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Apocynin prevents isoproterenol-induced cardiac hypertrophy in rat

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Abstract

Oxidative stress is implicated in the pathogenesis of a plethora of cardiovascular diseases including interstitial fibrosis, contractile dysfunction, ischemia-reperfusion injury, and cardiac remodeling. However, antioxidant therapies targeting oxidative stress in the progression of those diseases have largely been unsuccessful. The current study evaluated the effects of a NADPH oxidase inhibitor, apocynin (Apo), on the production of reactive oxygen species and the development of pathological cardiac hypertrophy under sustained β-adrenergic stimulation in male Wistar rats. As evident from the HW/BW ratio, HW/TL ratio, echocardiography, and histopathology, hypertrophic responses induced by isoproterenol (Iso; 5 mg/Kg body weight, subcutaneous) were blocked by Apo (10 mg/Kg body weight, intraperitoneal). Iso treatment increased the transcript levels of *cybb* and *p22-phox*, the two subunits of Nox. Iso treatment also caused a decrease in reduced glutathione level that was restored by Apo. Increase in mRNA levels of a number of markers of hypertrophy, viz., ANP, BNP, β-MHC, and ACTA-1 by Iso was either partially or completely prevented by Apo. Activation of key signaling kinases such as PKA, Erk, and Akt by Iso was also prevented by Apo treatment. Our study thus provided hemodynamic, biochemical, and molecular evidences supporting the therapeutic value of Apo in ameliorating adrenergic stress-induced cardiac hypertrophy.

Keywords Redox signaling · NADPH oxidase · Cardiac hypertrophy · Isoproterenol · Apocynin

Abbreviations

β-MHC	β-Myosin heavy chain
ACTA-1	Actin alpha skeletal muscle 1
ANP	Atrial natriuretic peptide
Apo	Apocynin
BNP	Brain natriuretic peptide
CREB	cAMP response element binding protein
DMSO	Dimethyl sulfoxide
EF	Ejection fraction
ERK	Extracellular signal-regulated kinases
FS	Fractional shortening
Iso	Isoproterenol
IVS	Interventricular septal
LVPW	Left ventricular posterior wall
Nox	NADPH oxidase
PKA	Protein kinase A
ROS	Reactive oxygen species

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Introduction

Cardiac hypertrophy is a pathophysiological response to increased cardiac workload, resulting in increase in heart size [1]. While physiological hypertrophy is associated with normal cardiac function and occurs during development, pregnancy, and sustained exercise; pathological hypertrophy is promoted by hypertension, myocardial injury, excessive neurohumoral activation, etc, eventually leading to heart failure [2]. High and sustained β-adrenergic activity with elevated level of blood catecholamines is a hallmark of pathological hypertrophy in heart failure patients [3, 4]. Pathological hypertrophy is characterized by increase in myocyte size, increased rate of protein synthesis, and reinduction of fetal genes through multiple signaling pathways, viz., MAP-, PI3-, Akt kinases, and Ca^{++}-calcineurin system [5–7]. Transcription factors NFκB, NFAT, MEF2, GATA-4, etc, have also been associated with the reprogramming of gene expression in the hypertrophied heart [8]. Besides these canonical signal transduction pathways, reactive oxygen species (ROS) has also been attributed to the hypertrophic responses, but mechanisms are largely obscure [9, 10].

In recent years, NADPH oxidases (Noxes) have emerged as the major source of intracellular ROS generation under

various pathophysiological setups [10–12]. Out of seven isoforms of mammalian Noxes, Nox1/2/4 are expressed in heart [13]. While tightly regulated ROS generation is essential for the maintenance of various cardiac functions, over production of ROS leads to conditions like interstitial fibrosis, contractile dysfunction, ischemia-reperfusion injury, and cardiac remodeling [14]. Therapeutic targeting of Nox-mediated generation of ROS is currently an area of intense research [15]. We have recently demonstrated that the stimulation of β-adrenergic receptor co-stimulates Nox2 and the crosstalk between the kinase and the redox (Nox) signaling determines the downstream responses [16]. In the present study, we demonstrate that Apo, a widely used inhibitor of Nox, attenuates the hypertrophic response elicited by the β adrenergic receptor agonist isoproterenol (Iso). Apo prevents the decrease in reduced glutathione level and the upregulation of a number of marker genes of hypertrophy by Iso.

Materials and methods

Reagents

All chemicals were purchased from Sigma Aldrich, USA, unless otherwise specified. Rabbit monoclonal antibodies against Erk1/2, phospho-Erk1/2 (Thr202/Tyr204), Akt, phospho-Akt (Ser473), and GAPDH were purchased from Cell Signaling Technologies, USA. Rabbit monoclonal antibodies against CREB and phospho-CREB (Ser133) was purchased from Abcam, UK. Rabbit polyclonal antibodies against PKA and phospho-PKA (Ser96) were procured from Santa Cruz Biotechnology, USA. Horseradish peroxidase-conjugated anti-rabbit IgG antibody was obtained from Santa Cruz Biotechnology, USA. All the oligonucleotides used for the transcript analyses were synthesized from Sigma Aldrich (Table 1).

Animals

Animal studies were carried out using male Wistar rats of 6–8 weeks. Animal were kept under standard laboratory conditions (temperature; 25 ± 2 °C, relative humidity; $50 \pm 15\%$ and 12 h-dark/12-h light period) and provided feed and water *ad libitum*. All animal procedures were reviewed and approved by Institutional Animal Ethics Committee (IAEC code 09/2014) of the Jawaharlal Nehru University, New Delhi (Registration No. 19/GO/ReBi/S/99/CPCSEA Dated: 10.03.1999). All animal care and experimental protocols were performed in compliance with the National Institutes of Health (NIH) guidelines for the care and use of the Laboratory Animals (NIH Publication no. 85-23, revised 1996).

Rats were randomly divided into five groups ($n = 6$ in each group); (i) Control groups were administered

Table 1 Oligonucleotides used for real-time PCR

Name	Sequence
ANP	Forward 5′CAGGCCATATTGGAGCAAATC3′
ANP	Reverse 5′CTCATCTTCTACCGGCATCTT3′
BNP	Forward 5′CTAGCCAGTCTCCAGAACAATC3′
BNP	Reverse 5′AGCTGTCTCTGAGCCATTTC3′
β-MHC	Forward 5′TCCAGCTGAAAGCAGAAAGAG3′
β-MHC	Reverse 5′CCTGGGAGATGAACGCATAAT3′
ACTA-1	Forward 5′TATTCAGGCGGTGCTGTCT3′
ACTA-1	Reverse 5′TCCCCAGAATCCAACACGAT3′
cybb	Forward 5′CCCTTTGGTACAGCCAGTGAAGAT3′
cybb	Reverse 5′CAATCCCAGCTCCCACTAACATCA3′
p22-phox	Forward 5′ACCTGACCGCTGTGGTGAA3′
p22-phox	Reverse 5′ACAGCAGTAAGTGGAGGACAG3′
GAPDH	Forward 5′GACATGCCGCCTGGAGAAAC3′
GAPDH	Reverse 5′AGCCCAGGATGCCCTTTAGT3′

subcutaneously with saline, (ii) Iso groups were subcutaneously injected with isoproterenol (5 mg/kg body weight; in saline), (iii) Iso + Apo groups were administered intraperitoneally with Apo (10 mg/kg body weight; in 10% DMSO) followed by subcutaneous injection of isoproterenol (5 mg/kg body weight; in saline), (iv) Apo groups were administered intraperitoneally with Apo (10 mg/kg body weight; in 10% DMSO), and (v) DMSO groups were administered intraperitoneally with DMSO (10%; in saline). All the drugs were freshly prepared and given once daily for 14 days.

After 14 days, body weight and tail length were measured and rats were sacrificed by overdose of pentobarbitone. The hearts were carefully excised, washed in ice cold PBS, blotted dry with tissue paper, and weighed. The ratio of heart weight to body weight and the ratio of heart weight to tail length were taken as parameters of cardiac hypertrophy. Tissues were snap frozen in liquid nitrogen, and stored at −80 °C for later analysis.

Echocardiography

Rats were anesthetized with ketamine (50 mg/kg body weight) and xylazine (10 mg/kg body weight) and the chest hair was shaved. Transthoracic echocardiography was performed using a fully digitized Philips, USA system (Vivid 7 dimension) with a 10–11.5 MHz neonatal cardiac probe transducer. The transducer was placed on the left hemithorax. Two-dimensional M-mode images of left ventricle (LV) at the papillary muscle level were obtained from the parasternal short-axis view. LV posterior wall thickness (LVPWd), interventricular septal thicknesses (IVSd), and LV internal dimensions at the end of diastole (LVIDd) and systole (LVIDs) were measured from M-mode images.

Based on these measurements, various other parameters were also calculated using the following formula:

Fractional shortening (FS %) = {(LVIDd − LVIDs)/LVIDd} × 100

Ejection fraction (EF %)
$$= \left[\{(LVIDd)^3 - (LVIDs)^3\}/(LVIDd)^3\right] \times 100$$

Left ventricle mass (LVM gm)
$$= 0.8\left[1.04\{(LVIDd + IVSd + LVPWd)^3 - (LVIDd)^3\}\right] + 0.6$$

Hematoxylin and eosin (H&E) staining

Left ventricle sections were cut from the hearts and were fixed in 10% formalin overnight at room temperature followed by embedding in paraffin. Four to five micrometer sections were cut and subjected to H&E staining by following standard protocols. Slides were viewed with Nikon Eclipse TiS microscope and images were captured at 10X magnification.

Glutathione assay

Reduced glutathione (GSH) concentration was determined essentially as described by Rahman et al. [17]. Briefly, the LV section of heart was gently homogenized in 0.6% sulfosalicylic acid and 0.1% Triton X-100 solution in 0.1 M KPE (potassium phosphate buffer containing 5 mM EDTA disodium salt, pH 7.5). The homogenate was centrifuged at 8000 g for 10 min at 4 °C and the supernatant obtained was used for the assay.

Real-time PCR

Total RNA was isolated from LV section of heart tissues using TRI reagent according to the manufacturer's instructions. One microgram of RNA was reverse transcribed using Applied Biosystems reverse transcription kit as per the manufacturer's protocol. Gene-specific primers were used to analyze the transcript level using SYBR green master mix (Applied Biosystems) in an Applied Biosystems 7500 real-time PCR system. Data were normalized using the GAPDH as an internal control, and fold change over control was calculated.

Western blotting

The LV sections of heart were homogenized in RIPA buffer (50 mM Tris; pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) with phosphatase inhibitor cocktail, 1 mM phenylmethylsulphonyl fluoride (PMSF), and protease inhibitor cocktail followed by sonication. The homogenates were centrifuged

and supernatant was assessed for protein concentration by the Bradford method using BSA as standard. Proteins were separated (80 µg per well) on 10% SDS-polyacrylamide gel and transferred onto PVDF membrane (Millipore, USA). Membranes were blocked with 3% BSA in 0.1% TBST for 2 h at room temperature followed by incubation with specific primary antibodies at 4 °C overnight. Then the membranes were incubated with secondary antibody conjugated to peroxidase (HRP) for 40 min at room temperature. Membranes were exposed to enhanced chemiluminescence reagent and visualized on X-ray film (Kodak, USA). Quantification of band intensities was performed with the ImageJ software system. The signal intensity of each protein was normalized with the corresponding total protein signal and fold change over control was calculated.

Statistical analysis

Statistical analyses were performed using GraphPad Prism, PC version 5 (GraphPad software). Data are expressed as mean ± SEM. Experimental groups were compared with the use of one-way ANOVA if there was one independent variable (Tukey's test) or two-way ANOVA if there were two independent variables. Values of $P < 0.05$ were considered statistically significant.

Results

Isoproterenol-induced cardiac hypertrophy parameters are prevented by Apo

Sustained activation of β-adrenergic receptors is an important hallmark of pathological cardiac hypertrophy and ROS is attributed to it [18]. Isoproterenol (Iso), a non-selective β-adrenergic receptor agonist, is widely used for inducing cardiac hypertrophy in experimental rats while Apo, a non-toxic inhibitor of Nox is also used for inhibiting Nox2 activity in vivo [19, 20]. To explore the role of Nox, if any, in β-agonist-induced cardiac hypertrophy, we injected male Wistar rats with Iso (5 mg/kg body weight, subcutaneous) for 14 days. Apo was also co-administered (10 mg/kg body weight, intraperitoneal), 30 min prior to the injection of Iso [21]. Iso-induced cardiac hypertrophy, as evident from increase in heart weight-to-body weight ratio (HW/BW), heart weight-to-tail length ratio (HW/TL), and the echocardiogram, was prevented by Apo (Fig. 1a, b). As summarized in Table 2 and shown in Fig. 1b, Iso increased the IVSDd, LVPWd by ~ 2- and ~ 1.5-fold, respectively, indicating concentric hypertrophy. There was no significant change in LVIDd, LVIDs, FS, EF, and SV (stroke volume) in Iso-treated heart, suggesting absence of any cardiac dysfunction (Table 2).

Fig. 1 Isoproterenol-induced cardiac hypertrophy in rat is prevented by the Nox inhibitor apocynin. Wistar rats were administered with Iso (5 mg/kg body weight, subcutaneous) and Apo (10 mg/kg body weight, intraperitoneal) for 14 days. Upon completion of treatments, **a** Rats were sacrificed and the heart were excised. Upper panel: representative images of whole heart excised from treated rats. Lower panel: ratio of HW/BW (heart weight to body weight) and HW/TL (heart weight to tail length). **b** M-mode echocardiography was done on treated rats. Upper panel: representative echocardiogram of different groups on day 14. Lower panel: Quantitative representation of LVIDd and LVPWd on day 1 (baseline) and day 14. **(c)** Representative histology of H&E-stained LV sections of hearts captured at 10X magnification using Nikon TiS microscope. *LVIDd* left ventricular internal dimensions at diastole, *LVIDs* left ventricular internal dimensions at systole, *IVSd* interventricular septal thickness at diastole, *LVPWd* left ventricular posterior wall thickness at diastole. $**P \leq 0.01$ vs. Control; $***P \leq 0.001$ vs. Control; $^{\#}P \leq 0.05$ vs. Iso; $^{\#\#}P \leq 0.01$ vs. Iso; $^{\$\$}P \leq 0.01$ vs. Day 1; $^{\$\$\$}P \leq 0.001$ vs. Day 1
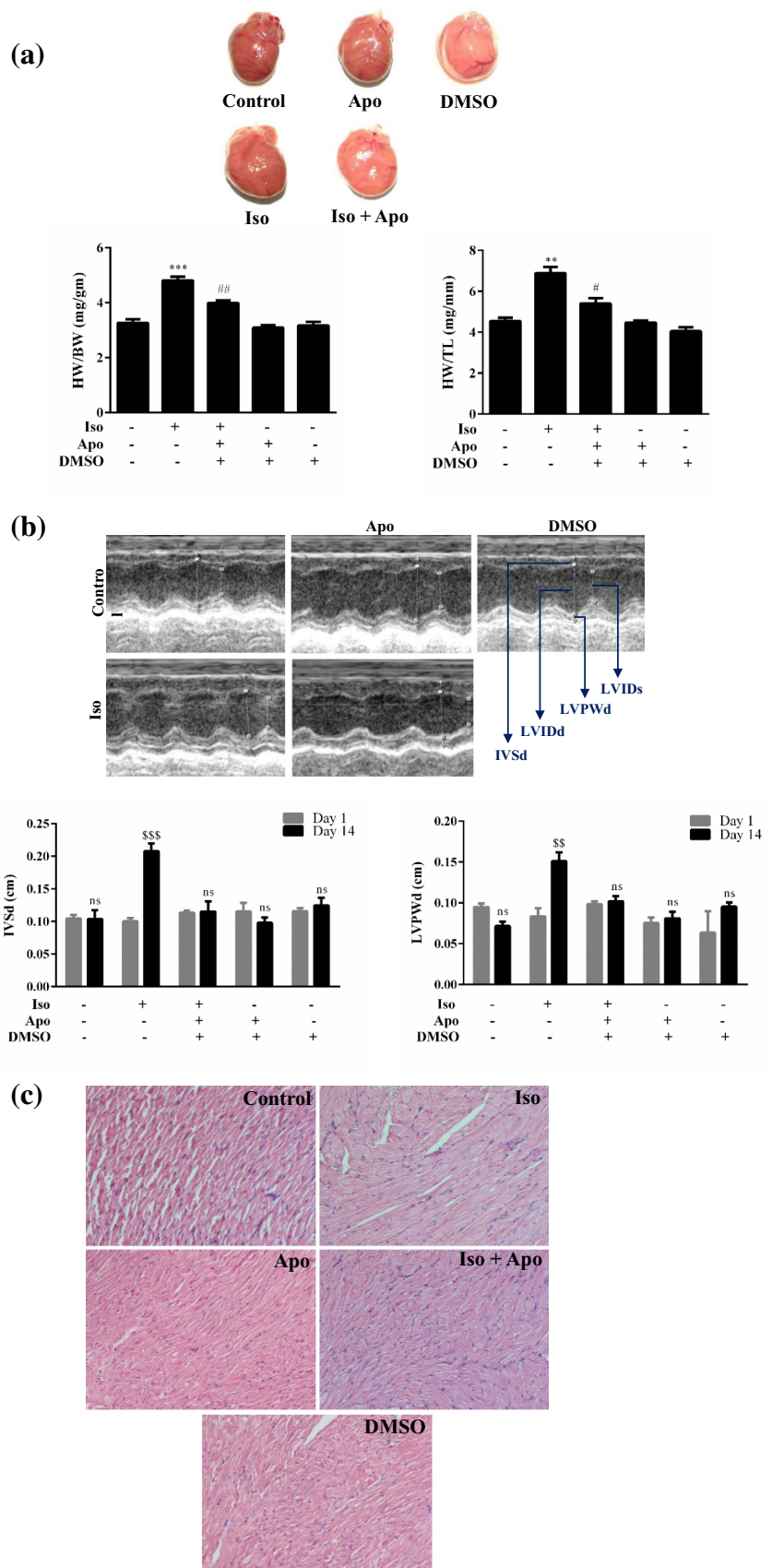
Also, there were no significant changes in Apo groups. Further, microscopic examinations of H&E-stained LV sections demonstrated enlarged cardiomyocytes under Iso treatment while concurrent treatment with Apo partially prevented the increase in the cardiomyocytes size (Fig. 1c). Taken together, these data suggest the involvement of Noxes in inducing cardiac hypertrophy under sustained adrenergic stimulation.

Isoproterenol upregulates the expression of *cybb* and *p22-phox* subunits of Nox

Activation of Nox2 at mRNA and protein levels by diverse stimuli has been demonstrated in different cell types [22]. To further establish the involvement of Nox2 in isoproterenol-induced cardiac hypertrophy, we measured the transcript levels of two of its subunits, i.e., *cybb* and *p22-phox*. The total RNA was extracted from the LV section of hearts and the expression of those subunits were assayed by Real-time PCR. As shown in Fig. 2a, in Iso-treated hearts, the levels of *cybb* and *p22-phox* mRNAs were increased by ~ 7.0- and ~ 3.0-fold, respectively. Concurrent treatment with Apo further increased the mRNA levels though it was not significant as compared to the Iso group. Since Apo is a pharmacological inhibitor of Nox activities [23], the levels of *cybb* and *p22-phox* mRNAs were unaffected with Apo treatment as expected and similar results have also been reported by others [24].

Iso treatment increases oxidative stress in heart and Apo reduces it

Reduced glutathione is a key constituent of cellular antioxidant defense and a well-established marker of oxidative stress. In many experimental setups, following the

generation of ROS, the level of GSH is reduced [25, 26]. We thus measured the level of reduced glutathione in Iso-treated heart and the effect of Apo on it. As shown in Fig. 2b, the level of GSH was reduced by ~ 30% in Iso-treated heart and Apo restored it by ~ 15%. Apo alone or the vehicle (DMSO) affected the GSH level only marginally. The data thus suggest that the induction of cardiac hypertrophy by Iso involves ROS generation and its inhibition by Apo reverses its effects.

Apocynin pretreatment prevents isoproterenol-induced expression of the markers of hypertrophy

To further establish the role of Nox-derived ROS in inducing cardiac hypertrophy by Iso, we assayed the mRNA levels of a number of well-established markers of hypertrophy. Total RNA were extracted from the LV section of hearts and the expression of markers genes were assayed by Real-time PCR [27]. As shown in Fig. 3, in Iso-treated hearts, the levels of transcripts for β-MHC, ANP, BNP, and ACTA-1 were increased by ~ 5.2-, ~ 2.5-, ~ 5.3-, and ~ 2.2-fold, respectively, that were prevented either partially (ANP, and BNP) or completely (β-MHC and ACTA-1) by the Apo treatment.

Apocynin prevents isoproterenol-induced activation of Akt and Erk

Cardiac hypertrophy involves upregulation of key signaling kinases including PKA, Akt, and Erk [7]. Although it has long been established that under adrenergic stress, these kinases are activated by the classical second messenger system, recent studies also suggest a role of ROS in their induction [28]. To understand if the regression of hypertrophy by Apo involves these kinases, we measured their levels by western blotting (Fig. 4). Iso treatment increased pPKA level by ~ 1.4-fold and pretreatment with Apo prevented that increase. The level of pAkt increased

Table 2 Echocardiography parameters evaluated from M-mode recording on day 14

Parameters	Control	Iso	Iso + Apo	Apo	DMSO
LVIDd (cm)	0.722 ± 0.027	0.687 ± 0.013	0.680 ± 0.032	0.786 ± 0.059	0.677 ± 0.048
LVIDs (cm)	0.421 ± 0.036	0.431 ± 0.027	0.348 ± 0.018	0.516 ± 0.075	0.428 ± 0.025
IVSd (cm)	0.104 ± 0.014	0.208 ± 0.012	0.115 ± 0.016	0.098 ± 0.008	0.124 ± 0.012
LVPWd (cm)	0.072 ± 0.005	0.151 ± 0.011	0.102 ± 0.006	0.081 ± 0.008	0.095 ± 0.005
FS (%)	38.15 ± 6.85	37.46 ± 2.71	48.43 ± 0.79	35.03 ± 5.19	36.75 ± 0.75
EF (%)	78.8 ± 7.14	73.07 ± 3.26	84.00 ± 3.72	68.97 ± 7.27	75.00 ± 1.70
SV (ml)	0.655 ± 0.040	0.536 ± 0.005	0.613 ± 0.110	0.730 ± 0.105	0.519 ± 0.107
LVM (gm)	0.888 ± 0.030	1.283 ± 0.029	0.939 ± 0.024	0.954 ± 0.073	0.955 ± 0.051

LVIDd left ventricular internal dimensions at diastole, *LVIDs* left ventricular internal dimensions at systole, *IVSd* interventricular septal thickness at diastole, *LVPWd* left ventricular posterior wall thickness at diastole, *FS* fractional shortening, *EF* ejection fraction, *SV* stroke volume, *LVM* left ventricular mass

The data are expressed as mean ± SEM; $n = 3$–4 rats per group
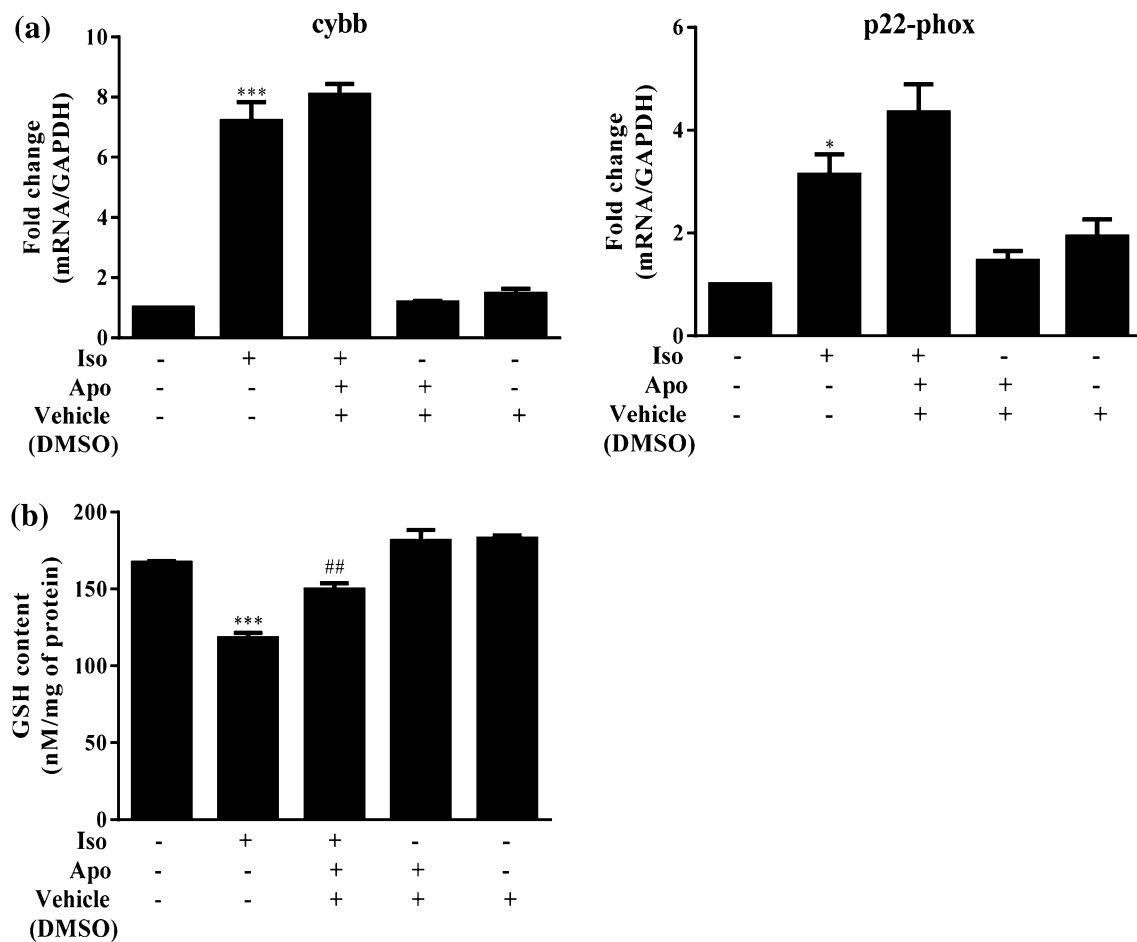
(a)

(b)

Fig. 2 Apocynin pretreatment prevents isoproterenol-induced activation of NADPH oxidase and subsequent oxidative stress. Wistar rats were administered with 5 mg/kg body weight (subcutaneous) of Iso and 10 mg/kg body weight of Apo (intraperitoneal). After 14 days, rats were sacrificed and heart was excised. **a** Total RNA was isolated from the left ventricle and assayed for *cybb* and *p22-phox* transcripts using gene-specific primers by Real-time PCR. Normalization of input RNA was done by using GAPDH level as an internal control. Fold differences of mRNA levels over control were calculated. **b** The concentration of GSH was measured in the LV homogenate. $*P \leq 0.05$ vs. Control; $***P \leq 0.001$ vs. Control; $^{##}P \leq 0.01$ vs. Iso

by ~ 2.0-fold under Iso treatment and Apo brought it to ~ 1.2-fold to the baseline. Similarly, in Iso-treated rats, pErk1/2 level was increased by ~ 1.2-fold and pretreatment with Apo abrogated the induction. Apart from the signaling kinases, we also assayed the level of the transcription factor CREB, a downstream target of PKA, which was unaffected under Iso treatment. Taken together, these data suggest that the activation of PKA, Akt, and Erk by the stimulation of β-adrenergic receptor is attenuated by the inhibition of Nox by apocynin.

Discussion

Cardiac hypertrophy is the common manifestation of several cardiovascular disorders. At the cellular level, there are several hallmarks like increased protein synthesis, increase in myocyte volume but not in number, and partial reactivation of fetal gene expression programs. Although oxidative stress has been attributed to it, failure of antioxidants in ameliorating those conditions necessitated a revisit of the concept of the cause and effect relationship [29]. Such re-examination has also been necessitated by the emergence of reactive oxygen species like superoxide and hydrogen peroxide as the mediators of physiological signals [30].

Noxes are the enzymes solely dedicated to the generation of intracellular superoxide and hydrogen peroxide.
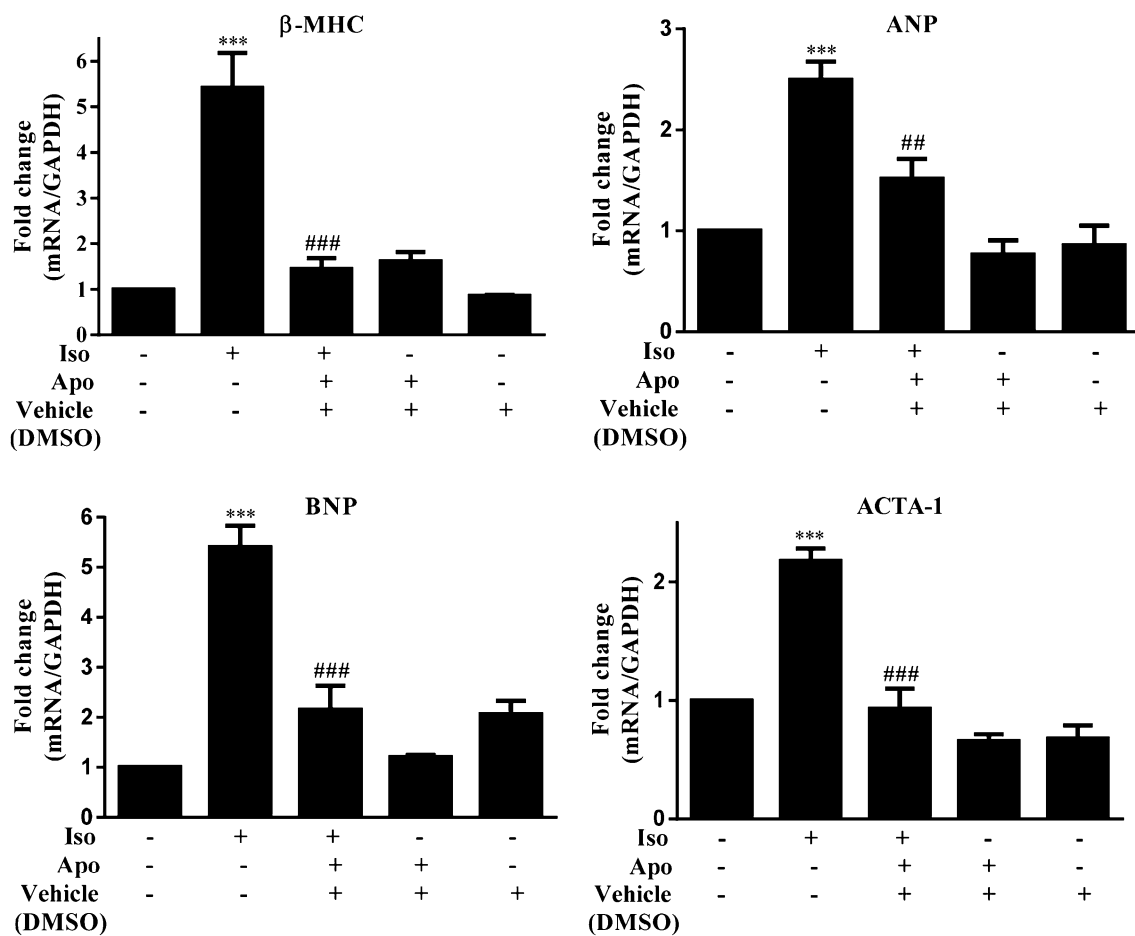
Fig. 3 Apocynin pretreatment prevents isoproterenol-induced alteration in transcripts level. Wistar rats were administered with 5 mg/kg body weight (subcutaneous) of Iso and 10 mg/kg body weight of Apo (intraperitoneal). After 14 days, rats were sacrificed and heart was excised. Total RNA was isolated from the left ventricle and assayed for β-MHC, ANP, BNP, and ACTA-1 transcripts using gene-specific primers by Real-time PCR. Normalization of input RNA was done by using GAPDH level as an internal control. Fold differences of mRNA levels over control were calculated. ***$P \le 0.001$ vs. control; ##$P \le 0.01$ vs. Iso; ###$P \le 0.001$ vs. Iso

With the emerging evidences on their pivotal role in the pathophysiology of various diseases, therapeutic inhibitors of Noxes are also being explored [20, 31]. Apocynin, a non-toxic plant metabolite, has been in use for treating respiratory, metabolic, and cardiovascular disorders even before its mode of action were known [32]. Later studies had described it as a general inhibitor of Nox [20]. In recent years, there has been a flurry of reports showing its beneficial effects under several pathobiological setups [20, 33]. Despite the ambiguities regarding the specificity of its actions, its beneficial effects have largely been well appreciated [34]. Based on our earlier study showing the involvement of Nox2 in mediating adrenergic signaling [16], we have tested whether Apo can ameliorate the hypertrophic response elicited by the adrenergic agonist Iso in male rats. As evident from the gross morphological

parameters (HW/BW and HW/TL ratio), echocardiography, histopathology, level of well-established marker of oxidative stress, and expression levels of the transcripts of the conventional markers of hypertrophy (β-MHC, ANP, BNP, and ACTA-1), Apo was quite effective in preventing the hypertrophic responses measured after two weeks of treatment. In full agreement, the activation of key nodal kinases mediating adrenergic signaling, viz., PKA, ERK, and Akt by Iso was also prevented by Apo; reiterating the well-anticipated crosstalk between the redox and kinase pathways [28]. Noticeably, transcription factor CREB, one of the downstream targets of β-AR/PKA signaling and a regulator of cardiac functions [35], was not affected either by Iso or by Iso + Apo. Such result is in agreement with earlier reports and thus suggests the pathway-specific effects of Apo [36].
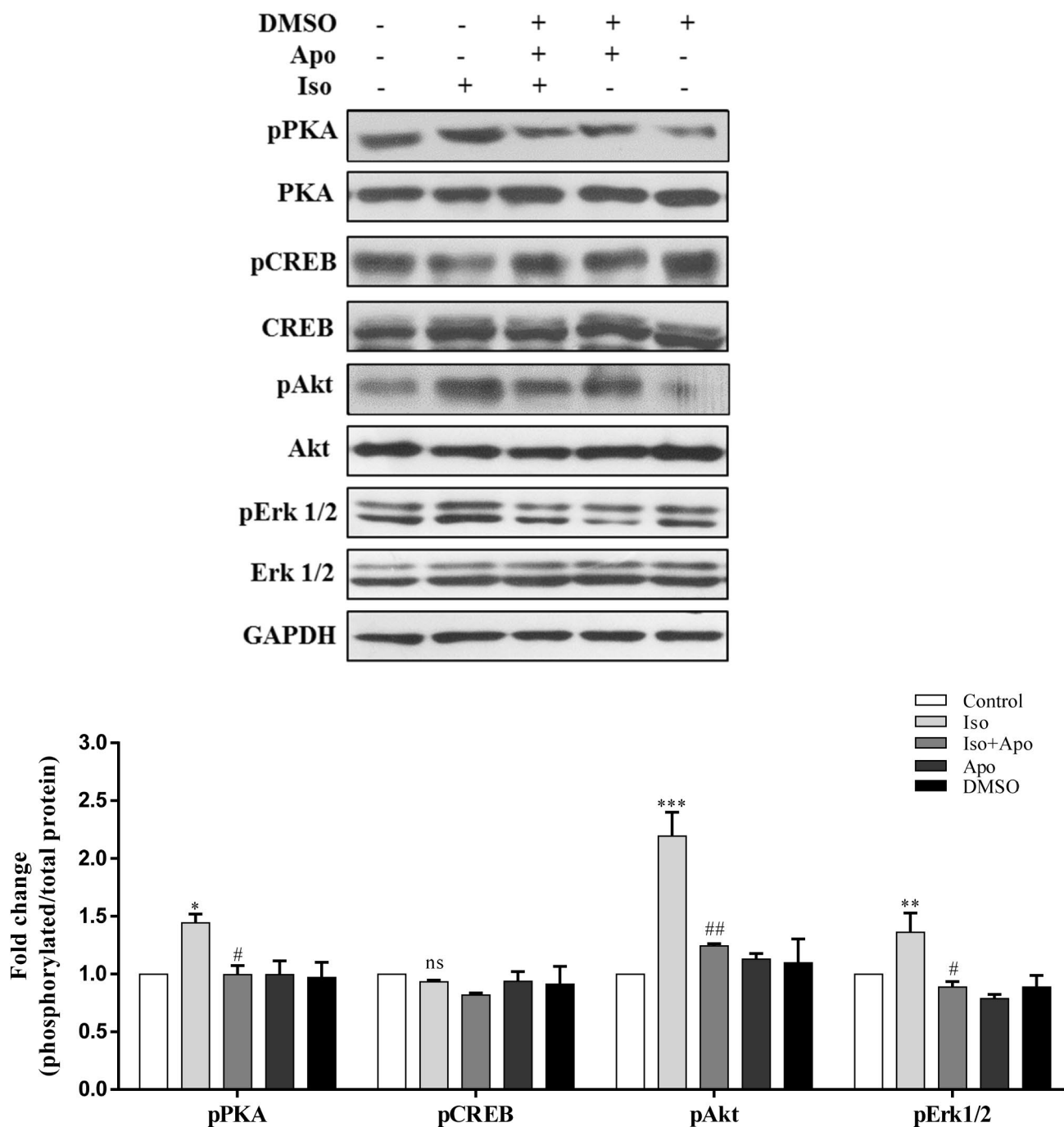
Fig. 4 Apocynin prevents isoproterenol-induced activation of downstream kinases. Wistar rats were administered with 5 mg/kg body weight (subcutaneous) of Iso and 10 mg/kg body weight of Apo (intraperitoneal). After 14 days, rats were sacrificed and heart was excised. Total protein was isolated from the left ventricle and equal amounts (80 μg) of lysates were assayed by western blot analysis using antibodies specific for phospho-PKA, phospho-CREB, phospho-Akt, and phospho-ERK1/2. Total PKA, total CREB, total Akt, and total ERK1/2 were used as respective controls. Also GAPDH level was the loading control. The mean density values of pPKA, pCREB, pAkt, and pERK were evaluated as a ratio relative to that of total PKA, total CREB, total Akt, and total ERK, respectively. Fold differences of relative ratio of protein levels over control were calculated. $*P \leq 0.05$ vs. control; $**P \leq 0.01$ vs. control; $***P \leq 0.001$ vs. control; $^{\#}P \leq 0.05$ vs. Iso; $^{\#\#}P \leq 0.01$ vs. Iso

Compliance with ethical standards

Conflict of interest We confirm that there are no conflicts of interest associated with this publication.

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