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Signaling pathway-focused gene expression profiling in pressure overloaded hearts

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Summary. The β-blocker propranolol displays antihypertrophic and antifibrotic properties in the heart subjected to pressure overload. Yet the underlying mechanisms responsible for these important effects remain to be completely understood. The purpose of this study was to determine signaling pathway-focused gene expression profile associated with the antihypertrophic action of propranolol in pressure overloaded hearts. To address this question, a focused real-time PCR array was used to screen left ventricular RNA expression of 84 gene transcripts representative of 18 different signaling pathways in C57BL/6 mice subjected to transverse aortic constriction (TAC) or sham surgery. On the surgery day, mice received either propranolol (80 mg/kg/day) or vehicle for 14 days. TAC caused a 49% increase in the left ventricular weight-to-body weight (LVW/BW) ratio without changing gene expression. Propranolol blunted LVW/BW ratio increase by approximately 50% while causing about a 3-fold increase in the expression of two genes, namely Brca1 and Cdkn2a, belonging to the TGF-beta and estrogen pathways, respectively. In conclusion, after 2 weeks of pressure overload, TAC hearts show a gene expression profile superimposable to that of sham hearts. Conversely, propranolol treatment is associated with an increased expression of genes which negatively regulate cell cycle progression. It remains to be established whether a mechanistic link between gene expression changes and the antihypertrophic action of propranolol occurs.

Key words: heart failure, β-blockers, gene expression.

Riassunto *(Analisi del profilo di espressione genica nell'ipertrofia cardiaca patologica)*. Il propranololo è il capostipite dei β-bloccanti, una classe di farmaci di ampio impiego clinico. In modelli sperimentali di ipertrofia cardiaca patologica, il propranololo ha dimostrato di possedere proprietà antipertrofiche e antifibrotiche. Sebbene sia opinione comune che questi importanti effetti siano dovuti all'interazione del farmaco con i recettori β-adrenergici, i meccanismi molecolari che ne sono alla base rimangono largamente sconosciuti. Lo scopo del presente studio è quello di caratterizzare il profilo di risposte intracellulari associate all'azione antipertrofica del propranololo. A tal proposito, abbiamo utilizzato un rt-PCR array per valutare il profilo di espressione cardiaca di 84 geni, rappresentativi di 18 differenti vie di segnalazione intracellulare, in topi sottoposti a coartazione dell'aorta toracica (TAC) e trattati con propranololo per 14 giorni al dosaggio di 80 mg/kg/die. Gli animali soggetti a TAC mostravano un marcato incremento della massa ventricolare sinistra (+49%), ma nessuna significativa variazione del livello di espressione dei geni esaminati in confronto al gruppo di controllo. Al contrario, gli animali trattati con propranololo presentavano un modesto incremento della massa ventricolare (+24%) associato ad una significativa incrementata espressione di 2 geni, propriamente Brca1 e Cdkn2a, appartenenti rispettivamente alla via di segnalazione del TGF-β e degli estrogeni. In conclusione, i risultati del presente studio indicano che i cuori soggetti a sovraccarico pressorio per 2 settimane hanno un profilo di espressione sovrapponibile a quello dei cuori di controllo. Sorprendentemente il trattamento con il β-bloccante propranolo è associato con un'incrementata espressione di geni che regolano negativamente il ciclo cellulare. Ulteriori studi sono necessari per stabilire se esiste un legame tra i cambiamenti nell'espressione genica e l'attività antipertrofica del farmaco.

Parole chiave: insufficienza cardiaca, β-bloccanti, espressione genica.

INTRODUCTION

When the heart is subjected to chronic pressure overload, it undergoes hypertrophic growth. Although left ventricular hypertrophy is thought to develop as an adaptive response, it ultimately results in pathological remodeling, deterioration of cardiac function, and subsequent transition toward congestive heart failure (HF). Thus, somewhat counterintuitively, limiting the

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hypertrophic response under work overload conditions could be beneficial.

To date, several signaling pathways are known to regulate heart mass under pressure overload conditions including the β-adrenergic receptor (β-AR)-mediated signaling. Specifically, previous studies indicate that chronic activation of the β-adrenergic system in the heart is not only sufficient to induce a pathological ventricular remodeling, but also necessary for the development of cardiac hypertrophy under hemodynamic overload conditions [1-5].

We and others have demonstrated previously that propranolol, a β-AR antagonist used for the management of systemic arterial hypertension, angina pectoris, and certain types of cardiac arrhythmias, significantly blunts cardiac hypertrophic growth in a murine model of cardiac hypertrophy and HF [6-8]. Although the conventional wisdow is that the antihypertyrophic action of propranolol depends on its ability to block β-AR activation, knowledge about the nature of molecular mechanisms involved in this important effect is still incomplete. Specifically, it remains to be determined whether propranolol would suppress intracellular signaling cascades which result in the enhanced expression of certain genes which positively regulate cardiac growth in response to pressure overload.

To address this issue, left ventricular hypertrophy was induced by transverse aortic constriction (TAC) in C57BL/6 mice, whereas the relative expression levels of 84 gene transcripts representative of 18 different signaling pathways were obtained by quantitative SYBR Green real time PCR. In the present study, we found that no gene was differentially expressed in TAC hearts 2 weeks after surgery. Additionally, we observed that the antihypertrophic action of propranolol was accompanied by a significant increased expression of two genes, namely Brca1 and Cdkn2a, belonging to the TGF-beta and estrogen pathways, respectively, and both involved in the regulation of the cell cycle progression.

MATERIALS AND METHODS

Chemicals

(±)-Propranolol hydrochloride, direct red 80 and acid picric were obtained from Sigma-Aldrich (St. Louis, MO), and isoflurane from Abbott (Pomezia, Italy).

Animals and chronic administration of drugs

Male C57BL/6 (Harlan, San Pietro al Natisone, Italy) mice of 12-14 week old were used for the most experiments. Animals were maintained in accordance with 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).

Propranolol was administered in drinking water at the dose of about 80 mg/kg/day for 14 consecutive days. The dosage was chosen on the basis of literature data [9]. At the end of the protocol, surviving animals were killed, hearts were quickly dissected, and all cardiac chambers were weighed. Left ventricles were frozen in liquid nitrogen and kept at -80 °C, or fixed in 10% buffered formalin solution for further analysis.

Mouse model of LV pressure overload

Pressure overload on the left ventricle was induced by TAC as reported previously [10], with some modifications. Animals were anesthetized with isoflurane (1.5-2.0% in 100% of oxygen) and the degree of aortic stenosis was about 60%. A control group of mice was subjected to a sham operation with an identical surgical procedure but the ligature was not tightened. To quantify the hemodynamic load imposed on the mouse LV after aortic banding, LV systolic pressure was measured with a 1.4-Fr micromanometer-tipped catheter (Millar Instruments, mod. SPR 839, Houston, TX, USA) by direct catheterization of the left ventricle at the end of experiments. Data were analyzed with a software package for cardiovascular analysis (IOX 1.7; EMKA Technologies, Paris, France).

Histological analysys

Histological analysis was performed as described previously [11]. Briefly, the left ventricle was isolated, weighed and normalized by body weight (BW) to determine LVW/BW ratio. The left ventricle was fixed in 10% buffered formalin solution, embedded in paraffin and sectioned (5 μm sections). Left ventricular collagen was evaluated in picrosirius red-stained cross-sections, whereas LV cardiomyocyte area was measured in hematoxylin-eosin stained cross sections. Morphometric analysis of each heart section was performed with a computer-based quantitative color image analysis system (Metamorph; Universal Image Corporation, Downingtown, PA).

Echocardiography

Two weeks after banding, echocardiographic examination was performed in mice intubated and anesthetized with isoflurane (1% in 100% of oxygen) as described [11]. Briefly, a SONOLINE G50 (Siemens AG, Erlangen, Germany) equipped with a 13-MHz imaging transducer was used. After good-quality 2D short-axis images of the left ventricle were obtained, M-mode freeze frames were printed on common echocardiographic paper and digitized. End-systolic (LVESD), end-diastolic (LVEDD) LV internal diameters, and posterior wall end-diastolic thickness (PWT) were measured by an image analysis system (Metamorph; Universal Image Corporation). Percent fractional shortening (FS) was calculated as (LVEDD- LVESD)/LVEDDx100.

RNA isolation and quantification

Total RNA was extracted from mouse left ventricles by using SV Total RNA Isolation System (Promega, Madison, WI), and retrotranscribed by RT^2 First

Table 1 | *Echocardiographic and hemodynamic analyses in propranolol treated mice*

Group	LVIDd, mm	LVIDs, mm	FS, %	HR, b/min	PWT, mm	LVSP, mmHg
Sham	3.21 ± 0.12	1.85 ± 0.09	42.1 ± 2.1	416 ± 23	0.56 ± 0.04	65 ± 3
TAC	3.30 ± 0.10	1.96 ± 0.08	40.1 ± 2.2	407 ± 19	0.95*‡ ± 0.06	101 ± 5*
Pro	3.28 ± 0.11	1.89 ± 0.09	41.8 ± 2.3	387 ± 24	0.55 ± 0.04	62 ± 3
Pro-TAC	3.30 ± 0.15	1.92 ± 0.10	41.5 ± 2.0	391 ± 16	0.69* ± 0.05	95 ± 4*

*Data are given as mean ± SEM; LVIDd, left ventricular end-diastolic diameter; LVIDs, left ventricular end-systolic diameter; FS, fractional shortening; HR, heart rate; PWT, posterior wall thickness in diastole; LVSP, left ventricular systolic pressure; Sham, sham operated mice; TAC, transverse aortic constriction; Pro, propranolol-treated sham mice; Pro-TAC, propranolol-treated TAC mice. (n = 5-6 per group); *p < 0.05 vs the respective sham control; ‡p < 0.05 vs Pro-TAC group*

Strand Kit (SABiosciences Corporation, Frederick, MD). Mouse Signal Transduction Pathway Finder RT^2 Profiler PCR Array (PAMM-014A) and RT^2 Real-Timer SyBR Green/ROX PCR Mix were also used (SABiosciences Corporation, Frederick, MD). The full list of analyzed genes is shown in *Table 1*. Real-time PCR was performed on ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA). The specificity of the SYBR Green assay was confirmed by melting point analysis. For data analysis the $2^{-\Delta\Delta Ct}$ method was used with 5 housekeeping genes as controls. The fold of change was determined by dividing the mean of $2^{-\Delta Ct}$ of the treated group for the mean of $2^{-\Delta Ct}$ of the sham group [12]. Significant changes were determined using an unpaired two-tailed Student's t-test on $2^{-\Delta Ct}$ values according to Schmittgen and Livak [12].

Statistical analysis

Group means (± SEM) were calculated for all relevant variables. Statistical analysis was performed by ANOVA with Bonferroni's multiple comparison test for post hoc analyses when applicable or by Student's t-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Propranolol attenuates TAC-induced cardiac hypertrophy

We used the TAC mouse model of LVH to evaluate the antihypertrophic property of propranolol. Mice were therefore subjected to TAC, that provided an approximate 60% reduction in the lumen of the aortic arch, or sham surgery. On the surgery day, mice received either propranolol dissolved in drinking water (Pro-TAC and Pro groups) or tap water (TAC and Sham groups). Pressure overload was tolerated well by all TAC groups with no signs of cardiovascular compromise or postoperative increased mortality. As expected, left ventricular weight to body weight (LVW/BW) ratio significantly increased by ≈ 49% in TAC group *(Figure 1A)*. In contrast, cardiac hypertrophic growth was significantly blunted in Pro-TAC mice, with approximately a 24% increase in the LVW/BW ratio *(Figure 1A)*.

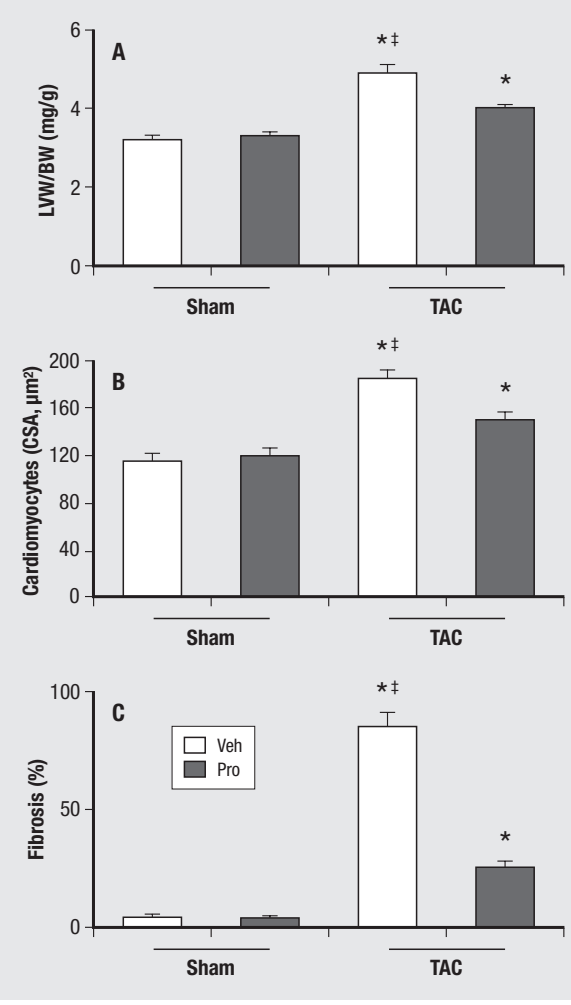

Fig. 1 | *Attenuated hypertrophic response of propranolol-treated hearts to TAC. (A) Left ventricular weight to body weight (LVW/BW) ratio (mg/g). A significant attenuation of hypertrophic response to TAC was observed in propranolol-treated mice (n = 8 for each group). (B) Cross-sectional area (CSA) of cardiomyocytes. About 200 cardiomyocytes per group were analyzed (n = 3 for each group). (C) Perivascular fibrosis is expressed as fibrosis area related to total vessel area. Collagen accumulation is prominent in untreated mice 14 days after TAC (n = 3 for each group). Veh, tap water; Pro, propranolol; Sham, sham operated mice; TAC, transverse aortic constriction; *p < 0.05 vs the respective sham control; ‡ p < 0.05 vs propranolol-treated TAC group.*

Histomorphometric analysis revealed a significant increase in the cardiomyocyte cross-sectional area in TAC hearts compared with Sham ones *(Figure 1B)*. Again, a significant decrease was observed in Pro-TAC mice in comparison with TAC ones, confirming the gross pathological data *(Figure 1B)* and suggesting that the diminished cardiac growth reflects reduction of cardiomyocyte hypertrophy. Evident signs of perivascular fibrosis were observed in all TAC hearts, but the ratio of collagen area to total vessel area, an index of perivascular fibrosis, in the left ventricle was greater in TAC than in Pro-TAC mice *(Figure 1C)*. This suggests that propranolol may negatively affect the mechanisms controlling collagen deposition under pressure overload conditions.

Echocardiographic measurements revealed that the TAC hearts developed significant thickening of LV posterior wall (PWT) 2 weeks after TAC *(Table 1)*. Conversely, a significant attenuation in PWT increase was observed in Pro-TAC mice when compared with TAC mice *(Table 1)*. With the exception of its ability to attenuate the increase of PWT, propranolol did not significantly affect heart rate, fractional shortening or ventricular chamber size in anesthetized mice *(Table 1)*. Also, propranolol did not alter ventricular afterload as assessed by measurement of LV systolic pressure *(Table 1)*. Overall, these results indicate that orally administered propranolol is able to significantly attenuate the LV concentric hypertrophic response following TAC, but also to prevent LV decompensation under pressure overload conditions despite the blunted compensatory hypertrophic response.

Propranolol alters gene expression in TAC hearts

It is possible that the antihypertrophic effect of propranolol is attributable to its ability to suppress a critical growth pathway or to upregulate the expression of endogenous negative regulators. Left ventricles from sham or banded mice were also analyzed for gene expression by real-time quantitative RT-PCR. Specifically, we examined the expression profile of 84 genes representative of 18 different signal transduction pathways *(Table 2)*. The mean ± sd for each group was calculated from normalized individual data points using the $2^{-\Delta Ct}$ method [12]. For each group, at least three independent experiments run in duplicate were performed. Two weeks of pressure overload caused no significant difference in gene expression when compared to sham controls. Remarkably, Pro-TAC hearts only displayed a significant increase (≈ 3-fold) in Brca1 (breast cancer 1, early onset) and Cdkn2a (cyclin-dependent kinase inhibitor 2A) gene expression compared with sham ones *(Figure 2A,B)*. Propranolol alone did not affect cardiac gene expression *(Figure 2A,B)*. Collectively our data indicate that the antihypertrophic activity of propranolol is associated with changes of gene expression in hearts subjected to pressure overload.

Table 2 | *Signal transduction pathway PCR array*

Mitogenic Pathway: Egr1 (egr-1), Fos (c-fos), Jun (c-jun), Nab2.

Wnt Pathway: Birc5, Ccnd1 (cyclin D1), Cdh1, Fgf4, Jun (c-jun), Lef1, Myc (c-myc), Pparg, Tcf7, Vegfa, Wisp1.

Hedgehog Pathway: Bmp2, Bmp4, En1 (engrailed), Foxa2 (forkhead box A2 / HNF3B), Hhip, Ptch1 (patched 1), Wnt1, Wnt2.

TGF-β Pathway: Cdkn1a (p21Waf1, p21Cip1), Cdkn1b (p27), Cdkn2a (p16Ink4), Cdkn2b (p15 Ink2b).

Survival Pathway:

PI3 Kinase / AKT Pathway: Bcl2 (Bcl-2), Ccnd1, Fn1 (fibronectin), Jun (c-jun), Mmp7 (matrilysin), Myc (c-myc).
Jak / Src Pathway: Bcl2 (Bcl-2), Bcl2l1 (Bcl-XL).
NFκB Pathway: Birc1a, Birc2 (c-IAP2), Birc3 (c-IAP1), Tert.

p53 Pathway: Bax, Cdkn1a (p21Waf1, p21Cip1), Ei24 (Pig3), Fas (Tnfrsf6), Gadd45a (gadd45), Igfbp3, Mdm2.

Stress Pathway: Atf2, Fos (c-fos), Hsf1 (tcf5), Hspb1 (Hsp25), Myc (c-myc), Trp53 (p53).

NFκB Pathway: Ccl20, Cxcl1, Icam1, Ikbkb, Il1a, Il2, Lta (TNFb), Nfkbia, Nos2 (iNOS), Tank, Tnf (TNFa), Vcam1.

NFAT Pathway: Cd5, Fasl (Tnfsf6), Il2.

CREB Pathway: Cyp19a1, Egr1 (egr-1), Fos (c-fos).

Jak-Stat Pathway: Cxcl9 (MIG), Il4ra, Irf1, Mmp10 (stromelysin-2), Nos2 (iNOS).

Estrogen Pathway: Bcl2 (Bcl-2), Brca1, Greb1, Igfbp4, Nrip1.

Androgen Pathway: Cdk2, Cdkn1a (p21Waf1, p21Cip1), Pmepa1.

Calcium and Protein Kinase C Pathway: Csf2 (GM-CSF), Fos (c-fos), Il2, Il2ra (IL-2 R), Jun (c-jun), Myc (c-myc), Odc1, Tfrc.

Phospholipase C Pathway: Bcl2 (Bcl-2), Egr1, Fos (c-fos), Icam1, Jun (c-jun), Nos2 (iNOS), Ptgs2 (cox-2), Vcam1.

Insulin Pathway: Cebpb (C/EBP), Fasn (fatty acid synthase), Gys1 (GS, glycogen synthase), Hk2 (hexokinase II), Lep (Ob).

LDL Pathway: Ccl2, Csf2 (GM-CSF), Sele, Selp (P-selectin), Vcam1.

Retinoic Acid Pathway: En1 (engrailed), Hoxa1, Rbp1 (CRBPI).

DISCUSSION

In the present study, we demonstrate that propranolol administration in TAC mice alters the gene expression profile of pressure overloaded hearts by enhancing the expression of Brca1 and Cdkn2a genes which belong to a class of genes known as tumor suppressors and involved in the regulation of cell cycle and the maintenance of genomic integrity. We also report that at this stage of cardiac hypertrophy development no gene was differentially expressed between TAC and sham mice.

Previous studies have evidenced that chronic activation of the β-AR signaling pathway in the heart is sufficient to induce a pathological ventricular remodeling, but also necessary for the cardiac growth in response to pressure overload. Specifically, chronic treatment with isoproterenol, a β-AR agonist, as well as cardiac-specific overexpression of β_1- and β_2-ARs, Gsα, and protein kinase A results in cardiac hypertrophy and fibrosis [1-4], and, at least in Gsα overexpressing mice, this effect is blocked by propranolol [9]. The results of the present study fur-

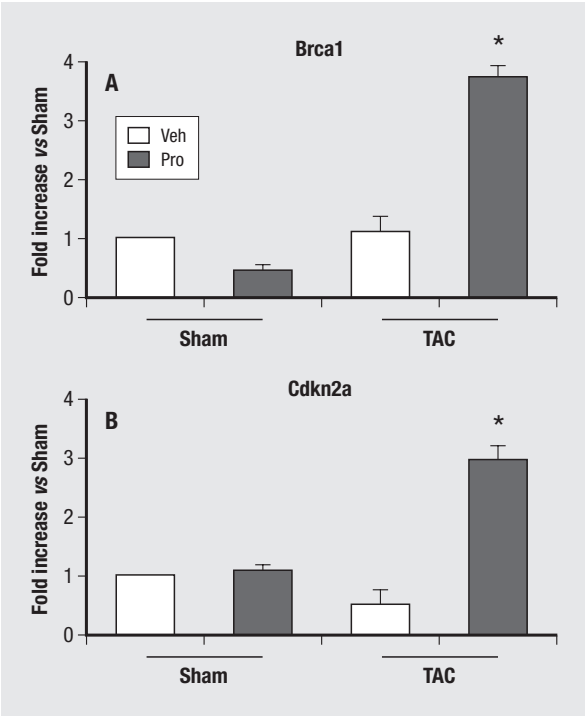

Fig. 2 | *Enhanced expression of Brca1 and Cdkn2a genes in propranolol-treated mice subjected to TAC. Expression levels of Brca1 (A) and Cdkn2a (B) genes were significantly increased in TAC mice treated with propranolol. Transcripts from left ventricles were detected by real-time RT-PCR 14 days after TAC. Values indicate expression level relative to the sham operated group. For each group, summary data of at least three independent experiments run in duplicate are shown. Brca1, breast cancer 1, early onset; Cdkn2a, cyclin-dependent kinase inhibitor 2a; *p<0.05 vs. sham.*

ther support this notion. Indeed we found that propranolol, a widely used β-blocker, negatively affects the extent to which cardiac hypertrophy develops in response to pressure overload.

Real-time PCR analysis of gene expression in TAC hearts demonstrates that propranolol significantly increases the expression of Brca1 and Cdkn2a, which belong to a class of genes known as tumor suppressors and involved in the regulation of cell cycle and the maintenance of genomic integrity. Indeed, Brca1 gene encodes a nuclear phosphoprotein that plays a role in maintaining genomic stability and acts as a tumor suppressor [13]. Also, the product of the Cdkn2a gene, $p16^{Ink4a}$, inhibits the retinoblastoma protein (Rb) phosphorilation and induces cell cycle arrest, which is considered its main tumor suppressor function [14, 15]. Involvement of cell cycle regulatory molecules in cardiomyocyte hypertrophy has already been reported. For example, in rat neonatal cardiomyocytes, cyclin D-cdk4/6-dependent phosphorylation of pRb and activation of E2F is necessary for hypertrophic growth [16]. Additionally, some G1 phase cyclins and cyclin-dependent kinases (CDKs) are upregulated during the early stages of pressure overload-induced hypertrophy (17), whereas a significant downregulation of the CDK inhibitors p21 and p27 was observed during the development of pressure overload-induced LVH [18]. Using transgenic mouse models, it has been found that inhibition of the cyclin D-dependent kinases by overexpression of $p16^{Ink4a}$ impairs hypertrophic growth in the TAC-induced LVH model [19]. Although it has been hypothesized that activation of cell cycle regulators by hypertrophic stimuli can promote protein synthesis and growth, but not DNA synthesis in cells that can not replicate such as adult cardiomyocytes [19], the relationship between the antihypertrophic activity of propranolol and the enhanced expression of these cell cycle regulators in pressure overloaded hearts remains to be clarified.

Finally, additional features of our experiments are worth commenting on. Firstly, we did not perform microarray-based gene expression profiling. However, it has to be considered that profiling 84 genes using real-time PCR configured in 96-well reaction plates is a convenient method to generate high-quality data on several dozens of genes and to analyze the expression of various genes within a particular signaling pathway. Secondly, despite the decreased hypertrophic response to pressure overload in propranolol-treated TAC mice, no signs of ventricular dysfunction were observed. However, future long-term experiments will be required to assess whether blunting cardiac hypertrophy is beneficial or deleterious under pressure overload conditions or whether propranolol provides greater cardioprotection when compared to other β-blockers. Thirdly, heart consists primarily of myocytes, fibroblasts, and endothelial cells. Although myocytes make up the largest cellular volume, fibroblasts are the most numerous cell type. Recently, it was reported that cardiac fibroblast activation under pressure overload conditions is associated with fibrosis, hypertrophy and cardiac dysfunction suggesting a primary role for cardiac fibroblasts in myocardial disease [20]. Whether the antihypertrophic effects of propranolol also derive from actions on cardiac fibroblasts remains to be established. Fourthly, propranolol was able to attenuate perivascular fibrosis suggesting that β-adrenergic signaling has an important role in cardiac collagen accumulation under pressure overload conditions. Previously, in a 10 week study, it was reported that cardiac fibrosis was abolished by sympathectomy but left unchanged by β-AR blockade in rats subjected to abdominal aortic banding [21]. This discrepancy may be related to the different experimental procedures used to produce LVH (abdominal aortic banding in the previous study versus TAC in this study). TAC is a more severe model of chronic pressure overload and, in contrast to abdominal aortic banding, is a renin angiotensin system-independent model.

In summary, propranolol produces an antihypertrophic effect which is associated with the enhanced

expression of genes which regulate the cell cycle. Although it remains to be established a mechanistic link between gene expression changes and the antihypertrophic action of propranolol, we believe, however, that the results of the present study are sufficient to warrant further investigation, in order to confirm or disprove this notion.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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