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Local Atrial Natriuretic Peptide Signaling Prevents Hypertensive Cardiac Hypertrophy in Endothelial Nitric-oxide Synthase-deficient Mice*

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The crucial functions of atrial natriuretic peptide (ANP) and endothelial nitric oxide/NO in the regulation of arterial blood pressure have been emphasized by the hypertensive phenotype of mice with systemic inactivation of either the guanylyl cyclase-A receptor for ANP (GC-A−/−) or endothelial nitric-oxide synthase (eNOS−/−). Intriguingly, similar levels of arterial hypertension are accompanied by marked cardiac hypertrophy in GC-A−/−, but not in eNOS−/−, mice, suggesting that changes in local pathways regulating cardiac growth accelerate cardiac hypertrophy in the former and protect the heart of the latter. Our recent observations in mice with conditional, cardiomyocyte-restricted GC-A deletion demonstrated that ANP locally inhibits cardiomyocyte growth. Abolition of these local, protective effects may enhance the cardiac hypertrophic response of GC-A−/− mice to persistent increases in hemodynamic load. Notably, eNOS−/− mice exhibit markedly increased cardiac ANP levels, suggesting that increased activation of cardiac GC-A can prevent hypertensive heart disease. To test this hypothesis, we generated mice with systemic inactivation of eNOS and cardiomyocyte-restricted deletion of GC-A by crossing eNOS−/− and cardiomyocyte-restricted GC-A-deficient mice. Cardiac deletion of GC-A did not affect arterial hypertension but significantly exacerbated cardiac hypertrophy and fibrosis in eNOS−/− mice. This was accompanied by marked cardiac activation of both the mitogen-activated protein kinase (MAPK) ERK 1/2 and the phosphatase calcineurin. Our observations suggest that local ANP/GC-A/cyclic GMP signaling counter-regulates MAPK/ERK- and calcineurin/nuclear factor of activated T cells-dependent pathways of cardiac myocyte growth in hypertensive eNOS−/− mice.

The heart is involved in cardiovascular homeostasis by the secretion of two natriuretic peptides, atrial natriuretic peptide (ANP)[1] and B-type natriuretic peptide (1). Natriuretic peptides activate a common guanylyl cyclase A (GC-A) receptor expressed in a wide variety of tissues, which results in elevation of intracellular cGMP concentrations (2, 3). ANP is secreted from atrial granules into the circulation in response to acute or chronic atrial stretch, where it functions as an antihypertensive and antihypervolemic factor through GC-A in distant organs (3). In chronic hemodynamic overload, there is a significant increase in ANP and, in particular, B-type natriuretic peptide expression in the cardiac ventricles (4). It is postulated that in this situation natriuretic peptides not only act as endocrine factors but also exert local antihypertrophic (ANP) and antifibrotic (B-type natriuretic peptides) actions (5). For example, ANP counters hypertrophic growth of cultured cardiac myocytes and proliferation of fibroblasts via GC-A (6). In addition, targeted overexpression of GC-A in cardiomyocytes exerts antihypertrophic effects *in vivo* (7). Conversely, mice with a genetic, systemic disruption of the GC-A gene (GC-A−/− mice) not only have increased systemic blood pressure but also display a marked cardiac hypertrophy that is disproportionate to their increased blood pressure and is resistant to antihypertensive medication (8, 9). By comparison, other mutant mice with similar increases in blood pressure, *i.e.* mice with systemic disruption of endothelial nitric-oxide synthase (eNOS−/−), do not have this pronounced hypertrophic phenotype (10–12). Thus, hearts from GC-A-deficient mice seem to be abnormally susceptible to the development of cardiac hypertrophy in response to pressure overload, corroborating the hypothesis that ANP, via GC-A, counterregulates cardiomyocyte growth in a local, paracrine/autocrine way. Indeed, our observation that mice with conditional, (αMHC-CRE recombinase/loxP-mediated) cardiomyocyte-restricted deletion of GC-A (CM GC-A KO mice) exhibit blood pressure-independent cardiac hypertrophy, provided final proof to this concept (13). However, the mechanisms underlying the antihypertrophic effects of ANP remain completely uncharacterized.

Here we sought to determine the mechanisms by which local ANP/GC-A signaling moderates basal cardiomyocyte growth and the cardiac growth response to chronic arterial

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[1] The abbreviations used are: ANP, atrial natriuretic peptide; GC-A, guanylyl cyclase-A; CM GC-A KO, cardiomyocyte-restricted deletion of GC-A; eNOS, endothelial nitric-oxide synthase; MAPK, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinase; NFAT, nuclear factor of activated T cells; MHC, myosin heavy chain; α-sk-actin, α-skeletal actin; JNK, c-Jun NH_2-terminal kinase; CM, cardiomyocyte; HW, heart weight; LVW, left ventricular weight; BW, body weight.

hypertension. To investigate the impact of a loss of function of cardiomyocyte GC-A on the development of hypertensive cardiac hypertrophy, cardiomyocyte (CM)-specific GC-A KO mice (13) were crossed with mice deficient in endothelial nitric-oxide synthase (eNOS−/−), a model with chronic arterial hypertension (12). This genetic approach was based on the observations that eNOS−/− mice exhibit modest cardiac hypertrophy (10–12) together with markedly elevated cardiac ANP levels (Ref. 14 and this study), suggesting that increased local ANP can ameliorate hypertensive cardiac remodeling. Indeed, our studies indicate that ANP/GC-A/cyclic GMP signaling functions as a local cardiac system that can inhibit both MAPK/ERK- and calcineurin/NFAT-dependent pathways of cardiomyocyte growth.

EXPERIMENTAL PROCEDURES

Animals—CM GC-A KO and eNOS−/− mice were generated by methods described previously (12, 13). Control (floxed GC-A mice with normal GC-A and eNOS expression levels), CM GC-A KO (floxed GC-A mice harboring the αMHC-CRE recombinase), eNOS−/− (floxed GC-A mice with normal GC-A expression levels and systemic disruption of eNOS), and double KO mice (eNOS−/−, CM GC-A KO) used in the present study were generated from heterozygous mice after crossbreeding single eNOS−/− (12) and CM GC-A KO mice (13). The animals were in a mixed genetic background (C57BL/6 × 129SV). Male and female littermates ~6–8 months old were examined. The animals were housed under a 12-h day/night cycle and fed a standard diet containing 0.6% NaCl (normal salt conditions). Genotypes were identified by PCR analyses of tail-tip DNA. All experimental protocols were approved by the local animal care committee and conform with the Guide for the Care and Use of Laboratory Animals published by the U. S. National Institutes of Health (Publication No. 85–23, revised 1996).

Hemodynamic Measurements and Tissue Harvesting—Heart rate and diastolic and systolic blood pressure were measured in conscious mice by tail cuff (Softron, Tokyo) (15). Mice were then sacrificed, the hearts were weighed, and the atria and right ventricular free wall (RV) were dissected away from the left ventricle (LV) inclusive of the septum (13, 15). The ratios of whole heart and left ventricular weights to the body weight (HW/BW and LVW/BW) were calculated and used as an index of cardiac enlargement. The left ventricles were bisected and frozen in liquid nitrogen (for RNA or protein extraction) and fixed in 4% buffered formaldehyde (for histology).

Histology and Morphometrical Analyses—Formaldehyde-fixed ventricles were embedded in paraffin, and 5-μm sections were stained with hematoxylin eosin, periodic acid Schiff (to discriminate cardiomyocyte cell borders), or 0.1% picrosirius red (for collagen). Photomicrographs of the sections were evaluated by a computer-assisted image analysis system (VIDAS 25; Zeiss) (13, 15), with the investigator blinded to the genotypes. The mean cross-sectional cardiomyocyte diameters were calculated by measuring 100 cells with a centrally located nucleus per specimen. Interstitial collagen fractions were obtained by calculating the ratio, in percent, between the collagen area and the total ventricular area in the corresponding section (13, 15).

Northern Blot Analysis—Total RNA was prepared from the left ventricle by use of TRIzol (Invitrogen). Ventricular mRNA levels of ANP, α-skeletal actin (α-sk-actin), α- and β-myosin heavy chain (α/β-MHC) were determined from 20 μg of total RNA (13, 15) using Northern blot analysis. Glyceraldehyde-3-phosphate dehydrogenase was used for normalization. Specific mouse ANP, α-sk-actin, and glyceraldehyde-3-phosphate dehydrogenase cDNA probes (15) were labeled with [α-^{32}P]dCTP. Oligonucleotides for α-MHC and β-MHC were labeled with [γ-^{32}P]ATP. Radioactive signals were visualized in a PhosphorImager™ and quantified by the ImageQuant software (13, 15).

Western Blot Analysis—Frozen left ventricles were homogenized and proteins were solubilized in SDS sample buffer and separated on 10% polyacrylamide gels. The primary antibodies were against phosphorylated forms of the mitogen-activated protein kinases (MAPK) ERK 1/ERK 2, p38 and c-Jun NH_2-terminal kinase (JNK) and against the MAPK phosphatase MKP-1 (all from Cell Signaling Technology, Beverly, MA). The secondary antibodies were horseradish peroxidase-conjugated anti-rabbit Igs. All signals were normalized to the cardiomyocyte-specific protein junctin (16). To analyze the activation status of endogenous calcineurin, the relative phosphorylation status of its endogenous transcriptional effector, nuclear factor of activated T cells (NFAT), was determined as described previously in detail (17). In brief, left ventricular frozen samples were lysed in ice-cold buffer (0.5% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 g/ml leupeptin, 10 g/ml phenylmethylsulfonyl fluoride (Sigma), 2 g/ml soybean trypsin inhibitor). Protein concentration in lysates was determined using a protein dye assay (Bio-Rad). 400 mg of ventricular protein extract was immunoprecipitated overnight with either a monoclonal anti-NFATc2 antibody (sc-7925; Santa Cruz) or a monoclonal anti-NFATc4 antibody (H-74; Santa Cruz) and protein A/G-agarose. Immunoprecipitates were separated on gradient gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Bio-Rad). Filters were blocked for 1 h at room temperature using 10% nonfat dry milk dissolved in Tris-buffered saline with 0.1% Triton X-100 (Sigma). Primary antibody was a rabbit polyclonal anti-phospho-serine antibody (ab9332; Abcam). To evaluate the total amount of immunoprecipitated NFATc2 and c4, the blots were stripped and reprobed with polyclonal antibodies against c2 or c4, respectively. Immunoreactive proteins were visualized by chemiluminescence (ECL; Amersham Biosciences) (17).

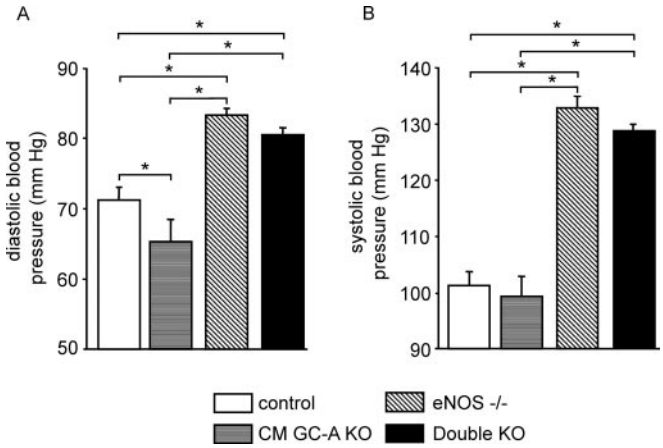

FIG. 1. **Diastolic (*A*) and systolic (*B*) arterial blood pressure of control (*n* = 10), CM GC-A KO (*n* = 7), eNOS−/− (*n* = 15), and double KO mice (*n* = 15).** Measurements were made in awake mice by tail cuff. *, $p < 0.05$.

Data Analysis—Results are expressed as means ± S.E. All data were analyzed by analysis of variance followed by Student-Newman-Keuls test, with the exception of the results from Northern and Western blot analyses, which were evaluated by Student's *t*-Test. Results were considered statistically significant in all analyses at $p < 0.05$.

RESULTS

Cardiomyocyte-restricted Deletion of GC-A Does Not Affect Arterial Hypertension but Provokes Cardiac Enlargement in eNOS−/− Mice—In accordance with our previous study (13), the average diastolic and systolic blood pressure level of CM GC-A KO mice tended to be slightly lower as compared with control mice (Fig. 1). Both eNOS−/− and double KO mice were clearly hypertensive, with increased diastolic blood pressure (by 12 ± 3 and 9 ± 1.7 mm Hg, respectively) and systolic blood pressure levels (by 31 ± 6 and 28 ± 2 mm Hg) as compared with controls (Fig. 1). This increase is comparable with that originally seen in eNOS−/− mice (12). There was no significant difference in heart rate between control and CM GC-A KO mice (635 ± 21 and 597 ± 9 bpm). In contrast, arterial hypertension in eNOS−/− and double KO mice was accompanied by significant bradycardia (519 ± 15 and 535 ± 5 bpm, respectively; both $p < 0.01$ *versus* controls). There was no difference in blood pressure or heart rate between sexes.

Even under conditions of slight arterial hypotension, CM GC-A KO mice showed increased HW/BW and LVW/BW ratios as compared with controls (by 28.5 and 22%, respectively; see Fig. 2). HW/BW and LVW/BW were only marginally, but not significantly, increased in eNOS−/− mice (by 12.7 and 11.1%) but markedly increased in double KO mice (by 61.4 and 48%) as compared with control mice (Fig. 2). Thus, although eNOS−/− mice have chronic arterial hypertension, they only display moderate cardiac enlargement. In contrast, cardiomyocyte-

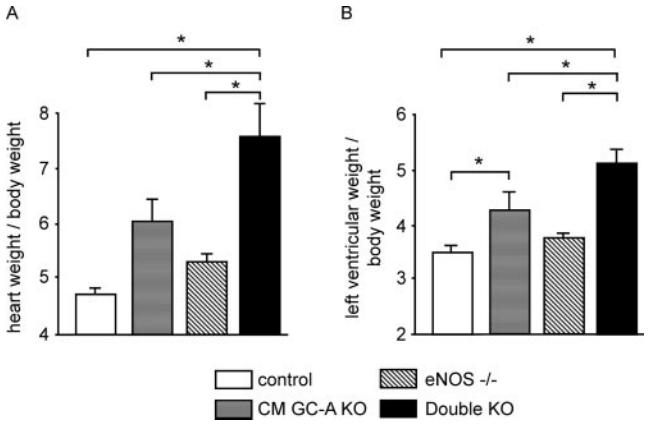

FIG. 2. **HW/BW (mg/g) (*A*) and LVW/BW (mg/g) (*B*) ratios of control (n = 10), CM GC-A KO (n = 7), eNOS−/− (n = 15), and double KO mice (n = 15).** *, $p < 0.05$.

restricted deletion of GC-A leads to significant left ventricular enlargement already under normal (CM GC-A KO) and even more under increased pressure load conditions (double KO mice).

Cardiomyocyte-restricted Deletion of GC-A Causes Excessive Left Ventricular Remodeling in eNOS−/− Mice—To determine whether cardiomyocyte GC-A deletion and/or systemic eNOS ablation influenced individual cardiomyocyte size, we next measured myofiber diameters on periodic acid Schiff-stained left ventricular paraffin sections. The average diameter was significantly increased in CM GC-A KO mice (15.9 ± 0.01 mm) as compared with controls (12.6 ± 0.2 mm), whereas cardiomyocytes from eNOS−/− mice were slightly, but not significantly, enlarged (13.3 ± 1 mm) (Fig. 3). Cardiomyocytes from double KO mice were markedly hypertrophic (average diameter 17.7 ± 0.3 mm) as compared with all three other genotypes (Fig. 3). Sirius red stainings showed that left ventricular interstitial collagen fractions were not different between control, CM GC-A KO, and eNOS−/− mice (Fig. 4). Notably, in left ventricles of double KO mice interstitial collagen fractions were almost doubled (Fig. 4), indicating increased proliferation and/or collagen production of fibroblasts. Thus, hypertensive eNOS−/− mice are protected from cardiac hypertrophy and fibrosis, and abolition of the local effects of ANP removes this protection.

Cardiac Hypertrophy in Double KO Mice Is Accompanied by Increased Expression of Hypertrophy Marker Genes—Because cardiac hypertrophy is accompanied by an activation of the cardiac fetal gene program in the ventricle, we next examined ventricular mRNA expression of ANP, α- and β-MHC, and α-sk-actin. Left ventricular ANP mRNA levels in eNOS−/− mice were ~3-fold higher as compared with controls (Fig. 5). The levels of α-sk-actin and α-MHC were not significantly altered, but the expression levels of β-MHC and the ratios β-MHC/α-MHC were significantly increased (by ~1.6- and 1.4-fold, respectively) (Fig. 5). These changes in gene expression levels were even more pronounced in the double KO mice (increases in cardiac ANP mRNA by 2.5-fold, β-MHC mRNA by 2.4-fold, and β/α-MHC by 5-fold as compared with eNOS−/− mice) (Fig. 5). Thus, ablation of cardiomyocyte ANP/GC-A signaling enhances the reactivation of the fetal gene program in hearts of eNOS−/− mice.

Cardiac Hypertrophy in Double KO Mice Is Accompanied by Increased Activation of the Mitogen-activated Protein Kinases ERK 1/2 and the Phosphatase Calcineurin—To characterize the intermediate intracellular signaling factors that ultimately drive the hypertrophic response in CM GC-A KO and double KO mice, we first examined the phosphorylation state of the

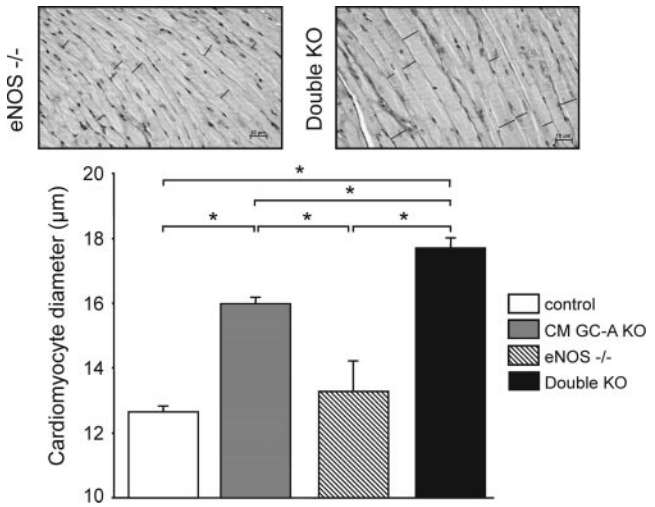

FIG. 3. **Morphometrical analyses of cardiac sections.** Cardiomyocyte diameters were estimated by quantitative morphometry of periodic acid Schiff-stained ventricular sections. *Black lines* (*top*) indicate cardiomyocyte diameters in the region of the cell nucleus, which were significantly increased in hearts of CM GC-A KO (n = 7) and double KO mice (n = 11) as compared with control (n = 8) and eNOS−/− mice (n = 12) (*bottom*). *, $p < 0.05$.

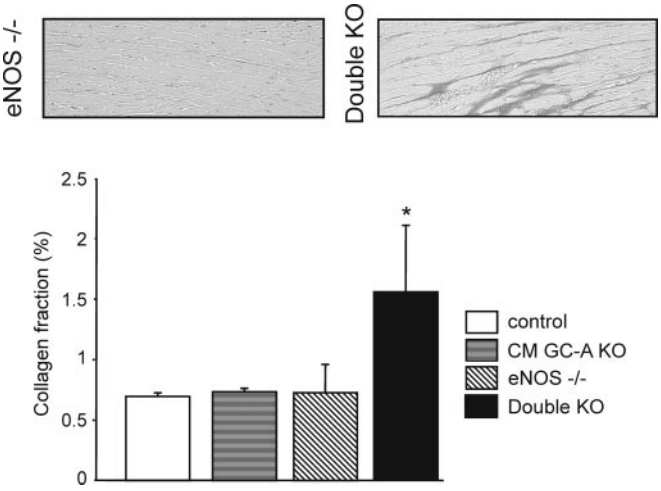

FIG. 4. **Morphometrical analyses of cardiac sections.** Total interstitial collagen fractions were estimated by quantitative morphometry of sirius red-stained ventricular sections of control (n = 8), CM GC-A KO (n = 7), eNOS−/− (n = 12), and double KO mice (n = 11). *, $p < 0.05$ *versus* all other genotypes.

mitogen-activated protein kinases (MAPK) ERK 1/2, JNK 1/2, and p38. As shown in Fig. 6, the cardiac expression levels of phosphorylated p38 and JNK 1/2 were not different between controls and transgenic mice of the three different genotypes (Fig. 6, *A–C*). However, we observed a significant increase in the cardiac levels of the dually phosphorylated (Thr-202/Tyr-204), active forms of ERK 1 and ERK 2 in hearts of CM GC-A KO mice (by ~1.4-fold, see Fig. 6*A*), and of double KO mice (by 3.2-fold, Fig. 6*C*) as compared with controls. In contrast, no significant changes in ERK 1/2 phosphorylation levels were detected in hearts from eNOS−/− as compared with control mice (Fig. 6*B*). Because ANP was shown to induce the expression levels of MKP-1, a specific phosphatase that suppresses MAPK activity (18), we also examined cardiac MKP-1 expression but failed to observe significant genotype-dependent variations (Figs. 6, *A–C*).

Lastly, we studied myocardial calcineurin activity. Because activated calcineurin dephosphorylates members of the NFAT family, two endogenous NFAT isoforms, NFATc2 and NFATc4,

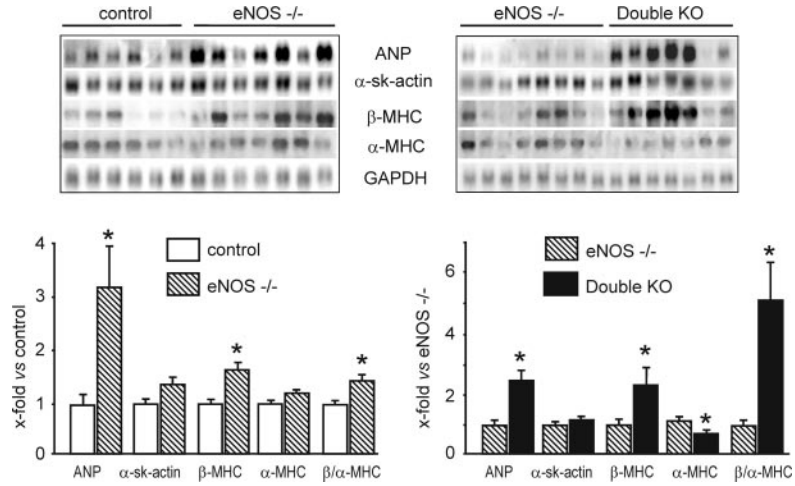

FIG. 5. *Top*, representative Northern blots. *Bottom*, messenger RNA expression levels of ANP, α-sk-actin, α-MHC, and β-MHC in left ventricles of eNOS−/− ($n = 7$) as compared with control mice ($n = 6$) (*left panel*) and double KO ($n = 7$) as compared with eNOS−/− mice ($n = 8$) (*right panel*). All levels normalized to glyceraldehyde-3-phosphate dehydrogenase. *, $p < 0.05$.

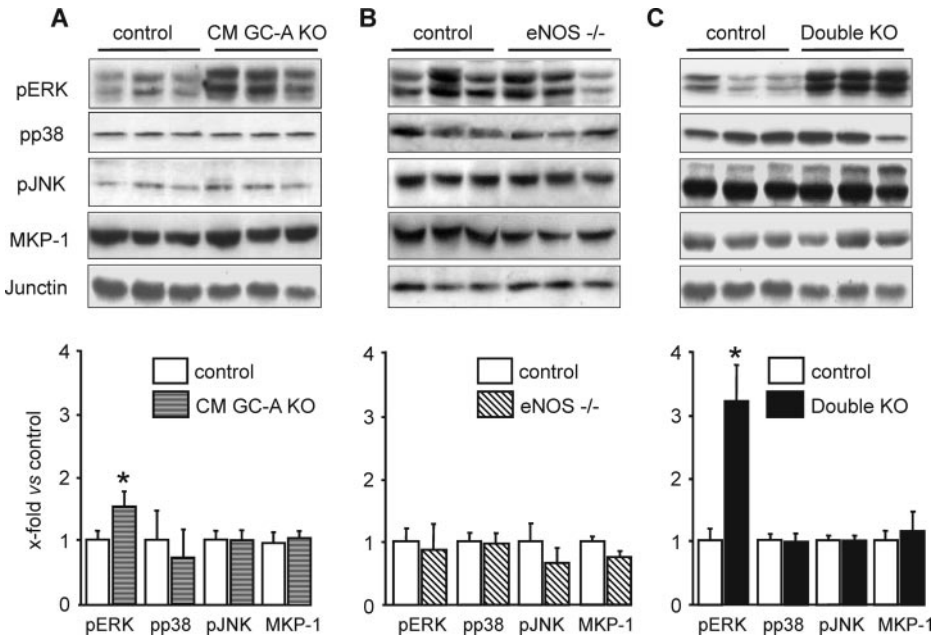

FIG. 6. *Top*, representative Western blots of ERK 1/2, p38, and JNK1/2 MAPK activation status. *Bottom*, left ventricular phosphorylation levels of ERK 1/2 (*pERK*) were significantly increased in CM GC-A KO mice ($n = 5$) as compared with controls ($n = 5$) (*A*) and even more in double KO mice ($n = 6$) as compared with controls ($n = 6$) (*C*). pERK levels in eNOS−/− hearts ($n = 6$) were not different from controls ($n = 6$) (*B*). The levels of phosphorylated p38 and JNK as well as the expression levels of MKP-1 were not different between genotypes. All signals were normalized to the cardiomyocyte-specific protein junctin. *, $p < 0.05$.

were analyzed for their relative phosphorylation status. Both NFATc2 and NFATc4 were present in a hyperphosphorylated (inactive) state in hearts of control, CM GC-A KO, and eNOS−/− mice, consistent with a relatively low calcineurin activity (Fig. 7). In contrast, the cardiac levels of phospho-NFATc2 and phospho-NFAT-c4 were decreased by ~77 and 59% in double KO mice, respectively, consistent with relatively higher endogenous calcineurin activity (Fig. 7).

Collectively, these results indicate that cardiac hypertrophy in normotensive CM GC-A KO mice is accompanied by activation of ERK 1/2, whereas calcineurin-NFAT signaling is unaltered. In contrast, the more pronounced cardiac hypertrophy and fibrosis in hypertensive double KO mice is associated with marked activation of both the MAPK ERK 1/2 and the calcineurin-NFAT signaling pathways.

DISCUSSION

We used a genetic approach to study the impact of a loss of function of the local, cardiac ANP/GC-A system on cardiac remodeling under normotensive and hypertensive conditions and to uncover the intracellular mechanisms responsible for its purported protective effects. Despite normal or even diminished arterial blood pressure, CM GC-A KO mice exhibit subtle but significant cardiac hypertrophy associated with slight MAPK/ERK 1/2 activation and unaltered calcineurin-NFAT activity. Despite elevated arterial blood pressure, eNOS−/− mice do not show cardiac remodeling or increased cardiac activity of the MAPK and calcineurin-NFAT cascades. However, selective inactivation of the GC-A receptor in cardiomyocytes of hypertensive eNOS−/− mice (double KO) results in pronounced cardiac hypertrophy and fibrosis, which is accompanied by marked activation of both ERK 1/2 and the phosphatase calcineurin. The selective activation of the MAPK ERK 1/2 is consistent with a series of recent reports indicating that the stress-activated protein kinases JNK and p38 may actually serve as negative (not positive) regulators of pathologic cardiac hypertrophy (comprehensively reviewed in Refs. 19 and 20). In contrast, the ERK 1/2 and calcineurin-NFAT signaling pathways likely occupy a central co-regulatory, cooperative, and even interconnected position in almost every form of reactive, *i.e.* in load-induced cardiac hypertrophy (21, 22). Taken together, these results indicate that local ANP/GC-A signaling counter-regulates MAPK/ERK-mediated cardiomyocyte growth already under basal, normal pressure load conditions. Even more, ANP, via GC-A, can prevent cardiac activation of ERK 1/2 and calcineurin in response to enhanced pressure load. Lastly, our observations also suggest that increased cardiac ANP expression in eNOS−/− mice provides an endogenous protective pathway preventing hypertensive heart disease.

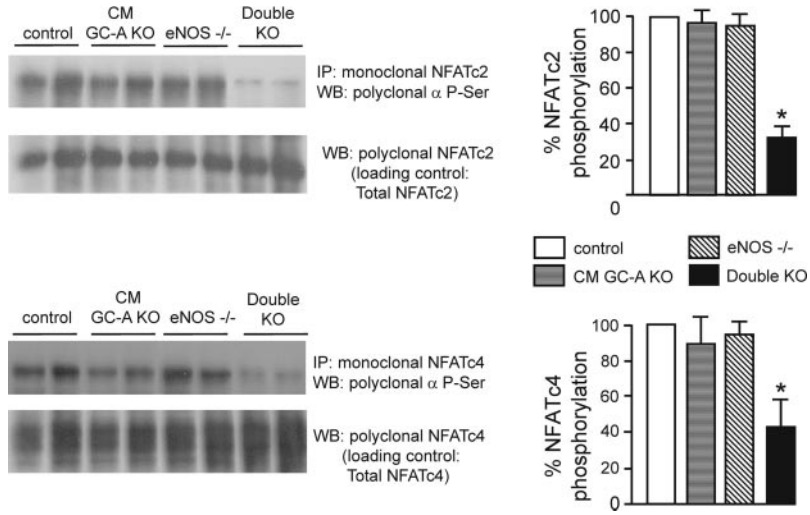

FIG. 7. *Left*, representative Western blots for the ventricular levels of hyperphosphorylated NFATc2 and NFATc4. *Right*, phosphorylation levels of NFATc2 and NFATc4 were significantly decreased in double KO mice as compared with all three other genotypes. Signals were calculated as percent *versus* controls. Data are from 8 animals/genotype; *, $p < 0.05$ *versus* controls.

In past years, the role of nitric oxide in the regulation of cardiac remodeling has been extensively investigated. Both neuronal and endothelial (eNOS) nitric-oxide synthases are constitutively expressed within the heart, in cardiomyocytes and adjacent cells (23, 24). *In vitro* studies with cultured cardiac fibroblasts and myocytes suggested that nitric oxide can inhibit cardiac fibrosis (6, 25) and cardiomyocyte hypertrophy (6, 26). However, the relevance of these findings to the *in vivo* situation is unclear. Three independent eNOS−/− mouse lines have been generated by gene targeting (10–12), and studies in all three lines consistently demonstrated that the lack of eNOS increases mean arterial blood pressure, on average by ~30 mm Hg. However, very discrepant observations regarding the cardiac phenotype of eNOS−/− mice have been made. Surprisingly, the mouse lines generated by Huang *et al.* (10) and Gödecke *et al.* (12) do not consistently develop cardiac hypertrophy under baseline conditions, despite chronic arterial hypertension (27). In contrast, the eNOS−/− line established by Shesely *et al.* (11) exhibits significant cardiac hypertrophy. The reasons for this discrepancy remain unclear, but taken together they seem to indicate that either nitric oxide is not a critical local modulator of cell growth within the heart and/or that other endogenous antihypertrophic mechanisms are able to compensate for a loss of function of eNOS *in vivo*. Based on published (14) and own observations of increased, ectopic ANP mRNA expression levels in the left ventricular myocardium of eNOS−/− mice (see Fig. 5), we hypothesized that enhanced local ANP/GC-A signaling may confer endogenous protection. Indeed, our genetic experiment, in which absence of cardiomyocyte GC-A markedly exacerbated cardiac hypertrophy and fibrosis in eNOS−/− mice, indicates that the increased local ANP levels play a significant local role in preventing hypertensive ventricular remodeling in the latter.

The downstream signaling pathways that mediate the effects of the ANP/GC-A/cGMP system on ERK 1/2 and calcineurin activity in cardiomyocytes remain unclear and will be an important question for our future studies. In cultured vascular smooth muscle cells ANP directly inhibits MAPK activation via cGMP (28). In addition, ANP was shown to induce the expression of MKP-1, a specific phosphatase that suppresses MAPK and, in particular, ERK 1/2 activity (18). However, we did not observe decreases in cardiac MKP-1 expression in double KO as compared with control mice. Neuroendocrine growth factors that typically stimulate ERK 1/2 signaling in cardiac myocytes via G protein-coupled receptors are angiotensin II, endothelin, and catecholamines. ANP inhibits not only the vasoconstrictor and hypertrophic effects of these factors but also their levels of expression and release (29). It is therefore conceivable that cardiomyocyte GC-A dysfunction and the subsequent local functional imbalance between ANP and these neuroendocrine growth factors leads to increased activation of ERK 1/2 and thereby to pressure-independent cardiac hypertrophy.

Notably, although the normotensive CM GC-A KO mice showed only moderate cardiac MAPK/ERK 1/2 activation, unaltered calcineurin-NFAT activity, and modest cardiac hypertrophy, hypertensive double KO mice exhibited greater MAPK/ERK 1/2 activation together with elevated calcineurin activity, and this was associated with pronounced cardiac hypertrophy. Moreover, similar levels of arterial hypertension in eNOS−/− mice were not accompanied by cardiac ERK 1/2 and/or calcineurin activation or by cardiac hypertrophy. Taken together, these results suggest that increased local ANP/GC-A signaling (*e.g.* in eNOS−/− mice) can prevent the activation of ERK 1/2 and calcineurin in response to hemodynamic load and thereby ameliorate hypertensive hypertrophy. Abolition of this local protective pathway (*e.g.* in double KO mice) facilitates load-induced activation of ERK 1/2 and calcineurin in cardiomyocytes, where these pathways directly coregulate the hypertrophic growth response (22). In fact, it has been suggested that calcineurin is inactive in an unstimulated heart, given the observation that dominant negative calcineurin-expressing transgenic mice have similar calcineurin phosphatase activity compared with wild-type controls (31). However, how GC-A/cGMP signaling counteracts load-induced activation of ERK 1/2 and calcineurin is unclear. Activation of cGMP-dependent protein kinase I might be involved because it was shown that activation of this kinase inhibits calcineurin-NFAT signaling in cultured cardiomyocytes in part by inhibiting L-type Ca^{2+} channels (32).

Deletion of cardiomyocyte GC-A in eNOS−/− mice (double KO) not only accentuated cardiac hypertrophy but also markedly enhanced the fibrotic response to pressure overload. These findings extend our previous observations of increased interstitial fibrosis in CM GC-A KO mice after transverse aortic banding (13). GC-A receptors are present on fibroblasts and mediate anti-fibrotic effects (6, 29) and should not have been affected by our strategy of conditional, cardiomyocyte-restricted gene deletion. Thus, increased fibrosis after deletion of cardiomyocyte GC-A is most likely mediated by an effect on cardiomyocyte production of pro- or anti-fibrotic factors. Cardiomyocytes produce angiotensin II (33), transforming growth factor-β (34), and maybe even aldosterone (35), and these and other profibrotic and proinflammatory factors may be regulated by natriuretic peptides via GC-A (29, 35). Thus, in future

studies this model provides an opportunity to explore the effects of ANP on cardiomyocyte-fibroblast interactions.

ANP is a robust marker of cardiac hypertrophy, which always increases early during induction and decreases during regression of hypertrophy (Ref. 36, and many others). Together with our previous report (13), our present extended analyses in mice with cardiomyocyte-restricted deletion of the ANP receptor emphasize that the induction of ANP expression in response to ventricular stretch is not only part of the reactivation of the fetal gene program but also has an important endogenous counter-regulatory, growth-moderating function. The observation that two molecules (ANP, NO), which both signal via cGMP, exert different effects on cardiomyocyte growth is striking. It is conceivable that spatial confinement of different guanylyl cyclase receptors (GC-A, soluble GC) within distinct microdomains of cardiomyocytes allows cGMP to locally regulate different effector molecules and, ultimately, to exert different effects on cardiomyocyte growth and contractile functions. For instance, it has been shown that GC-A, but not soluble GC, has potent effects on plasma membrane control of the calcium ATPase pump (37). Interestingly, a recent study demonstrated that ANP/GC-A, but not NO/soluble GC, stimulates the translocation of cGMP-dependent protein kinase I to the plasma membrane (30). Hence, the mechanisms for compartmentation of cGMP-mediated signaling within cardiomyocytes, and in particular the specific immediate downstream targets ultimately mediating the ERK 1/2 and calcineurin-inhibiting, antihypertrophic effects of ANP/GC-A/cGMP, remain intriguing issues.

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