ATRIAL NATRIURETIC PEPTIDE LOCALLY COUNTERACTS THE DELETERIOUS EFFECTS OF CARDIOMYOCYTE MINERALCORTICOID RECEPTOR ACTIVATION

Nakagawa et al., Cardiac imbalance between ANP and aldosterone

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Abstract

Background. The endocrine balance between atrial natriuretic peptide (ANP) and the renin-angiotensin-aldosterone system is critical for the maintenance of arterial blood pressure and intravascular volume homeostasis. The present study investigated whether a local, cardiomyocyte - restricted imbalance between ANP and aldosterone contributes to adverse cardiac remodeling in response to pressure overload.

Methods and Results. The effect of transverse aortic constriction (TAC)-induced left ventricular (LV) pressure overload was examined in mice with selective inactivation (KO) of the ANP receptor (guanylyl cyclase (GC)-A) or the downstream cGMP-dependent protein kinase I (cGKI) in myocytes. In response to 21-days of TAC, both strains of KO mice developed exacerbated pathologic LV hypertrophy and fibrosis together with contractile dysfunction. Blockade of the MR with eplerenone fully prevented these changes. TAC induced the expression of profibrotic CTGF and attenuated the expression of SERCA in KO, but not in control, hearts, and these molecular changes were also prevented by eplerenone. In HEK 293 cells, ANP, via GC-A/cGKI, attenuated the aldosterone-induced nuclear translocation of MRs. Even more, co-immunoprecipitation and FRET experiments demonstrated that a population of MRs is membrane-associated, in close interaction with GC-A and cGKI. And, moreover, that aldosterone causes a conformational change of this membrane MR/GC-A protein complex, which is prevented by ANP.

Conclusions. ANP attenuates the aldosterone-induced nuclear MR translocation. Imbalanced cardiomyocyte signaling of the ANP/GC-A and aldosterone/MR systems contributes to the progression of hypertrophy to heart failure.

Keywords. Heart Failure; Natriuretic Peptides; Angiotensin; Aldosterone

Introduction

Compensated hypertensive left ventricular hypertrophy can progress to adverse remodeling and heart failure (HF) with poor prognosis. Little is known about the mechanisms causing this transition. Investigations over the past three decades have demonstrated that neurohormones play an important role in the complex multiorgan and cellular adaptations in HF. Excessive activation of vasoconstricting and pro-proliferative neurohumoral systems such as the sympathetic and the renin-angiotensin II (Ang II)-aldosterone (RAA) systems has detrimental effects. In particular, cardiac expression and activity of the mineralcorticoid receptor (MR; a ligand-dependent transcription factor) is increased in heart failure (HF) (1). MR antagonism limits the transition to HF in experimental models of hypertensive or ischemic cardiac remodeling and diminishes morbidity and mortality in patients with severe HF (2). Although MR is expressed in both cardiac myocytes and fibroblasts, myocyte-MR possibly plays the major pathophysiological role (3). Hence, in mice, genetic myocyte-restricted ablation of the MR improves ventricular function and remodeling in ischemic or hypertensive heart failure (3,4); whereas myocyte-MR overexpression induces arrhythmias (5) and worsens Ang II-induced remodeling (6). The mechanisms mediating the deleterious effects of myocyte MR activation are largely unclear.

The cardiac hormones atrial (ANP) and B-type natriuretic peptides (BNP) are the main endogenous antagonists of the RAA system. They were originally isolated from the heart and brain as "natriuretic factors" (7,8). But subsequent research of many scientists demonstrated that they exert pleiotropic effects which all together are critical for the maintenance of arterial blood pressure and intravascular volume homeostasis. Via their shared cyclic GMP (cGMP) - producing guanylyl cyclase (GC)-A receptor they attenuate juxtaglomerular renin as well as adrenal aldosterone release and counteract the vasoconstrictory and antinatriuretic actions of Ang II and aldosterone (9). In humans, even small decreases in the plasma levels of

ANP/BNP are associated with increased blood pressure (10), emphasizing that the delicate balance between NPs and the RAA system is essential for pressure/volume homeostasis. In addition, experimental and clinical studies indicate that ANP/BNP not only exert endocrine but also local, cardioprotective actions. Experimentally, cardiac overexpression of GC-A in mice attenuated hypertensive or ischemic heart disease (11). Conversely, conditional, cardiomyocyte (CM)-specific inactivation of the ANP/BNP receptor (CM GC-A KO mice) exacerbated hypertensive cardiac remodeling (12). Similar alterations were observed in mice with CM disruption of cGMP-dependent protein kinase (CM cGKI KO mice), indicating that this kinase is one downstream target mediating the protective actions of NPs in the heart (13-15). Based on these and many other observations, a number of established and investigational treatments of heart failure enhance intracellular cGMP signaling (16).

Cardiac hypertrophy is accompanied by GC-A desensitization (17,18) and increased MR activity (1,2). Here we hypothesized that the resulting local, cardiac imbalance between the myocyte activities of the ANP/GC-A and aldosterone/MR systems contributes to the progression from hypertrophy to heart failure. Accordingly, the goal of this study was (1) to test whether a selective MR antagonist, eplerenone, prevents pressure overload - induced adverse cardiac remodeling in mice with myocyte-restricted inactivation of the NP/GC-A/cGKI signaling pathway; and (2) to dissect the molecular mechanism(s) involved in the cardiac balance between the NP/GC-A and aldosterone/MR systems as well as in the deleterious consequences of a local imbalance between them.

Methods

Detailed methods are described in the online-only Data Supplement.

Genetic mouse models

Mice with cardiomyocyte-restricted deletion of either GC-A (CM GC-A KO) of cGKI (CM cGKI KO) and their respective control (CTR) littermates (GC-$A^{fl/fl}$, $cGKI^{fl/fl}$) were generated by *αMHC-Cre / lox P* technology as described previously (12,13). All study mice were aged 2-3 months. KO and respective control (CTR) littermates were compared in all experiments. The experiments complied with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) and were approved by the animal care committee of the University of Würzburg.

Animal studies

Surgical transverse aortic constriction (TAC, 21 days) or sham-operation were performed as described previously (12,13). Two weeks before TAC, littermates were randomly assigned to regular chow or chow containing the MR antagonist eplerenone (100 mg/kg body weight (BW) per day). Arterial blood pressure was measured in awake mice by tail cuff (12). Echocardiography was performed under light isoflurane anaesthesia before and after 3-weeks TAC (13). Mice were then sacrificed, the left ventricles (LV), lungs and tibias were dissected, and LV samples were frozen in liquid nitrogen (for protein or mRNA extraction) and fixed in 4 % buffered formaldehyde (for histology and immunohistochemistry) (12,13).

Histology

Cardiomyocyte cross-sectional areas were determined by immunohistochemistry with antibodies directed against pan-cadherin (Sigma) (19). The extent of myocardial fibrosis was determined on parallel LV sections stained with 0.1% picrosirius red (13). Connective tissue growth factor (CTGF) expression was examined by immunohistochemistry (13).

Protein and gene expression

Western blotting was performed to analyze left ventricular expression levels of SERCA2a (antibody from Badrilla, Leads, UK), phosphorylated and total ERK1/2 and GAPDH (antibodies from Cell Signaling). CTGF mRNA expression levels were quantified by real time RT-PCR (13).

Studies in transfected HEK 293 cells

HEK 293 cells stably expressing FLAG-tagged GC-A (or mock) were transfected with plasmids encoding for enhanced green fluorescent protein (EGFP)-tagged MR and cGKI with Fugene (Roche). The cells were maintained in charcoal-stripped (steroid-reduced) medium. For confocal microscopy or FRET (Fluorescence resonance energy transfer) studies, 24 h after transfection the cells were seeded on coverslips, and the incubation experiments with aldosterone ± ANP were performed 24 h later. For immunoprecipitation studies, 48 h after transfection the membrane and cytosolic proteins were extracted and the former were incubated with anti-FLAG antibody coupled to agarose beads (M2; Sigma) during 2 hours at 4°C under rotation. Aliquots of the cytosolic and membrane fractions and of the immunoprecipitated proteins were subjected to western blotting with antibodies against mineralcorticoid receptor (DSHB, Iowa, USA), *Hsp90* (BD Biosciences), cGKI (Cell Signaling) and GC-A (13).

Statistics

Results are presented as mean ± SEM. Comparisons between 2 groups were performed using the unpaired Student *t* test. Group data were compared using 2-way ANOVA (with genotype and treatment as categories) followed by the multiple comparison Bonferroni test to evaluate differences between groups.

Results

In HEK 293 cells, ANP inhibits the aldosterone stimulated nuclear translocation of the mineralcorticoid receptor

To investigate whether ANP influences aldosterone-stimulated nuclear translocation of MRs, HEK 293 cells stably expressing GC-A were cotransfected with EGFP-tagged MR and cGKI. The nuclear *vs* cytoplasmic distribution of MRs was analyzed by confocal microscopy. As shown in Figure 1, aldosterone (500 pmol/L, 1 h) induced a marked nuclear accumulation of MR-EGFP, which was significantly inhibited by ANP (10 nmol/L, pretreatment during 30 min). Based on these observations, we hypothesized that the ANP/GC-A system, via cGMP/cGKI signaling, might counterregulate local cardiac deleterious MR-mediated genomic actions of aldosterone *in vivo*.

Eplerenone prevented pressure load - induced adverse left ventricular remodeling in CM GC-A KO and CM cGKI KO mice

To test directly the role of the ANP-aldosterone interaction in regulating the cardiac responses to LV pressure overload *in vivo*, we subjected separate cohorts of mice with cardiomyocyte (CM)-restricted deletion of either GC-A (CM GC-A KO mice) or cGKI (CM cGKI KO mice) and respective control (CTR) littermates ($GC\text{-}A^{fl/fl}$; $cGKI^{fl/fl}$) to TAC for 21 days in the presence and absence of the selective MR blocker eplerenone. Eplerenone treatment (100 mg/kg BW/day) was started 2 weeks before TAC or sham operation. Since the morphological and also the functional and molecular responses to TAC were identical in both groups of CTR mice, these results are combined in all sections and figures throughout the manuscript.

CM GC-A KO and CM cGKI KO mice have normal Mendelian inheritance, grow normally, and have a normal life span (12,13). Under resting conditions, their systemic arterial blood pressure as well as cardiac morphology and function are unaltered (Suppl. Table 1). Also, systolic blood pressure levels in CM GC-A KO, CM cGKI KO and control (CTR) mice

subjected to TAC were similar (110 ± 2.0, 108 ± 2.8 and 109 ± 2.6 mmHg, respectively; n=6-10 per group) and were not altered by eplerenone (111 ± 2.0, 107 ± 2.0 and 108 ± 1.8 mmHg, respectively; n=6-10). As shown in Figures 2A-C, in CTR mice, TAC induced significant increases in LV weight (LVW) - to – tibia length (TL) ratios and myocyte cross sectional areas, and these mild hypertrophic responses were not influenced by eplerenone treatment. In CM GC-A KO and CM cGKI KO mice, TAC provoked exacerbated LV hypertrophy, as shown at the organ (Figure 2A) and cellular level (Figure 2B). Even more, in both KO strains, TAC also increased the wet lung/TL ratios (Figure 2C), suggesting that they developed pulmonary congestion, a consequence of left-sided heart failure. Eplerenone significantly prevented the development of exacerbated LV hypertrophy and fully prevented pulmonary congestion in response to 21-days TAC (Figures 2A-C).

Picrosirius red stainings of cardiac sections showed pronounced LV interstitial fibrosis in CM GC-A KO and CM cGKI KO mice after TAC (Figure 3A), which was associated with enhanced expression of the profibrotic cytokine connective tissue growth factor (CTGF; shown by RT-PCR and confirmed by immunohistochemistry; Figure 3B). In both, CM GC-A KO and CM cGKI KO mice, eplerenone fully prevented pressure-load dependent LV fibrosis and induction of CTGF.

Eplerenone prevented pressure overload-induced LV dilatation and dysfunction in CM GC-A KO and CM cGKI KO mice

Echocardiography showed that LV contractility did not differ between genotypes under baseline conditions, before TAC (Figure 4; Suppl. Table 1). TAC did not alter LV geometry and function in CTR mice (Figure 4, left column). However, CM GC-A KO and CM cGKI KO mice developed severe LV dilatation in response to TAC, with LV enddiastolic and endsystolic areas (EDA and ESA) markedly increasing and fractional shortening (FS) decreasing (Figure 4). Of note, eplerenone almost fully prevented these morphological and

functional alterations. Heart rates in CTR, CM GC-A KO or CM cGKI KO mice were similar (574 ± 13, 578 ± 6 and 589 ± 16 bpm, respectively) and were not altered by TAC (562 ± 12, 576 ± 15, 552 ± 12 bpm, respectively) or eplerenone (562 ± 11, 603 ± 6, 575 ± 8 bpm, respectively).

Eplerenone prevented blunted LV expression of SERCA2a in CM GC-A KO and CM cGKI KO mice subjected to TAC

Based on these morphological and functional data, we hypothesized that ANP, via GC-A/cGKI signaling in cardiac myocytes, counterregulates deleterious effects of MR signaling. Impaired ANP/GC-A/cGKI signaling in myocytes enhances the actions of aldosterone contributing to load-dependent adverse cardiac remodeling and dysfunction. We therefore examined myocardial SERCA2a expression which has been demonstrated by others to mediate LV decompensation in response to excessive RAA activation (20). As shown in Figure 5 by immunoblot analyses, LV SERCA2a protein levels were unaltered in CTR mice after 3 weeks of TAC, but were significantly reduced in both groups of KO mice. Notably, this inhibition was significantly prevented by eplerenone treatment. As also shown in Figure 5, TAC provoked significant increases in LV levels of phosphorylated ERK1/2, without differences between genotypes and treatment groups.

Association of plasma membrane GC-A and MR proteins in HEK293 cells

Lastly, we investigated whether a GC-A/MR colocalization may partly underlie the functional antagonism between the GC-A receptor and the MR by coimmunoprecipitation (coIP) and FRET in transfected HEK 293 cells. For coIP experiments, MR was transiently coexpressed in HEK cells together with FLAG-tagged GC-A or mock (21). The membrane fraction of transfected cells was used to enrich GC-A by immunoprecipitation with anti-FLAG (M2) antibody. Western blotting demonstrated that a small fraction of MR is indeed localized at the cell membrane (Figure 6A, right). Even more, membrane MR coimmunoprecipitates with

GC-A, cGKI and *hsp*90, indicating that these proteins are part of a protein complex. MR immunoprecipitation was not obtained in cells transfected with MR and empty vector (Figure 6A, left).

Single cell FRET analysis demonstrates that ANP and aldosterone modulate the interaction between GC-A and MR in HEK 293 cells

FRET microscopy can be used as a tool to monitor protein-protein interactions in intact living cells. Intriguingly, co-expression of GC-A-CFP (18) and MR-EGFP resulted in a substantial basal FRET (Figure 6B), suggesting close proximity between both receptors. Even more, their ligands, ANP and aldosterone, both induced significant decreases in FRET by 2-3 % within 5-10 min after hormone stimulation (Figures 6B and C), indicative of an agonist-induced rearrangement within the GC-A/MR complex. Pretreatment with ANP (10 min) prevented the responses to subsequent application of aldosterone (Figures 6 B and C). These data corroborate the results of the coIP experiments indicating that GC-A and a subpopulation of membrane MR interact with each other in a dynamic, hormone-responsive way.

Discussion

Our observations demonstrate that impaired ANP signalling in cardiomyocytes (here provoked by myocyte-restricted GC-A or cGKI inactivation) does not alter baseline cardiac growth and function, but provokes dilatative cardiomyopathy and pronounced interstitial fibrosis in response to pathological pressure-load (induced by TAC). These morphological and functional alterations were associated with enhanced expression of CTGF and diminished expression of SERCA2a. Notably, myocyte hypertrophy was partly, and cardiac fibrosis as well as dysfunction were almost completely prevented by treatment with the selective MR blocker eplerenone. MR antagonism also prevented the pressure-load induced increases in left ventricular CTGF expression and decreases of SERCA2a, which may have contributed to the improvement in remodeling and LV contractile function. Together, these results indicate that the delicate local, cardiac myocyte balance between ANP/GC-A/cGKI and MR signaling is crucial to prevent adverse cardiac remodeling and failure in response to chronically increased afterload.

The cardiac antagonistic roles of ANP and aldosterone were suggested by previous studies in mice with global, systemic inactivation of ANP or GC-A (22,23). However, these studies have the limitation that systemic arterial hypertension, increased sympathetic tone and systemic RAA activity together with vascular abnormalities markedly impact the cardiac responses of $ANP^{-/-}$ and $GC\text{-}A^{-/-}$ mice to pressure overload. Another limitation is that these studies did not explore the specific cardiac cell types through which ANP antagonizes the pathologic responses to aldosterone. Hence, one strength of our studies in mice with CM-restricted inactivation of GC-A or cGKI is that they allowed us to address the clinically relevant question of a functional interaction of the GC-A and mineralcorticoid receptors specifically in cardiac myocytes.

MR binds aldosterone and glucocorticoids with similar affinity (24). The MR selectivity for aldosterone is achieved by the coexpression of 11β-hydroxysteroid dehydrogenase (11β-HSD2), which inactivates glucocorticoids (25). The activity of this enzyme is very low in myocytes and, moreover, circulating concentrations of glucocorticoids are much higher than those of aldosterone (25,26). Thus, the beneficial effects of MR antagonists in our experimental as well as in published clinical studies may result from blockade of glucocorticoids binding to myocyte MR. However, a recent study in mice dissected specific gene targets of aldosterone in cardiomyocytes, demonstrating that myocyte CTGF is directly and specifically induced by aldosterone via the MR, and not by corticosterone (27). As shown in the present study, both CM GC-A KO and CM cGKI KO hearts responded to pressure load with marked induction of CTGF, which was totally prevented by eplerenone. Together, these results indicate that ANP counterregulates the myocyte effects of aldosterone. An imbalance between these systems, favouring aldosterone/MR signaling, enhances myocyte expression and release of the fibrogenic stimulus CTGF and thereby promotes cardiac interstitial fibrosis during pressure load.

Remarkably, eplerenone treatment also completely prevented LV dilatation and contractile dysfunction in CM GC-A KO and CM cGKI KO mice, again indicating involvement of MR signaling in these pathological responses. Conversely, mice deficient in myocyte MR are protected from cardiac dilatation and failure after TAC (3). These and other published results demonstrate that enhanced MR activity in myocytes leads to functional deterioration, although the mechanisms remain unclear. The myocyte-mediated profibrotic, CTGF-mediated actions of MR signaling might be involved, since myocardial fibrosis can result in excessive muscle fiber entrapment, myocyte atrophy and/or abnormal diastolic and systolic stiffness of the myocardium, each of which is sufficient for the development and progression of LV dysfunction (28). In addition, our study suggests that MR activation during pressure load impairs the expression of the SR calcium-regulatory protein SERCA2a. Although SERCA

was not among the genes shown to be specifically regulated by aldosterone in cardiomyocytes (27), other studies showed that impaired myocardial SERCA2a protein expression contributes to LV decompensation in response to excessive RAA activation (20). Ultimately, the exact link between MR activation and diminished SERCA2a expression remains unclear.

Non-genomic signaling pathways of the MR, such as the transactivation of the epidermal growth factor receptor (EGFR) and phosphorylation/activation of the MAPK ERK1/2 (29), have also been implicated in the transition to heart failure (30). Although we observed increased LV levels of phosphorylated ERK1/2 in mice subjected to TAC, this response was not different between control and knockout mice, and was not modulated by eplerenone. In agreement with these results, mice with myocyte-restricted MR inactivation did not exhibit diminished, but even increased cardiac pERK1/2 levels after TAC (3). Together with our CTGF data, these results suggest that enhanced activation of classical (genomic) MR signaling pathways mainly contributes to adverse remodeling in the CM GC-A/cGKI KO mice with TAC. This hypothesis is corroborated by the observation that ANP, via GC-A, significantly inhibited the aldosterone-stimulated nuclear translocation of the MR in a heterologous expression system, in HEK 293 cells. Corroborating our study, ANP, via cGMP/cGKI signaling, attenuated aldosterone-induced MR nuclear import and subsequent changes of ion transport in colonic epithelial cells (31). Interestingly, cGMP inhibits nuclear translocation of another steroid receptor, the vitamin D receptor (VDR), in fibroblasts by altering the interaction of VDR with the cytoskeletal protein tubulin (32). Since cGKI can phosphorylate tubulin and microtubule-associated proteins, it was postulated that cGMP prevents activated VDR dissociation from tubulin (32). Thus, it is possible that ANP, via cGMP/cGKI, inhibits MR nuclear translocation by altering the interaction of the MR with the cytoskeletal protein transport system.

Both, the GC-A receptor and the MR, have been shown to be associated with the chaperone *hsp*90 (33,34). Even more, a small population of MR is cell membrane-associated, possibly within cholesterol-rich domains (29,35). This population detaches from the membrane in response to aldosterone (29), and might therefore contribute to the genomic actions of the hormone. A subpopulation of GC-A receptors is also localized in caveolae microdomains (36). Therefore we postulated that the functional antagonism between GC-A and MR might be mediated in part by a direct interaction between both proteins. Indeed, co-immunoprecipitation and FRET experiments demonstrated that GC-A, MR, cGKI and *hsp90* are within a macromolecular complex at the cell membrane of HEK 293 cells. Intriguingly, ANP binding to GC-A or aldosterone binding to the MR both caused a conformational change of the MR/GC-A complex (as shown by FRET). Even more, the effect of aldosterone was prevented by ANP pretreatment. Together, these data suggest the existence of a signaling microdomain at the cells membrane which harbours a subpopulation of GC-A receptors and MRs. This interaction might contribute to the moderation of the genomic actions of aldosterone by ANP.

Perspectives. In heart failure (HF) patients, plasma levels of aldosterone, BNP and (less) ANP are elevated, correlating with the severity of the disease (1,37,38). However, despite these high NP levels, HF is in fact a state of combined deficiency of the active processed form of BNP and resistance to both NPs (9,17,21,39). Hence GC-A-mediated cGMP formation and the endocrine as well as cardiac effects of NPs are markedly blunted, due to desensitization of the receptor (17). The present experimental study indicates that a local, cardiac imbalance between the activities of the NP/GC-A (inhibition) and aldosterone/MR systems (activation) can critically contribute to myocyte hypertrophy and secretion of profibrotic factors, and ultimately to the transition to HF. Our experimental observations corroborate current clinical concepts (16) that RAAS inhibition combined with natriuretic peptide system augmentation could represent a novel therapeutic concept to combat heart failure.

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Novelty and Significance

The antagonistic endocrine actions of the cardiac hormones ANP/BNP and the RAA system maintain arterial blood pressure and volume homeostasis. Our experimental study demonstrates that independently from this endocrine crosstalk, cardiac interactions between NPs and aldosterone are critically involved in the regulation of myocyte growth and contractile functions, and the myocyte secretion of profibrotic factors such as CTGF. Patients with heart failure have a complex neurohumoral imbalance, which involves high ANP/BNP and aldosterone levels, but resistance to the endocrine and cardiac effects of the former, which favours even more an excessive activation of mineralcorticoid receptors. The present study indicates that dysbalanced actions of the NP/GC-A (inhibition) and aldosterone/MR systems (activation) on cardiomyocytes contributes to the progression of myocyte hypertrophy and dysfunction and secretion of profibrotic factors, and ultimately to the aggravation of heart failure. MR blockade combined with natriuretic peptide system augmentation could represent a novel therapeutic concept to combat this fatal disease.

Legends to Figures

Figure 1. ANP inhibits the aldosterone-stimulated nuclear translocation of the MR in HEK 293 cells. GC-A - expressing HEK 293 cells were co-transfected with EGFP-tagged MR and cGKI. The nuclear-to-cytosolic ratio of EGFP-MR (as fluorescence intensity ratio) was studied by confocal microscopy. Aldosterone (500 pmol/L, 1 h) increased the nuclear localization of the EGFP-MR. ANP (10 nmol/L, pretreatment during 30 min) attenuated this effect (*P<0.05 vs aldosterone; n=3 independent experiments; 100 cells were analyzed in each experiment).

Figure 2. Eplerenone prevented adverse left ventricular hypertrophy and lung congestion in CM GC-A KO and CM cGKI KO mice with TAC. (**A**) Ratios of left ventricular (LV) weight to tibia length (TL); (**B**) LV cross sectional myocyte areas; and (**C**) wet lung weight / TL from CTR, CM GC-A KO and CM cGKI KO mice after TAC or sham-operation. Mice with TAC received control food or eplerenone starting 2 weeks prior to surgery. For determination of myocyte areas, PAS stainings and immunohistochemistry with an anti-Pan-cadherin antibody were combined, to simultaneously highlight the intercalated disks and the cellular membrane (19). Length and width of myocytes with a centrally located nucleus were measured (right panels in **B**). n = 6-10 per group; $^{*}P < 0.05$ vs sham, $^{\dagger}P < 0.05$ vs TAC without eplerenone treatment.

Figure 3. Eplerenone prevented cardiac fibrosis induced by TAC in CM GC-A KO and CM cGKI KO mice. (A) LV interstitial collagen fractions of CTR, CM GC-A KO and CM cGKI KO mice after sham- or TAC-operation (± eplerenone); and **(B)** real time RT-PCR and immunohistochemical analyses of left ventricular CTGF expression. Right panels: representative pictures. n = 6-10 per group; $P < 0.05$ *vs sham, †vs TAC without eplerenone.

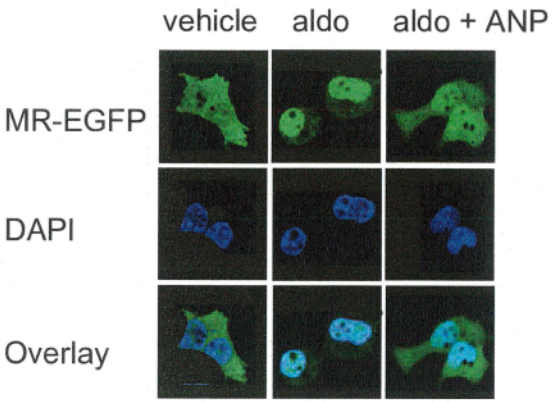
Figure 4. Eplerenone prevented left ventricular dilatation and dysfunction in CM GC-A KO and CM cGKI KO mice after TAC. LV end-diastolic area (EDA), end-systolic area (ESA) and fractional shortening in percentage (FS %) from CTR, CM GC-A KO and CM cGKI KO mice before and 21-days after TAC, measured by echocardiography. Mice received eplerenone (black circles) or control food (white circles) starting 2 weeks prior to surgery (n = 6-10 per group); $^{*}P < 0.05$ vs basal values, before TAC, $^{\dagger}P < 0.05$ vs TAC/vehicle.

Figure 5. Left ventricular expression levels of the $Ca^{2+}{}_i$ regulating protein, SERCA2a, and of phosphorylated ERK1/2 in CTR, CM GC-A KO and CM cGKI KO mice after TAC or sham operation. *Top*, Representative Western blots. *Bottom*, Protein levels of SERCA2a were normalized to GAPDH; levels of phosphorylated ERK1/2 were normalized to total ERK. Ratios were calculated as x-fold respective sham-operated, vehicle (v) - treated CTR mice. (n = 6-8 per group); $^{*}P < 0.05$ vs sham, $^{\dagger}P < 0.05$ vs TAC/vehicle.

Figure 6. Coimunoprecipitation experiments and FRET reveal the association of GC-A and a subpopulation of MRs at the membrane of HEK 293 cells. (A) Right: Coimmunoprecipitation of MR, cGKI and Hsp90 with FLAG-GC-A from membranes of cotransfected HEK 293 cells. Cytosolic (C) and membrane fractions (M) as well as immunoprecipitates from the membrane fractions (IP) with anti-FLAG antibody were separated on SDS-PAGE and blotted with antibodies against GC-A, cGKI, MR, and *Hsp*90. FT: flow through; w: wash step. Representative Western Blots of three independent experiments. Inputs were 1/10 – 1/20 of the protein used for IP. The left side of the blot shows that control IPs with HEK 293 cells without GC-A expression (mock transfection) do not give similar reactions. (**B**) Representative ratiometric recordings of single cell FRET signals in HEK cotransfected with GC-A-CFP and MR-EGFP at baseline and during superfusion of ANP, aldosterone or both. Left: Decrease of FRET between GC-A-CFP and MR-GFP in single HEK 293 cells treated with ANP. Right: Likewise, stimulation with aldosterone leads

to a decrease of FRET (black trace) which is inhibited after pretreatment with ANP for 10 min (grey trace). (**C**) Quantification of agonist-induced decreases in FRET (n = 9-13 cells from 3 independent experiments, for each group; *$p<0.05$ vs aldosterone alone).

Figure 1


vehicle
aldo
aldo + ANP
MR-EGFP
DAPI
Overlay

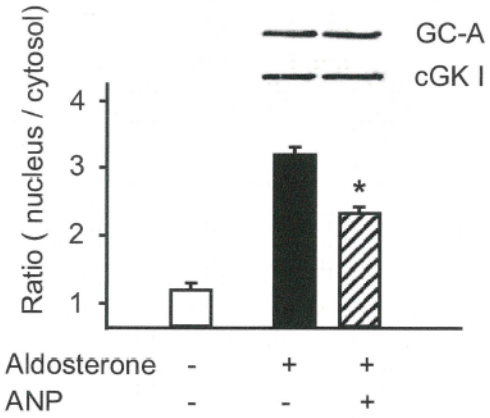

GC-A
cGK I
Ratio (nucleus / cytosol)
4
3
2
1
*
Aldosterone - + +
ANP - - +

Figure 2

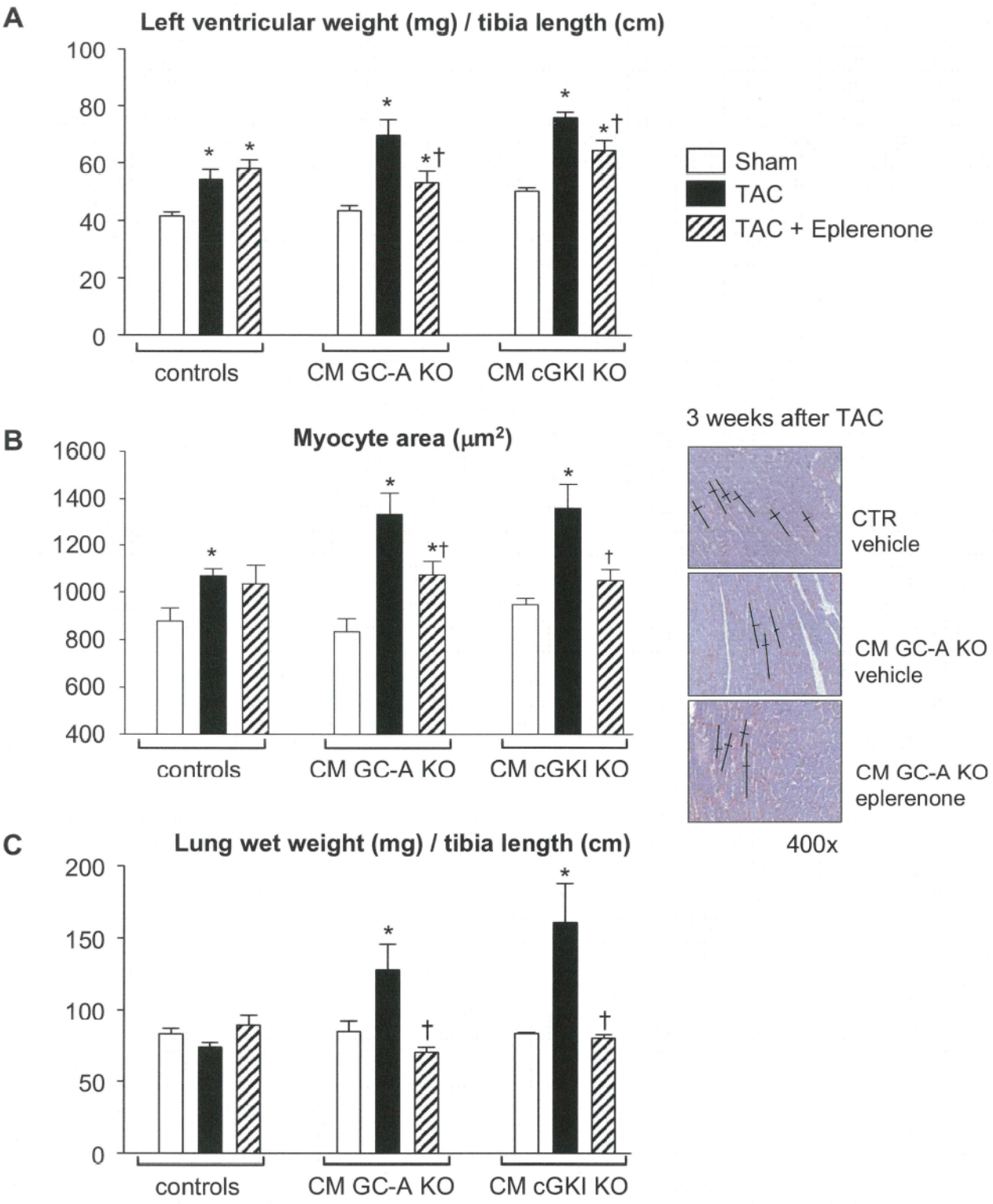

A
Left ventricular weight (mg) / tibia length (cm)
100
80
60
40
20
0
controls
CM GC-A KO
CM cGKI KO
Sham
TAC
TAC + Eplerenone
B
Myocyte area (μm²)
1600
1400
1200
1000
800
600
400
controls
CM GC-A KO
CM cGKI KO
3 weeks after TAC
CTR
vehicle
CM GC-A KO
vehicle
CM GC-A KO
eplerenone
400x
C
Lung wet weight (mg) / tibia length (cm)
200
150
100
50
0
controls
CM GC-A KO
CM cGKI KO

Figure 3

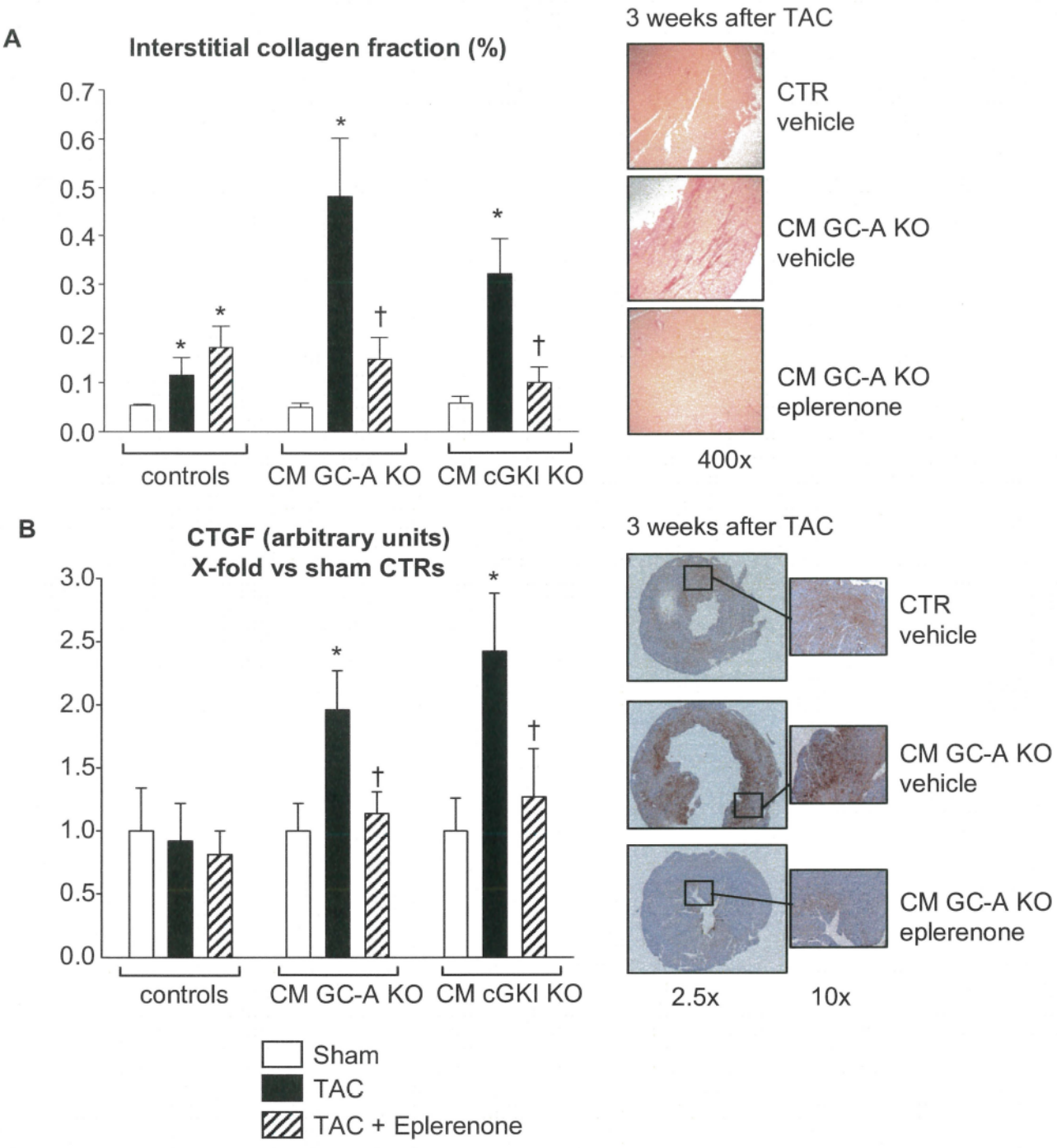

A
Interstitial collagen fraction (%)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
controls
CM GC-A KO
CM cGKI KO
3 weeks after TAC
CTR
vehicle
CM GC-A KO
vehicle
CM GC-A KO
eplerenone
400x
B
CTGF (arbitrary units)
X-fold vs sham CTRs
3.0
2.5
2.0
1.5
1.0
0.5
0.0
controls
CM GC-A KO
CM cGKI KO
Sham
TAC
TAC + Eplerenone
3 weeks after TAC
CTR
vehicle
CM GC-A KO
vehicle
CM GC-A KO
eplerenone
2.5x
10x

Figure 4

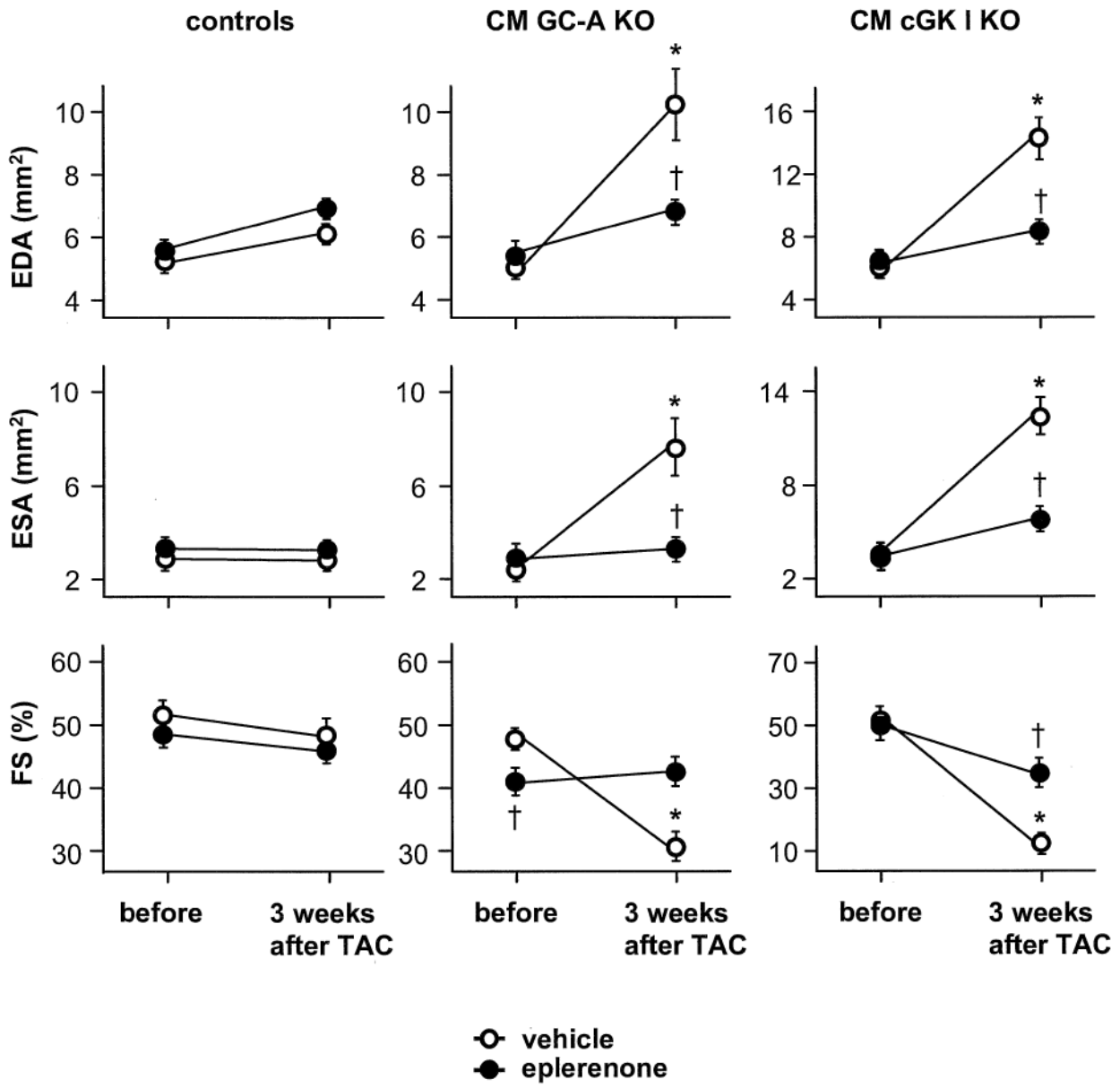

controls
CM GC-A KO
CM cGK I KO
EDA (mm²)
ESA (mm²)
FS (%)
before
3 weeks after TAC
vehicle
eplerenone

Figure 5

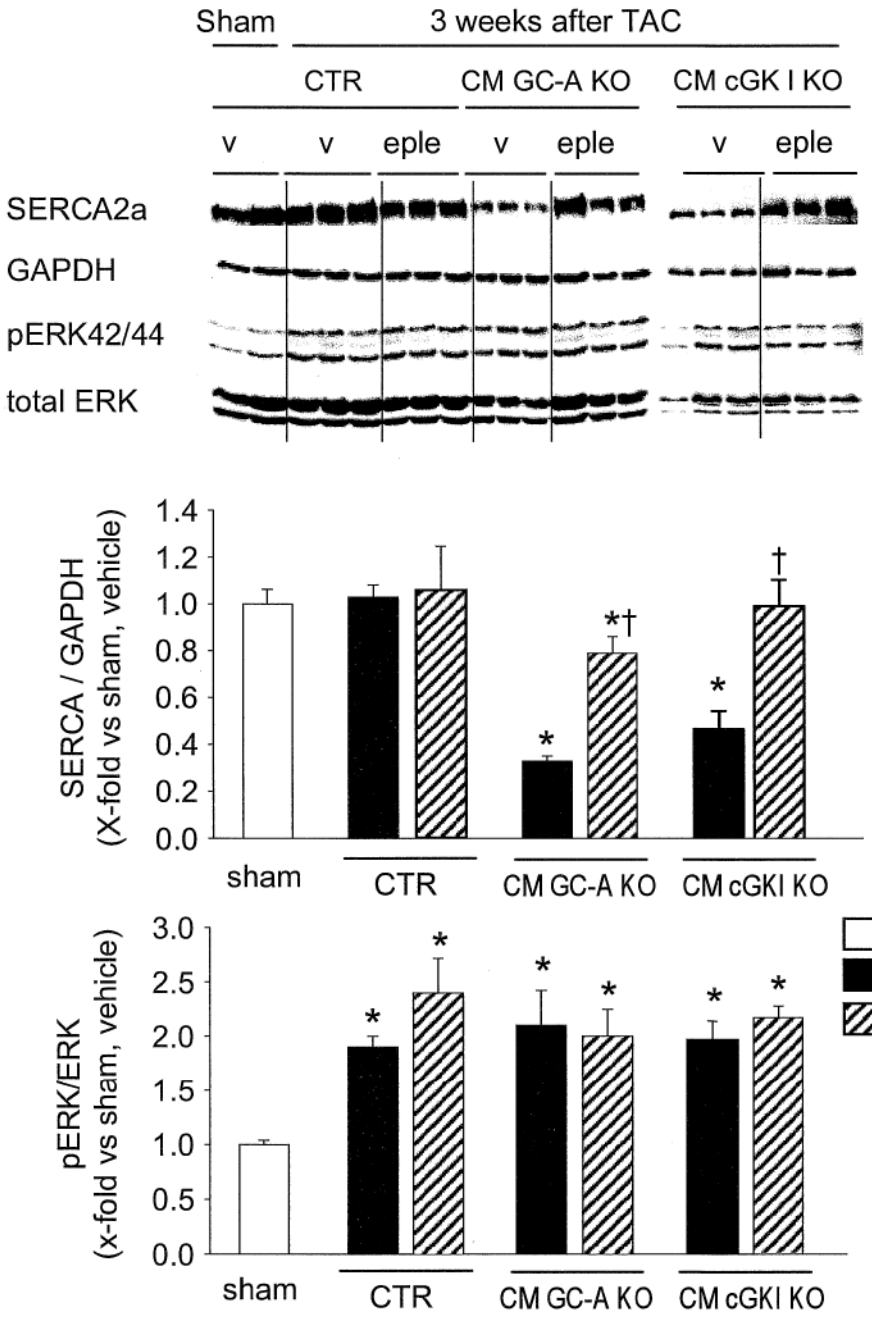
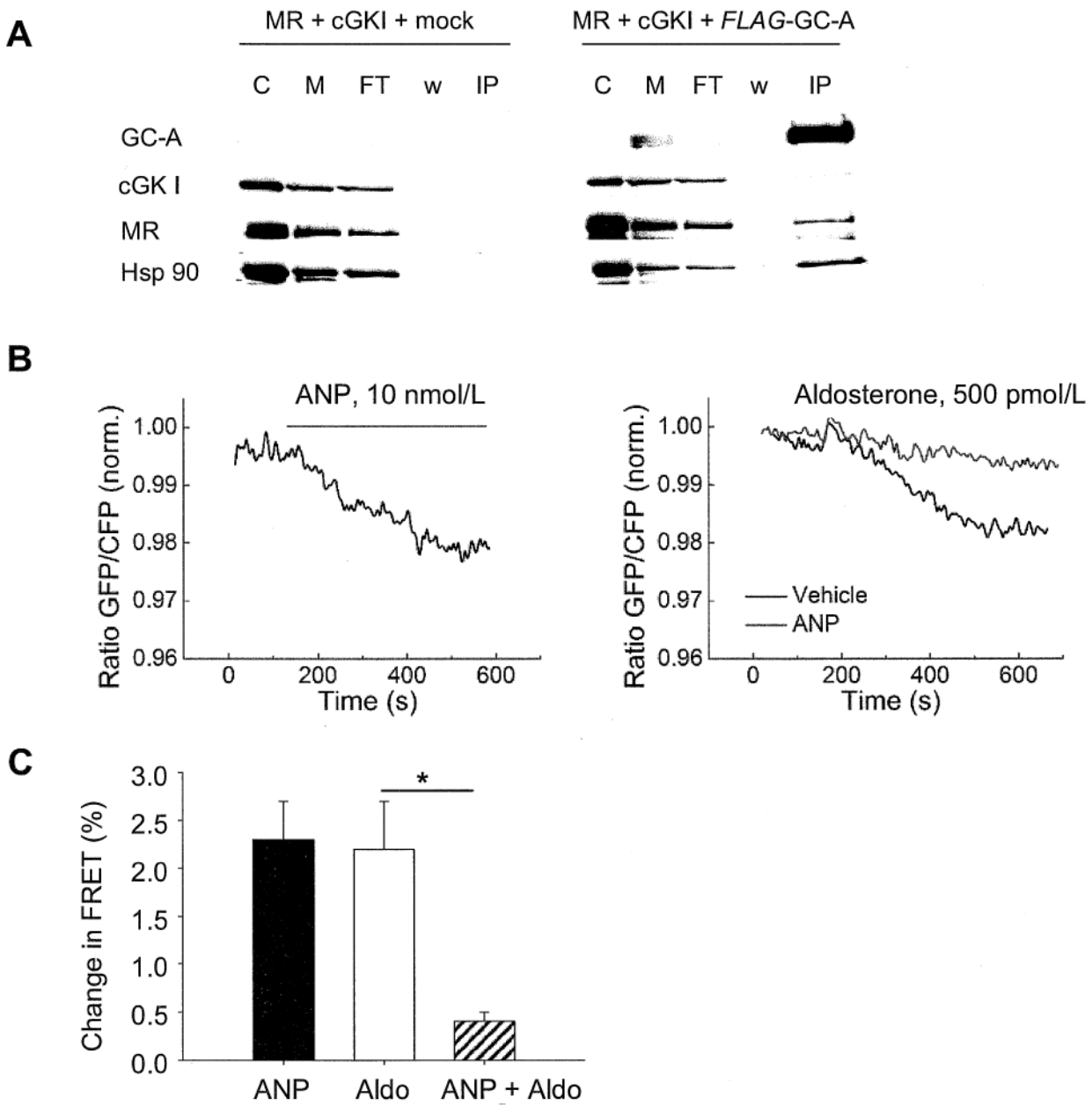

Sham
3 weeks after TAC
CTR
CM GC-A KO
CM cGK I KO
v
v
eple
v
eple
v
eple
SERCA2a
GAPDH
pERK42/44
total ERK
SERCA / GAPDH
(X-fold vs sham, vehicle)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
sham
CTR
CM GC-A KO
CM cGKI KO
pERK/ERK
(x-fold vs sham, vehicle)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Sham
TAC, vehicle
TAC, eplerenone

Figure 6


A
MR + cGKI + mock
MR + cGKI + FLAG-GC-A
C M FT w IP
C M FT w IP
GC-A
cGK I
MR
Hsp 90
B
ANP, 10 nmol/L
Aldosterone, 500 pmol/L
Ratio GFP/CFP (norm.)
1.00
0.99
0.98
0.97
0.96
0 200 400 600
Time (s)
Vehicle
ANP
C
Change in FRET (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
ANP
Aldo
ANP + Aldo