

**PKC ζ -MEDIATED G α Q STIMULATION OF THE ERK5 PATHWAY IN
CARDIOMYOCYTES AND CARDIAC FIBROBLASTS**

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Running title: G α q/PKC ζ signaling in cardiac cells

ABSTRACT

Gq-coupled G protein-coupled receptors (GPCR) mediate the actions of a variety of messengers that are key regulators of cardiovascular function. Enhanced Gαq-mediated signaling plays an important role in cardiac hypertrophy and in the transition to heart failure. We have recently described that Gαq acts as an adaptor protein that facilitates PKCζ-mediated activation of ERK5 in epithelial cells. Since the ERK5 cascade is known to be involved in cardiac hypertrophy, we have investigated the potential relevance of this pathway in cardiovascular Gq-dependent signaling using both cultured cardiac cell types and chronic administration of angiotensin II in mice. We find that PKCζ is required for the activation of the ERK5 pathway by Gq-coupled GPCR in neonatal and adult murine cardiomyocyte cultures and in cardiac fibroblasts. Stimulation of ERK5 by angiotensin II is blocked upon pharmacological inhibition or siRNA-mediated silencing of PKCζ in primary cultures of cardiac cells and in neonatal cardiomyocytes isolated from PKCζ-deficient mice. Moreover, upon chronic challenge with angiotensin II, these mice fail to promote the changes in the MEK5/ERK5 pathway, in gene expression patterns and in hypertrophic markers observed in wild-type animals.

Overall, our data put forward that PKCζ is essential for Gq-dependent ERK5 activation in cardiomyocytes and cardiac fibroblasts and indicate a key cardiac physiological role for this recently described Gαq/PKCζ/MEK5 signaling axis.

INTRODUCTION

G protein-coupled receptors (GPCR) signaling through Gq proteins and MAPK cascades are known to play an important role in cardiovascular function and disease (1) (2) (3) (4). Enhanced angiotensin II, endothelin-1 and catecholamine (α_1 -adrenergic) signaling

correlate with pathological cardiac hypertrophy. Cardiac overexpression of a constitutively active α_{1B} -AR or of wild-type angiotensin AT₁-receptor (AT₁R) is sufficient to trigger this process (5) (6), as does chronic administration of angiotensin II to wild-type mice (7) (8). On the contrary, agents that block angiotensin synthesis prevent the development of pressure-overload-induced cardiac hypertrophy in animal models and in humans (9) (10). The direct involvement of Gαq-mediated signaling is further stressed by reports showing that cardiac-specific overexpression of Gαq in transgenic mice triggers cardiac hypertrophy in a similar manner to pressure overload and enhances expression of foetal genes, whereas the over-expression of a dominant-negative Gαq peptide in the heart renders mice resistant to pressure overload-triggered hypertrophy [(11) (12) (4), and references therein].

Gαq stimulation can initiate a number of downstream signaling pathways. By coupling to its classic effector phospholipase Cβ (PLCβ), activated Gq proteins promote diacylglycerol and inositol-1,4,5-triphosphate production, leading to the activation of conventional and novel protein kinase C (PKC) isoforms and to the mobilization of internal Ca²⁺, respectively (11) (12). This in turn leads to the activation of additional intracellular pathways relevant to hypertrophy such as the calcineurin/NFAT or the calmodulin kinase II cascades (11) (12) (13).

However, Gαq can also activate other signaling pathways independently of PLCβ stimulation. Gαq triggers the RhoA cascade, also required for a Gq-induced hypertrophic response through a direct interaction with p63RhoGEF (14) (15) (16) (17). More recently, we have reported a novel role for Gαq as a scaffold protein capable of recruiting both protein kinase C zeta (PKCζ) and the kinase MEK5 in an active complex, leading to the activation of the ERK5 pathway in epithelial cells (18). ERK5 is a member of the MAPK family that is activated by the upstream MEK5 kinase in response to different growth factors and cellular stressors (19). Activation of Gαq is a potent inducer of MAPK signaling in cardiac myocytes. The complex role of ERK1/2, JNK and p38 MAPK cascades in promoting compensated

cardiac hypertrophy and/or transition to heart failure has been extensively investigated (1) (12). Additionally, an important cardiac role for ERK5 has been recently identified (20). Whilst genetic deletion of ERK5 or MEK5 has indicated a role for this pathway in angiogenesis, endothelial cell physiology and cardiovascular development (21) (22), recent data also point to a role of ERK5 in cardiac hypertrophy and cardiomyocyte survival (23) (24) (25). Notably, the transcription factors MEF2A and 2C, well-known downstream targets of ERK5, promote cardiac hypertrophy in transgenic mice (26) whereas targeted deletion of this kinase attenuates the hypertrophic response mediated by MEF2 activation *in vivo* (25).

However, despite the important role of both Gq and ERK5 in cardiovascular function, the occurrence of a functional relationship between these pathways in cardiac cells has not been fully elucidated. In this report, we identify the atypical PKC ζ as a key link underlying G α q-coupled GPCR-mediated stimulation of the ERK5 cascade in cardiomyocytes and cardiac fibroblasts and show that this new signaling axis is relevant for angiotensin-induced hypertrophic pathways *in vivo*.

EXPERIMENTAL PROCEDURES

Materials.

The affinity-purified rabbit polyclonal antibody G α q/11 (C19) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The rabbit polyclonal antibody that recognizes ERK5 and the monoclonal antibody against PKC ζ (C24E6) were from Cell Signaling Technologies (Billerica, MA, USA). The anti-phospho (pT/pY^{218/220}) ERK5 polyclonal antibody was purchased from Invitrogen (Carlsbad, CA, USA). Angiotensin II was obtained from Sigma (St. Louis, MO, USA) and the myristoylated PKC ζ pseudosubstrate peptide (Myr-SIYRRGARRWRKL) from Biosource (Camarillo, CA, USA). Pertussis toxin was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). All other reagents were of the highest commercially-available grades.

Cell culture and treatments.

Primary cultures of neonatal mouse cardiomyocytes and fibroblasts were prepared from C57BL/6J mice or PKC ζ -deficient mice or wild-type animals (SV129J background) by dissociation of neonatal (1-2 days) hearts. After plating to remove fibroblasts for two hours, cardiomyocytes were plated in M12 multiwell precoated with 0.02% gelatin (300,000 cells per well) and cultured in DMEM plus 10% serum for 48 h. Cardiomyocytes were cultured for 2 days before use whereas cardiac fibroblasts were expanded and passed twice before the assay in order to remove contamination by endothelial cells. Both cell types were cultured in DMEM (D5648, Sigma-Aldrich) supplemented with 10% FBS.

In all cases, the desired cell type was stimulated with angiotensin II (100 nM) in serum-free media, during the indicated time periods. The cells were serum-starved for 5-6 h before ligand addition to minimize basal kinase activity. Treatment with the PKC ζ pseudosubstrate inhibitor (10 μ M) was initiated 30 min before agonist stimulation. For the inactivation of Gi proteins, cells were pretreated with pertussis toxin (100 ng/ml) for 16 h. Isolation of cardiac myocytes was performed as previously described (27). Adult male C57BL6 mice or Wistar rats were heparinized (4U/g) and anaesthetized with sodium pentobarbital (50mg/kg). The hearts were removed and mounted on a Langerdorff-perfusion apparatus. The ascending aorta was cannulated and a retrograde perfusion was set up. The hearts were successively perfused with the following oxygenated solutions at 36°C: (1) standard nominally Ca²⁺-free Tyrode solution (3min), (2) standard nominally Ca²⁺-free Tyrode solution (15min) containing type II collagenase. The hearts were removed from the Langerdorff apparatus, and after removal of atria, the ventricles were cut off and gently shaken for 3 min in a standard Tyrode solution containing 0.1mM CaCl₂ to disperse the isolated cells. The resulting cell suspensions were filtered through a 250 μ m nylon mesh and centrifuged for 4 min at 20g. Finally, the cell pellets were stored in Tyrode solution (1mM CaCl₂) and stimulated with angiotensin II (100nM) shortly after extraction.

PKC ζ knock-down.

Primary neonatal mouse cardiomyocytes were reverse-transfected using siRNA specific oligos for PKC ζ (Mouse PRKCZ, NM_008860, on-target plus SMARTpool L-040823-00-0005, sequences GAUCGACCAGUCCGAAUUU, CAAGGCCUCACACACGUCUUA, CAUCAAGUCUCAUGCCUUC and GGGCAUGCCUUGUCCUGGA) were purchased from Dharmacon (Roche, Palo Alto, CA). Scrambled oligos were purchased from Ambion to serve as negative control. siRNAs were transfected using DharmaFECT 1 reagent (Dharmacon) to reach a final concentration of 50nM. A PKC ζ specific antibody (Cell Signaling) was utilized to assess effective knockdown of the kinase by Western blot.

Determination of MAPK stimulation.

The activation state of ERK5 was measured by Western blot analysis of cell lysates by using specific anti-phospho-ERK5 (1:500) or anti-ERK5 (1:500) antibodies. In the latter case, the stimulation of ERK5 can be detected by the presence of a band with slower electrophoretic mobility that represents the active, phosphorylated form of the protein (28). Both approaches have been used by us (18) and by other laboratories (29) (30) (31) as a reliable method for determining ERK5 activation. To obtain cell lysates, cells were washed with ice-cold PBS-buffer plus 1mM sodium orthovanadate and subsequently solubilized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% (w/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA, 1 mM NaF, supplemented with 1 mM sodium orthovanadate plus a mixture of protease inhibitors). Lysates were resolved by 6-10% SDS-PAGE and subjected to immunoblot analysis as previously described (32). Bands were quantified by laser-scanner densitometry and the amount of phospho-ERK5 protein normalized to the amount of the total loaded protein (ERK5 or GAPDH/actin/ α -tubulin), as assessed by the specific antibodies. Statistical analysis was performed using the two-tailed Student's t test, as indicated.

Chronic Angiotensin treatment in vivo.

The generation of PKC ζ knock-out mice (SV129J background) has been previously described (33). Littermate wild-type and

PKC ζ $-/-$ male mice (32 weeks of age) were subjected to continuous infusion of angiotensin II (or PBS as a control) for 14 days, a well established model for the induction of cardiac hypertrophy (8) (34). Angiotensin II dissolved in PBS was continuously and subcutaneously infused at a rate of 432 μ g/kg/day using Alzet osmotic minipumps (model 2002, Alza Corp., Mountain View, CA) implanted dorsally under isofluorane anesthesia as reported (34). Heart rate and electrocardiogram (ECG) components were measured using a non-invasive recording electrocardiogram method in conscious mice [ECGenie™ ECG Screening System (Mouse specifics, Inc., Boston MA)] at 14 days after pump implantation as reported (35). Before sacrifice, blood plasma samples were obtained to assess circulating levels of pro ANP (1-98), which reflects chronic levels of ANP secretion (36) (37), by using an established immunoassay (proANP EIA, Alpco Diagnostics, Windham, NH). Finally, hearts from wild-type or knockout mice were excised, cleaned of blood, the weight of the whole heart was measured (HW) and the ratio to body weight (HW/BW) or tibia length was calculated. Mice were maintained under pathogen-free conditions and all the experiments were performed in accordance with guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Directive 86/609/EEC) and with the authorization of the Bioethical Committee of the University Autónoma of Madrid (CEI-21-440).

Immunohistochemistry and histological analysis.

For histological analysis, hearts from wild-type or PKC ζ knockout mice were fixed in formalin and embedded in paraffin wax. Sections of 5 μ m were processed for immunohistochemistry. To compare the expression of Ets-1 and activated MEF2C and MEK5 in wild-type versus knockout mice a high temperature antigen unmasking technique (10-minute microwaving of slides in Tris-EDTA pH 8.0 for 90 seconds) was carried out. Antigen retrieval was performed after deparaffinization to enhance staining. Sections were then incubated with 5% horse serum for 30 minutes, and then washed three

times with sterile PBS (pH 7.5) prior to incubation with the appropriate primary antibodies at 1:50/1:100 dilutions. Biotin-conjugated secondary antibodies were purchased from Jackson ImmunoResearch and used at 1/4000 dilution. For all antibodies, signal was amplified using avidin peroxidase (ABC elite kit Vector) and visualized using diaminobenzidine as a substrate (DAB kit Vector). Finally, sections were stained with haematoxylin. Image analysis was performed with ImageJ 1.46a software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Four images were acquired from a randomly selected location in each slide. Random RGB images were converted to 8 bit images for the quantification of the DAB signal to obtain the quantification staining parameters.

Quantitative Real-Time PCR.

Total RNA from hearts of PBS- or angiotensin-treated animals was extracted with using RNeasy kit (Qiagen, Valencia, CA) following manufacturers' recommendations. The integrity of the RNA populations was tested in the Bioanalyzer (Agilent, Santa Clara, CA) showing 28S/18S ratios above 1.7. Gene expression levels for *Nppa*, *Col1a2* and *MAPK7* were determined using *quantitative real-time PCR* (qRT-PCR). Total RNA (1 µg) was reverse-transcribed into cDNA by the Gene Ampkit (Perkin Elmer). qRT-PCR reactions were performed in triplicates in 10 µl final volume with the cDNA amount equivalent to 5 ng of total RNA, 250 nM of each primer and 5 µl of Power Sybr Green PCR Master Mix (Applied Biosystems PN 4367659) using a CFX 384 Real Time System, C1000 Thermal Cycler (Bio-Rad). A melting curve was included at the end of the program to verify the specificity of the PCR product. PCR primer sequences were selected using Universal Probe Library (Roche Applied Science). The expression values were normalized using the average of the housekeeping genes 18S ribosomal RNA, HPRT (hypoxanthine-guanine phosphoribosyltransferase) and GAPDH.

Gene expression microarray analysis.

Total RNA was purified from mice hearts using RNeasy Fibrous Tissue Mini Kit, following the manufacturer's instructions

(Qiagen). RNA was hybridized to Mouse Expression MOE 430 2.0 Affymetrix GeneChips, containing more than 45,000 probesets covering the mouse transcriptome. CEL files obtained after biochip scanning were used to normalize gene expression values using RMA (38) (39). Log2 transformed values were mean centered (mean=0, stdv=1). In order to compare different mouse models of heart hypertrophy, the series matrix files corresponding to the experiments GSE7781 (40), GSE5500 (41), GSE12337 (42), and GSE8771 were downloaded from GEO website and performed in the same Affymetrix GeneChip version (MOE430 2.0). After removing irrelevant experiments, log2 transformed values were also mean centered (mean=0, stdv=1) independently. This transformation allowed the comparison of expression values from different datasets in a unique multi-experiment dataset. Differential expression analysis between control and hypertrophic samples was done using a Ttest and probesets were selected if they displayed >1.8 fold change with p-val <0.008. Using these thresholds, a total number of 1,536 probesets were found to be differentially expressed, being 823 under-expressed in hypertrophy and 713 over-expressed. Hierarchical clustering of the samples performed from the selected probesets was done using Pearson distance and average linkage. To assure a proper grouping, samples and genes were resampled 100 times using bootstrapping. Gene expression microarray data generated has been submitted to GEO database with the Accession number GSE29145, and accomplishes MIAME guidelines.

Functional analysis.

We performed enrichment analysis of functions of the differentially expressed genes using the Gene Ontology (GO) database and the web tool Database for Annotation, Visualization and Integrated Discovery (DAVID) (<http://david.abcc.ncifcrf.gov/>) (43) (44). Briefly, probesets lists of either Group I or Group II genes in the differential expression analysis of hypertrophy performed were uploaded in DAVID, which computes enrichment in GO "biological processes". The enriched functions or "terms" were ordered

by statistical relevance, displaying only those functions with <0.05 FDR value of corrected p-val. Protein interaction networks were obtained using the Pathway Studio software (Ariadne Genomics).

RESULTS

The angiotensin Gq-coupled receptor activates ERK5 in cardiomyocytes and cardiac fibroblasts in a PKC ζ -dependent manner.

We have recently shown that activation of the m1-muscarinic Gq-coupled receptor in epithelial cells promoted the stimulation of the ERK5 pathway by biochemical routes not involving the classical G α q effector PLC β , nor cytoplasmic tyrosine kinases or EGF receptor transactivation (18). Since both Gq-coupled GPCR and ERK5 play an important role in the cardiovascular system function and dysfunction (see Introduction), we sought to determine whether a key messenger acting through Gq-coupled GPCR such as angiotensin II also triggered the ERK5 cascade in different cardiac cell types. Primary cultures of neonatal mouse cardiomyocytes were incubated with angiotensin II for different periods of time and endogenous ERK5 activation was assessed by immunoblot analysis using an ERK5 antibody that detects ERK5 stimulation by the appearance of a band of slower electrophoretic mobility, corresponding to the phosphorylated, stimulated kinase (Fig. 1A). A clear, time-dependent increase in endogenous ERK5 activation was detected in response to this agonist.

Our previous results in epithelial cells indicated that a functional association between G α q and PKC ζ was essential for stimulation of the ERK5 pathway by G α q-coupled GPCR agonists (18). To establish whether this novel pathway was also operating in cardiomyocytes, primary neonatal cell cultures were treated either with scrambled or PKC ζ -targeting siRNA oligos. Interestingly, whereas a clear ERK5 activation (assessed using a specific phospho-ERK5 antibody) was noted in cardiomyocytes treated with scrambled siRNA (Fig. 1B), angiotensin-mediated endogenous ERK5 stimulation was not detected upon siRNA-

induced PKC ζ downregulation. We also investigated ERK5 stimulation by angiotensin in cardiomyocytes isolated from PKC ζ -deficient mice or wild-type littermates (SV129J background) (33). As in cardiomyocytes obtained from the C57BL/6J strain, angiotensin promoted a clear ERK5 activation in cells from wild-type, with an earlier peak of activation, whereas this response was absent in PKC ζ -/- cardiomyocytes (Fig. 1C). Furthermore, we found that angiotensin-dependent ERK5 stimulation could also be observed in cardiomyocytes isolated from adult mouse (Fig. 1D) or rat (Fig. 1E) and that this process was blocked in the presence of a specific PKC ζ inhibitor (Fig. 1E). Collectively, these data indicated that PKC ζ is strictly required for Gq-mediated ERK5 stimulation in cardiomyocytes.

Interestingly, angiotensin II-mediated stimulation of endogenous ERK5 was also observed in cardiac fibroblasts (Fig. 2A), with slight differences in the time-course of activation. As we observed in epithelial cells for other Gq-coupled GPCRs (18), ERK5 activation by angiotensin II in cardiac fibroblasts was not affected in the presence of pertussis toxin (PTX) (supplemental Fig. 1), thus indicating that this process does not involve paracrine transmodulation of Gi-coupled- GPCR. Moreover, in line with the data in cardiomyocytes, the pretreatment of primary neonatal mouse (Fig. 2B) or rat (Fig. 2C) cardiac fibroblasts prior to agonist challenge with a PKC ζ inhibitor (the cell-permeable myristoylated PKC ζ pseudosubstrate peptide (45) (46)) markedly decreased endogenous ERK5 phosphorylation in response to angiotensin II, thus suggesting that PKC ζ is also required for Gq-mediated ERK5 activation in cardiac fibroblasts.

Role of the G α q/PKC ζ pathway in angiotensin-mediated heart signaling and gene expression patterns in vivo.

To assess the potential relevance of the G α q/PKC ζ pathway in cardiac cells *in vivo*, we subjected homozygous null mice (PKC ζ -/-) and matched control littermates (wild-type) to chronic angiotensin II (or control vehicle) infusion for 14 days using osmotic minipumps. This is a classical, well-established procedure to assess angiotensin effects in

cardiac functions and to promote cardiac hypertrophy in mice models (34) (8). Interestingly, immuno-histological analysis of the hearts at the end of the chronic infusion period using phosphospecific antibodies showed that MEK5 (the upstream activator of ERK5) and MEF2C (a well-established downstream target of ERK5) display a higher phosphorylation state upon angiotensin treatment in wild-type animals that is absent in PKC ζ -deficient mice (Fig. 3). These data further indicate that PKC ζ is an important mediator of ERK5 activation by G α q-coupled GPCR in murine heart in vivo and support that the Gq/PKC ζ /ERK5 pathway plays a relevant role in the response triggered by angiotensin.

Enhanced signaling by G α q-coupled GPCR often underlies the development of pathological cardiac hypertrophy. Therefore, we conducted a global microarray gene expression pattern analysis using total RNA isolated from wild-type or PKC ζ -deficient mice treated with PBS or with chronic angiotensin II-infusion, and compared these data with the results published for different mouse models of heart hypertrophy (TAC or AT1 receptor transgenic mice), using the same Affymetrix GeneChip version. Test analysis was performed in order to find differences in gene expression patterns between experimentally assessed hypertrophic hearts and controls (see Experimental Procedures). A total number of 1,536 probesets that did accomplish statistically significant criteria for differential expression were identified and listed as either over-expressed in hypertrophy (Group I) or under-expressed (Group II) compared to control animals (supplemental Tables S1 and S2). This information was used to group genes and samples using unsupervised hierarchical clustering (Fig 4A). Our gene profiling analysis demonstrated that the expression patterns of animals developing hypertrophy as a consequence of TAC, AT1R over-expression or angiotensin II infusion, were clearly separated from control treatments. Importantly, whereas our wild-type angiotensin II-treated mice consistently segregated with the “hypertrophic” expression profile group, the angiotensin II-treated PKC ζ -deficient mice were included among the control group animals, together with PBS-

treated animals. These results indicate that, upon angiotensin II treatment, PKC ζ -deficient mice do not display a gene expression pattern characteristic of hypertrophic hearts.

Consistently, amongst Group I genes we found several whose products are considered hypertrophic markers, as ANP (*Nppa*) and brain natriuretic peptide (*Nppb*), as well as type I α 2 procollagen (*Col1a2*) and procollagen type IV alpha (*col4a*), that are overexpressed in cardiac fibrosis (47) (48) (49). These genes were not over-expressed in samples from angiotensin II-treated PKC ζ -deficient mice (supplemental Table S1 and S2). Additionally, the biological functions of the differentially expressed genes were studied using DAVID software. Genes over-expressed in hypertrophy (Group I) included functions related to cell cycle and cellular division, cellular adhesion, cytoskeleton organization, phosphate transport and development (Fig. 4B), some of which can be related to cellular changes required for the induction of the hypertrophic phenotype. The under-expressed genes (Group II) represented functions involved in metabolism, oxidative phosphorylation, cell respiration and electron transport, which have been described to be down-regulated in hypertrophic hearts (50) (48).

Microarray data were further analyzed using Pathway Studio (Ariadne Genomics) to identify and characterize possible targets whose alteration in gene expression could be related to the activation of the ERK5 signaling pathway. This analysis revealed that expression of ERK5 (*Mapk7*) itself and of its direct target ETS-1 (*Ets-1*) (51) was up-regulated in angiotensin-treated wild-type mice compared to angiotensin II-treated PKC ζ -deficient mice or control mice (Fig. 5A). The same was true for gene products downstream the ETS-1 transcription factor (*Col1a2*, *Bcl2*). Consistently, immunohistological analyses of heart sections using an ETS-1 specific antibody confirmed a marked increase in ETS-1 protein expression in angiotensin II-treated wild-type mice compared to angiotensin II-treated PKC ζ -/- animals (Fig. 5B). In this regard, it is interesting to point out that some genes regulated by other known downstream targets

of ERK5 were also up-regulated. These are *Ccna2* (directly controlled by c-Myc), *Nppa*, and *Col1a2* (directly controlled by c-jun) and *Bcl2* (directly controlled by CREB) (Fig. 5A, see also supplemental Tables S1 and S2). A quantitative RT-PCR analysis in cardiac tissue confirmed an increase in the mRNA expression of several of the relevant genes highlighted in Fig. 5A in angiotensin II-treated wild-type mice compared to angiotensin II-treated PKC ζ -/- littermates, such as the hypertrophic biomarker atrial natriuretic peptide (*Nppa*), collagen, type I, alpha 2 (*Col1a2*) and mitogen-activated protein kinase 7 (*MAPK7/ERK5*) (Fig. 5B).

Overall, these data indicated the absence of an altered gene expression pattern related to cardiac hypertrophy in PKC ζ -deficient mice upon angiotensin II treatment in contrast to that observed in wild-type mice, suggesting an important role for the G α q/PKC ζ axis in cardiac physiology. Consistent with these data, an initial analysis indicated that several markers that correlate with cardiac hypertrophy were altered upon angiotensin treatment in wild-type, but not in PKC ζ -deficient mice. Plasma levels of ANP were markedly enhanced upon angiotensin infusion in wild-type, but not in PKC ζ -/- mice (Fig. 6A), in agreement with heart expression levels (Fig. 5C). Similarly, angiotensin-treated wild-type mice showed a significant increase in heart weight to-body weight or tibial length ratios compared to vehicle-infused animals that was not observed in PKC ζ -deficient mice (Fig. 6B). In addition, an electrocardiographic (ECG) analysis performed at the end of the chronic infusion period indicated that angiotensin II promoted a clear increase in the duration of the QRS and QT components in wild-type animals (what has been reported to correlate with left ventricular hypertrophy (52) (53) (54)) compared to vehicle-treated mice. However, no noticeable ECG changes were induced by angiotensin-II in PKC- ζ deficient animals (Fig. 6C). Taken together, these data suggest that the absence of PKC ζ impairs the induction of cardiac hypertrophy by the G α q-coupled GPCR agonist angiotensin II.

DISCUSSION

In this report, we show that PKC ζ is required for the stimulation of the ERK5 pathway by

the Gq-coupled GPCR angiotensin II receptor in cardiomyocytes and cardiac fibroblasts. Moreover, we suggest an important pathophysiological role for this G α q/PKC ζ /ERK5 axis in the triggering of signaling and gene expression pathways related to cardiac hypertrophy in response to angiotensin *in vivo*.

We observe that angiotensin II can promote ERK5 activation in both neonatal and adult murine cardiomyocytes and in neonatal cardiac fibroblasts, although the extent and kinetics of ERK5 stimulation can vary with the cell type or the mice strain. In agreement with our previous findings for other Gq-coupled GPCR in epithelial cells (18), this process does not appear to involve cross-talk with Gi-coupled signaling, as has been described for angiotensin II-mediated ERK1/2 activation in cardiac fibroblasts (55). On the other hand, pharmacological inhibition or siRNA-mediated silencing of PKC ζ completely abrogated angiotensin-induced ERK5 activation in cardiomyocytes or cardiac fibroblasts, consistent with the lack of activation of this cascade in cardiomyocytes isolated from PKC ζ -deficient mice. Overall, these data suggest that PKC ζ is required for angiotensin-induced ERK5 stimulation in these cell types, most likely by mechanisms involving the scaffold role of G α q and the formation of G α q/PKC ζ /MEK5 complexes as we have previously demonstrated (18).

Recent reports have indicated that angiotensin II is able to induce hypertrophy through activation of ERK5 in aortic smooth muscle cells (56) (57) and in rat neonatal cardiomyocytes (58), suggesting the functional involvement of PKC/PKD or PKC ϵ , respectively. Although these differences might be explained by the different cell types used, the possibility exists that several PKC isoforms might be required for full activation of the ERK5 pathway by AT1 receptors.

The finding that PKC ζ -deficient mice do not undergo the changes induced in wild-type animals upon chronic challenge with angiotensin II in the MEK5/ERK5/MEF2C pathway, in heart global gene expression and in hypertrophic markers suggest an important pathophysiological role for the G α q/PKC ζ axis. Although PKC ζ was known to be

present in cardiac cells and to translocate in response to Gq-coupled GPCR agonists (59), no cardiac-related phenotype had been reported in PKC ζ -deficient mice in the absence of agonist challenge (33). It is worth noting that PKC ζ deficiency does not alter the levels of the other PKC isoforms in different tissues (33) thus making unlikely that changes in such PKC isoforms (11) may underlie the observed effects on G α q signaling. The heart hypertrophy parameters determined herein do not allow establishing whether they are the consequence of cardiomyocyte hypertrophy, increased fibroblast proliferation/fibrosis, enhanced proliferation of other cardiac cell populations or a combination of some of these processes. Since we report for the first time that ERK5 can also be activated by angiotensin II in neonatal cardiac fibroblasts, and given the emerging role of cardiac fibroblasts in cardiovascular function and dysfunction (60) (61), the contribution of different heart cell types to the observed hypertrophic phenotype clearly merits future investigation.

The induction of cardiac hypertrophy and eventually cardiac failure by agents acting through Gq-coupled GPCR appears to be a complex process involving changes in highly interconnected signaling pathways (including calcium homeostasis, PKC, MAPK and PI3K/AKT cascades) triggered upon receptor stimulation (1) (62) (11) (12). The primary downstream actions of Gq-coupled GPCR have been tied to the activation of its canonical effector PLC β , leading to the stimulation of conventional and novel PKC isoforms, calcium mobilization and to the regulation of the calcineurin/NFAT cascade. In particular, the splicing variant PLC β 1 seems to be involved in the hypertrophic responses initiated by Gq-coupled α 1-adrenergic receptors in cardiomyocytes (63). However, other reports have shown a lack of correlation between PLC β activation and the cardiac hypertrophy phenotype induced in transgenic mouse lines expressing activated G α q, suggesting a role for additional pathways in this process (64). In line with this notion, our data suggest that the functional link between G α q and PKC ζ is also necessary for the development of G α q-induced cardiac hypertrophy programmes.

Our data are consistent with the idea that the absence of PKC ζ impairs the ability of Gq-coupled angiotensin receptors to promote cardiac hypertrophy programmes by disrupting G α q signaling to ERK5. The immunohistochemical data in hearts of PKC ζ -deficient mice following chronic angiotensin II infusion, demonstrate a decrease in the phosphorylation / activation status of its upstream kinase MEK5, as well as of its well-known downstream target, MEF2C, compared to wild-type littermates. Moreover, global gene expression analysis indicate that ERK5 (*MapK7*) itself and several direct (*Ets-1*) and indirect (*NppA*, *Colla2*, *Ccna2*, *Bcl2*) ERK5 targets are up-regulated in angiotensin-treated wild-type animals but not in PKC ζ -deficient mice. These results are in agreement with previous reports showing that angiotensin II infusion promotes ERK5 activation in mice myocardium (8), that over-expression of either upstream activators (MEK5) or downstream targets (MEF2A and C) of ERK5 induce cardiac hypertrophy in mice (23) (26), and that the activity of ERK5 is increased during left ventricular hypertrophy (24) (59), whereas targeted deletion of ERK5 attenuates the hypertrophic response in the heart (25). It is also worth noting that high levels of the antiapoptotic protein Bcl2 have been found in early stages of hypertrophy (65) and that arterial wall thickness, peri-vascular fibrosis and cardiac hypertrophy induced by angiotensin II treatment are significantly reduced in *Ets-1* deficient mice (66).

Although our data support the idea that the G α q/PKC ζ / ERK5 axis plays a relevant role in the development of cardiac hypertrophy signaling and gene expression programmes triggered by angiotensin II, the possibility that PKC ζ deficiency may alter additional G α q signaling pathways downstream of PKC ζ cannot be ruled out. PKC ζ has been shown to increase atrial natriuretic factor in ventricular cardiomyocytes (67) and more recently, to be involved in cardiac sarcomeric protein phosphorylation (68), which can be relevant during mechanical heart stress (69). Interestingly, PKC ζ directly participates in the activation of MAPK and NF κ B in several cell types (reviewed in (70,71)), pathways widely known to be involved in cardiac hypertrophy (1) (12). The potential triggering

of PKC ζ downstream signaling pathways other than the ERK5 cascade upon Gq-coupled GPCR activation is being actively investigated in our laboratory.

In summary, we unveil that the novel G α q/PKC ζ signaling axis plays a key role in the development of cardiac hypertrophy programmes in response to angiotensin. Since G α q and PKC ζ protein levels have been reported to increase in cardiomyocytes after volume overload-induced hypertrophy (72) it is tempting to suggest that such pathway would be particularly active in such

pathological settings, providing new targets for therapeutic intervention.

Acknowledgements

Our laboratory is funded by grants from Ministerio de Educación y Ciencia (SAF2008-00552), Fundación Ramón Areces, The Cardiovascular Network (RECAVA) of Instituto de Salud Carlos III (RD06-0014/0037), Comunidad de Madrid (S-SAL-0159-2006) to F.M. and Instituto de Salud Carlos III (PI080461) to C.R. We thank Dr. Michel Herranz (Salamanca, Spain) for help with the ECG experiments.

REFERENCES

1. Dorn, G. W., 2nd, and Force, T. (2005) *J Clin Invest* **115**, 527-537
2. Harris, D. M., Eckhart, A. D., and Koch, W. J. (2006) *J Mol Cell Cardiol* **40**, 589-592
3. Wang, Y. (2007) *Circulation* **116**, 1413-1423
4. Rohini, A., Agrawal, N., Koyani, C. N., and Singh, R. *Pharmacol Res* **61**, 269-280
5. Hein, L., Stevens, M. E., Barsh, G. S., Pratt, R. E., Kobilka, B. K., and Dzau, V. J. (1997) *Proc Natl Acad Sci U S A* **94**, 6391-6396
6. Paradis, P., Dali-Youcef, N., Paradis, F. W., Thibault, G., and Nemer, M. (2000) *Proc Natl Acad Sci U S A* **97**, 931-936
7. Bueno, O. F., Wilkins, B. J., Tymitz, K. M., Glascock, B. J., Kimball, T. F., Lorenz, J. N., and Molkentin, J. D. (2002) *Proc Natl Acad Sci U S A* **99**, 4586-4591
8. Ikeda, Y., Aihara, K., Sato, T., Akaike, M., Yoshizumi, M., Suzaki, Y., Izawa, Y., Fujimura, M., Hashizume, S., Kato, M., Yagi, S., Tamaki, T., Kawano, H., Matsumoto, T., Azuma, H., and Kato, S. (2005) *J Biol Chem* **280**, 29661-29666
9. Zhu, Y. C., Zhu, Y. Z., Gohlke, P., Stauss, H. M., and Unger, T. (1997) *Am J Cardiol* **80**, 110A-117A
10. Hunyady, L., and Catt, K. J. (2006) *Mol Endocrinol* **20**, 953-970
11. Liggett, S. B. (2006) *J Clin Invest* **116**, 875-877
12. Heineke, J., and Molkentin, J. D. (2006) *Nat Rev Mol Cell Biol* **7**, 589-600
13. Mishra, S., Ling, H., Grimm, M., Zhang, T., Bers, D. M., and Brown, J. H. *J Cardiovasc Pharmacol* **56**, 598-603
14. Chikumi, H., Vazquez-Prado, J., Servitja, J. M., Miyazaki, H., and Gutkind, J. S. (2002) *J Biol Chem* **277**, 27130-27134
15. Lutz, S., Freichel-Blomquist, A., Yang, Y., Rumenapp, U., Jakobs, K. H., Schmidt, M., and Wieland, T. (2005) *J Biol Chem* **280**, 11134-11139
16. Lutz, S., Shankaranarayanan, A., Coco, C., Ridilla, M., Nance, M. R., Vettel, C., Baltus, D., Evelyn, C. R., Neubig, R. R., Wieland, T., and Tesmer, J. J. (2007) *Science* **318**, 1923-1927
17. Rojas, R. J., Yohe, M. E., Gershburg, S., Kawano, T., Kozasa, T., and Sondek, J. (2007) *J Biol Chem* **282**, 29201-29210
18. Garcia-Hoz, C., Sanchez-Fernandez, G., Diaz-Meco, M. T., Moscat, J., Mayor, F., and Ribas, C. *J Biol Chem* **285**, 13480-13489
19. Obara, Y., and Nakahata, N. *Mol Pharmacol* **77**, 10-16
20. Wang, X., and Tournier, C. (2006) *Cell Signal* **18**, 753-760
21. Nishimoto, S., and Nishida, E. (2006) *EMBO Rep* **7**, 782-786
22. Roberts, O. L., Holmes, K., Muller, J., Cross, D. A., and Cross, M. J. (2009) *Biochem Soc Trans* **37**, 1254-1259

23. Nicol, R. L., Frey, N., Pearson, G., Cobb, M., Richardson, J., and Olson, E. N. (2001) *EMBO J* **20**, 2757-2767
24. Kacimi, R., and Gerdes, A. M. (2003) *Hypertension* **41**, 968-977
25. Kimura, T. E., Jin, J., Zi, M., Prehar, S., Liu, W., Oceandy, D., Abe, J., Neyses, L., Weston, A. H., Cartwright, E. J., and Wang, X. *Circ Res* **106**, 961-970
26. Xu, J., Gong, N. L., Bodi, I., Aronow, B. J., Backx, P. H., and Molkentin, J. D. (2006) *J Biol Chem* **281**, 9152-9162
27. Fernandez-Velasco, M., Goren, N., Benito, G., Blanco-Rivero, J., Bosca, L., and Delgado, C. (2003) *J Physiol* **553**, 395-405
28. Xu, B. E., Stippec, S., Lenertz, L., Lee, B. H., Zhang, W., Lee, Y. K., and Cobb, M. H. (2004) *J Biol Chem* **279**, 7826-7831
29. Nakamura, K., and Johnson, G. L. *Methods Mol Biol* **661**, 91-106
30. Diaz-Rodriguez, E., and Pandiella, A. *J Cell Sci* **123**, 3146-3156
31. Ohnesorge, N., Viemann, D., Schmidt, N., Czymai, T., Spiering, D., Schmolke, M., Ludwig, S., Roth, J., Goebeler, M., and Schmidt, M. *J Biol Chem* **285**, 26199-26210
32. Elorza, A., Sarnago, S., and Mayor, F., Jr. (2000) *Mol Pharmacol* **57**, 778-783
33. Leitges, M., Sanz, L., Martin, P., Duran, A., Braun, U., Garcia, J. F., Camacho, F., Diaz-Meco, M. T., Rennert, P. D., and Moscat, J. (2001) *Mol Cell* **8**, 771-780
34. Braz, J. C., Bueno, O. F., Liang, Q., Wilkins, B. J., Dai, Y. S., Parsons, S., Braunwart, J., Glascock, B. J., Klevitsky, R., Kimball, T. F., Hewett, T. E., and Molkentin, J. D. (2003) *J Clin Invest* **111**, 1475-1486
35. Chu, V., Otero, J. M., Lopez, O., Morgan, J. P., Amende, I., and Hampton, T. G. (2001) *BMC Physiol* **1**, 6
36. Missbichler, A., Hawa, G., Schmal, N., and Woloszczuk, W. (2001) *Eur J Med Res* **6**, 105-111
37. Molhoek, S. G., Bax, J. J., van Erven, L., Bootsma, M., Steendijk, P., Lentjes, E., Boersma, E., van der Laarse, A., van der Wall, E. E., and Schalij, M. J. (2004) *Heart* **90**, 97-98
38. Bolstad, B. M., Irizarry, R. A., Astrand, M., and Speed, T. P. (2003) *Bioinformatics (Oxford, England)* **19**, 185-193
39. Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. J., Scherf, U., and Speed, T. P. (2003) *Biostatistics (Oxford, England)* **4**, 249-264
40. Kuba, K., Zhang, L., Imai, Y., Arab, S., Chen, M., Maekawa, Y., Leschnik, M., Leibbrandt, A., Markovic, M., Schwaighofer, J., Beetz, N., Musialek, R., Neely, G. G., Komnenovic, V., Kolm, U., Metzler, B., Ricci, R., Hara, H., Meixner, A., Nghiem, M., Chen, X., Dawood, F., Wong, K. M., Sarao, R., Cukerman, E., Kimura, A., Hein, L., Thalhammer, J., Liu, P. P., and Penninger, J. M. (2007) *Circ Res* **101**, e32-42
41. Bisping, E., Ikeda, S., Kong, S. W., Tarnavski, O., Bodyak, N., McMullen, J. R., Rajagopal, S., Son, J. K., Ma, Q., Springer, Z., Kang, P. M., Izumo, S., and Pu, W. T. (2006) *Proc Natl Acad Sci U S A* **103**, 14471-14476
42. Smeets, P. J., de Vogel-van den Bosch, H. M., Willemsen, P. H., Stassen, A. P., Ayoubi, T., van der Vusse, G. J., and van Bilsen, M. (2008) *Physiol Genomics* **36**, 15-23
43. Hosack, D. A., Dennis, G., Jr., Sherman, B. T., Lane, H. C., and Lempicki, R. A. (2003) *Genome Biol* **4**, R70
44. Dennis, G., Jr., Sherman, B. T., Hosack, D. A., Yang, J., Gao, W., Lane, H. C., and Lempicki, R. A. (2003) *Genome Biol* **4**, P3
45. Parmentier, J. H., Smelcer, P., Pavicevic, Z., Basic, E., Idrizovic, A., Estes, A., and Malik, K. U. (2003) *Hypertension* **41**, 794-800
46. Godeny, M. D., and Sayeski, P. P. (2006) *Am J Physiol Cell Physiol* **291**, C1297-1307
47. Liao, Y., Asakura, M., Takashima, S., Ogai, A., Asano, Y., Shintani, Y., Minamino, T., Asanuma, H., Sanada, S., Kim, J., Kitamura, S., Tomoike, H., Hori, M., and Kitakaze, M. (2004) *Circulation* **110**, 692-699
48. Mirotsov, M., Dzau, V. J., Pratt, R. E., and Weinberg, E. O. (2006) *Physiol Genomics* **27**, 86-94

49. Fielitz, J., Kim, M. S., Shelton, J. M., Qi, X., Hill, J. A., Richardson, J. A., Bassel-Duby, R., and Olson, E. N. (2008) *Proc Natl Acad Sci U S A* **105**, 3059-3063
50. Larkin, J. E., Frank, B. C., Gaspard, R. M., Duka, I., Gavras, H., and Quackenbush, J. (2004) *Physiol Genomics* **18**, 152-166
51. Hayashi, M., and Lee, J. D. (2004) *Journal of molecular medicine (Berlin, Germany)* **82**, 800-808
52. Dhingra, R., Ho Nam, B., Benjamin, E. J., Wang, T. J., Larson, M. G., D'Agostino, R. B., Sr., Levy, D., and Vasan, R. S. (2005) *J Am Coll Cardiol* **45**, 685-689
53. Salles, G., Leocadio, S., Bloch, K., Nogueira, A. R., and Muxfeldt, E. (2005) *Hypertension* **46**, 1207-1212
54. Domenighetti, A. A., Boixel, C., Cefai, D., Abriel, H., and Pedrazzini, T. (2007) *J Mol Cell Cardiol* **42**, 63-70
55. Yamazaki, T., and Yazaki, Y. (1999) *J Hum Hypertens* **13 Suppl 1**, S43-47; discussion S49-50
56. Geng, J., Zhao, Z., Kang, W., Wang, W., Liu, G., Sun, Y., Zhang, Y., and Ge, Z. (2009) *Biochem Biophys Res Commun* **388**, 517-522
57. Zhao, Z., Geng, J., Ge, Z., Wang, W., Zhang, Y., and Kang, W. (2009) *Mol Cell Biochem* **322**, 171-178
58. Zhao, Z., Wang, W., Geng, J., Wang, L., Su, G., Zhang, Y., Ge, Z., and Kang, W. *J Cell Biochem* **109**, 653-662
59. Takeishi, Y., Huang, Q., Abe, J., Glassman, M., Che, W., Lee, J. D., Kawakatsu, H., Lawrence, E. G., Hoit, B. D., Berk, B. C., and Walsh, R. A. (2001) *J Mol Cell Cardiol* **33**, 1637-1648
60. Takeda, N., Manabe, I., Uchino, Y., Eguchi, K., Matsumoto, S., Nishimura, S., Shindo, T., Sano, M., Otsu, K., Snider, P., Conway, S. J., and Nagai, R. *J Clin Invest* **120**, 254-265
61. Kakkar, R., and Lee, R. T. *Circ Res* **106**, 47-57
62. Harris, D. M., Cohn, H. I., Pesant, S., and Eckhart, A. D. (2008) *Clin Sci (Lond)* **115**, 79-89
63. Filtz, T. M., Grubb, D. R., McLeod-Dryden, T. J., Luo, J., and Woodcock, E. A. (2009) *FASEB J* **23**, 3564-3570
64. Mende, U., Semsarian, C., Martins, D. C., Kagen, A., Duffy, C., Schoen, F. J., and Neer, E. J. (2001) *J Mol Cell Cardiol* **33**, 1477-1491
65. Ecartot-Laubriet, A., Assem, M., Poirson-Bichat, F., Moisan, M., Bernard, C., Lecour, S., Solary, E., Rochette, L., and Teyssier, J. R. (2002) *Biochim Biophys Acta* **1586**, 233-242
66. Zhan, Y., Brown, C., Maynard, E., Anshelevich, A., Ni, W., Ho, I. C., and Oettgen, P. (2005) *J Clin Invest* **115**, 2508-2516
67. Decock, J. B., Gillespie-Brown, J., Parker, P. J., Sugden, P. H., and Fuller, S. J. (1994) *FEBS Lett* **356**, 275-278
68. Wu, S. C., and Solaro, R. J. (2007) *J Biol Chem* **282**, 30691-30698
69. Borges, L., Bigarella, C. L., Baratti, M. O., Crosara-Alberto, D. P., Joazeiro, P. P., Franchini, K. G., Costa, F. F., and Saad, S. T. (2008) *Biochem Biophys Res Commun* **374**, 641-646
70. Moscat, J., Rennert, P., and Diaz-Meco, M. T. (2006) *Cell Death Differ* **13**, 702-711
71. Moscat, J., and Diaz-Meco, M. T. *Nat Immunol* **12**, 12-14
72. Sentex, E., Wang, X., Liu, X., Lukas, A., and Dhalla, N. S. (2006) *Can J Physiol Pharmacol* **84**, 227-238

FIGURE LEGENDS

Figure 1.- Stimulation of Gq-coupled GPCR by angiotensin II promotes ERK5 activation in cardiomyocytes in a PKC ζ -dependent way

A, Neonatal mouse primary cardiomyocytes cell cultures were incubated with 100 nM Angiotensin II for the indicated times, and endogenous ERK5 activation determined with an ERK5-antibody that recognizes both the phosphorylated (upper band) and un-phosphorylated (lower band) forms of ERK5 as detailed in Experimental Procedures. Data (mean \pm SEM of 3 independent experiments) were expressed as fold-activation compared to the absence of agonist. * $p < 0.05$, two tailed t-test when compared to basal conditions. *B,C*, Angiotensin II-induced ERK5 activation is abrogated upon PKC ζ down-regulation in neonatal mouse cardiomyocytes. *B*, Primary cardiomyocytes were treated with scrambled or PKC ζ -targeting siRNA oligos as detailed in Experimental Procedures, and then challenged with angiotensin 100 nm for the indicated times. ERK5 activation was determined in cell lysates with a phospho-ERK5 specific antibody. Along with PKC ζ expression, total ERK5 and alpha-tubulin levels were also determined as loading controls. *C*, Primary cultures of cardiomyocytes were obtained from wild-type (wt) or PKC ζ -deficient (PKC ζ ^{-/-}) mice as detailed in Experimental Procedures, and challenged with angiotensin 100 nM for different time periods. ERK5 activation was monitored and quantified by laser densitometry and data (mean \pm SE of 3 independent experiments, with activation in the absence of angiotensin taken as control condition) expressed as percentage of the phosphorylated kinase (p-ERK5) versus total ERK5. *D, E*, Angiotensin triggers ERK5 stimulation in adult murine cardiomyocytes. Primary cultures of adult mouse (*D*) or rat (*E*) cardiomyocytes were isolated as detailed in Experimental Procedures and challenged with Angiotensin 100 nM for 15 min. *E*, Adult rat cardiomyocytes were preincubated with vehicle or a specific PKC ζ pseudosubstrate inhibitor (10 μ M). ERK5 activation was detected as in panel *B*, and data normalized using GAPDH as loading control and expressed as fold-induction over basal conditions. Data are mean \pm SE of 3 independent experiments. * $p < 0.05$, two tailed t-test when compared to basal (*D*) or control (*E*) conditions. Representative blots are shown in all panels.

Figure 2.- PKC ζ is required for Angiotensin II-mediated stimulation of the ERK5 pathway in cardiac fibroblasts.

A, Neonatal mouse cardiac fibroblasts were challenged with angiotensin and ERK5 stimulation determined as in Figure 1B. Data (mean \pm SE of 3 independent experiments) were normalized using actin as a loading control, and expressed as fold-induction compared to the absence of agonist * $p < 0.05$, ** $p < 0.01$. *B,C*, Mouse (*B*) or rat (*C*) cardiac fibroblasts were preincubated with vehicle or with a myristoylated PKC ζ pseudosubstrate inhibitor (10 μ M) as detailed in Experimental Procedures, challenged with angiotensin 100 nM for the indicated times, and ERK5 activation determined as in panel *A*. Blots are representative of 2-3 experiments.

Figure 3.- Activation of MEK5 and MEF-2C upon chronic angiotensin administration is inhibited in PKC ζ -deficient mice.

PKC ζ -deficient mice (PKC ζ ^{-/-}) and matched wild-type (WT) control littermates were subjected to chronic angiotensin II infusion (or phosphate-buffered saline, PBS, as a control vehicle) for 14 days using osmotic minipumps as detailed in Experimental Procedures. The activation status of MEK5 and MEF2C was assessed by immunohistochemistry using phosphospecific antibodies. Brown colour indicates positive DAB staining. Signal intensity was quantified as detailed in Experimental Procedures, and data plotted below represents the average of 3 independent experiments. * $p < 0.05$, two tailed t-test when compared control with angiotensin II-treated wild-type mice.

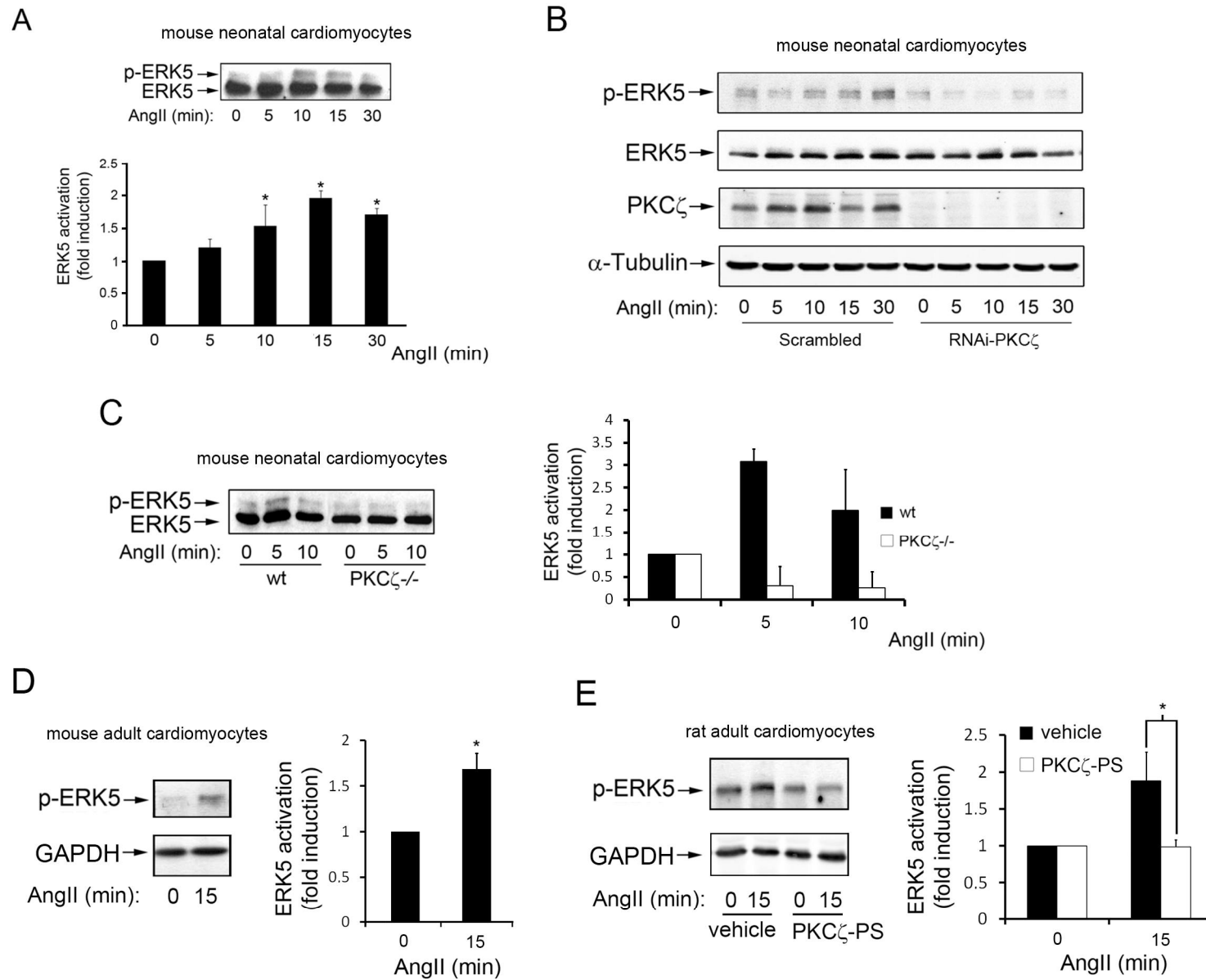
Figure 4.- Differential gene expression patterns between control conditions and cardiac hypertrophy induced by different methods in mouse models.

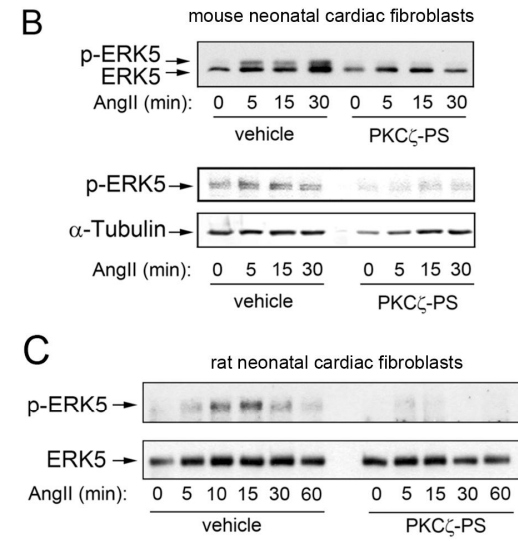
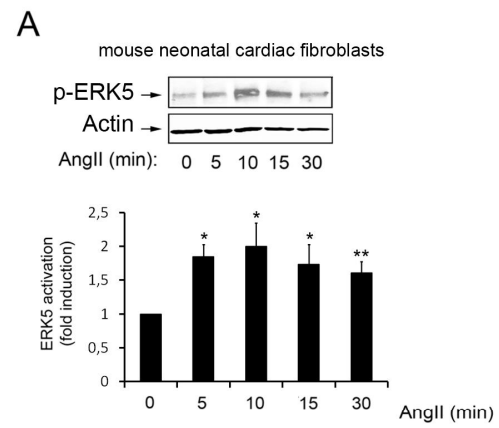
A, Global gene expression patterns in wild-type (WT) or PKC ζ -deficient (KO) mice chronically infused with angiotensin (AngII) or vehicle (PBS), were compared as indicated in Experimental Procedures section and in the text to those reported in three different transverse aortic constriction (TAC) experiments with their corresponding controls (SHAM), and in one group of mice over-expressing the AT1R versus non-transgenic controls. Groups of genes either over-expressed (Group I) or under-expressed (Group II) in hypertrophic versus control conditions were identified. Samples were grouped using hierarchical clustering analysis. Expression values are shown in colour code as log₂, mean centered per gene (mean=0, SD=1). B, Enrichment analysis of functions overrepresented in differentially expressed genes. Shown are Gene Ontology biological processes (GOBP) terms that are significantly enriched in Group I genes (red bars) and Group II genes (green bars), as assessed by using DAVID web tool. Significance is shown as $-\log_{10}$ of p-val.

Figure 5.- Alterations in gene expression related to the activation of the ERK5 signaling pathway differ in wild-type and PKC ζ -deficient mice. A, Microarray data were analyzed using Pathway Studio to identify changes in the expression of genes functionally related to ERK5. Several genes that are part of the ERK5 signaling network were found up-regulated (red color) in angiotensin II-treated mice compared to PBS-treated mice or angiotensin II-treated PKC ζ -deficient animals. Genes whose expression does not change are shown in grey. The interaction map shows reported positive (+) and negative (-) relationships between related proteins. *Mapk7* (ERK5), *jun* (c-jun), *Ets1* (ETS1), *myc* (c-Myc), *Nppa* (ANP, Atrial Natriuretic factor), *Nppb* (BNP, Brain type Natriuretic peptide), *Colla2* (collagen type I, alpha 2), *Ccna2* (cyclin A2), *Creb1* (CREB, cAMP responsive element binding protein 1), *Bcl2* (BCL2). B, Immunohistochemical analysis with a specific antibody shows the over-expression of Ets-1 in heart sections from angiotensin II-treated wild-type but not PKC ζ -deficient animals. Staining intensity was quantified and plotted as in Fig.3. C-E Quantitative RT-PCR analysis in heart tissue showing enhanced mRNA expression of *Nppa*, *Colla2*, *Mapk7* in angiotensin II-treated wild-type mice compared to angiotensin II-treated PKC ζ -/- mice. Data are mean \pm SE of 3 independent experiments. *p<0.05, **p<0.01, two tailed t-test when compared to untreated animals.

Figure 6.- Analysis of hypertrophic markers in wild-type and PKC ζ deficient mice upon chronic Angiotensin infusion.

PKC ζ -deficient mice (PKC ζ -/-) and matched wild-type (WT) control littermates were subjected to chronic angiotensin II infusion (or phosphate-buffered saline, PBS, as a control vehicle) for 14 days using osmotic minipumps as in previous figures. A, Circulating plasma levels of the hypertrophic marker atrial natriuretic peptide (ANP) were determined, as detailed in Experimental Procedures. Heart to body weight (B, left panel) or to tibia length ratios (B, right panel) were also calculated. C, *In vivo* electrocardiographic analysis indicates a significant increase in the duration of QRS and QT components (what has been shown to correlate with cardiac hypertrophy) in angiotensin versus PBS-treated wild-type animals. PBS-treated PKC ζ -deficient mice display parameters similar to PBS-treated wt mice, but angiotensin fails to promote an increase in QRS and QT components duration in this PKC ζ knockout model, as in wild-type animals. HR, heart rate; bpm, beats per min; ms, millisecond. Data in bar diagrams are expressed as mean \pm SE of 4 animals in each group. * p < 0.05, ***p<0.001 (two-tailed Student *t*-test) compared to PBS-treated animals.

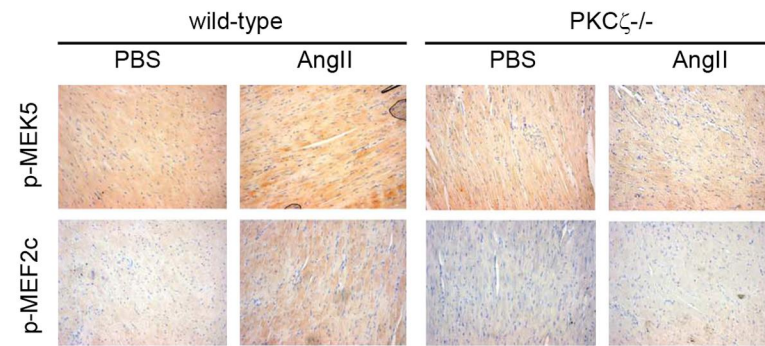




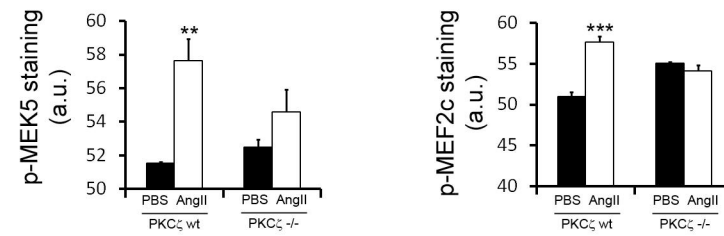
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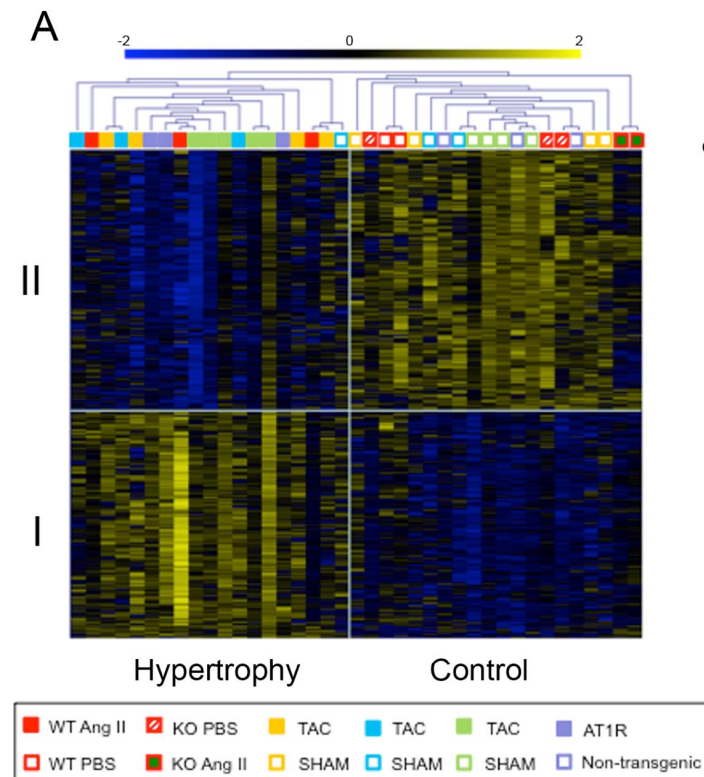
Figure 2

A



B





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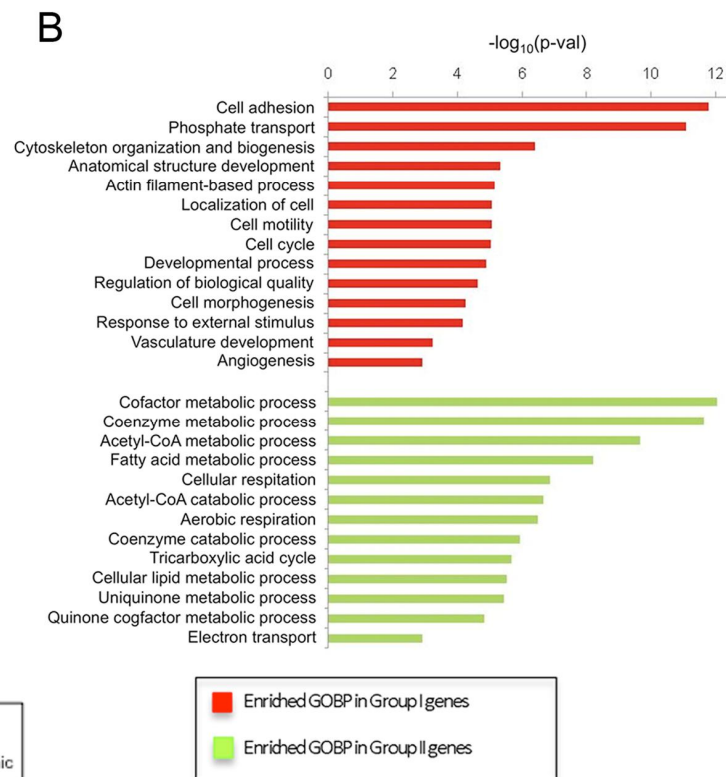
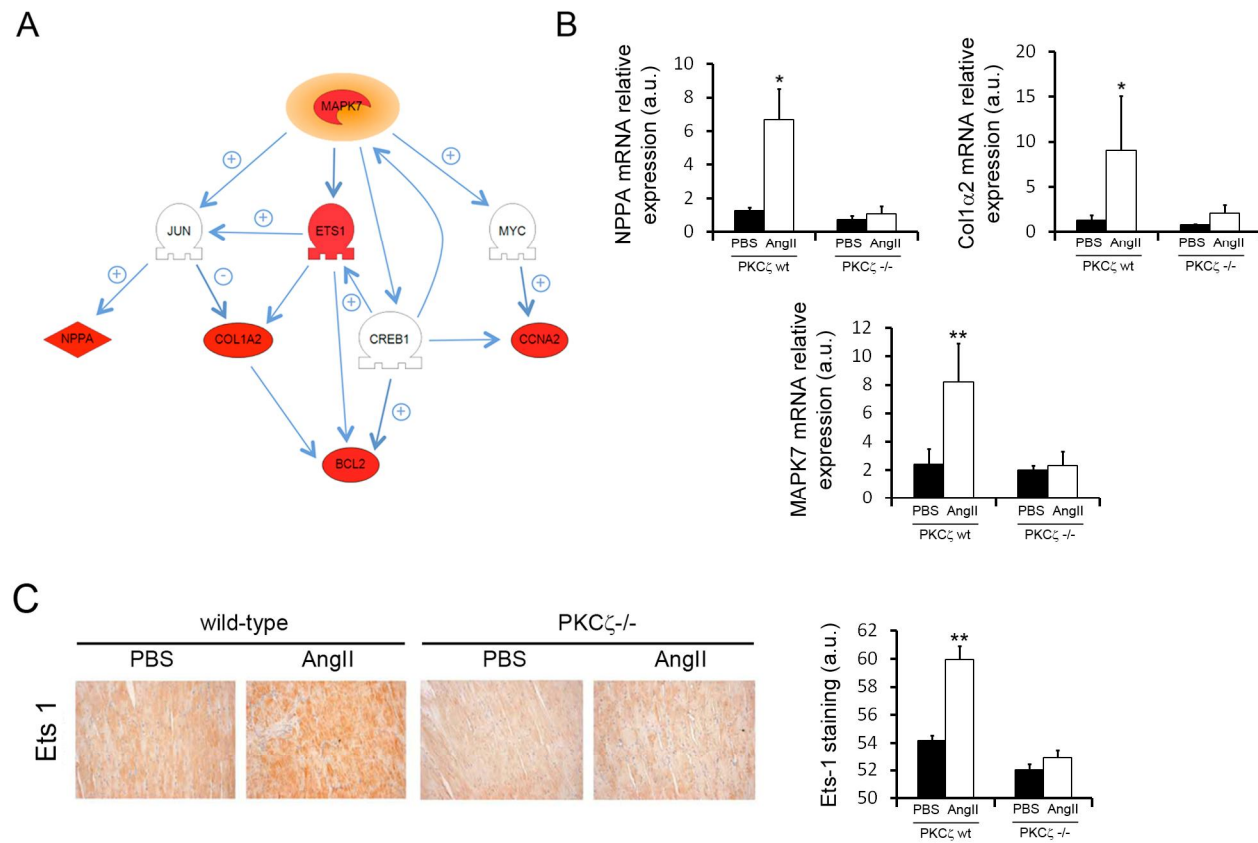
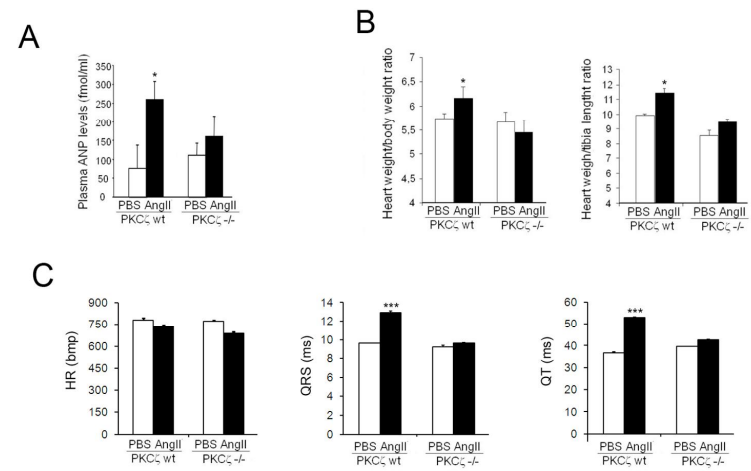


Figure 4





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Figure 6

