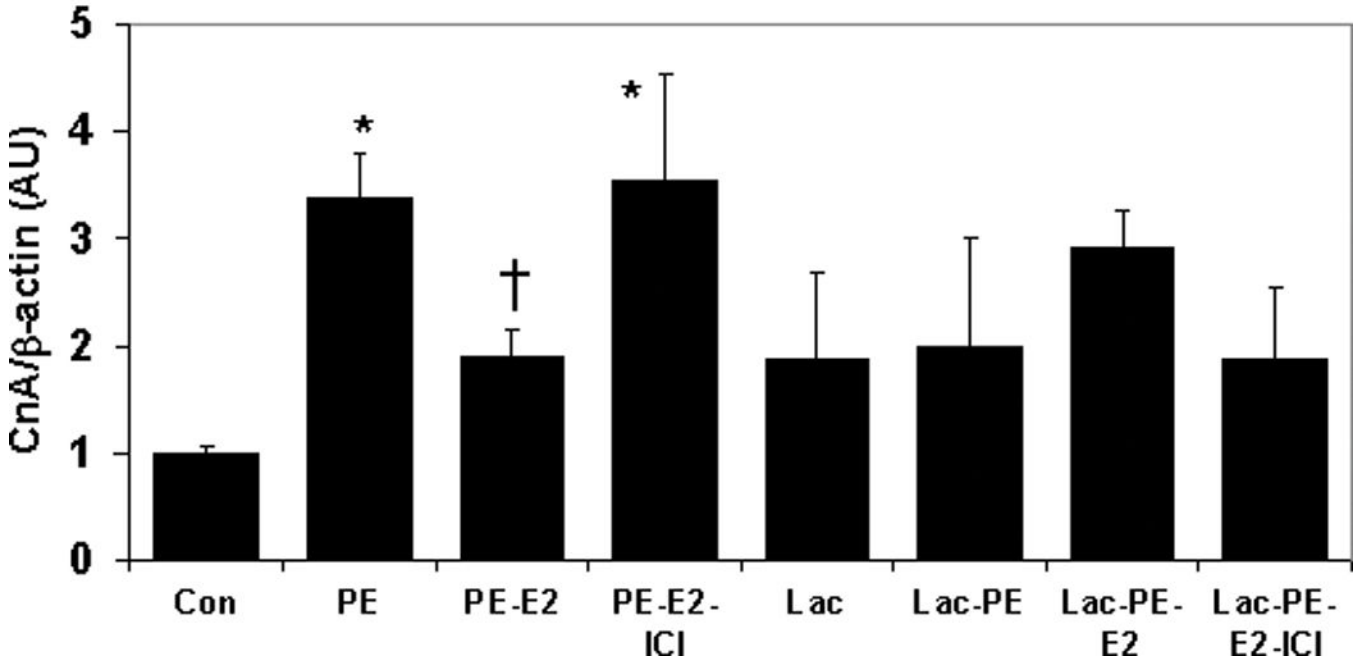


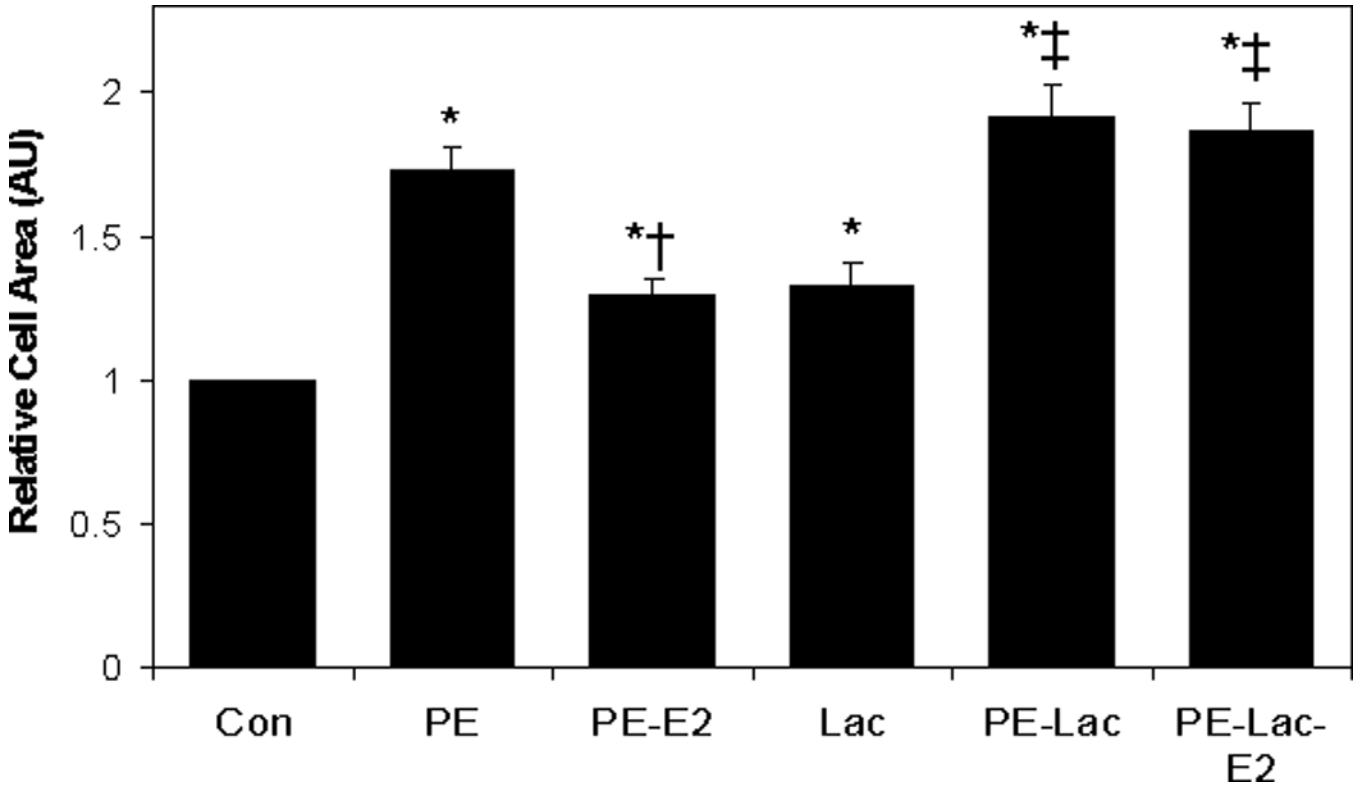

**Figure 4. Calcineurin Signaling Pathway as a Target of E2-mediated inhibition *in vitro***

**A. Left: Calcineurin Protein Expression.** NRVMs were harvested 24 hours following PE stimulation and CnA expression was measured by Western blotting using an antibody that recognizes both the α and β isoforms of CnA. PE (50uM) caused a significant rise in CnA protein expression that was limited by E2 pretreatment and reversed by the ER antagonist, ICI. These data represent 5 independent experiments. **Right: NFAT activation:** NRVMs were transfected with an NFAT promoter-luciferase reporter plasmid along with an L7RH-β-galactosidase plasmid for signal normalization. Upon serum deprivation, cells were

pretreated with E2 (10nM) or vehicle. PE was added on the following day and cells harvested 24 hours later. PE (50uM) induced a more than 3 fold rise in NFAT activation that was significantly limited by E2 and CSA pretreatment. * $p<0.01$ vs. control cells; † $p<0.05$ vs. PE. **B. A constitutively active mutant of CnA (caCnA) blocks the inhibition by E2 of cardiomyocyte growth and NFAT-activation. Left:** Cell size data in NRVMs infected with Adv-GFP as a control, or Adv-caCnA. E2 pretreatment inhibits hypertrophy of GFP expressing cells stimulated with PE, but has no effect on NRVM hypertrophy stimulated by caCnA. Similar loss of E2 effect was seen in ARVMs expressing caCnA. **Right:** Similar results are seen with activation of NFAT in which the inhibition by E2 of PE-induced NFAT activation is maintained in GFP expressing cells but is lost in cells expressing caCnA. Both graphs are representative of 3 independent experiments. * $p<0.01$ vs. control cells; † $p<0.05$ vs. PE. **C. E2 has no additive effects in NRVMs pretreated with the CnA inhibitor, Cyclosporine (500ng/ml).** Following 24 hours of growth in serum containing media, NRVMs were serum deprived overnight in the presence of vehicle, E2, cyclosporine, or their combination and stimulated with PE for an additional 48 hours. * $p<0.01$ vs. control cells; † $p<0.05$ vs. PE. **D. PE reduces CnA degradation that is normalized by E2 pretreatment.** NRVMs were treated with E2, E2 plus ICI, or vehicle upon serum deprivation. On the following day, cells were pretreated with cycloheximide (40ug/ml-based on preliminary experiments demonstrating complete inhibition of GFP-tagged ERα translation delivered by adenoviral transfection). 2 hours later, cells were stimulated with PE and harvested at 6, and 24 hours. CnA expression was normalized to β-actin. The line graph (left) shows quantified CnA levels. PE significantly prevents CnA degradation at 24 hours which is normalized by E2 replacement and reversed by the ER antagonist, ICI. A representative western blot of the control and 24 hour samples is shown to the right. These data are representative of 4 independent experiments. * $p<0.01$ vs. control cells; † $p<0.05$ vs. PE.





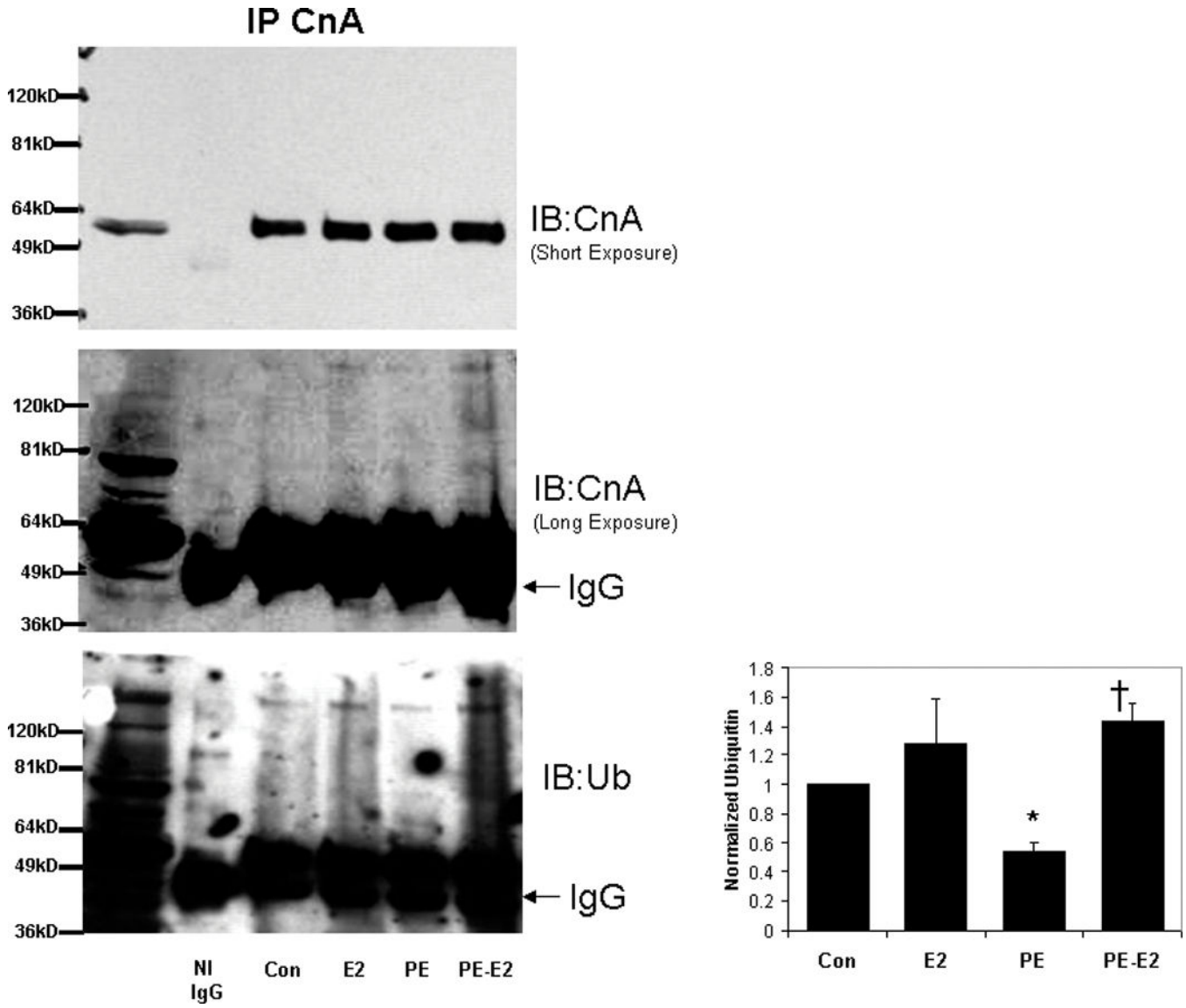



**Figure 5. Proteasome inhibition abolishes the inhibitory effect of E2 on CnA expression and cardiomyocyte hypertrophy**

**A.** Lactacystin (10uM) prevents both the agonist-induced increase in CnA and the inhibitory effects of E2. Representative western blot of CnA with corresponding bar graph. **B.** Lactacystin abolishes the E2-mediated inhibition of NRVM hypertrophy induced by PE. * $p<0.01$ vs. control cells; † $p<0.05$ vs. PE. ‡ $p<0.05$ vs. Lactacystin alone. **C. Phenylephrine diminishes CnA ubiquitination that is restored by E2 pretreatment.** NRVMs were pretreated overnight with E2 upon serum deprivation, and stimulated with PE. Cells were harvested 4 hours later and IPs of CnA were performed and analyzed by SDS-PAGE. Negative controls consisted of non-immune mouse IgG precipitated in exactly the same way as CnA. PE stimulation reduced CnA ubiquitination that is restored by E2 pretreatment. The bar graph represents data from 5 separate experiments. * $p<0.01$ vs. Control; † $p<0.05$ vs. PE.

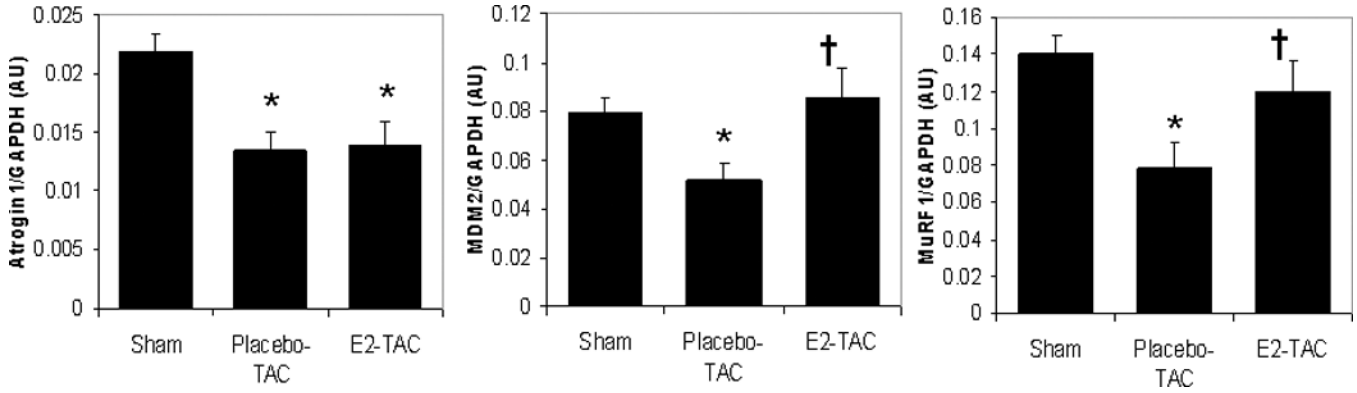



**Figure 6. Influence of TAC and E2 Replacement on Myocardial E3 Ubiquitin Ligase Expression**

Total cellular RNA was isolated from myocardial samples 2 weeks following TAC or Sham procedure and ubiquitin ligase mRNA expression quantified by real time rtPCR. TAC significantly reduced the expression of Atrogin 1 (left), Mouse double minute 2 (MDM2 – middle) and muscle-specific ring finger protein 1 (MuRF1 – right). E2 replacement normalized MDM2 and MuRF1 but had no effect on atrogin1 mRNA abundance. * $p<0.01$ vs. Sham; † $p<0.05$ vs. placebo-TAC.

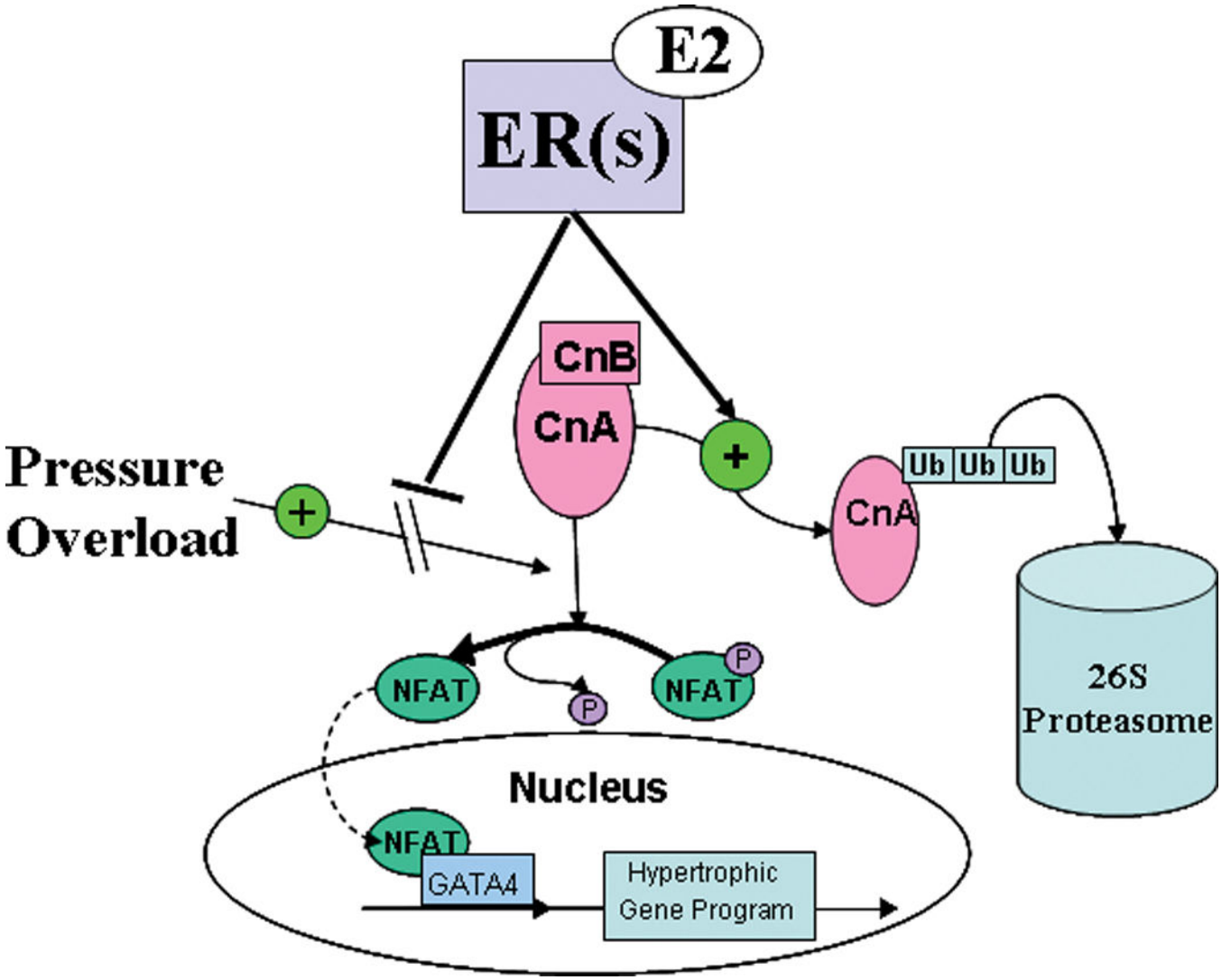



**Figure 7. Proposed model of estrogen and ER-mediated inhibition of cardiomyocyte hypertrophy**

**Table 1**

Morphometric, Hemodynamic Data and Echocardiographic

| | Sham (N=8) | Placebo-TAC (N=9) | E2-TAC (N=9) |
|---|---|---|---|
| Body Weight (gr) | 22.0±0.3 | 21.6±0.3 | 21.5±0.7 |
| Tibial Length (TL-mm) | 17.0±0.1 | 16.7±0.2 | 17.0±0.1 |
| LV Mass (mg/mm TL) | 3.58±0.10 | 5.77±0.25 | 4.97±0.26 |
| LV EDD (mm) | 3.30±0.08 | 3.89±0.06* | 3.40±0.08† |
| LV ESD (mm) | 2.09±0.08 | 2.86±0.09* | 2.14±0.07† |
| Fractional Shortening (%) | 37.0±1.1 | 26.6±1.7* | 37.2±1.4† |
| HR (bpm) | 555±12 | 568±10 | 540±18 |
| LV Systolic Pressure (mmHg) | 92.4±4.3 | 130.2±3.7* | 138.6±5.9* |
| Gradient (mmHg) | – | 73.9±5.5 | 72.5±5.1 |
| LVEDP (mmHg) | 6.4±1.6 | 22.9±2.2* | 11.3±2.8*† |
| LV +dP/dt (mmHg/sec) | 8467±333 | 5699±402* | 6499±415* |
| Stroke Volume (ul) | 20.2±1.6 | 8.1±1.0* | 14.9±1.6*† |
| Cardiac Output (ml/min) | 11.20±0.86 | 4.63±0.58* | 7.89±0.92*† |
| ESPVR (mmHg/ul) | 5.36±0.58 | 3.23±0.38* | 4.99±0.49† |
| PRSW (mmHg) | 60.0±6.9 | 23.9±3.7* | 62.1±8.1† |

All data are means±sem. E2=17β-estradiol; TAC=transverse aortic constriction; LV=left ventricular; EDD=end diastolic diameter; ESD=end systolic diameter; HR=heart rate; EDP= end diastolic pressure; +dP/dt=peak positive first derivative of LV pressure during steady state recording. ESPVR=slope of the end systolic pressure volume relationship; PRSW=preload recruitable stroke work.

* $p<0.05$ vs. Shams

† $p<0.05$ vs. Placebo-TAC.