Atrial Natriuretic Peptide Locally Counteracts the Deleterious Effects of Cardiomyocyte Mineralocorticoid Receptor Activation

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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 volume homeostasis. This study investigated whether a cardiac imbalance between ANP and aldosterone, toward increased mineralocorticoid receptor (MR) signaling, contributes to adverse left ventricular remodeling in response to pressure overload.

Methods and Results—We used the MR-selective antagonist eplerenone to test the role of MRs in mediating pressure overload–induced dilative cardiomyopathy of mice with abolished local, cardiac ANP activity. In response to 21 days of transverse aortic constriction, mice with cardiomyocyte–restricted inactivation (knockout) of the ANP receptor (guanylyl cyclase [GC]-A) or the downstream cGMP-dependent protein kinase I developed enhanced left ventricular hypertrophy and fibrosis together with contractile dysfunction. Treatment with eplerenone (100 mg/kg/d) attenuated left ventricular hypertrophy and fully prevented fibrosis, dilatation, and failure. Transverse aortic constriction induced the cardiac expression of profibrotic connective tissue growth factor and attenuated the expression of SERCA2a (sarco/plasmic reticulum Ca²⁺-ATPase) in knockout mice, but not in controls. These genotype-dependent molecular changes were similarly prevented by eplerenone. ANP attenuated the aldosterone-induced nuclear translocation of MRs via GC-A/cGMP-dependent protein kinase I in transfected HEK 293 (human embryonic kidney) cells. Coimmunoprecipitation and fluorescence resonance energy transfer experiments demonstrated that a population of MRs were membrane associated in close interaction with GC-A and cGMP-dependent protein kinase I and, moreover, that aldosterone caused a conformational change of this membrane MR/GC-A protein complex which was prevented by ANP.

Conclusions—ANP counter-regulates cardiac MR activation in hypertensive heart disease. An imbalance in cardiac ANP/GC-A (inhibition) and aldosterone/MR signaling (augmentation) favors adverse cardiac remodeling in chronic pressure overload. (Circ Heart Fail. 2014;7:814-821.)

Key Words: aldosterone ■ angiotensins ■ atrial natriuretic factor ■ heart failure

Compensated hypertensive left ventricular (LV) hypertrophy can progress to adverse remodeling and heart failure (HF) with poor prognosis. Neurohormones play an important role in the complex multiorgan and cellular alterations in HF. Excessive activation of vasoconstricting and proproliferative 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 mineralocorticoid receptor (MR; a ligand-dependent transcription factor) is increased in HF. 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. Although MR is expressed in both cardiac myocytes and fibroblasts, it seems that myocyte MR plays the major pathophysiological role. This is reflected following genetic myocyte–restricted ablation of the MR in mice, which improves ventricular function and remodeling in ischemic or hypertensive HF. In contrast, cardiomyocyte MR overexpression induces arrhythmias and worsens Ang II–induced remodeling. The mechanisms mediating the deleterious effects of cardiomyocyte MR activation remain poorly understood.

Clinical Perspective on p 821

The cardiac hormones atrial (ANP) and B-type natriuretic peptides (BNP) are the main endogenous antagonists of
the RAA system. They share a cGMP-producing guanyllyl cyclase (GC)-A receptor. ANP and BNP, via GC-A, attenuate juxtaglomerular renin as well as adrenal aldosterone release and counteract the vasoconstrictive and antinatriuretic actions of Ang II and aldosterone. Therby NPs moderate the hypertensive and hypervolemic actions of the RAA system. 

In addition, experimental and clinical studies indicate that ANP/BNP exert not only endocrine but also local, cardioprotective actions. Experimentally, overexpression of GC-A in cardiomyocytes attenuated hypertensive or ischemic heart disease. Conversely, conditional, cardiomyocyte-specific inactivation of GC-A exacerbated hypertensive cardiac remodeling. Similar alterations were observed in mice with cardiomyocyte disruption of cGMP-dependent protein kinase I (cGKI), indicating that this kinase is one downstream target mediating the protective actions of NPs in the heart. 

Of note, cardiac hypertrophy is accompanied by GC-A desensitization which impairs the systemic and also the local actions of ANP and BNP. As already mentioned, aldosterone levels and MR activity are concomitantly enhanced. Here, we hypothesized that the resulting shift in the local cardiac balance between NPs and aldosterone toward increased MR signaling contributes to the progression from hypertrophy to HF. Accordingly, the goals of this study were (1) to test whether a selective MR antagonist, eplerenone, prevents pressure overload–induced adverse cardiac remodeling in mice with cardiomyocyte–restricted inactivation of the NP/GC-A/cGKI signaling pathway and (2) to dissect the molecular mechanism(s) favoring adverse hypertensive cardiac remodeling subsequent to a cardiac imbalance between ANP/GC-A (attenuation) and aldosterone/MR (augmentation) signaling.

**Methods**

**Genetic Mouse Models**

Mice with cardiomyocyte–restricted deletion of either GC-A (CM GC-A KO) or cGKI (CM cGKI KO) and their respective control littermates (GC-A^fl/fl, cGKI^fl/fl) were generated by αMHC-Cre/lox P technology as described previously. All study mice were aged 2 to 3 months. KO and respective control mice were compared in all experiments. The studies 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. Two weeks before TAC, littermates were randomly assigned to regular chow or chow containing the MR antagonist eplerenone (100 mg/kg body weight per day). Arterial blood pressure was measured in awake mice by tail cuff. Echocardiography was performed under light isoflurane anesthesia before and 3 weeks after TAC. Mice were then euthanized, the LVs, lungs, and tibias were dissected, and LV samples were frozen in liquid nitrogen (for protein or mRNA extraction) or fixed in 4% buffered formaldehyde (for histology and immunohistochemistry).

**Histology and Immunohistochemistry**

Because dilatative cardiomyopathy is accompanied by increases in myocyte length rather than thickness, we measured the longitudinal instead of cross-sectional areas of single cardiomyocytes. Longitudinal cardiomyocyte areas (length × width) were determined on LV sections stained by diastase-periodic acid Schiff (which stains the sarcolemma) and simultaneously subjected to immunohistochemistry with antibodies directed against pan-cadherin (Cell Signaling, Frankfurt, Germany). This highlighted the intercalated disks and allowed discrimination of longitudinal margins, as previously described. The extent of myocardial fibrosis was determined on sequential LV sections stained with 0.1% picrosirius red. LV expression of connective tissue growth factor (CTGF) protein was examined by immunohistochemistry (antibody from Abcam, Cambridge, United Kingdom).

**Protein and Gene Expression**

Western blotting was performed to analyze LV expression levels of sarcomplasmic reticulum Ca2+-ATPase (SERCA2a; antibody from Badrilla, Leads, United Kingdom), phosphorylated versus total extracellular regulated kinase 1/2 (ERK1/2), and GAPDH (antibodies from Cell Signaling). LV CTGF mRNA expression levels were quantified by real-time reverse transcription polymerase chain reaction as described recently.

**Studies in Transfected Human Embryonic Kidney 293 Cells**

Because the commercially available anti-MR antibodies were not suitable for immunohistochemical stainings of murine hearts or isolated cardiomyocytes, we studied the expression and interactions of GC-A with MR in overexpressing human embryonic kidney (HEK) 293 cells. As previously described, HEK 293 cells (Invitrogen, Karlsruhe, Germany) stably expressing GC-A, FLAG-tagged GC-A, or cyan fluorescent protein–tagged GC-A were transfected with plasmids encoding for enhanced green fluorescent protein–tagged MR and cGKI with Fugene (Roche, Mannheim, Germany). The cells were maintained in charcoal-stripped (steroid-reduced) medium. For confocal microscopy or fluorescence resonance energy transfer (FRET), 24 hours after transfection, the cells were seeded on coverslips, and the incubation experiments with aldosterone±ANP were performed 24 hours later. For immunoprecipitation studies, the membrane and cytosolic proteins were extracted 48 hours after transfection and the membrane proteins were incubated with anti-FLAG antibody coupled to agarose beads (M2; Sigma, Taufkirchen, Germany) for 2 hours at 4°C under rotation as described before. Aliquots of the cytosolic and membrane fractions and of the immunoprecipitated proteins were subjected to Western blotting with antibodies against MR (DSHB, Iowa), Hsp90 (BD Biosciences, Heidelberg, Germany), cGKI (Cell Signaling), and GC-A.

**Statistics**

The statistical distribution of the necropsy and histological data sets obtained in the in vivo studies is illustrated with boxes and whisker plots. All other data are expressed as means±SEM. Data were analyzed by using SPSS. Comparisons between 2 groups were performed using the unpaired Student t test. Cardiac and lung changes in response to TAC and eplerenone were analyzed by nonparametric ANOVA (Kruskal–Wallis test) followed by a Mann–Whitney test to evaluate differences between groups. Repeated-measures ANOVA was used to analyze the echocardiography data, followed by the nonparametric Friedman test. We considered P values <0.05 as statistically significant.

**Results**

**In HEK 293 Cells, ANP Inhibits the Aldosterone-Stimulated Nuclear Translocation of the MR**

We investigated whether ANP influences aldosterone-stimulated nuclear translocation of MRs in HEK 293 cells expressing GC-A, cGKI, and enhanced green fluorescent protein–tagged MR. The nuclear versus cytoplasmic distributions of fluorescently labeled MRs were analyzed by confocal microscopy. Immunoblotting confirmed the expression of...
Eplerenone Prevented Adverse LV Remodeling in CM GC-A KO and CM cGKI KO Mice

To directly test the role of the ANP–aldosterone interaction in regulating the cardiac responses to LV pressure overload in vivo, we subjected CM GC-A KO mice or CM cGKI KO mice and respective control littermates to TAC for 21 days in the presence and absence of the selective MR blocker eplerenone. Because the morphological, functional, and molecular responses to TAC were identical in both groups of control mice, these results are combined in all sections and figures throughout the article.

CM GC-A KO and CM cGKI KO mice have normal Mendelian inheritance, grow normally, and have a normal life span. Under resting conditions, their arterial blood pressure as well as cardiac morphology and function are unaltered (Table I in the Data Supplement). Also, systolic blood pressure levels in CM GC-A KO, CM cGKI KO, and control mice subjected to TAC were similar (110±2.0, 108±2.8, and 109±2.6 mm Hg, respectively; n=6–10 per group) and were not altered by eplerenone (111±2.0, 107±2.0, and 108±1.8 mm Hg, respectively; n=6–10). As shown in Figure 2A and 2B, TAC in control mice induced only mild but significant increases in LV weight-to-tibia length ratios and myocyte longitudinal areas, and these responses were not influenced by eplerenone treatment. In CM GC-A KO and CM cGKI KO mice, the cardiac hypertrophic responses to TAC were greater than in control mice, as shown at the organ (Figure 2A) and cellular level (Figure 2B). Even more, in both KO strains, TAC also increased the wet lung/tibia length ratios (Figure 2C), indicative of pulmonary congestion. In both genotypes, eplerenone significantly attenuated the development of exacerbated LV hypertrophy and fully prevented pulmonary congestion after TAC (Figure 2A–2C).

Picrosirius red stainings showed mild LV interstitial fibrosis after TAC in both eplerenone-treated and untreated control mice (Figure 3A). In contrast, CM GC-A KO and CM cGKI KO mice with TAC developed enhanced LV fibrosis (Figure 3A). This was associated with increased expression of the profibrotic cytokine CTGF (quantitated by reverse transcription polymerase chain reaction and corroborated by immunohistochemistry; Figure 3B and 3C). In both genotypes, eplerenone...
Eplerenone Prevented Left Ventricular 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; Table I in the Data Supplement). TAC (with and without eplerenone) did not alter LV geometry or function in control mice (Figure 4, left column). However, CM GC-A KO and (even more so) CM cGKI KO mice developed severe LV dilatation secondary to TAC, with increases in LV end-diastolic and end-systolic areas and decreased fractional shortening (Figure 4). Of note, eplerenone fully prevented these morphological and functional alterations. Heart rates in control, CM GC-A KO, or CM cGKI KO mice were similar (574±13, 578±6, and 589±16 beats per minute, respectively; see Table I in the Data Supplement) and were not altered by TAC (562±12, 576±15, and 552±12 beats per minute, respectively) or eplerenone (562±11, 603±6, and 575±8 beats per minute, respectively).

Eplerenone Prevented the Decrease of LV SERCA2a Expression in CM GC-A KO and CM cGKI KO Mice Subjected to TAC

Immunoblot analyses showed that LV SERCA2a protein levels were unaltered in control mice after TAC and were not affected by eplerenone (Figure 5). In contrast, LV SERCA2a expression levels were markedly attenuated in both groups of KO mice 3 weeks after TAC (Figure 5). Notably, this decrease was almost fully prevented by eplerenone treatment (Figure 5). As 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 HEK 293 Cells

Finally, we investigated whether a GC-A/MR colocalization may partly underlie the antagonistic actions of these receptors. For coimmunoprecipitation experiments, MR was transiently coexpressed in HEK 293 cells together with FLAG-tagged GC-A and the membrane proteins were subjected to immunoprecipitation with anti-FLAG (M2) antibody. Western blotting demonstrated that a small fraction of MR is indeed localized at the cell membrane (Figure 6A). Even more, membrane MRs coimmunoprecipitated with GC-A, cGKI, and hsp90. Finally, we investigated whether a GC-A/MR colocalization may partly underlie the antagonistic actions of these receptors.
alter baseline cardiac growth and function, but provokes dilative cardiomyopathy and pronounced LV interstitial fibrosis in response to pathological pressure overload (induced by TAC). These morphological and functional alterations induced by TAC were associated with enhanced expression of CTGF and diminished expression of SERCA2a. Notably, myocyte hypertrophy was partially attenuated, and LV fibrosis as well as LV contractile dysfunction were almost fully prevented by treatment of the knockout mice with the selective MR blocker eplerenone. MR antagonism also prevented the pressure overload–induced increases in LV CTGF and decreases of SERCA2a expression levels, which may have contributed to the observed improvement in remodeling and LV contractile function. These results provide direct evidence that MRs are involved in mediating pressure overload–evoked HF in mice with selective disruption of cardiac ANP/BNP signaling and indirect evidence for the involvement of aldosterone.

**Opposite Effects of ANP/GC-A and Aldosterone/MR Signaling on Myocardial CTGF Expression and Fibrosis**

The MRs bind aldosterone and glucocorticoids with similar affinity. The selectivity for aldosterone is achieved by the coexpression of 11β-hydroxysteroid dehydrogenase which inactivates glucocorticoids. The activity of this enzyme is low in cardiomyocytes, and moreover, circulating concentrations of glucocorticoids are much higher than those of aldosterone. Thus, the beneficial effects of MR antagonists in our experimental, as well as in published clinical studies, may partly result from blockade of glucocorticoids binding to cardiomyocyte MR. However, a recent study by Messaoudi et al in mice dissected specific genes regulated by aldosterone in cardiomyocytes and demonstrated that CTGF is induced by aldosterone via the MR, whereas corticosterone had no effect. Conversely, ANP was shown to attenuate CTGF expression and CTGF-dependent fibrosis. In line with these observations, both CM GC-A KO and CM cGKI KO hearts responded to pressure overload with marked induction of CTGF which was totally prevented by eplerenone. Based on these findings, we suggest that ANP, via GC-A/cGKI signaling in cardiomyocytes, counter-regulates the previously established stimulatory effect of aldosterone on myocyte CTGF expression. An imbalance between these systems, favoring aldosterone/MR signaling, enhances myocyte expression of the fibrogenic stimulus CTGF.

**Cardiac Imbalance Between GC-A and MR Activities Favors HF After Chronic Pressure Overload**

Control mice responded to chronic pressure overload with only mild and functionally well-compensated cardiac hypertrophy. In contrast, both CM GC-A KO and CM cGKI KO mice developed dilative cardiomyopathy in response to TAC, emphasizing the protective role of local NP signaling. Remarkably, eplerenone treatment had no effect on the adaptive responses of control hearts to pressure overload, but completely prevented LV dilatation and contractile dysfunction in CM GC-A KO and CM cGKI KO mice. This indicates the involvement of enhanced MR signaling in these pathological responses. In line with our observations, mice deficient in cardiomyocyte MR are

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**Figure 5.** Left ventricular (LV) expression levels of the Ca²⁺-regulating protein, sarcoplasmic reticulum Ca²⁺-ATPase (SERCA2a), and of phosphorylated extracellular regulated kinase 1/2 (ERK1/2) in control (CTR), cardiomyocyte (CM) guanylyl cyclase-A (GC-A) knockout (KO), and CM cGMP-dependent protein kinase I (cGKI) KO mice after transverse aortic constriction (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 to 8 per group; *P<0.05 vs sham; †P<0.05 vs TAC/vehicle.

**Discussion**

**Principal Findings**

Our observations emphasize that impaired ANP/BNP signaling in cardiomyocytes (here achieved by genetic, cardiomyocyte-restricted GC-A, or cGKI inactivation in mice) does not

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protected from cardiac dilatation and failure after TAC. The mechanisms by which enhanced MR activity in cardiomyocytes leads to functional deterioration remain unclear. The profibrotic actions of aldosterone might be involved, because myocardial fibrosis can result in excessive muscle fiber entrapment, myocyte atrophy, or abnormal diastolic and systolic stiffness of the myocardium, each of which is sufficient for the development and progression of LV dysfunction. In addition, eplerenone almost fully prevented the pressure overload–dependent reduction of SERCA2a expression in CM GC-A/cGKI KO hearts. Similarly, blockade of the RAA system with angiotensin-converting enzyme inhibitors improved cardiac function in experimental ischemic HF by preventing the depression in SERCA2a expression and activity. Together, these observations suggest that a cardiac imbalance between NP and aldosterone activities impairs myocyte SERCA2a expression and stimulates interstitial fibrosis, both of which may contribute to HF.

NPs, via GC-A, Counter-Regulate Genomic MR Signaling

Nongenomic signaling pathways of the MR, such as the transactivation of the epidermal growth factor receptor and subsequent phosphorylation/activation of the ERK1/2, have also been implicated in the transition to HF. However, although we observed increased LV levels of phosphorylated ERK1/2 in mice subjected to TAC, this response was similar in control and KO mice and was not prevented by eplerenone. Furthermore, mice with cardiomyocyte-restricted MR inactivation did not exhibit diminished cardiac pERK1/2 levels after TAC, indicating that ERK1/2 activation is not linked to MR signaling. Together with the aforementioned CTGF data, these results suggest that classical (genomic) MR signaling pathways participate in adverse cardiac remodeling of CM GC-A/cGKI KO mice with TAC. This hypothesis is corroborated by the observation that ANP, via GC-A/cGMP, significantly inhibited the aldosterone-stimulated nuclear translocation of the MR in HEK 293 cells (in our study) as well as in colonic epithelial cells. Interestingly, cGMP inhibits the nuclear translocation of another steroid receptor, the vitamin D receptor, in fibroblasts by altering its interaction with the cytoskeletal protein tubulin. Because cGKI can phosphorylate tubulin and microtubule-associated proteins, it was postulated that cGMP prevents dissociation of the activated vitamin D receptor from tubulin. 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 hsp90. Even more, a small population of MR is cell membrane associated, possibly within cholesterol-rich domains. This population detaches from the membrane in response to aldosterone and might therefore contribute to the genomic actions of the hormone. A subpopulation of GC-A receptors is also localized in caveolae microdomains. Therefore, we postulated that the functional antagonism between GC-A receptors and MRs might be mediated in part by a direct interaction between these proteins. Indeed, coimmunoprecipitation and FRET experiments demonstrated that GC-A and a portion of MRs, cGKI, and

**Figure 6.** Coimmunoprecipitation experiments and fluorescence energy transfer (FRET) reveal the association of guanylyl cyclase-A (GC-A) and a subpopulation of mineralocorticoid receptors (MRs) at the membrane of human embryonic kidney (HEK) 293 cells. A (right), Coimmunoprecipitation of MR, cGMP-dependent protein kinase I (cGKI), and Hsp90 with FLAG-GC-A from membranes of cotransfected HEK 293 cells. Cytosolic (C) and membrane fractions (M) as well as membrane proteins immunoprecipitated (IP) with anti-FLAG antibody were separated on SDS-PAGE and blotted with antibodies against GC-A, cGKI, MR, and Hsp90. FT indicates flow through; and w, wash step. Representative Western blots of 3 independent experiments. Inputs were 1/10 to 1/20 of the protein used for IP. The left side of the blot (A) shows that control IPs with HEK 293 cells without GC-A expression (mock transfection) do not give similar IP reactions. B, Representative ratiometric recordings of single-cell green fluorescent protein (GFP)/cyan fluorescent protein (CFP) FRET ratio signals in HEK 293 cells cotransfected with CFP-tagged GC-A and enhanced GFP (EGFP)-tagged MR at baseline and during superfusion of 10 nmol/L ANP (left) or 500 pmol/L aldosterone (aldo; right), as indicated by horizontal black lines. The recordings were normalized to baseline FRET. Left, Decrease of FRET between CFP-GC-A and EGFP-MR in single HEK 293 cells treated with ANP suggests hormone-induced conformational rearrangement in the preformed GC-A/MR complex. Right, Likewise, stimulation with aldo leads to a decrease of FRET (black trace) which is inhibited after pretreatment with ANP for 10 minutes (gray trace). C, Quantification of agonist-induced decreases in FRET as a percent change of FRET ratio from the basal level (n=9–13 cells from 3 independent experiments, for each group; *P<0.05 vs aldo alone).
Cardiac Cell Types Mediating the Protective Effects of Eplerenone

Eplerenone antagonizes the MR-mediated effects of aldosterone in different cardiac cell types. Hence, although these studies in CM GC-A/cGKI KO mice were designed to dissect specific cardiomyocyte interactions between NPs and aldosterone, we cannot rule out that the beneficial cardiac effects of eplerenone were mediated in part by other cells such as fibroblasts, endothelial, and inflammatory cells.

Perspectives

In patients with HF, plasma levels of aldosterone, BNP, and (less) ANP are elevated, correlating with the severity of the disease. However, despite these high NP levels, HF is characterized by a decreased activity of the active form of processed BNP and resistance to both NPs. Because of desensitization of the GC-A receptor, NP-dependent intracellular CGMP formation and subsequent cardiovascular effects are markedly blunted. Of course the mice with genetic cardiomyocyte GC-A or cGKI ablation studied herein present an artificial situation of nonexistent myocyte NP signaling. However, our observations in these mice suggest that a local, cardiac imbalance between the activities of the NP/GC-A (inhibition) and aldosterone/MR systems (augmentation), as occurs in patients with HF, can critically contribute to adverse cardiac remodeling. Our observations support current therapeutic concepts that inhibition of the RAA system combined with augmentation of the natriuretic peptide system (with synthetic analogs or by stimulation of the NGF/M-cAMP/PKA pathway of cyclic GMP-independent signaling of guanylyl cyclase A, the receptor for atrial natriuretic peptide) is inactive in the failed cardiomyocyte pathway of cyclic GMP-dependent protein kinase I. Low-dose nesiritide in human anterior myocardial infarction suppresses aldosterone and preserves ventricular function and structure: a proof of concept study. Heart. 2009;95:1315–1319.}

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Disclosures

None.

References


The antagonistic endocrine actions of the cardiac hormones atrial and B-type natriuretic peptides and the renin–angiotensin–aldosterone system are critically involved in the maintenance of arterial blood pressure and volume homeostasis. Our studies in genetic mouse models show that selective inactivation of natriuretic peptide signaling in cardiomyocytes causes adverse cardiac remodeling in response to pressure overload. Together with published studies, these observations emphasize that local, cardiac effects of these peptides counter-regulate pathological remodeling. Notably, the morphological and functional cardiac alterations provoked by defective cardiomyocyte atrial natriuretic peptide/B-type natriuretic peptide signaling were fully prevented by blockade of the mineralocorticoid receptor with eplerenone. These experimental observations indicate that apart from their endocrine cross-talk, cardiac paracrine interactions between natriuretic peptides and aldosterone are critically involved in the regulation of cardiomyocyte growth and contractile functions and in the secretion of profibrotic factors such as connective tissue growth factor. Patients with heart failure have complex neurohumoral alterations, which involve high atrial natriuretic peptide/B-type natriuretic peptide and aldosterone levels, but resistance to the endocrine and cardiac effects of the former. The present study indicates that disbalanced cardiac actions of the atrial natriuretic peptide/B-type natriuretic peptide (inhibition) and aldosterone/mineralocorticoid receptor systems (activation) favor adverse cardiac remodeling and dysfunction in chronic pressure overload. Restoration of this balance by mineralocorticoid receptor blockade (eg, with eplerenone) combined with natriuretic peptide system augmentation (through administration of synthetic analogs or stabilization of the endogenous hormones) could represent a therapeutic approach to prevent the fatal progression of hypertensive cardiac hypertrophy to heart failure.
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SUPPLEMENTAL MATERIAL

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

Hitoshi Nakagawa et al.

Expanded Methods

Transverse aortic constriction

Surgical transverse aortic constriction (TAC, 21 days) or sham-operation were performed as described previously (1,2). In brief, 8 - 12 weeks old mice with a body weight of 20-25 g were anesthetized with isoflurane (2.5%), intubated, and put on a mechanical small-animal ventilator. After thoracotomy the aorta was ligated between the innominate and left carotid artery with an overlying 27-gauge needle to generate a reproducible, discrete stenosis. After ligation, the needle was withdrawn. Sham mice underwent the same procedure without aortic ligation. Buprenorphine (0.05 - 0.1 mg/kg BW) was used for postoperative analgesia. Two weeks before TAC, littermates were randomly assigned to regular chow or chow containing the mineralcorticoid receptor (MR) antagonist eplerenone (100 mg/kg body weight (BW) per day). Arterial blood pressure was measured in awake mice by tail cuff (1,2) before, one week after starting the eplerenone treatment and 2 weeks after TAC (± eplerenone). Echocardiography was performed under light isoflurane anaesthesia before and 3 weeks after TAC (2).

Histology and immunohistochemistry

Hearts tissues were fixed in 4% paraformaldehyde, embedded in paraffin and 3 µm sections were used for stainings. The extent of myocardial fibrosis was determined on sections stained
with picrosirius red and analysed with an imaging system (KAS 300, Zeiss, Germany). Five representative fields per section at a magnification x100 were analyzed (3 sections per heart). Collagen area was determined under polarization microscopy by quantification of the red-yellow and green illuminated area due to the fact that fibrillar collagen shows birefringent properties. The total tissue area was quantified by determination of the stained area under bright field microscopy. The collagen fraction was calculated as the collagen area in % of the corresponding total tissue area (1,2).

For immunohistochemical analyses of CTGF expression, after deparaffinization without pre-treatment the antibody against CTGF (Abcam ab6992, Cambridge, UK) was applied in a dilution of 1:100 overnight at 4°C. For pan-cadherin immunostaining, deparaffinized sections were pretreated in a buffer (Dako S2367, pH 9.0) at 98°C. The antibody against pan-cadherin (Cell Signaling #4068, Frankfurt, Germany) was applied in a dilution of 1:50 at room temperature for 60 min. Both antibodies were detected by the SignalStain® Boost IHC Detection Reagent (Cell Signaling #8114), incubated for 30 min at room temperature, developed with DAB and counterstained with hematoxilin. Negative controls were performed by using the appropriate immunoglobulin or by omitting the primary antibody.

To measure longitudinal cardiomyocyte areas, slides were simultaneously subjected to immunohistochemistry with antibodies directed against pan-cadherin (which helps highlight the intercalated disks and allows discrimination of the longitudinal margins (3)) and diastase-periodic acid Schiff (PAS) reaction for cell membrane staining and removal of glycogen to allow discrimination of cell margins other than intercalated disks (3). With the combination of the two staining methods, the cell margins of the cardiomyocytes could clearly be seen in areas in which they were longitudinally oriented. Areas were estimated by tracing the fibres length and diameter (in the region of the cell nucleus). 30 - 50 myocytes with centrally located
nucleus were measured in each LV section by a computer-assisted image analysis system as described before (1-3), with the investigator blinded to the genotypes.

**Plasmids**

The generation of plasmids for expression of FLAG-tagged (FLAG-GC-A) and fluorescent (C-terminal CFP-tagged) GC-A in HEK 293 cells was described recently (4). The plasmid for expression of the MR with a C-terminal EGFP tag was generated by PCR-mutagenesis using the cDNA encoding human MR as template (generous gift from Dr. Somekawa, Dept. of Cardiology, University of Nara, Japan). The following primers were used to generate the cDNA coding for MR flanked by Bgl II and Hind III restriction sites: fwd—5′- T AGA TCT ACC ATG GAG ACC AAA GGC TAC CAC AGT C -3′; and rev—5′- A AAG CTT CTT CCG GTG GAA GTA GA G CGG CTT GGC G -3′. The resulting fragment was subcloned into TOPO TA cloning vector (Invitrogen, Karlsruhe, Germany) for sequence verification. After double digestion with Bgl II and Hind III the fragment was ligated into pCMV-EGFP expression vector (Takara Bio Inc., Shiga, Japan).

**Analyses of nuclear MR translocation in HEK 293 cells**

For confocal microscopy and coimmunoprecipitation studies, HEK 293 cells stably expressing GC-A (4, 5) were transfected with plasmids (2 μg of each plasmid per 10 cm dish) encoding for MR-EGFP and cGKI with FuGene (Roche, Mannheim, Germany). The cells were maintained in DMEM medium containing 10% (v/v) charcoal-stripped (steroid-reduced) serum (Invitrogen). 24 h after transfection, cells were seeded on poly-d-lysine-coated round glass coverslips, and the incubation experiments were performed 24 h later. To study the effect of ANP on aldosterone-induced nuclear MR translocation, cells were first pretreated with 10 nmol/L ANP (30 min; Bachem, Bubendorf, Switzerland) or vehicle and thereafter with 500 pmol/L aldosterone (1 h; Sigma, Taufkirchen, Germany). The cells were then fixed
with 4% paraformaldehyde, permeabilized with 0.1 % Triton X-100 and mounted in DAPI containing mowiol medium (Dianova, Hamburg, Germany; for staining of cell nuclei). Fluorescent images (100 cells per condition in each of 3 independent experiments) were acquired using a Leica TCS Confocal microscope equipped with the 63x objective and excitation at 488 nm. Intensity ratios of nuclear-to-cytosolic MR-EGFP were determined using the Adobe Photoshop CS5 software.

**Immunoprecipitation of GC-A and MR from HEK 293 cells**

For coimmunoprecipitation studies, transfected HEK 293 cells were cultured in medium with 10% charcoal-stripped serum for 2 days. A cell fractionation kit was applied to separate the membrane and cytosolic proteins (nano-TOOLS Antikörpertechnik, Teningen, Germany) (4,5). Membrane proteins were incubated with anti-FLAG antibody coupled to agarose beads (M2; Sigma) during 2 hours at 4°C under rotation. The beads were washed and resuspended in SDS-sample buffer. Aliquots of the cytosolic and membrane fractions and of the immunoprecipitated proteins were subjected to western blotting with antibodies against MR (DSHB, Iowa, USA; dilution 1:500), Hsp90 (BD Biosciences, Heidelberg, Germany; dilution 1:5000), cGKI (Cell Signaling; dilution 1:1000) and GC-A ((5); dilution 1:1000). An ECL system (Amersham-Pharmacia, Freiburg, Germany) was used for detection and results were quantitated by densitometry (ImageQuant software; Molecular Dynamics, Krefeld, Germany).

**Fluorescence resonance energy transfer (FRET) between GC-A and MR in HEK 293 cells**

For FRET, CFP-tagged GC-A (4) and EGFP-tagged MR were cotransfected into HEK 293 cells as described above. 24 h later, cells were seeded on poly-d-lysine-coated round glass coverslips, and the FRET microscopy experiments were performed 48 h after transfection (4, 6). Fluorescence was recorded from entire living single cells in GFP and CFP emission.
channels using 510 ± 15 nm and 475 ± 20 nm emission filters, respectively, separated by a
dcrx 505 nm beam splitter. FRET was monitored as the emission GFP/CFP ratio, which was
corrected offline for the spillover of CFP into the GFP channel and the direct GFP excitation.
This ratio is proportional to the degree of FRET and spatial proximity between CFP (donor)
and GFP (acceptor) fluorophores and sensitive to the degree of protein-protein interaction and
conformational rearrangements in the protein-protein complex. To study ligand-induced
changes in FRET and GC-A/MR interaction, cells were treated with FRET buffer
supplemented with ANP or aldosterone.

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Supplemental Table 1. Baseline cardiovascular parameters of CM GC-A KO mice vs control littermates and CM cGKI KO mice and respective control littermates. Systolic (SBP) and diastolic (DBP) blood pressure levels were determined in awake, trained mice by tail cuff. Heart rate and LV ejection fraction (EF) were determined by echocardiography.

<table>
<thead>
<tr>
<th></th>
<th>CTR (GC-A^{fl/fl}) n = 8</th>
<th>CM GC-A KO n = 10</th>
<th>CTR (cGKI^{fl/fl}) n = 9</th>
<th>CM cGKI KO n = 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBP, mmHg</td>
<td>66 ± 2.6</td>
<td>67 ± 3.4</td>
<td>68 ± 2.4</td>
<td>69 ± 3.5</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>109 ± 2.8</td>
<td>110 ± 2.0</td>
<td>109 ± 2.4</td>
<td>108 ± 2.8</td>
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<tr>
<td>Body weight (BW, g)</td>
<td>20.3 ± 0.9</td>
<td>20.4 ± 0.6</td>
<td>20.3 ± 0.9</td>
<td>21.4 ± 0.7</td>
</tr>
<tr>
<td>Heart weight (HW, mg)</td>
<td>100.5 ± 4.5</td>
<td>102.8 ± 5.1</td>
<td>101.5 ± 2.9</td>
<td>104.9 ± 0.7</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>4.9 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>5.0 ± 0.2</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>HW/tibia length (mg/cm)</td>
<td>58 ± 3</td>
<td>63 ± 2.8</td>
<td>59 ± 2</td>
<td>64 ± 1.8</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>576 ± 8</td>
<td>578 ± 6</td>
<td>572 ± 17</td>
<td>589 ± 16</td>
</tr>
<tr>
<td>LV EF (%)</td>
<td>55 ± 3</td>
<td>59 ± 3</td>
<td>54 ± 3</td>
<td>53 ± 4</td>
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