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# Murine cardiac growth, TRPC channels, and cGMP kinase I

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**Abstract** Signaling via cGMP-dependent protein kinase I (cGKI) and canonical transient receptor potential (TRPC) channels appears to be involved in the regulation of cardiac hypertrophy. Recent evidence suggests that TRPC channels are targets for cGKI, and phosphorylation of these channels may mediate the antihypertrophic effects of cGMP signaling. We tested this concept by investigating the role of cGMP/cGKI signaling on angiotensin II (A II)-induced cardiac hypertrophy using a control group (Ctr), *trpc*$6^{-/-}$, *trpc*$3^{-/-}$, *trpc*$3^{-/-}/6^{-/-}$, βRM mice, and *trpc*$3^{-/-}/6^{-/-}\times$βRM mice. βRM mice express cGKIβ only in the smooth muscle on a cGKI$^{-/-}$ background. The control group was composed of littermate mice that contained at least one wild type gene of the respective genotype. A II was infused by minipumps (7 days; 2 mg/kg/day) in Ctr, *trpc*$6^{-/-}$, *trpc*$3^{-/-}$, *trpc*$3^{-/-}/6^{-/-}$, βRM, and *trpc*$3^{-/-}/6^{-/-}\times$βRM mice. Hypertrophy was assessed by measuring heart weight per tibia length (HW/TL) and fibrosis by staining of heart slices. A II-induced increase in HW/TL and fibrosis was absent in *trpc*$3^{-/-}$ mice, whereas an increase in HW/TL and fibrosis was evident in Ctr and *trpc*$6^{-/-}$, minimal or absent in *trpc*$3^{-/-}$, moderate in βRM, and dramatic in *trpc*$3^{-/-}/6^{-/-}$ βRM mice. These results suggest that TRPC3 may be necessary for A II-induced cardiac hypertrophy. On the other hand, hypertrophy and fibrosis were massively increased in βRM mice on a TRPC3/6× cGKI$^{-/-}$KO background, indicating an "additive" coupling between both signaling pathways.

**Keywords** Cardiac myocytes · Endothelium/fibrocytes · Nitric oxide/PKG-I · Signal transduction · TRPC channels

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## Introduction

A number of publications pointed out that activation of canonical transient receptor potential (TRPC) channels is an essential part of angiotensin II (A II)-induced cardiac hypertrophy [2, 3, 17, 26, 30, 37]. TRPC3/6 channels have been identified in cardiomyocytes (CMs) [9] and cardiac myofibroblasts (MFBs) [34]. TRPC3/6 channels are a potential target for cGMP-dependent protein kinase I (cGKI) in the heart. Phosphorylation of the TRPC3 or TRPC6 channels by cGKI inhibits the opening of these channels, reduces $Ca^{2+}$ inflow, and stops the induction of genes associated with cardiac hypertrophy [15, 16, 25, 29, 32]. This cGKI-dependent effect has been invoked to be essential for the beneficial effects of ANP/BNP on cardiac hypertrophy. Recently, TRPC6 has been implicated as a necessary molecule in TGFβ-induced transformation from fibroblasts (FBs) to MFBs [6]. FBs express a number of TRP channels, namely TRPC3 [11], TRPC6 [6], TRPV4 [1], and TRPM7 [8]. We have investigated the involvement of TRPC3/6 channels in the A II-induced cardiac hypertrophy model in freely moving mice that were negative for TRPC3 (C3KO), TRPC6 (C6KO), TRPC3/TRPC6 (DKO), TRPC3/TRPC6/cGKI (TKOβRM), and cGKI (βRM).

## Experimental procedures

*Mice* All animals were maintained and bred in the animal facility of the Institut für Pharmakologie und Toxikologie, Technische Universität München, and had free access to tap water and standard chow. All procedures relating to animal care and treatment conformed to the institutional and governmental guidelines (Directive 2010/63/EU of the European Parliament) and were approved by local authorities (Regierung von Oberbayern). The following mouse lines were generated as described previously [19, 36] and used for the experiments: A control group (Ctr) that was composed of littermate mice containing at least one allele of the wild type genes, mice that express cGKIβ only in the smooth muscle on a cGKI$^{-/-}$ background (βRM [36]), mice lacking TRPC3 (C3KO mice [12]), mice lacking TRPC6 (C6KO mice [7]), mice lacking both TRPC3 and TRPC6 (DKO mice [19]), and mice lacking TRPC3 and TRPC6 on a βRM-cGKI$^{-/-}$ background [36] (TKOβRM mice). Male mice were used for the experiments at the age of 2 to 3 months and were sacrificed by cervical dislocation.

*Angiotensin II administration* Osmotic minipumps (Model 1007D; Alzet) were implanted subcutaneously to deliver A II (2 mg/kg/day) for 7 days. Anesthesia was induced (4 % isoflurane) and maintained (1.5 % isoflurane) by continuous oxygen/isoflurane inhalation. Animals that did not receive A II were sham-operated. Heart weight (HW), body weight (BW), and tibia length (TL) were determined as described [21, 27]. The change in BW during A II infusion was recorded.

*Cardiac histology and fibrosis* Paraffin-embedded mouse hearts were sectioned at 10-μm intervals. For fibrosis measurements, sections were stained with Sirius Red and Fast Green. Whole-section images were taken with a digital camera mounted on an optic microscope, and then the percentage of fibrosis was measured using software-assisted image analysis (MetaMorph).

*Statistical analysis* Results are exemplified by photos and presented as mean±SEM. Statistical comparisons of data sets were performed by ANOVA followed by Bonferroni post hoc test using Prism 5 (www.graphpad.com) or by Student's *t* test. ANOVA was used for comparison of more than two groups, whereas *t* test was used for comparison of two groups. Differences were considered significant at $p<0.05$.

## Results

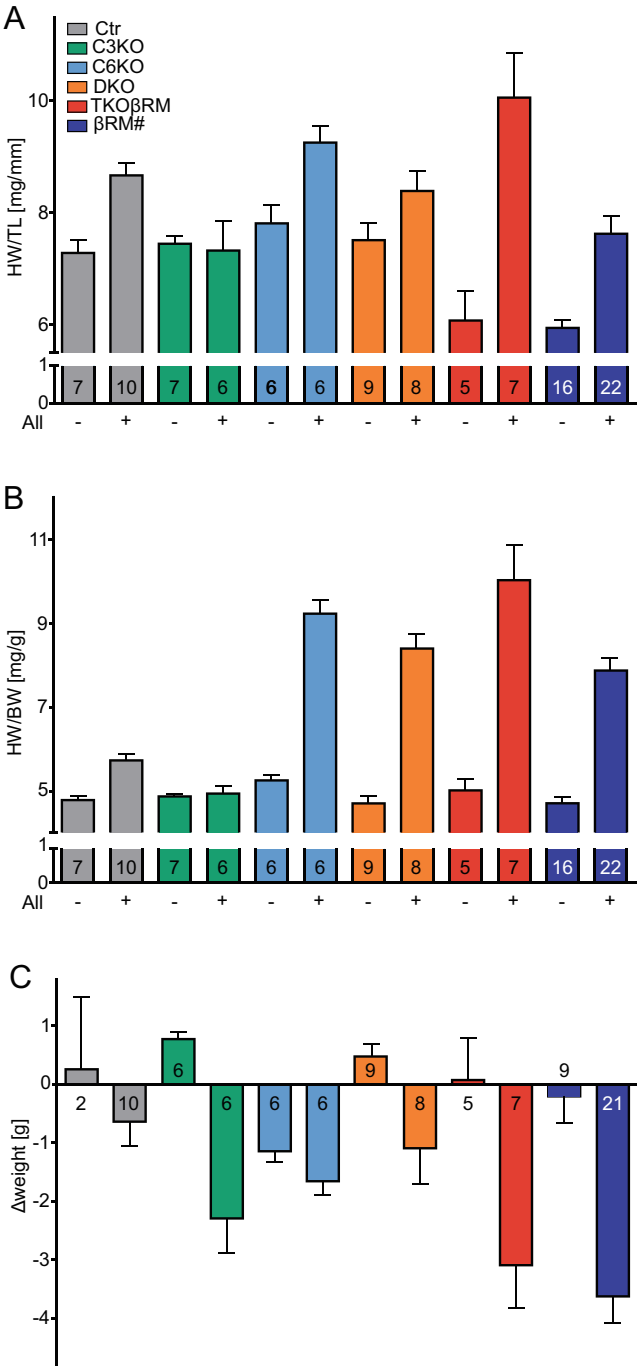
TRPC channels, especially the TRPC6 channel, have been proposed to play a central role in A II-induced cardiac hypertrophy [2, 3, 9, 17, 26, 30, 37]. In agreement with other groups, we have reported that infusion of A II (2 mg/kg/day) for 7 days significantly increased the ratio of heart weight/tibia length (HW/TL) in mice [27]. Figure 1 shows that A II increased the HW/TL ratio in the control (Ctr) group, TRPC6-KO (C6KO), βRM, and TKOβRM mice, while in the TRPC3-KO (C3KO) and in the double-KO TRPC3/TRPC6 (DKO), there was no significant increase, though DKO showed a tendency for an increased HW/TL ratio (Fig. 1a). C3KO showed no increase at all after A II infusion, neither for HW/TL index nor for fibrosis (Figs. 1a and 2), supporting a previous report that TRPC3 is involved in the induction of cardiac growth [2, 24]. The HW/TL ratio was largest in TKOβRM. For comparison, we included the data for βRM in a TRPC3/6-positive background [27]. It is evident from this comparison that deletion of cGKI and the TRPC3/6 channels removed two potential antihypertrophic factors, i.e., cGKI and one or both TRPC channels.

Figure 1b shows the HW/BW ratio of the same groups. This normalization confirms the above data and indicates that even the double deletion of TRPC3 and TRPC6 (DKO) leads to a significant A II-induced increase in the HW/BW ratio. However, we suggest to interpret the HW/BW data with caution because we observed a decrease in BW during the A II infusion (Fig. 1c). No such change was observed for TL [27]. Therefore, we think only the HW/TL ratio should be considered. We did extensive significance calculation of the data presented in Fig. 1a using *t* test for comparison of two groups and ANOVA for the comparison of more than two groups (Tables 1, 2, and 3). Table 1 shows that A II infusion increased significantly the HW/TL ratio in the Ctr, C6KO, TKOβRM, and βRM mice as revealed by the *t* test. The A II-induced HW/TL increase was significantly different between βRM and TKOβRM mice (Table 2), supporting the notion that deletion of the TRPC3/6 channels removed protein(s) with an antihypertrophic effect. This difference was also obtained by the ANOVA test (Table 3). The outcome of these tests strongly supports the notion that A II induced a significant increase in the HW/TL ratio, if the TRPC6 or the TRPC3/6 and the cGKI were deleted (see also Fig. 1a TKOβRM mice and Tables 1, 2, and 3).

Analysis of the hearts for fibrosis showed an increase in collagen fibers for all A II-treated animals with the exception of the TRPC3-KO mice (Fig. 2). These fibrosis data agree very well with the HW/TL ratio. However, the increase in fibrosis reached statistical significance only for the βRM, the double-KO TRPC3/6, and the TKOβRM mice.

## Discussion

The results of these experiments are a surprise. In agreement with a previous report [2, 24], deletion of the TRPC3 channel




◀ **Fig. 1** A II-induced cardiac growth. A II (+A II) (2 mg/kg/day) was infused for 7 days. Animals not infused with A II (−A II) were sham-operated. These animals had the same genetic background as the A II-infused mice. **a** Cardiac hypertrophy was measured as heart weight (*HW* in mg) per tibia length (*TL* in mm). **b** Cardiac hypertrophy was measured as heart weight (*HW* in mg) per body weight (*BW* in g). **c** Weight change during A II infusion. Abbreviations are as follows: *A II* mice infused with A II solution, *Ctr* control group of heterozygous littermate animals, *C3KO* negative for TRPC3, *C6KO* negative for TRPC6, *DKO* negative for TRPC3 and TRPC6, *TKOβRM* negative for TRPC3/6 and cGKI on a βRM background, *βRM* mice negative for cGKI except for expression of cGKIβ in the smooth muscle and positive for TRPC3/6 [27]. For better comparison, the results for βRM# mice are included. They are from [27]. Please note that the βRM mice are smaller than normal WT mice. As shown in [27], this is caused by the deletion of cGKI in the CNS, but these mice show the same cardiac hypertrophy as WT animals

completely prevented A II-induced cardiac hypertrophy and fibrosis. Deletion of TRPC6 alone resulted in a significant increase in the HW/TL ratio after A II. The increased ratio of HW/TL is reflected by an increased fibrosis. For DKO mice, there was a slight tendency to an increased HW/TL ratio and a significant increase in fibrosis after A II infusion. Considering previous publications, we expected the opposite outcome, i.e., no hypertrophy in the TRPC6-KO and a mild hypertrophy in the TKOβRM mice. Please note that the TRPC3- and TRPC6-KO mice were provided by the same laboratory to all research groups that reported on the effect of TRPC3 or TRPC6 deletion on cardiac hypertrophy. Therefore, it is impossible that the reported differences are caused by distinct gene modifications.

Previously, it was shown that deletion of the TRPC1 channel reduced A II-induced cardiac hypertrophy [31]. We may speculate that TRPC3 forms a heteromeric complex with TRPC1, explaining its effect on cardiac growth. However, Seth and coworkers did not report an alteration of the

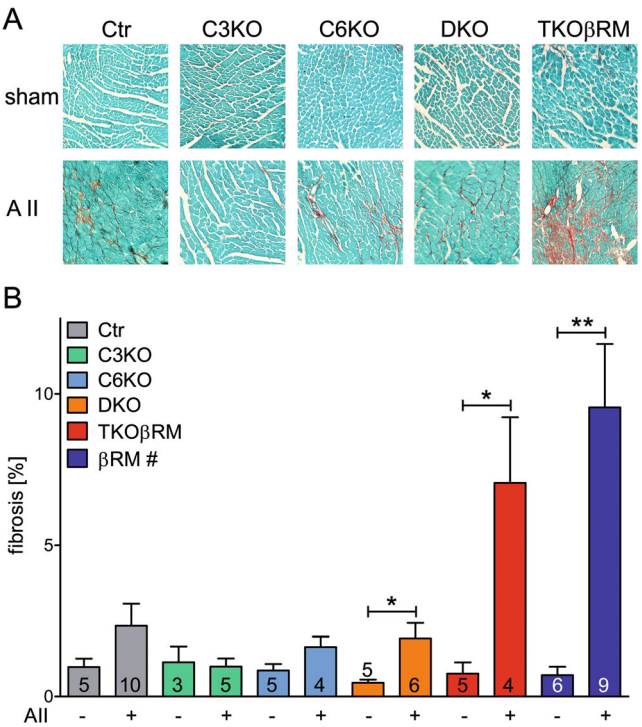



**Fig. 2** Left ventricular fibrosis. **a** Representative sections through the indicated left ventricle. Magnification is 20-fold. **b** Statistics for the indicated individual heart sections. The number of hearts analyzed is shown in the *columns*. Abbreviations and statistical analysis are as in Fig. 1. *$p<0.05$; **$p<0.01$. Representative staining and statistics for cardiac sections of βRM mice have been published in [27]

**Table 1** Significance calculation for results shown in Fig. 1a (HW/TL)

| | Ctr | | C3KO | | C6KO | | DKO | | TKOβRM | | βRM | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A II | − | + | − | + | − | + | − | + | − | + | − | + |
| *p* value | 0.0015 | | 0.8217 | | 0.0119 | | 0.0812 | | 0.0037 | | 0.0003 | |
| | ** | | n.s. | | * | | n.s. | | ** | | *** | |

Results of *t* test. Values obtained±A II are compared in each group. Significance level was set as follows: *<0.05; **<0.01; ***<0.001

TRPC3 protein concentration during TAC-induced hypertrophy in wild type or TRPC1$^{-/-}$ mice [31]. TRPC3/C6 deletion in the absence of cGKI as present in the TKOβRM mice increased dramatically heart weight and fibrosis. This increase in the HW/TL ratio was significantly higher than for βRM mice that have a TRPC3/6-positive background, suggesting an "additive" coupling between both signaling pathways. Furthermore, one or both of these two TRPC channels might have an antihypertrophic impact on A II-induced cardiac hypertrophy.

The necessity of TRPC3 for an increased HW/TL ratio, however, seems to be weak because deletion of both TRPC6 and TRPC3 increased cardiac weight and fibrosis. This was also observed for TRPC6-KO only. Further work needs to be done to nail down the potential involvement of TRPC3 channels in the development of cardiac hypertrophy. The results for TKOβRM mice also imply that there is an interaction between cGKI and TRPC3/6 during cardiac growth because the cardiac phenotype of the TKOβRM mice was even more pronounced than that in the βRM mice with intact TRPC3 and 6 channels. These results do not exclude the notion that cGKI may phosphorylate TRPC3 or TRPC6, decreases the influx of $Na^+$/$Ca^{2+}$, and inhibits thereby cardiac hypertrophy under different conditions. The difference between the βRM and the TKOβRM mice suggests that the channel(s) and cGKI interact in the heart to reduce A II-induced cardiac growth. Please note that the βRM mice lack cGKI in all cells with the exception of smooth muscle cells but have unaltered *trpc*3 and *trpc*6 genes. Since A II induced less hypertrophy in βRM mice, we may speculate that TRPC3/6 channels—that are not phosphorylated by cGKI—may ameliorate A II-induced cardiac growth.

Completely unclear is the cell type in which these proteins interact in such a way that cardiac growth is reduced. Previous work raised the possibility that an essential part of the cGMP system, PDE5, may not be present in CMs [21, 27]. On the other hand, a physiologically significant interaction between TRPC6 and cGKI has been identified in endothelial cells [19]. These cells produce CNP, an antihypertrophic factor, which may be regulated by cGKI and TRPC channel activity [20, 35]. An alternative cell type is MFBs that express soluble guanylyl cyclase, PDE5, cGKI, and TRPC channels in wild type cells and produce the extracellular matrix, i.e., fibrosis. cGKI has been shown to reduce fibrosis in wild type hearts [27].

The presented results are partially in contrast to the previously reported effects of TRPC channels in the heart. However, most of the previous results suggesting a positive involvement of TRPC channels in cardiac hypertrophy did not rely on whole animal experiments. Thus, these results raise concern about the proposed involvement of TRPC6 as a necessary component of A II-induced cardiac hypertrophy [9, 16, 25]. As reported previously [16, 27, 32, 33], hypertrophy of Ctr mice was affected by cardiac cGKI, but in contrast to these reports [15, 16, 25, 32], cGKI did not ameliorate hypertrophy through phosphorylation of TRPC3 or TRPC6 channels because the hypertrophic response was largest in the absence of these channels and cGKI, i.e., in the TKOβRM mice. Therefore, it remains to be established which molecular target is modified by cGKI during A II-induced cardiac

**Table 2** Significance calculation for results shown in Fig. 1a (HW/TL)

| | Ctr A II | C3KO A II | C6KO A II | DKO A II | TKOβRM A II | βRM A II |
|---|---|---|---|---|---|---|
| *p* (Ctr A II) | – | 0.0206* | 0.1752 n.s. | 0.5255 n.s. | 0.0760 n.s. | 0.0557 n.s. |
| *p* (C3KO A II) | 0.0206* | – | 0.0108* | 0.1169 n.s. | 0.0195* | 0.6960 n.s. |
| *p* (C6KO A II) | 0.1752 n.s. | 0.0108* | – | 0.1205 n.s. | 0.4003 n.s. | 0.0224* |
| *p* (DKO A II) | 0.5255 n.s. | 0.1169 n.s. | 0.1205 n.s. | – | 0.0711 n.s. | 0.2114 n.s. |
| *p* (TKOβRM A II) | 0.0760 n.s. | 0.0195* | 0.4003 n.s. | 0.0711 n.s. | – | 0.0029** |
| *p* (βRM A II) | 0.0557 n.s. | 0.6960 n.s. | 0.0224* | 0.2114 n.s. | 0.0029** | – |

Results of *t* test. Values obtained+A II are compared between the different groups. Significance level was set as follows: *<0.05; **<0.01

**Table 3** Results for multiple testing with ANOVA followed by Bonferroni's multiple comparison test

| | | | |
|---|---|---|---|
| One-way analysis of variance | | | |
| *p* value | 0.0017 | | |
| *p* value summary | ** | | |
| Are means significantly different? ($p<0.05$) | Yes | | |
| Number of groups | 6 | | |
| *F* | 4505 | | |
| *R* squared | 0.2983 | | |
| Bonferroni's multiple comparison test | Mean difference | *t* | Summary |
| C3KO+A II vs TKOβRM+A II | −2705 | 3485 | * |
| TKOβRM+A II vs βRM+A II | 2428 | 4011 | ** |

Comparison was done for animal groups infused with A II (+A II). Please note that only significant differences are shown. As is evident from Fig. 1a, ANOVA did not reveal differences between the groups in the absence of A II with the exception of the values obtained for the βRM and TKOβRM mice (−A II) versus all other groups. Significance level was set as follows: *<0.05; **<0.01

growth. A number of cGKI targets alternative to TRPCs have been reported for cardiomyocytes, among them the L-type calcium channel [22, 28], $Na^+/H^+$ exchanger [14], transmission of cardioprotective signals from the cytosol to the mitochondria [4], decreased apoptosis in the presence of enhanced nuclear accumulation of zyxin and Akt [13], decreased apoptosis by interference with the TAB1-p38 mitogen-activated protein kinase pathway [10], decreased necrosis and apoptosis after ischemia/reoxygenation [5, 23], PDE 5 [38], and interruption of profibrotic TGF signaling by cGK-dependent phosphorylation of Smad-3 in MFBs [18]. At present, it is unclear whether any of these diverse mechanisms is involved in the antihypertrophic effect of cGMP in the intact animal.

At the end, we would like to add a note of caution. The background of most mice was C57/Bl6. We have tested the A II-induced hypertrophy in Sv129 and C57/BL6 mice. No difference was recorded (unpublished results, but provided to the reviewers). Although the TRPC3- and TRPC6-KO mice were provided from the same laboratory, we have not ruled out completely that distinct results will be obtained with a different background as used by other groups. If this suggestion is correct, it implies that we need to identify the additional factors that prevent or allow the association of the TRPC3/6 channels with A II-induced cardiac hypertrophy. This possibility implies further that TRPC3/6 is not the mono- or digenetic cause of A II-induced hypertrophy. The question remains: Can we generalize the previous findings [3, 16, 17, 29] if unidentified factors are necessary components of this signaling pathway?

Recent data of one of us show that deletion of all TRPC1–7 channels does not preclude birth of apparently normal pups. Thus, we would like to propose that TRPC channels are important proteins kept through evolution but that their precise necessity for biology and/or pathophysiology needs quite a bit of future research.

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**Conflict of interest** None declared

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