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Inhibition of ADP-ribosyl cyclase attenuates angiotensin II-induced cardiac hypertrophy

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Aims Here, we report the discovery of a small molecule inhibitor, 2,2′-dihydroxyazobenzene (DAB), of ADP ribosyl cyclase (ADPR-cyclase) and showed that this inhibitor attenuated angiotensin (Ang) II-induced hypertrophic responses.

Methods and results The intracellular concentration of free Ca^{2+} $[Ca^{2+}]_i$ in adult rat cardiomyocytes was measured by using a confocal microscope. Cardiac hypertrophy was induced by the two-kidney one-clip (2K1C) method. Hypertrophy was determined by *de novo* protein synthesis, cell volume, echocardiography, nuclear translocation of nuclear factor of activated T-cells, and transforming growth factor-β1 protein expression. Treatment of cardiomyocytes with Ang II generated a biphasic $[Ca^{2+}]_i$ increase that included an initial Ca^{2+}peak and sustained Ca^{2+} rise via inositol trisphosphate and cyclic ADP-ribose (cADPR) formation, respectively. A cADPR antagonistic analogue, 8-Br-cADPR, and an ADPR-cyclase inhibitor, DAB, blocked the sustained Ca^{2+} signal, but not the initial Ca^{2+} rise. Furthermore, DAB significantly inhibited Ang II-mediated cADPR formation and hypertrophic responses *in vitro*. Echocardiography and histological examination revealed significant cardiac hypertrophy in 2K1C rats that was potently inhibited by treatment with DAB. In addition, the hypertrophic responses induced by Ang II *in vitro* were significantly increased by 2K1C, and DAB treatment reversed these hypertrophic responses to the levels of sham Control.

Conclusion ADPR-cyclase is an important mediator of cardiac hypertrophy, and inhibition of ADPR-cyclase by DAB may provide a new therapeutic strategy for cardiac diseases.

1. Introduction

ADP ribosyl cyclase (ADPR-cyclase) produces a Ca^{2+} mobilizing second messenger, cyclic ADP-ribose (cADPR), from β-NAD^+.[1] A prototype of mammalian ADPR-cyclases is a lymphocyte antigen CD38, which possesses ADPR-cyclase and cADPR hydrolase enzyme activities involved in the synthesis and degradation of cADPR, in a variety of cells.[2] Mounting evidence indicated that CD38/cADPR signalling plays a pathophysiological role in diabetes,[3,4] airway hyperresponsiveness,[5] and autism.[6] These conditions could result from changes in CD38 expression and/or the enzymatic activities associated with CD38, leading to alterations in the production of cADPR and calcium homeostasis. Studies with $CD38^{-/-}$ mice suggested the existence of other ADPR-cyclase(s), since cADPR content of $CD38^{-/-}$ tissues such as kidney, brain, or heart was nearly the same as that of the wild-type tissues.[7] Reducing agent-insensitive and Zn^{2+}-sensitive ADPR-cyclase(s), which are characteristically different from CD38, are expressed in smooth muscle cells, brain, kidney, and heart.[1,8–10] Moreover, our recent study revealed that Ang II-stimulated Ca^{2+} signals were not significantly different between $CD38^{-/-}$ and $CD38^{+/+}$ cardiomyocytes, and that 8-Br-cADPR completely inhibited the Ang II-induced sustained Ca^{2+}increase indicating that cADPR is generated by a novel ADPR-cyclase, but not CD38.[11]

Among the regulators of cardiac growth, the renin–angiotensin system (RAS) appears to play an important role in the maintenance of blood pressure, fluid, and sodium homeostasis through the activity of the vasoactive peptide angiotensin (Ang) II.[12] It is well known that increased circulating Ang II levels can lead to short-term vascular

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effects such as contraction, and to long-term biological effects such as cell growth, migration, extracellular matrix deposition, and inflammation.[13] The effects of Ang II are mainly mediated by its binding directly to G_q protein-coupled Ang II type 1 receptor (AT1R) that in turn leads to the sequential activation of Src, phosphatidylinositol-3-kinase (PI3K)/Akt, phospholipase C (PLC)-γ1 and inositol triphosphate (IP_3)-mediated Ca^{2+} release, resulting in activation of ADPR-cyclase and cADPR formation.[11,14] cADPR induces sustained Ca^{2+} increase by Ca^{2+} influx and activation of ryanodine receptor via Ca^{2+}-induced Ca^{2+} release.[11,15] The release of Ca^{2+} from intracellular stores and sarcoplasmic reticulum is one of the key signal transduction mechanisms in the regulation of numerous cellular functions, including contractility, protein synthesis and turnover, hormone secretion, and proliferation.[14,16] Dipp and Evans[17] demonstrated that increased cADPR levels in the pulmonary vascular smooth muscle led to increased $[Ca^{2+}]_i$ (intracellular concentration of free Ca^{2+}) levels and vasoconstriction, that were completely blocked *in situ*, by an antagonist of cADPR, 8-Br-cADPR, and Rakovic *et al*.[18] showed that microinjection of another cADPR antagonist, 8-amino-cADPR, into the cardiomyocyte cytosol reduced cardiac muscle contraction and the $[Ca^{2+}]_i$ peak. Moreover, recent studies have shown that Ang II-mediated $[Ca^{2+}]_i$ increase via the activation of ADPR-cyclase triggered calcineurin, which dephosphorylates NFAT3 (nuclear factor of activated T-cells) to promote cardiac hypertrophy[11,19] and cell proliferation,[14] and that these effects were completely reversed by the use of an antagonist of cADPR, 8-Br-cADPR, *in vitro*. These studies suggest that ADPR-cyclase is indispensable for Ang II-induced hypertrophic responses and inhibition of ADPR-cyclase may selectively block Ca^{2+} increase and hypertrophy in response to Ang II.

Studies have shown that polyphenols are potent antioxidants and treatment with the polyphenolic compounds inhibits cardiac hypertrophy,[20] proliferation in murine mesangial cells (MMCs),[21] and vascular smooth muscle cells.[22] In our previous studies, we have demonstrated that a bisphenyl compound, 4,4′-dihydroxyazobenzene (DHAB) inhibits proliferation in MMCs through its inhibitory effects on Ang II-induced kidney ADPR-cyclase activation.[9,14] In this study, we have shown that a structural analogue of DHAB, 2,2′-dihydroxyazobenzene (DAB) inhibits Ang II-induced heart ADPR-cyclase activition and thereby attenuates cardiac hypertrophy. To elucidate the role of ADPR-cyclase in Ang II-induced cardiac hypertrophy *in vivo*, we tested the effects of DAB on a renin Ang-dependent model of hypertension, 2K1C (two-kidney one-clip), in which constriction of the renal artery causes an increase in the Ang II levels via the activation of RAS leading to the development of hypertension and consequently cardiac hypertrophy.[16] Treatment with DAB significantly reversed the hypertrophic responses in 2K1C rats to the sham Control levels. These results clearly indicate that ADPR-cyclase is an important effector in the pathway in which Ang II signalling leads to hypertension and cardiac hypertrophy.

2. Methods

2.1 Animals

Sprague–Dawley male rats were obtained from Orientbio Inc.; (Seoungnam, Korea). Animals were housed in a 12 h light–dark schedule with food and water *ad libitum*. All studies conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Care and Use Committee of the Chonbuk National University Medical School.

2.2 Preparation of cardiomyocytes

Cardiomyocytes were isolated from (200–220 g) Sprague–Dawley male rats by enzymatic digestion as described previously.[10,11] See Supplementary methods online for further details.

2.3 Measurement of intracellular concentration of free Ca^{2+}

$[Ca^{2+}]_i$ in quiescent cardiomyocytes was measured as described previously.[14,15] See Supplementary methods online for further details.

2.4 Measurement of ADP ribosyl cyclase activity and cyclic ADP-ribose concentration

For ADPR-cyclase activation assay, samples were incubated with 200 μM nicotinamide guanine dinucleotide in 0.1 M sodium phosphate buffer (pH 7.2) at 37°C for 10 min. Fluorescence of cGDPR produced was determined at excitation/emission wavelengths of 297/410 nm (Hitachi F-2000).[10] Cyclic enzymatic assay was used to measure cADPR level as described previously.[15,23] Fluorescence was measured at excitation/emission wavelength of 544/590 nm using a fluorescence plate reader (Molecular Devices Corp., SpectraMax GEMINI).

2.5 Surgical preparation

Renovascular hypertension was produced by 2K1C.[16] Sprague–Dawley male rats (7–9 weeks old and weighing 200–220 g) were anaesthetized with ketamine (100 mg/kg, intraperitoneally) and rumpen (5 mg/kg, intraperitoneally). The left kidney was exposed through the median abdominal incision, and the renal artery was separated from the renal vein with caution. Then, a silver clip with 0.15 mm slit was placed around the renal artery. The sham procedure was performed, including the entire surgery with an exception of arterial clipping. To examine the effect of DAB (Sigma-Aldrich; St. Louis, MO, USA) in the 2K1C model, we injected DAB intraperitoneally with a dose of 1.5 μL/g body weight (428 μg/200 g/day) at days 7 after the surgery for 7 weeks. Sham group and Control 2K1C received vehicle treatment. The schematic diagram of the different number of animal used is each experiment is shown in *Table 1*.

2.6 Physiological studies

Systolic blood pressure was measured by using tail plethysmography in conscious rats once a week, from the day prior to surgery until the day of sacrifice. Left ventricular (LV) dimensions were assessed by echocardiography using a GE Vivid 4 ultrasound machine (GE Medical Systems, Waukesha, WI, USA) equipped with a 13 MHz

Table 1 Experimental design

Group	Number of LV samples used in western blot analysis	Number of LV samples used in ADPR-cyclase/cADPR measurement	Number of animals used in haemodynamic analysis
Sham	5	5	15
2K1C	6	6	10
Sham + DAB	5	5	15
2K1C + DAB	6	6	14

phase array linear transducer. M-mode images were used for measurement of wall thickness, chamber dimension, and fractional shortening and ejection fraction (EF) in anaesthetized rats. EF provides a global assessment of LV systolic performance and can be estimated by the following equation.

$$EF(\%) = \left\{ \frac{[\text{Left ventricular end-diastolic diameter (LVEDd)}^2 - \text{Left ventricular end-systolic diameter (LVESd)}^2]}{\text{LVEDd}^2} \right\} \times 100$$

Fractional shortening (FS) is the percentage of change in the LV cavity dimension with systole. It can be calculated from the equation.

$$\text{FS}\ (\%) = \frac{(\text{LVEDd} - \text{LVESd})}{\text{LVEDd}} \times 100$$

LV mass was calculated by American Society of Echocardiography method.

$$\text{Left ventricular mass} = 1.04[(\text{Left ventricular end-diastolic diameter} + \text{Posterior wall thickness} + \text{Interventricular thickness})^3 - \text{Left ventricular end-diastolic diameter}^3] \times 0.8 + 0.6$$

2.7 Angiotensin II plasma levels

Ang II levels from ethylene diamine tetraacetic acid (EDTA) plasma were determined with a commercially available radioimmunoassay (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) according to manufacturer's protocol.

2.8 Immunoblotting

Protein extraction and immunoblotting were performed as previously described.[15] See Supplementary methods online for further details.

2.9 [^{3}H]leucine incorporation

Cardiomyocytes were incubated with 150 nM Ang II in the presence or absence of inhibitors for 24 h. See Supplementary methods online for further details.

2.10 Histological analysis

Heart sections were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned at 6 μm thickness, and stained with haematoxylin and eosin for overall morphology. See Supplementary methods online for further details.

2.11 Statistical analysis

Data represent means ± SEM of at least three separate experiments. Differences in the multiple groups were compared by analysis of variance followed by Bonferroni's multiple comparison test. Two-group analysis was performed by Student's *t*-test (paired or unpaired as appropriate). A value of $P < 0.05$ was considered significant.

3. Results

3.1 2,2′-Dihydroxyazobenzene inhibits angiotensin II-induced sustained increase of intracellular concentration of free Ca^{2+} in cardiomyocytes

Treatment of cardiomyocytes with Ang II induced a long-lasting increase in $[Ca^{2+}]_i$, consisting of an initial Ca^{2+} rise followed by a sustained Ca^{2+} rise (*Figure 1A*). Ang II-induced sustained Ca^{2+} rise was completely blocked by pretreatment of the cells with 8-Br-cADPR (*Figure 1F*), indicating that sustained Ca^{2+} rise is generated by cADPR/ADPR-cyclase. On the basis of these results, we evaluated the dose-dependent effects of DAB (*Figure 1B–E*) on Ang II-induced Ca^{2+} signal and compared its effects with kidney-specific ADPR-cyclase inhibitor, DHAB, previously discovered (*Figure 1G–J*). DAB (1 μM) blocked Ang II-induced sustained Ca^{2+} increase, but not the initial rise of $[Ca^{2+}]_i$, to an extent similar to that of 8-Br-cADPR (*Figure 1E*), whereas DHAB showed complete inhibition of sustained $[Ca^{2+}]_i$ increase at a minimal dose of 30 μM (*Figure 1J*). These results indicate that DAB inhibits Ang II-induced sustained Ca^{2+}increase in cardiomyocytes at a dose lower than that of DHAB.

3.2 2,2′-Dihydroxyazobenzene inhibits angiotensin II-induced increase in ADP ribosyl cyclase activity and cyclic ADP-ribose formation in cardiomyocytes

To further confirm the previous observations, the effects of DAB and DHAB on Ang II-induced ADPR-cyclase activity and cADPR formation were evaluated. Treatment of cardiomyocytes with different concentrations of Ang II showed a dose-dependent increase in cADPR formation and ADPR-cyclase activity, (*Figure 2A* and *B*) with a maximal effect occurring at 150 nM. In order to ascertain whether DAB is the real inhibitor of heart ADPR-cyclase, we selected a concentration of 150 nM Ang II for our study, because this concentration showed a significant increase in ADPR-cyclase activity and cADPR formation. DAB significantly inhibited Ang II-induced ADPR-cyclase activity as well as cADPR production at 1 μM concentration, whereas DHAB at 30 μM concentration produced similar extent of effects on ADPR-cyclase activity and cADPR production (*Figure 2C* and *D*). These data together indicate that DAB blocks Ang II-induced ADPR-cyclase activation, and that the efficacy of DAB to inhibit Ang II-induced ADPR-cyclase activation is higher than that of DHAB in cardiomyocytes.

3.3 Angiotensin II-induced Src phosphorylation and phosphatidylinositol-3-kinase/Akt activation are unaffected by 2,2′-dihydroxyazobenzene in cardiomyocytes

We have previously shown that Ang II-induced ADPR-cyclase involves the upstream activation of Src and PI3K/Akt.[11] To corroborate, we tested the effects of DAB on Ang II-induced Src and PI3K/Akt activation. Ang II-mediated Src activation was determined by immunoblotting using anti-phospho-Src Tyr416 (p-Src) antibody. Ang II treatment significantly increased the levels of p-Src that was significantly reduced by pretreatment with a selective Src inhibitor, PP2 (10 μM), whereas, unaffected by DAB pretreatment (see Supplementary material online, *Figure S1A*). Similarly, the effect of DAB on Ang II-induced PI3K/Akt activation was evaluated by immunoblotting using anti-phospho-Akt Ser473 (p-Akt) antibody. Ang II-induced Akt phosphorylation was significantly inhibited by pretreatment with a PI3K inhibitor, wortmannin (100 nM), however, DAB did not alter the effects of Ang II on Akt phosphorylation (see Supplementary material online, *Figure S1B*). Taken together, these data indicate that ADPR-cyclase is activated at the downstream of Src and PI3K/Akt.

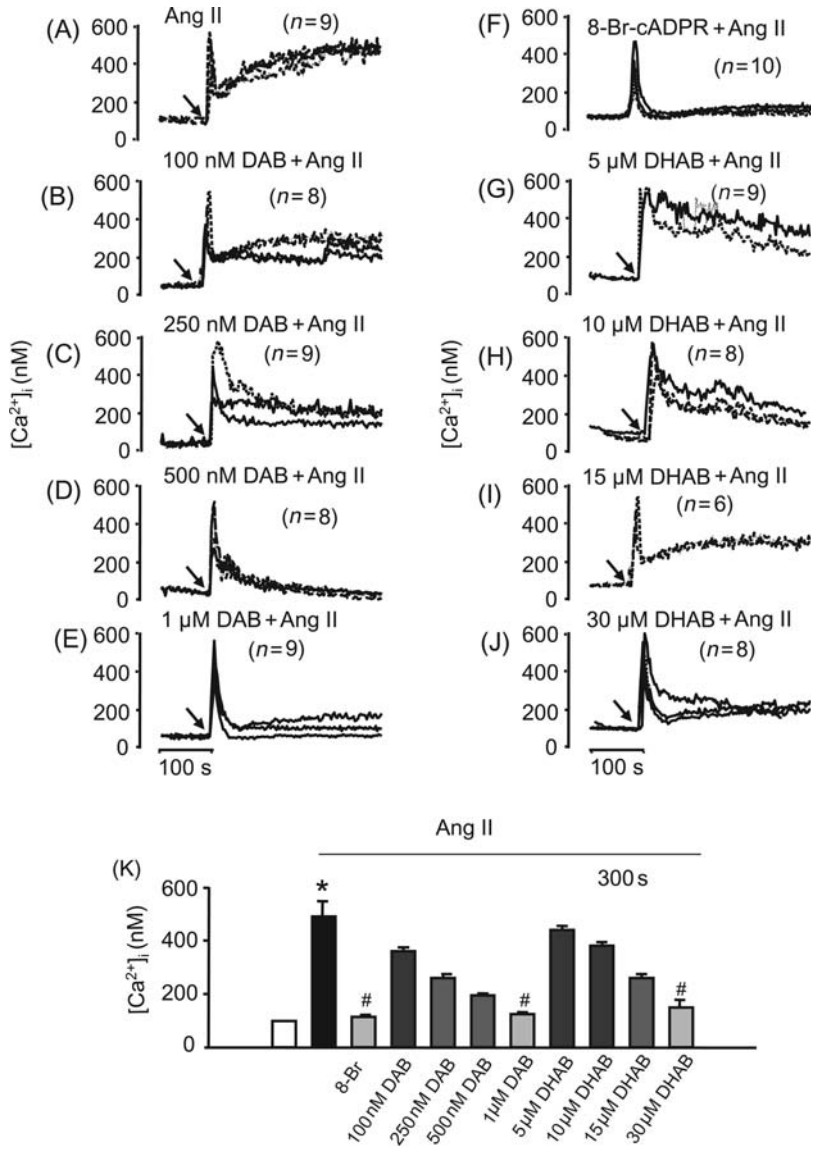

Figure 1 Effect of 2,2′-dihydroxyazobenzene (DAB) and 4,4′-dihydroxyazobenzene (DHAB) on angiotensin (Ang) II-induced $[Ca^{2+}]_i$ (intracellular concentration of free Ca^{2+}) increase in adult rat cardiomyocytes. (*A*) Representative tracings of Ca^{2+} response to 150 nM Ang II. (*F*) Typical response to Ang II after pretreatment with 100 μM 8-Br-cADPR (cyclic ADP-ribose). (*B–E*) Typical response to Ang II after pretreatment with 0.1, 0.25, 0.5, and 1 μM DAB. (*G–J*) Typical response to Ang II after pretreatment with 5, 10, 15, and 30 μM DHAB. (*K*) A direct comparison of mean $[Ca^{2+}]_i$ during sustained increases of $[Ca^{2+}]_i$. The data shown are analysed at 300 s. Cardiomyocytes were treated with indicated concentrations of blockers and incubated at 37°C for 30 min prior to Ang II treatment. $^*P < 0.01$ vs. Control, $^{\#}P < 0.01$ vs. Ang II. Results are mean ± SEM of three independent experiments.

3.4 2,2′-Dihydroxyazobenzene administration prevents angiotensin II-induced cardiomyocyte hypertrophy *in vitro*

Previous studies have shown that Ang II-induced Ca^{2+} increase is involved in cardiac hypertrophy. To study the possible role of ADPR-cyclase in the development of cardiac hypertrophy, factors that have been known to be involved in cardiac hypertrophy were evaluated. The principal downstream effector of Ca^{2+} increase in heart is calcineurin that induces nuclear translocation of NFAT3, which in turn up-regulates cardiac-specific gene expression to induce cardiac hypertrophy.[19] Therefore, to clarify whether ADPR-cyclase inhibition influences calcineurin-NFAT-dependent hypertrophy, we assessed the effects of calcineurin inhibitor, cyclosporin A, 8-Br-cADPR, or DAB on Ang II-induced NFAT3 translocation. As shown in *Figure 3A*, treatment of cardiomyocytes with Ang II increased NFAT3 nuclear translocation, however, pretreatment with cyclosporin A, 8-Br-cADPR, or DAB significantly suppressed the Ang II-induced NFAT3 nuclear translocation. These results indicate that Ang II-induced NFAT3 nuclear translocation is dependent upon ADPR-cyclase activation, which activate calcineurin to induce NFAT3 nuclear translocation via cADPR-mediated sustained Ca^{2+} increase.

Next, we tested the involvement of ADPR-cyclase in the development of Ang II-induced cardiac hypertrophy by determining [^{3}H]leucine incorporation. Thus, cardiomyocytes were pretreated with cyclosporin A, DAB or 8-Br-cADPR for 30 min prior to stimulation with 150 nM Ang II, and [^{3}H]leucine incorporation was determined after 48 h. As expected, Ang II induced a strong increase in [^{3}H]leucine incorporation, however, pretreatment with cyclosporin A, DAB or 8-Br-cADPR markedly reduced Ang

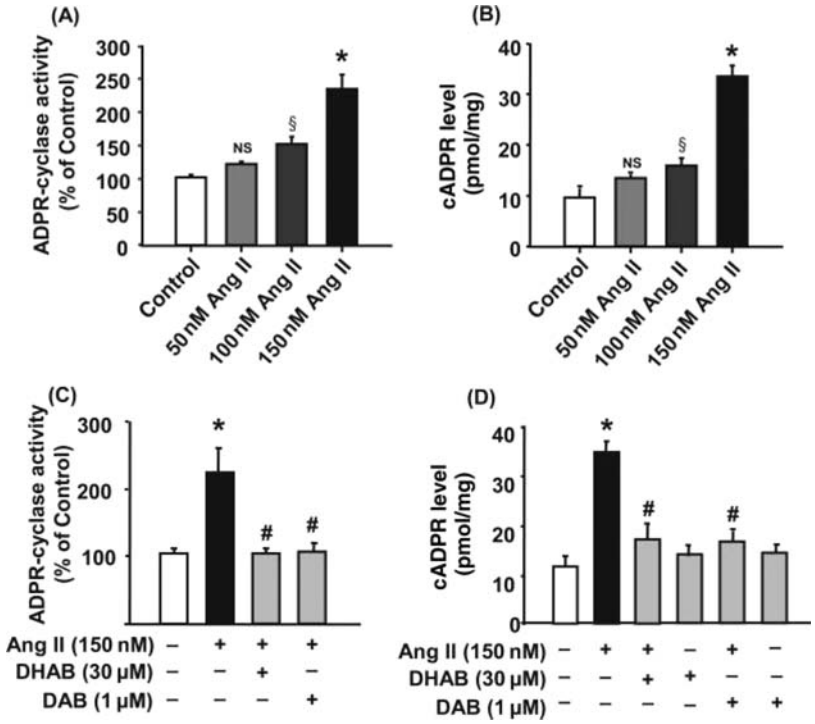

Figure 2 Effect of 2,2′-dihydroxyazobenzene (DAB) and 4,4′-dihydroxyazobenzene (DHAB) on angiotensin (Ang) II-induced ADP ribosyl cyclase (ADPR-cyclase) activation and cyclic ADP-ribose (cADPR) formation. (*A* and *B*) Ang II-stimulated ADPR-cyclase activity and cADPR production in a dose-dependent manner. (*C* and *D*) Ang II-stimulated ADPR-cyclase activity and cADPR production in the presence of 1 μM DAB and 30 μM DHAB. Cardiomyocytes were treated with indicated concentrations of blockers and incubated at 37°C for 30 min prior to Ang II treatment. $P < 0.01$ (*) and $P < 0.03$ (§) vs. Control, $P < 0.01$ (#) vs. Ang II. Results are mean ± SEM of three independent experiments.

II-induced [^{3}H]leucine incorporation (*Figure 3B*), indicating that ADPR-cyclase is involved in Ang II-induced increase in protein synthesis. The inhibitory effects of DAB on Ang II-induced cardiac hypertrophic response were further demonstrated by evaluating the protein expression levels of transforming growth factor, TGF-β1.[24] As shown in *Figure 3C*, Ang II increased the level of TGF-β1 protein, whereas pretreatment of cardiomyocytes with 8-Br-cADPR and DAB significantly reduced the protein expression, indicating that ADPR-cyclase signalling is associated with TGF-β1 expression. These results indicate that Ang II-induced ADPR-cyclase activation can ultimately lead to cardiomyocyte hypertrophy, and that DAB attenuates Ang II-induced cardiac hypertrophy at a range of concentrations similar to that observed in the inhibition of the sustained Ca^{2+} signal, thus suggesting that cADPR-induced sustained Ca^{2+} signal regulates cardiomyocyte hypertrophy.

3.5 Protective effects of 2,2′-dihydroxyazobenzene on cardiac hypertrophy in Goldblatt hypertensive rats

To corroborate whether our *in vitro* results have any physiological relevance, we investigated the effects of ADPR-cyclase inhibitor, DAB, on an Ang II-dependent model of hypertension (2K1C). In the untreated 2K1C rats, hypertension was established in 1–2 weeks after clipping and was stable during the rest of 8 weeks study period. DAB was administered to both sham-operated and 2K1C rats 1 week after the surgery. Four weeks after clipping, DAB treatment decreased the blood pressure by 25 ± 5 mmHg compared with untreated 2K1C rats and was sustained during the rest of the study period (8 weeks). Eight weeks after clipping, the systolic blood pressure in the vehicle-treated 2K1C rats was 181 ± 3 mmHg, whereas the systolic blood pressure in the DAB-treated 2K1C rats was on an average 155 ± 5 mmHg (see Supplementary material online, *Figure S2*). No significant difference in the systolic blood pressure was observed in sham rats treated with DAB compared with vehicle-treated sham rats. To verify if the type of hypertension developed in 2K1C rats was Ang II-dependent, plasma Ang II levels were determined. As shown in *Table 2*, Ang II levels were significantly elevated in vehicle- and DAB-treated 2K1C rats compared with vehicle- and DAB-treated sham rats indicating that increase in Ang II levels lead to the development of hypertension in 2K1C rats. Elevated systolic blood pressure in 2K1C rats resulted in the development of cardiac hypertrophy (see Supplementary material online, *Figure S2*). However, DAB treatment completely blocked the increase in cardiac mass. To exclude the possibility of deleterious influences of DAB on animal growth that could represent a confusing factor in these experiments, heart weight to body weight ratios were calculated, and the ratio showed consistent DAB-dependent inhibition of cardiac growth (*Table 2*). Furthermore, no significant differences were observed in body weights among vehicle treated sham, and DAB treated sham or 2K1C rats. However, vehicle treated 2K1C rats showed a decrease in their body weights compared with sham Control (*Table 2*). Echocardiographic data revealed that 2K1C resulted in anatomical and functional changes that were consistent with cardiac hypertrophy, such as increases in the LV mass, posterior wall thickness and LV end-diastolic diameter, and decrease in fractional shortening and EF

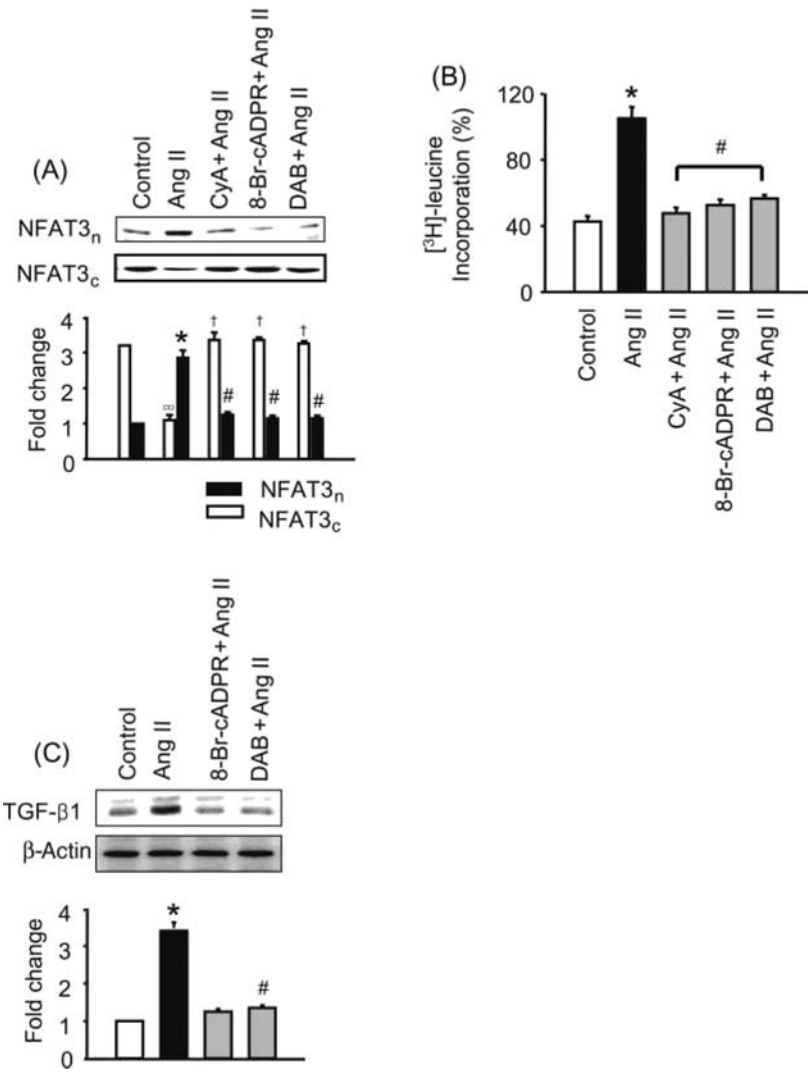

Figure 3 Prevention of angiotensin (Ang) II-induced cardiac hypertrophy by 2,2′-dihydroxyazobenzene (DAB) treatment *in vitro*. (*A*) Immunoblot with summary quantifications of Ang II-stimulated NFAT3 (nuclear factor of activated T-cells) expression in nuclear (NFAT3n) and cytosolic (NFAT3c) fraction after pretreatment with cyclosporin A (CyA), 8-Br-cADPR (cyclic ADP-ribose), and DAB. $^{*}P < 0.01$ vs. Control, $^{\#}P < 0.01$ vs. Ang II in nuclear fractions. $^{\infty}P < 0.01$ vs. Control, $^{\dagger}P < 0.01$ vs. Ang II in cytosolic fractions. (*B*) Ang II-induced [3H]-leucine incorporation after pretreatment with CyA, 8-Br-cADPR, and DAB. (*C*) Immunoblot of TGF-β1 (transforming growth factor) with summary quantifications. $^{*}P < 0.01$ vs. Control, $^{\#}P < 0.01$ vs. Ang II. Results are mean ± SEM of three independent experiments.

Table 2 Effects of 2,2′-dihydroxyazobenzene (DAB) on sham and two-kidney one-clip (2K1C) rats 8 weeks after surgery

Parameters	Sham ($n = 15$)	2K1C ($n = 19$)	Sham + DAB ($n = 15$)	2K1C + DAB ($n = 20$)
BW (g)	385 ± 10	324 ± 8[a]	375 ± 7	380 ± 10[b]
HW/BW (mg/g)	3.0 ± 0.3	4.4 ± 0.8[a]	2.9 ± 0.6	3.1 ± 0.5[b]
Ang II levels (pg/mL)	60 ± 3	170 ± 9[a]	62 ± 4	160 ± 4[a]
Heart rate (bpm)	292 ± 22	266 ± 20	255 ± 30	256 ± 32

BW, body weight; HW/BW, heart weight/body weight; Ang, angiotensin.
[a]$P < 0.05$ vs. sham.
[b]*P*0.05 vs. 2K1C.

compared with sham-operated rats. On the contrary, however, DAB treatment significantly attenuated the LV mass, posterior wall thickness and LV end-diastolic diameter, and increased fractional shortening and EF in 2K1C rats (*Figure 4*). Furthermore, histochemical data also showed an enlargement in cross-sectional area of cardiomyocytes in 2K1C group, which was significantly shortened by DAB treatment (*Figure 5A* and *B*). These results indicate that DAB significantly attenuates cardiac hypertrophy induced in 2K1C rat model. To further evaluate the

inhibitory effects of DAB on cardiac hypertrophy, NFAT3 nuclear translocation and TGF-β1 protein expression were measured as markers of cardiac hypertrophy. 2K1C rats showed an increase in NFAT3 nuclear translocation and TGF-β1 protein expression in cardiac tissues as compared with sham Controls (*Figure 5C* and *D*). On the other hand, DAB-treatment blocked NFAT3 nuclear translocation and TGF-β1 protein expression in 2K1C rats.

3.6 Attenuation of ADP ribosyl cyclase activation by 2,2′-dihydroxyazobenzene diminishes the expression of hypertrophic markers in two-kidney one-clip model of rat

To further confirm our observation that DAB prevents cardiac hypertrophy through the inhibition of ADPR-cyclase activation, we examined ADPR-cyclase activity and cADPR formation in cardiac tissues. Interestingly, 2K1C rats showed an increase in both ADPR-cyclase activity and cADPR formation compared with sham rats, however, 2K1C rats treated with DAB showed a significant reduction in both ADPR-cyclase activity and cADPR formation which is consistent with our *in vitro* findings (*Figure 6A* and *B*). No apparent changes in the ADPR-cyclase activity and cADPR formation were observed in sham rats treated with DAB compared with sham rats receiving vehicle. These data provide a clear indication that ADPR-cyclase activity is enhanced in hypertrophied myocardium of 2K1C rats and DAB treatment prevents cardiac hypertrophy by blocking ADPR-cyclase activation.

4. Discussion

We have previously elucidated the molecular mechanism of ADPR-cyclase activation in Ang II signalling using adult rat primary cardiomyocytes, indicating that ADPR-cyclase

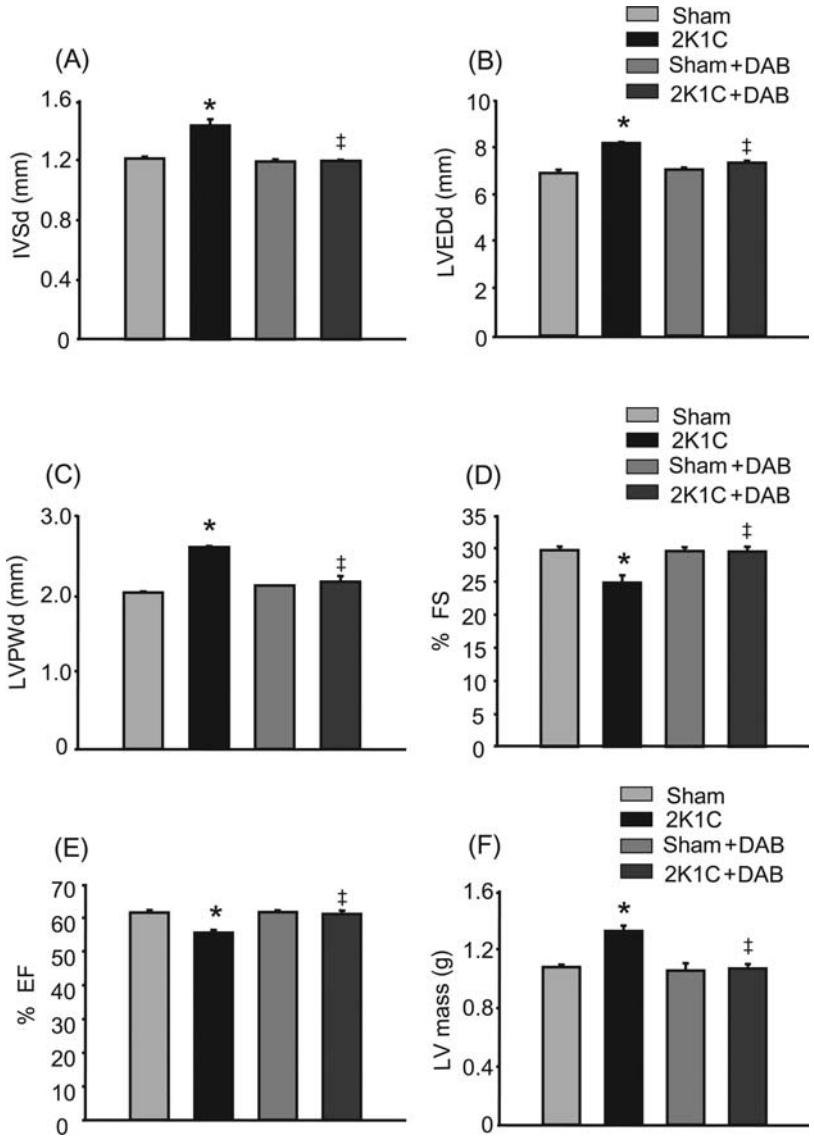

Figure 4 Echocardiographic measures in sham and two-kidney one-clip (2K1C) rats 8 weeks after 2,2′-dihydroxyazobenzene (DAB) treatment. (*A*) Diastolic thickness of interventricular septum (IVSd); (*B*) left ventricular (LV) end-diastolic diameter (LVEDd); (*C*) diastolic thickness of LV posterior wall (LVPWd); (*D*) fractional shortening (FS); (*E*) ejection fraction (EF); and (*F*) LV mass. $^{*}P < 0.05$ vs. sham, $^{‡}P < 0.05$ vs. 2K1C. Results are expressed as mean ± SEM of six to seven rats per experimental group.

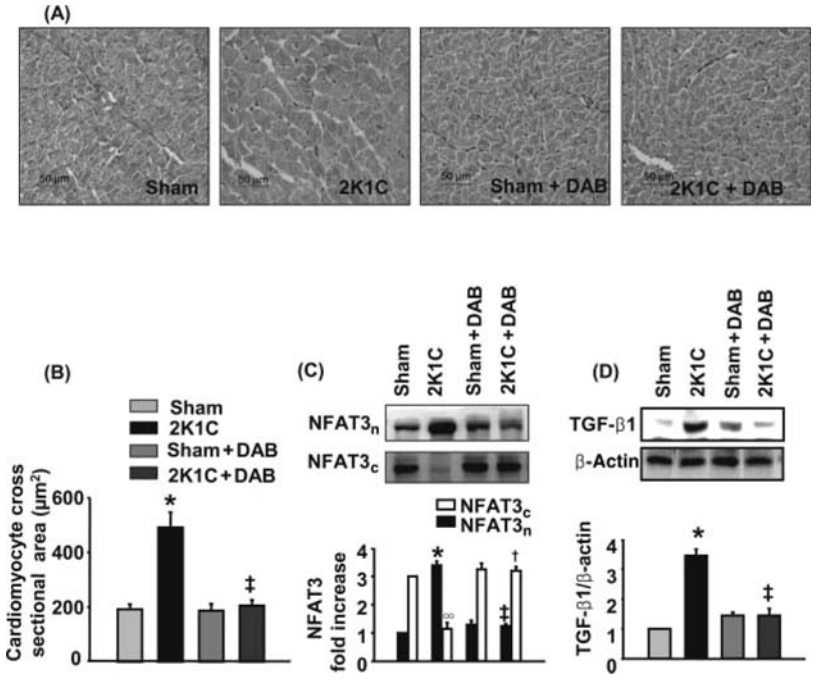

Figure 5 Prevention of two-kidney one-clip (2K1C)-induced cardiac hypertrophy by 2,2′-dihydroxyazobenzene (DAB) injection *in vivo*. (*A*) Left ventricular (LV) cardiomyocyte cross-sectional areas in vehicle or DAB treated sham rats and vehicle or DAB-treated 2K1C rats examined by haematoxylin–eosin staining (scale bar 50 μm). (*B*) Represents the quantitative analysis of cross-sectional area. (*C*) Immunoblot of NFAT3 (nuclear factor of activated T-cells) in nuclear and cytosolic fraction with summary quantification. $^{*}P < 0.01$ vs. Control, $^{\#}P < 0.01$ vs. angiotensin (Ang) II in nuclear fractions. $^{\infty}P < 0.01$ vs. Control, $^{\dagger}P < 0.01$ vs. Ang II in cytosolic fractions. (*D*) Immunoblot of TGF-β1 (transforming growth factor) with summary quantification. $^{*}P < 0.05$ vs. sham, $^{\ddagger}P < 0.05$ vs. 2K1C. Results are expressed as mean ± SEM of six to seven rats per experimental group.

activation involves a sequential activation of Src, PI3K/Akt, and PLC-γ1/IP_3, that results in up-regulation of hypertrophic responses.[11,14] In the present study, we demonstrated that inhibition of ADPR-cyclase by a small molecule inhibitor DAB blocked Ang II-induced sustained Ca^{2+} increase and production of cADPR in adult rat cardiomyocytes. In addition, the results showed that inhibition of ADPR-cyclase by DAB attenuated Ang II-induced hypertrophic responses, as indicated by decrease in NFAT nuclear translocation, TGF-β1 protein expression, and [^{3}H]leucine incorporation. Consistent with these findings, DAB was capable of preventing the development of cardiac hypertrophy in rat renovascular hypertension model (2K1C).

Ang II stimulation of AT1R rapidly induces intracellular calcium mobilization and ADPR-cyclase activation in cardiomyocytes.[11] Ang II generates a biphasic $[Ca^{2+}]_i$ response comprising of a rapid initial phase and a sustained phase. Ang II-induced second $[Ca^{2+}]_i$ phase, which appears to contribute to the sustained increase, was completely blocked by pretreatment of cardiomyocytes with a cADPR antagonist, 8-Br-cADPR. These observations indicated that the Ang II-induced sustained rise of $[Ca^{2+}]_i$ in cardiomyocytes was mediated by cADPR. On the basis of the result that stimulation of AT1R by Ang II activates ADPR-cyclase in cardiomyocytes, we evaluated the effects of our previously discovered ADPR-cyclase inhibitor DHAB and its structural analogue DAB on Ang II-induced Ca^{2+} signalling.[9,14] Interestingly, Ang II-induced sustained Ca^{2+} increase, but not initial peak, was completely blocked by pretreatment of the cells with DAB with maximal inhibition occurring at 1 μM, whereas DHAB showed maximal inhibition at 30 μM. Consistent with these observations, Ang II-induced ADPR-cyclase activity and production of cADPR were also blocked by pretreatment of the cells with DAB at similar ranges of concentration. From these data, it was apparent that DAB treatment blocks Ang II-induced activation of ADPR-cyclase/cADPR and sustained Ca^{2+} increases. Inhibitory effects of DAB on Ang II-induced sustained Ca^{2+} signal, made us possible to use DAB as a tool for prevention of Ang II-induced cardiac hypertrophy.

The importance of Ca^{2+} in the development of cardiac hypertrophy has recently been highlighted. Studies have shown that Ang II-mediated hypertrophy of cardiomyocytes is dependent upon an increase in $[Ca^{2+}]_i$.[25] Spontaneous Ca^{2+} sparks with increased amplitude were found in hypertrophied spontaneously hypertensive rats.[26] These studies indicate that increased Ca^{2+} plays a key regulatory role in the development of pathological cardiac growth.[27] One such pathway is regulated by calcium/calmodulin-dependent phosphatase calcineurin. Ang II-induced cardiomyocyte hypertrophy *in vitro* was inhibited by cyclosporin A, an inhibitor of calcineurin.[19] Overexpression of constitutively active mutants of a Ca^{2+}-dependent phosphatase calcineurin and of its downstream transcription factor NFAT3 induced marked cardiac hypertrophy in transgenic mice.[28] To understand the role of ADPR-cyclase-mediated sustained Ca^{2+} increase in cardiac hypertrophy, we examined the effects of DAB on Ang II-induced NFAT3 translocation. Treatment of cardiomyocytes with Ang II increased nuclear NFAT3 expression that was significantly inhibited by cyclosporin A, 8-Br-cADPR or DAB. The blockade of the NFAT3 nuclear translocation by 8-Br-cADPR and DAB indicate that ADPR-cyclase/cADPR signalling pathway plays an important role in Ang II-induced hypertrophic responses, and that the effects of ADPR-cyclase/cADPR are associated with the activation of calcineurin. Supporting the

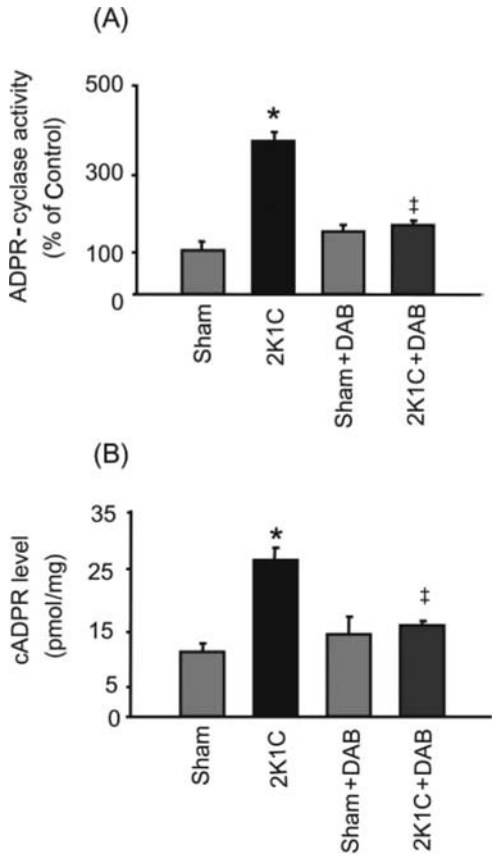

Figure 6 Effects of 2,2′-dihydroxyazobenzene (DAB) administration on ADP ribosyl cyclase (ADPR-cyclase) activity and cyclic ADP-ribose (cADPR) production in two-kidney one-clip (2K1C) model of rat. (*A* and *B*) ADPR-cyclase activity and cADPR formation, respectively, in 2K1C after DAB treatment. $^{*}P < 0.01$ vs. sham, $^{‡}P < 0.01$ vs. 2K1C. Results are expressed as mean ± SEM of six to seven rats per experimental group.

observation, Ang II-induced increase in protein synthesis, as reflected by [^{3}H]leucine-incorporation, was also significantly reduced by pretreatment with cyclosporin A, 8-Br-cADPR or DAB. Multiple lines of evidence indicate that TGF-β1 plays a critical role in Ang II-induced cardiac hypertrophy.[24] Rosenkranz *et al.*[29] recently demonstrated that overexpression of TGF-β1 in transgenic mice leads to cardiac hypertrophy. In the present study, we showed that treatment of cardiomyocytes with Ang II increased TGF-β1 protein expression that was substantially reduced by pretreatment with 8-Br-cADPR and DAB. Collectively, these data suggest that inhibition of ADPR-cyclase activation by DAB blocks sustained Ca^{2+} increase, thereby ameliorating the cardiac hypertrophy in response to Ang II. In order to further ascertain these observations, the effects of DAB on the development of cardiac hypertrophy were tested in 2K1C rat model of hypertension. In the untreated 2K1C rats, nuclear NFAT3 and TGF-β1 protein expression increased markedly, however, treatment with DAB resulted in significant decrease in these parameters. These observations suggest that the development of cardiac hypertrophy in the 2K1C rats is elicited by an increase in intracellular Ca^{2+} levels via the activation of ADPR-cyclase/cADPR signalling system, and that DAB can provide protection against 2K1C induced cardiac hypertrophy by inhibiting ADPR-cyclase activation.

The present results support our assumption that DAB attenuated Ang II-induced hypertrophy. Echocardiographic analysis showed an increase in LV mass, and wall thickness, and decrease in fractional shortening in untreated 2K1C rat model, compared with sham. In contrast, however, DAB not only ameliorated 2K1C-induced cardiac hypertrophy, but also enhanced cardiac functions by reducing LV end-diastolic dimensions, suggesting that ADPR-cyclase/cADPR signalling system plays an important role in cardiac hypertrophy. Surprisingly, we found that DAB attenuated increased blood pressure, although not completely the normotensive levels. However, no clear insights into the mechanism of anti-hypertension by DAB could be provided at present, and further studies are required to address this question.

A direct evidence to indicate the important role of ADPR-cyclase in hypertrophic responses in 2K1C was provided by measuring ADPR-cyclase activity and cADPR levels in cardiac tissues. In DAB-treated 2K1C rats, both cADPR content and ADPR-cyclase activity in LV myocardium were greatly reduced compared with that of untreated 2K1C rats, indicating that ADPR-cyclase is predominantly responsible for the induction of cardiac hypertrophy and that DAB specifically blocks ADPR-cyclase to prevent the development of hypertrophic response.

In summary, we have demonstrated for the first time that DAB prevents the development of cardiac hypertrophy by attenuating Ang II-induced ADPR-cyclase activation and subsequent cADPR-mediated sustained Ca^{2+} signalling pathways, which are associated with cardiac hypertrophy. These findings may have important clinical implications in developing new therapeutic strategies to prevent cardiac hypertrophy. However, this should facilitate future clarification for the utility of DAB to prevent hypertrophic heart disease.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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