FEBS Letters 586 (2012) 180–185

journal homepage: www.FEBSLetters.org

Phosphorylation of Akt/GSK-3β/eNOS amplifies 5-HT_{2B} receptor blockade mediated anti-hypertrophic effect in rats

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ARTICLE INFO

Article history:
Received 8 October 2011
Revised 16 November 2011
Accepted 14 December 2011
Available online 26 December 2011

Edited by Jesus Avila

Keywords:
5-HT_{2B}
Isoproterenol
Cardiac hypertrophy
Akt/GSK-3β
SB-204741
SB-216763

ABSTRACT

Herein, we studied the cross talk between 5-HT_{2B} receptor blocker (SB-204741) and GSK-3β inhibitor (SB-216763) in isoproterenol-induced cardiac hypertrophy for 28 days. SB-204741 treatment significantly ameliorated ($P < 0.05$) myocardial dysfunction, myocyte area, fibrosis and myocardial architecture in isoproterenol insulted myocardium. Moreover, this improvement in functional and morphological changes was associated with suppression of hypertrophic (BNP and CK-MB), inflammatory (IKK-β/NF-κB/TNF-α and CRP), and apoptotic markers (TUNEL positivity and Bax expression) along with phosphorylation of Akt/GSK-3β/β-catenin/eNOS. Intriguingly, co-treatment with GSK-3β inhibitor ($P < 0.01$) further amplified the anti-hypertrophic effect of SB-204741 ($P < 0.05$) such that the effect was indistinguishable from that of vehicle treated rats. Thus, 5-HT_{2B} receptor blockade mediated anti-hypertrophic effect is atleast in part is governed through phosphorylation of Akt/GSK-3β/β-catenin/eNOS via attenuating inflammatory and apoptotic pathways.

1. Introduction

Physiological and pathological stimulation of heart led to architectural remodeling that result in molecular reprogramming of various proteins. One of the direct consequences of this stimulation is an activation of various signaling pathways leading to cardiac hypertrophy. Especially, protein kinase B (Akt/PKB)-glycogen synthase kinase-3β (GSK-3β) is one of the pathways which actively participate in regulating hypertrophic effect in response to exogenous stimuli. GSK-3β being a direct substrate of Akt, arbitrates various cellular processes such as cardiac development, growth, protein synthesis, and gene transcription. Moreover, phosphorylation/inhibition of GSK-3β upregulates cellular defense mechanism by maintaining the cytoskeletal architecture, redox homeostasis, and shielding the cell from spontaneous or stimulated programmed cell death [1].

In recent years, several lines of evidence suggest that serotonin is an important neurohormonal factor that regulates cardiomyocyte growth, proliferation, contractility and hemodynamic functions [2]. Moreover, it has been demonstrated that, of all the 5-HT receptors subtypes, 5-HT_{2B} receptor blockade (5-HT_{2B}RB) emerged as the strongest candidate in ameliorating cardiac hypertrophy, though the molecular signaling pathways driving this activity are hitherto largely unknown [3].

On the basis of the aforementioned facts, we hypothesized that GSK-3β, a multifunctional Serine/Threonine kinase that significantly contributes in the survival and demise of cardiomyocytes; at least in part, plays a role in 5-HT_{2B}RB mediated anti-hypertrophic effect. Accordingly, this study was attempted to dissect the complex molecular, and functional pathophysiology of 5-HT_{2B}RB mediated anti-hypertrophic response by evaluating: (1) whether isoproterenol-induced myocardial hypertrophy is associated with 5-HT_{2B} over expression or downregulation, (2) if so, can its modulation by specific agonist or antagonist ameliorate cardiac hypertrophy and (3) whether this anti-hypertrophic response can be modulated by GSK-3β inhibition, through arbitrating Akt/GSK-3β/β-catenin/eNOS pathway.

2. Materials and methods

5-HT_{2B}R antagonist (SB-204741) and GSK-3β inhibitor, GSK-3βi, (SB-216763) were purchased from the Sigma Chemicals (St. Louis, MO, USA). NF-κB, MnSOD and Nox4 antibodies were purchased from Abcam Technology, USA and all other primary and secondary antibodies were purchased from Santa Cruz Biotechnology, USA. Rat TNF-α (Diaclone Tepnel Company, UK), Brain Natriuretic

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0014-5793/$36.00
doi:10.1016/j.febslet.2011.12.015

Peptide (BNP) (AssayPro, USA), Creatine kinase-MB (CK-MB) (Spinreact, Spain), and C-reactive protein (CRP) (Bender MedSystems Inc., A-1030 Vienna, Austria) ELISA kits were used. SB-204741 and SB-216763 were freshly prepared and dissolved in DMSO and propylene glycol (2:1) before administration.

2.1. Animals

The protocol was approved by the Institutional Animal Ethics Committee (Approval No. 567/IAEC/2010) and all study related activities conformed to the Indian National Science Academy Guidelines for the use and care of experimental animals in research.

2.2. Experimental protocol

Male wistar rats (150–170 g) were divided into five groups ($n = 8$) and were administered vehicle or drugs for 28 days. The doses of the test drugs were determined based on the previous studies [4,5].

Group 1 (NC): vehicle (0.3 ml, i.p.).
Group 2 (ISO): isoproterenol (3.0 mg/kg/day, s.c.).
Group 3 (ISO + SB): isoproterenol (3 mg/kg/day, s.c.) plus SB-204741 (1.0 mg/kg/day, i.p.).
Group 4 (ISO + SB + GSK-3βi): isoproterenol (3 mg/kg/day, s.c.) plus SB-204741 (1.0 mg/kg/day, i.p.) plus SB-216763 (0.6 mg/kg/day, i.p.).
Group 5 (SB + GSK-3βi): SB-204741 (1.0 mg/kg/day, i.p.) plus SB-216763 (0.6 mg/kg/day, i.p.), per se treated group.

The results of group 5 did not show any significant change in any of the parameters as compared to sham group; therefore we did not include the data of this group as it would add to the existing space.

2.3. Surgical procedures for evaluating hemodynamic parameters

The detailed surgical procedure for measuring hemodynamic parameters has been described in our previous study [6]. After recording the hemodynamic parameters, animals of all the groups were sacrificed with an overdose of anesthesia (sodium pentobarbitone 100 mg/kg, i.v.) and their hearts were excised and processed for various parameters. In addition, blood was withdrawn via cardiac puncture and centrifuged at 3000*g* for 5 min for measuring serum CK-MB, CRP and BNP levels.

2.4. Biochemical estimation

A 10% homogenate of myocardial tissue was prepared in ice-chilled phosphate buffer (50 mM, pH 7.4) and an aliquot was used to estimate malondialdehyde (TBARS) [7] and glutathione peroxidase (GSHPx) levels [8]. Furthermore, homogenate was centrifuged at 5000*g* for 20 min at 4 °C and the supernatant was assayed for protein content and TNF-α levels.

2.5. Western blot analysis

Left ventricular tissue protein samples (40 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane which was blocked for 2 h with 5% bovine serum albumin and incubated for 24 h at 4 °C with a rat primary antibody β-actin, 5-HT_{2B}, Bcl2, Bax (1:2500); eNOS, P-eNOS (Ser1177), Akt, P-Akt (Ser473) (1:2000); GSK-3β, P-GSK-3β (Ser9) (1:1500); NF-κBp65 chip grade, IKK-β (Ser180), Nox4, and MnSOD (1:1000). The primary antibody was detected with horse radish peroxidase-conjugated secondary antibody goat anti-rabbit/goat anti-mouse (1:1000). The blots so obtained were scanned and densitometry was performed to quantify the expression using Bio-Rad Quantity One 4.4.0 software (Bio-Rad, Hercules, CA).

2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assays were performed using an in situ cell death detection kit, POD (Roche, Germany) following the manufacturer's instructions.

2.7. Histopathological and transmission electron microscopy analysis

Histopathological and ultrastructural analysis of heart tissues were performed by the method described previously [6]. Briefly, the hearts were cut (3 μm thick) using microtome (Leica RM 2125, Germany) and stained with Hemotoxylin and Eosin for any histopathological changes or masson trichome staining (green-brown) for interstitial and fibrosis. For analyzing myocyte cross-sectional area, sections were stained for membranes with FITC-conjugated Wheat Germ Agglutinin (pictomicrographs not shown) (Nikon, Image Pro-Plus 4.0 software). The outline of 100 myocytes was traced in each section. The pathologist performing histopathological and ultrastructural analysis was blinded to the treatment protocol.

2.8. Statistical analysis

The data is presented as mean ± S.D. and one way ANOVA followed by Bonferroni post-hoc test was used to compare the groups. The histopathological grading was expressed as median score and Kruskal–Wallis test was employed to compare the groups. Value of $P < 0.05$ was considered significant.

3. Results and discussion

While there is strong evidence that 5-HT_{2B}RB negatively regulates cardiac hypertrophy [2–4], the molecular pathways driving this activity are hitherto largely unknown. It has been well documented that Akt/GSK-3β/β-catenin/eNOS is a crucial component of biochemical pathway responsible for cardiac hypertrophy induced by isoproterenol. Therefore, to establish whether this central hypertrophic pathway is the key regulator of cardioprotective potential of 5-HT_{2B}RB, it will be imperative to analyze its relation with Akt/GSK-3β/β-catenin/eNOS pathway. Accordingly, our findings demonstrated that (1) the protective effect of SB-204741, a 5-HT_{2B}RB is associated with the upregulation of P-Akt/P-GSK-3β/β-catenin/P-eNOS expressions and (2) which was coupled with shutting down of the inflammatory (decreased IKK-β/NF-κB/TNF-α expressions) and apoptotic markers (decreased Bax expression and TUNEL positivity and increased Bcl-2 expression). Intriguingly, we also found that SB-216763, a GSK-3βi further augmented this anti-hypertrophic effect, thereby, confirming the crucial role of GSK-3β in our hypothesis. Taken together, the anti-hypertrophic response of 5-HT_{2B}RB is at least in part is governed by augmented Akt/GSK-3β/β-catenin/eNOS phosphorylation.

In our study, 5-HT_{2B}RB abrogated the isoproterenol-induced cardiac hypertrophy as evidenced by significantly ($P < 0.05$) decreased HW/BW ratio (4.71 ± 0.58 vs. 7.51 ± 0.41 mg/g), LV/BW ratio (4.2 ± 0.7 vs. 5.92 ± 0.45 mg/g), myocyte area (213.64 ± 32.75 vs. 321.5 ± 55.89 μm^2) and interstitial fibrosis (2.9 ± 0.52 vs. 4.8 ± 0.65; fold change) as compared to isoproterenol controls (Fig. 1A–D). Moreover, this preserved cardiac architecture was well corroborated with cardiac performance as typified by significant

improvement in mean arterial pressure (129.4 ± 14.5 vs. 79.5 ± 10.2 mmHg), left ventricular end diastolic pressure (4.5 ± 0.4 vs. 7.24 ± 0.4 mmHg), inotropic status, +LVdP/dt_{max} (3683.75 ± 194.2 vs. 2899.5 ± 152.8 mmHg/s) and lusitropic status −LVdP/dt_{max} (2698.4 ± 168.2 vs. 2090.4 ± 220.5 mmHg/s) along with no effect on heart rate (362.5 ± 30.4 vs. 382.6 ± 28.5 beats/min) as compared to isoproterenol controls (1F–1J). Surprisingly, SB-216763, amplified the anti-hypertrophic effect of SB-204741 as manifested by a superior improvement ($P < 0.01$) in hemodynamic and ventricular functions. Likewise, histopathological and ultrastructural studies displayed lesser myonecrosis, interstitial fibrosis, infiltration of inflammatory cells, fiber disarray, mitochondrial swelling, widened I band, distorted Z line and chromatin condensed apoptotic nuclei as compared to SB-204741 treated group (Fig. 3: A1–A4, B1–B4, D1–D4 and Table 1B).

The corollaries of hypertrophied myocardium can range from damage to multiple loci within the cell to impaired contractile function. The former outcome is represented by down-regulation of various cytoprotective and cytoskeletal proteins and proteolysis of actin filaments (impaired excitation–contraction process); whereas the latter process is ensued by increased pre- and after load, inadequate ventricular emptying, reduced cardiac output and ultimately heart failure. So, to prevent the cardiomyocyte demise, phosphorylation of Akt/GSK-3β, a crucial switch gets upregulated and subsequently tunes various other cytoprotective genes to arrest the progression of cardiomyocyte insult [1]. This was exactly what we observed in our findings which might be a probable reason for superior anti-hypertrophic effect in SB-216763 group as compared to SB-204741 group. Firstly, this amplified phosphorylation/inhibition of Akt/GSK-3β activates sarcoplasmic reticulum Ca^{2+}ATPase 2a (SERCA2a) channel resulting in improved calcium homeostasis (by attenuating intracellular calcium overload), diastolic function and inotropic reserve of the myocardium [1,9,10]. Secondly, GSK-3βi prevents the decline in ATP and alleviates overall cell survival by inhibiting mitochondrial permeability transition pore [1,5,11]. Finally, Akt/GSK-3β phosphorylation/inhibition also preserves actin-myosin cross-bridge cycling; thus improving contractility and hemodynamic status of the recovered myocardium [1,9].

Therefore, the findings obtained from our study, allow us to postulate that enhanced phosphorylation of Akt/GSK-3β/eNOS by GSK-3βi may be a key step in anti-hypertrophic effect of 5-HT_{2B}RB. Notably, SB-216763 also enhanced β-catenin expression better than SB-204741 which might account for maintaining a more sustained cell–cell adhesive interaction in SB-216763 group as it has been demonstrated that mice overexpressing β-catenin had decreased myocyte area and a blunted hypertrophic response to angiotensin-II, with a resultant improved synergy of contraction [12,13]. Likewise, an eNOS deficient mouse exhibited significantly increased cardiac hypertrophy, proinflammatory cytokines and decreased SERCA2a mRNA levels [14]. Another unifying mechanism in the protective effect of SB-204741/SB-216763 lies in the inhibition of L-type calcium channel [4,15] or phosphorylation of Akt/GSK-3β/eNOS and stabilization of transcription factor β-catenin in the failing heart [9,15]. Perhaps, an overexpression of Bcl2 by SB-216763 over SB-204741 might also account for enhanced cardioprotection as augmented Bcl2 expression also attenuates calcium overload (Fig. 2) [4,16–17].

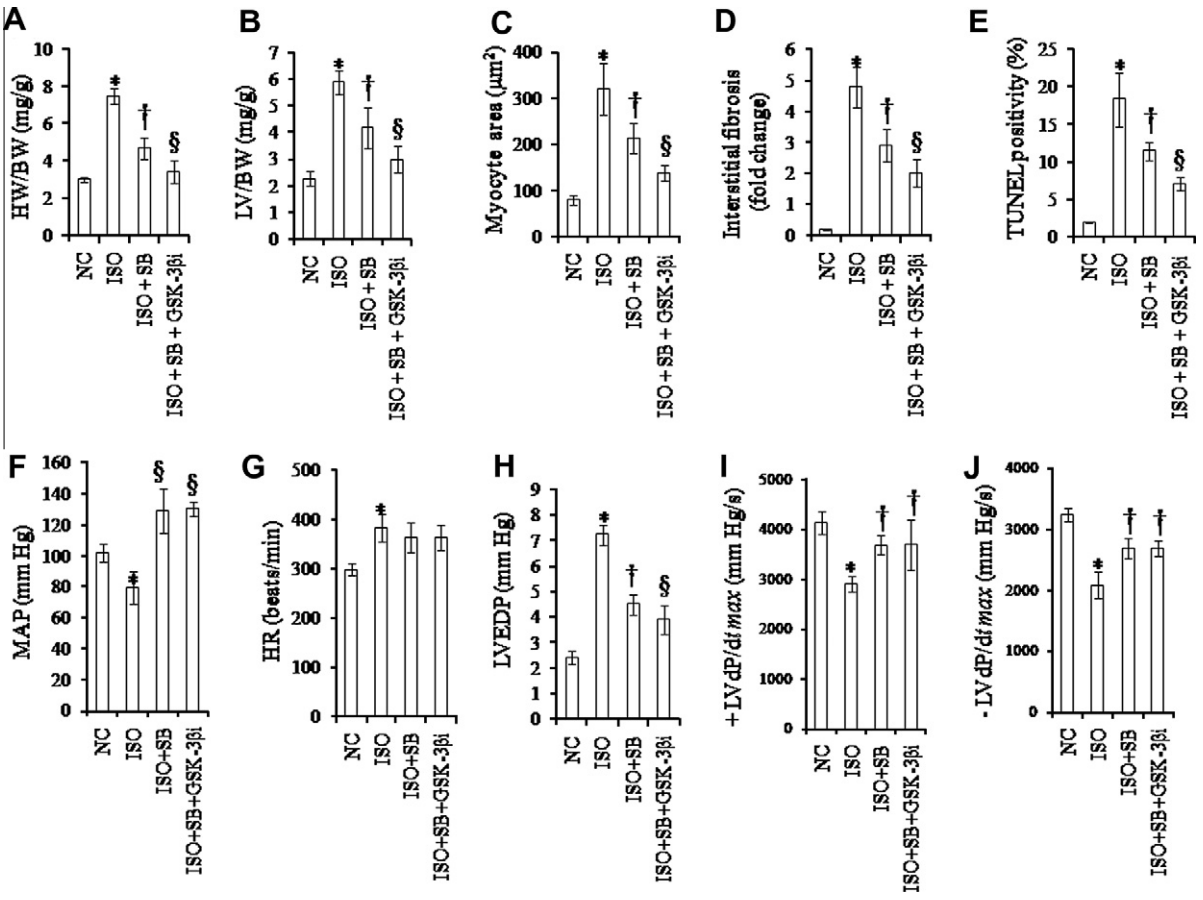

Fig. 1. Effect of drugs on (A) heart weight body weight ratio (HW/BW ratio); (B) left ventricular body weight ratio (LV/BW ratio); (C) myocyte area; (D) interstitial fibrosis; (E) apoptotic nuclei; (F) mean arterial pressure (MAP); (G) heart rate (HR); (H) left ventricular end diastolic arterial pressure (LVEDP); (I) rate of contraction (+LVdP/d*tmax*) and (J) rate of relaxation (−LVdP/dtmax) ($n = 8$). *$P < 0.01$ versus NC and †$P < 0.05$, §$P < 0.01$ versus ISO.

Table 1
(A) Effect of drugs on TBARS, CK-MB, GSHPx, TNF-α, CRP, and BNP ($n = 8$). (B) Histopathological grading 1: no change; 2: focal change; 3: patchy change; 4: confluent change; 5: massive change.

Parameters	TBARS (nmol/g tissue)	CK-MB (U/L)	GSPHx (U/mg protein)	TNF-α (pg/mg protein)	CRP (mg/ml)	BNP (pg/ml)
NC	41.4 ± 8.3	251 ± 25	0.81 ± 0.07	28.2 ± 4.2	2.1 ± 0.3	79.9 ± 6.1
ISO	212.4 ± 13.8*	690 ± 39*	0.28 ± 0.09*	149.3 ± 12.4*	8.5 ± 0.7*	381.5 ± 53.0*
ISO + SB	117.2 ± 6.2†	472 ± 49†	0.59 ± 0.09§	79.6 ± 9.2†	4.7 ± 0.32§	239.6 ± 36.8†
ISO + SB + GSK-3βi	73.5 ± 4.4§	259 ± 22§	0.62 ± 0.8§	51.1 ± 6.2§	4.4 ± 0.9§	132.8 ± 28.1§

Parameters (grading)	Myonecrosis (1–5)	Edema (1–5)	Inflammation (1–5)	Fiber disarray (1–5)	Total (4–20)
NC	1	1	1	1	4
ISO	4.5	4	4.5	4.5	17.5*
ISO + SB	2.5	2	2	2	8.5†
ISO + SB + GSK-3βi	2	1.5	1.5	2	7§

* $P < 0.01$ versus NC.
† $P < 0.05$ versus ISO.
§ $P < 0.01$ versus ISO.

Apart from the improvement in hemodynamic and contractile functions, 5-HT_{2B}RB negatively regulated the inflammatory and apoptotic pathways in isoproterenol-induced cardiac hypertrophy. Isoproterenol-infusion attenuated the activity of Bcl2 (3-fold) and amplified the activities of Bax (2.5-fold), IKK-β/NF-κB (2.5-fold), CRP (4-fold), TNF-α (4-fold) and TUNEL positivity (9-fold); which were subsequently attenuated by SB-204741 treatment ($P < 0.05$) (Figs. 1E, 2 and 3C1–C4 and Table 1A). This was almost normalized by co-treatment with SB-216763 ($P < 0.01$); thereby suggesting a decisive role between 5-HT_{2B}RB and GSK-3βi in tuning inflammatory/apoptotic pathways. Importantly, transcription factor NF-κB, orchestrates an array of cellular responses including inflammation, apoptosis and cellular proliferation by controlling a complex network of NF-κB dependent gene products (TNF-α/IL-6/IL-1β/IκB). One of the mechanisms for activation and intranuclear translocation of NF-κB is IκB kinase (IKK) complex induction in response to pro-inflammatory stimuli, leading to phosphorylation and degradation of IκBα and IκBβ [18,19]. It has also been delineated that the phosphorylation of Akt/GSK-3β pathway regulates NF-κB and β-catenin expressions [9], both of which are crucial regulators

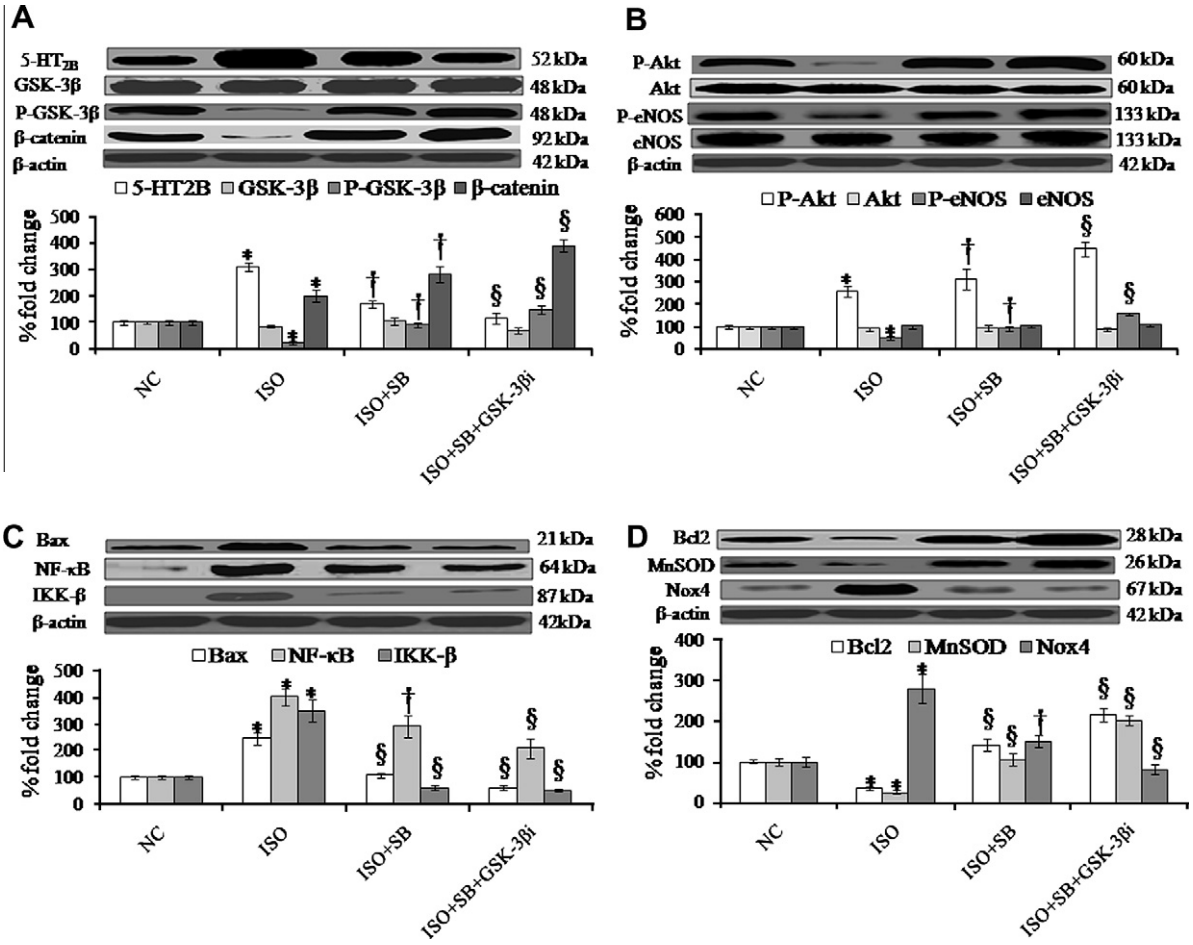

Fig. 2. Effect of drugs on (A) 5-HT_{2B}, GSK-3β, P-GSK-3β and β-catenin; (B) P-Akt, Akt, P-eNOS and eNOS; (C) Bax, NF-κBp65 and IKK-β and (D) Bcl2, MnSOD and Nox4 protein expressions. Data are expressed as a ratio of normal control value (set to 100) ($n = 8$). *$P < 0.01$ versus NC and †$P < 0.05$, §$P < 0.01$ versus ISO.

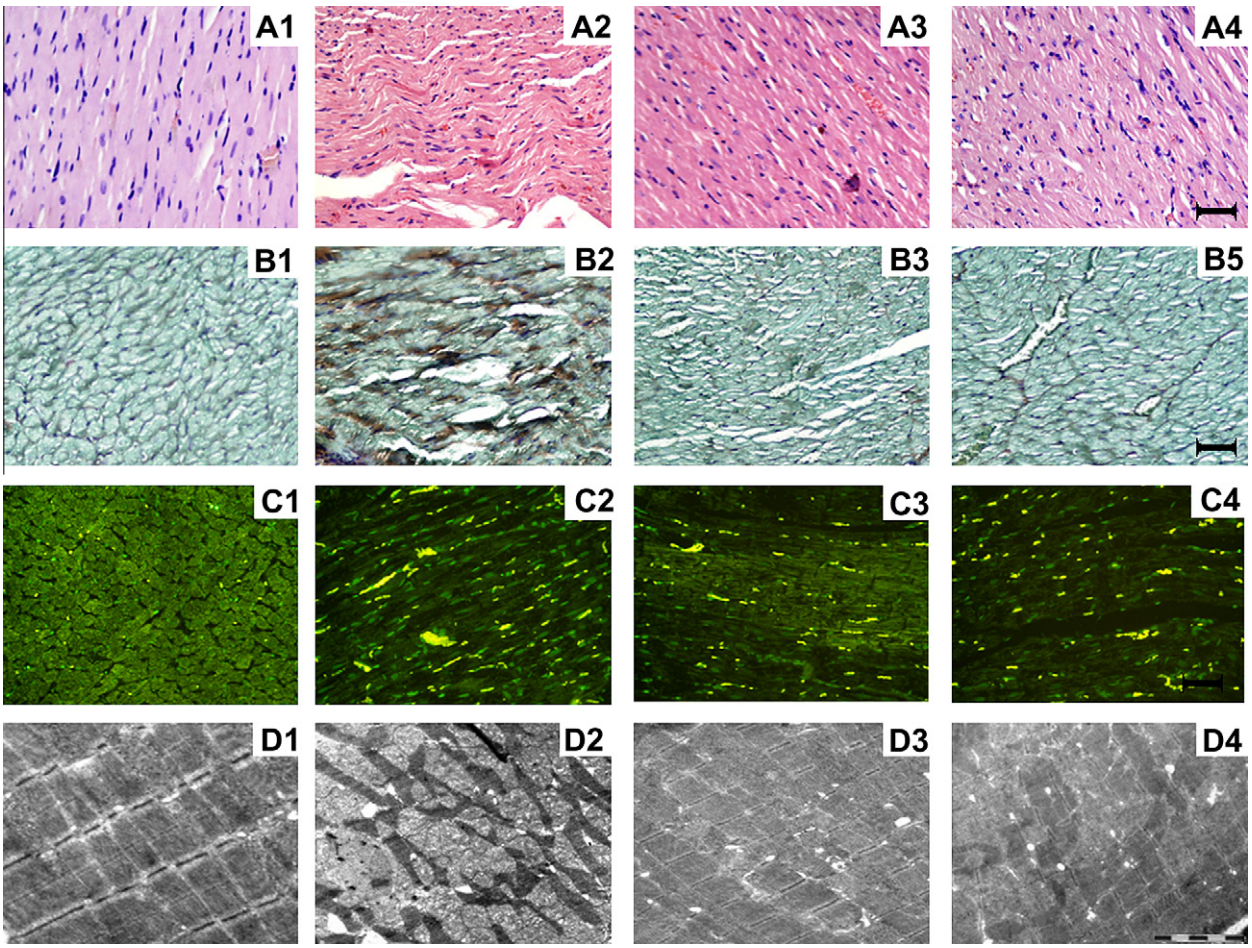

Fig. 3. Effect of drugs on light microscopic study (A1–A4, 20×, scale bar 50 μm, hematoxylin and eosin staining), interstitial fibrosis (B1–B4, 20×, scale bar 50 μm, masson trichome staining), TUNEL positivity (C1–C4, 20×, scale bar 50 μm) and electron microscopic study (D1–D4, 4000×, scale bar 1 μm) ($n = 8$). (1) Normal control; (2) isoproterenol control; (3) isoproterenol + SB-204741; (4) isoproterenol + SB-204741 + GSK-3βi.

of inflammation/apoptosis and cardiac hypertrophy. A higher anti-inflammatory/anti-apoptotic effect in SB-216763 treated group than SB-204741 group might be due to a higher phosphorylation of Akt/GSK-3β/eNOS or a direct suppression of IKK-β/NF-κB/TNF-α by SB-216763; as increased phosphorylation is cogently associated with abrogation of inflammatory/apoptotic cascade. Moreover, it might also be due to direct anti-inflammatory/anti-apoptotic activity of SB-204741 or synergistic activity of SB-204741 with SB-216763, as SB-216763 possesses strong anti-inflammatory/anti-apoptotic effect [4,5,15,17].

Isoproterenol also amplifies oxidative stress and ROS production leading to enhanced lipid peroxidation in heart tissue. In line with this assumption, we observed markedly ($P < 0.01$) increased TBARS (5-fold) and Nox4 (2.8-fold) activities, (parameters of lipid peroxidation and oxidant activity) accompanied by decreased MnSOD (4-fold) and GSHPx (2.8-fold) activities (parameters of antioxidant activity) in the isoproterenol challenged myocardium (Fig. 2 and Table 1A). On the other hand, both SB-204741 and SB-216763 attenuated oxidative stress, the effect being more pronounced in SB-216763 group, which could be due to the combined effect of 5-HT_{2B}RB and GSK-3βi in combating oxidative stress in the latter group; as GSK-3βi, has significantly shown to attenuate the oxidative stress in various animal models of drug discovery [1]. At a sub cellular level, the pronounced effect of SB-216763 can be attributed to a higher Akt/GSK-3β/eNOS phosphorylation and IKK-β/NF-kB suppression, reduced ROS production and/or amplification of myocardial antioxidant defense system in isoproterenol damaged heart, which scavenges ROS and lipid peroxyl radicals, and prevents myocardial damage [1,9,20].

Elevated serum BNP and CK-MB levels are the hallmark features of cardiac hypertrophy and have been employed to establish prognosis in cardiac hypertrophy and heart failure patients [21]. Recently, Liang and co-workers demonstrated that serotonin via 5-HT_{2B}R stimulation upregulates BNP levels through NF-κB pathway leading to cardiomyopathy, whilst 5-HT_{2B}RB showed the opposite results [22]. Consistent with this, our findings revealed that both 5-HT_{2B}RB ($P < 0.05$) and GSK-3βi ($P < 0.01$) improved BNP (239.6 ± 36.8 and 132.8 ± 28.1 vs. 381.5 ± 53.0 pg/ml) and CK-MB (472 ± 49 and 259 ± 22 vs. 690 ± 39 U/L) levels in isoproterenol-challenged myocardium, though the effect was more pronounced in GSK-3βi group. This protective effect suggests a superior and vital role of GSK-3βi in preserving the structural integrity and preventing the leakage of these cardiac enzymes (Table 1A).

To conclude, our molecular and functional findings strongly suggest that Akt/GSK-3β is a crucial component of the biochemical pathway responsible for 5-HT_{2B}RB mediated anti-hypertrophic effect and presents a possible future therapeutic strategy specifically aimed at treating myocardial hypertrophy.

Acknowledgements

The authors gratefully acknowledge Mr. B.M. Sharma for his technical assistance and the Department of Science and Technology,

Govt. of India for providing fellowship (JRF-Professional/IF10332) to Saurabh Bharti to carry out this research work, under the INSPIRE-DST-Fellowship programme.

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