Metoprolol and bisoprolol ameliorate hypertrophy of neonatal rat cardiomyocytes induced by high glucose via the PKC/NF-κB/c-fos signaling pathway

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Abstract. Hyperglycemia caused by diabetes mellitus could increase the risk of diabetic cardiomyopathy. However, to the best of our knowledge, the underlying mechanism of this process is still not fully explored. Thus, developing ways to prevent hyperglycemia can be beneficial for diabetic patients. The present study was designed to investigate the influence of metoprolol and bisoprolol on the cardiomyocytic hypertrophy of neonatal rat cardiomyocytes. Cardiomyocytes were cultured in two types of media: One with low glucose levels and one with high glucose levels. Cardiomyocytes cultured in high glucose were further treated with the following: A protein kinase C (PKC) inhibitor, an NF-κB inhibitor, metoprolol or bisoprolol. The pulsatile frequency, cellular diameter and surface area of cardiomyocytes were measured. Protein content and [^{3}H]-leucine incorporation were determined, atrial natriuretic peptide (ANP), α-myosin heavy chain (α-MHC) and β-myosin heavy chain (β-MHC) mRNA levels were calculated by reverse transcription-quantitative PCR, while the expression and activation of PKC-α, PKC-$β_2$, NF-κB, tumor necrosis factor-α (TNF-α), and c-fos were detected by western blotting. Metoprolol or bisoprolol were also used in combination with PKC inhibitor or NF-κB inhibitor to determine whether the hypertrophic response would be attenuated to a lower extent compared with metroprolol or bisoprolol alone. Cardiomyocytes cultured in high glucose presented increased pulsatile frequency, cellular diameter, surface area, and protein content and synthesis, higher expression of ANP and β-MHC, and lower α-MHC expression. High glucose levels also upregulated the expression and activation of PKC-α, PKC-$β_2$, NF-κB, TNF-α and c-fos. Metoprolol and bisoprolol partly reversed the above changes, while combined use of metoprolol or bisoprolol with PKC inhibitor or NF-κB inhibitor further ameliorated the hypertrophic response mentioned above to lower levels compared with using metroprolol or bisoprolol alone. In conclusion, metoprolol and bisoprolol could prevent hypertrophy of cardiomyocytes cultured in high glucose by the inhibition of the total and phospho-PKC-α, which could further influence the PKC-α/NF-κB/c-fos signaling pathway.

Introduction

As a common noncommunicable chronic disease, diabetes mellitus (DM) has become a global health issue. It is estimated that the morbidity of DM is expected to rise to 552 million by 2030 (1). Subjected to a relative or absolute deficiency of insulin secretion, patients with DM present numerous complications due to the long-term conditions of hyperglycemia, such as diabetic cardiomyopathy. DM may also cause abnormality in the heart metabolic signaling pathway. These mediators can result in diastolic dysfunction at the early stage of DM and damaged systolic function at the late stage of DM (2).

A number of studies have been performed to explore potential critical signaling pathways in DM (3-5). For example, in the pathogenesis of diabetic cardiomyopathy, the state of chronic hyperglycemia increases the formation of diacylglycerol (DAG), activates protein kinase C (PKC) and accelerates non-enzymatic formation of advanced glycated end products (6). These intracellular changes eventually cause structural and functional alteration of cardiomyocytes.

The PKC family comprises of more than ten different isozymes and was found to be associated with cardiovascular diseases such as cardiac hypertrophy and ischemia-reperfusion injury (7,8). It was reported that PKC inhibition could partly reverse both structural abnormalities and cardiac dysfunction in cardiac hypertrophy (9). PKC-α and PKC-$β_2$ are two highly expressed PKC isoforms in the heart. Previous studies

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showed that high glucose stimulation could activate PKC-α and PKC-β_2 at the early stage of diabetic cardiomyopathy (10). Such changes of those two isoforms suggested they may act as an important mediator in the pathological progress of diabetic cardiomyopathy (11). However, the exact role of the PKC family and its downstream signaling pathway in diabetic cardiomyopathy remains to be completely elucidated.

β-receptor blockers (β-blockers) are one of the most commonly used medicines for the treatment of cardiovascular diseases, including cardiac hypertrophy, heart failure, arrhythmia and angina (12,13). Use of β-blockers could effectively improve cardiac function, reverse the remodeling of left-ventricle and enhance the ability of physical exercise-related capacity (14). Although the prognostic benefits of β-blockers for patients with cardiovascular disease are commonly recognized, they are yet to be elucidated in patients with diabetes, as beta-blockers could delay the development of diabetes by counteracting hypoglycemia symptoms (15). However, β-blockers can also disrupt glycemic control and lipid metabolism of diabetic patients. In a Glycemic Effects in Diabetes Mellitus: Carvedilil-Metoprolol Comparison in Hypertensives study, patients with hypertension and type 2 DM (T2DM) treated with metaprolol showed a significant increase in hemoglobin A_{1c} levels compared with baseline levels (16). In another study, insulin-stimulated endothelial function in diabetic patients was significantly decreased following treatment with metaprolol (17). However, diabetic patients who have developed systolic heart failure (SHF) may still benefit from appropriate β-blocker therapy. A meta-analysis of large-scale clinical trials has verified that β-blockers could reduce mortality rates in patients with SHF with DM (18).

Metoprolol and bisoprolol, both β-antagonists, improve the prognosis of heart failure and prevent the progression of left ventricle remodeling (19). In ischemic or non-ischemic heart failure, both metoprolol and bisoprolol have been reported to reduce cardiac events, attenuate cardiac dysfunction, and reduce mortality (20,21). Therapy with beta-blockers, such as bisoprolol, has a favorable effect in clinical outcomes of reducing mortality and morbidity of heart failure patients with new-onset DM, either with heart failure with preserved or reduced ejection fraction (22). However, how metoprolol and bisoprolol attenuate left ventricle remodeling or dysfunction associated with diabetic cardiomyopathy remains to be determined.

In the present study, PKC and its downstream PKC/NF-κB/c-fos signaling pathway was hypothesized to be critical for the development of cardiac hypertrophy related to diabetic cardiomyopathy. Moreover, the current study also aimed to determine whether metoprolol and bisoprolol could attenuate cardiac hypertrophy induced by hyperglycemia and elucidate the underlying mechanism of this process.

Materials and methods

Experimental animals. Cardiomyocytes of each experiment were derived from 1 to 3-day-old neonatal Sprague-Dawley rats (total number of rats, 30; weight, 4-6 g; male rats, 15; female rats, 15), provided by the Laboratory Animal Center of Zhejiang University. Animals were housed in a temperature of 22-26°C and a humidity of 50-65% controlled environment with a 12-h light-dark cycle. The rats had free access to water and food. Procedures in the current study were all conducted according to the guidelines for animal care (23) and approved by the Ethics Committee of Zhejiang University.

Reagents, chemicals and drugs. DMEM, FBS and 0.25% trypsin were obtained from Gibco; Thermo Fisher Scientific, Inc.; type II collagenase, glucose and [³H]-leucine were provided by Sigma-Aldrich; Merck KGaA. Ro-31-8220 and BAY11-7082 were obtained from Selleck Chemcicals; metoprolol and bisoprolol were obtained from AstraZeneca. Primary antibodies for detection of PKC-α (cat. no. 2056), p-PKC-β_2 (cat. no. 9371), NF-κB p65 (cat. no. 8242), p-NF-κB (cat. no. 3033), Histone H3 (cat. no. 9717) and c-fos (cat. no. 2250) were purchased from Cell Signaling Technology. PKC-β_2 (cat. no. sc-210) and p-PKC-α (cat. no. sc-12356) were purchased from Santa Cruz Biotechnology. β-actin primary antibody (cat. no. 70-ab010-100) and horseradish peroxidase-linked goat anti-rabbit secondary antibodies (cat. no. 70-GAM0072) were purchased from MultiSciences Biotech Co., Ltd. Primers were designed according to our previous study (11) and synthesized by TSINGKE. TRIzon reagent (cat. no. CW0580) for extracting total RNA and UltraSYBR Mixture (cat. no. CW0957) for reverse transcription-quantitative PCR (RT-qPCR) assays were purchased from CWBIO Biosciences. PrimeScript RT-PCR kit was purchased from Takara Bio, Inc.

Isolation and culture of neonatal rat cardiomyocytes. Cardiomyocytes were obtained and cultured as follows. Neonatal Sprague-Dawley rats were sacrificed and hearts were dissected. A diluted solution made up of 0.125% trypsin and 0.1% type II collagenase was used to digest the ventricular tissues for 8 min at 37°C. Following each digestion, the supernatant was collected and added to DMEM with 10% FBS. The step was repeated six to eight times until the ventricles were completely digested. The collected mixture was then centrifuged at 55.9 x g for 5 min at room temperature. The supernatant was discarded and cells were resuspended in ACK Lysis Buffer (Beijing Solarbio Science and Technology Co., Ltd.) for 2 min. The cells were centrifuged as described above once again, and the collected cells were resuspended in DMEM with 10% FBS. Differential adhesion method was used for separating cardiomyocytes from myofibroblasts. Cardiomyocytes were diluted to $(3-5) \times 10^5$/ml in DMEM with 10% FBS and cultured in six-well plates in a humidified atmosphere with 5% CO_2 at 37°C. Culture medium was replaced with serum-free DMEM after 48 h.

Treatment groups. After the first 48 h, cardiomyocytes were cultured in serum-free DMEM with different concentrations of glucose and drugs for another 48, or 72 h for the [³H]-leucine incorporation measurements at 37°C with humidified 5% CO_2 as follows: Low glucose (LG; 5.5 mM), high glucose (HG; 25.5 mM), HG + metoprolol (0.5 or 10 μM), HG + bisoprolol (50 or 200 nM), HG + PKC inhibitor Ro-31-8220 (50 nM) and HG + NF-κB inhibitor BAY11-7082 (5 μM). A higher dose of metoprolol or bisoprolol was used in combination with PKC inhibitor (Ro-31-8220) or NF-κB inhibitor (BAY11-7082) to determine whether the hypertrophic response could be

attenuated to a lower extent compared with metoprolol or bisoprolol treatment alone. Cells were cultured in serum-free DMEM in the following conditions: HG + metoprolol (10 μmol/l) + Ro-31-8220 (50 nmol/l), HG + metoprolol (10 μmol/l) + BAY11-7082 (5 μmol/l), HG + bisoprolol (200 nmol/l) + Ro-31-8220 (50 nmol/l) and HG + bisoprolol (200 nmol/l) + BAY11-7082 (5 μmol/l).

Determination of cellular pulsatile frequency. Individual cellular pulsatile frequency of cardiomyocytes was determined using an inverted light microscope. The inverted microscope and 24-well plates were briefly placed in a homoiothermic air convection assembly, which was fully covered by convection air at 37°C. Five random fields from each group were selected and 20 individual cardiomyocytes of each field were counted for the pulsatile frequency at x400 magnification.

Measurement of cellular diameter. Cardiomyocytes on glass coverslips were washed with warm PBS twice, fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.5% Triton-100 for 5 min at room temperature. After washing, cells were incubated with 100 nM rhodamine-phalloidin (Beijing Solarbio Science and Technology Co., Ltd.) for 30 min at room temperature. Cells were then stained with DAPI (Beijing Solarbio Science and Technology Co., Ltd.) for 30 sec at room temperature. Images of five randomly selected fields of view were captured for each group and used to measure the cellular diameters of cardiomyocytes using a fluorescent microscope at x400 magnification.

Determination of cell protein content. Cardiomyocytes were washed with Hanks' balanced salt solution (HBSS) three times before being lysed with 1% SDS. Protein content measurement was then carried out in accordance with the Lowry method (24). A standard protein solution was utilized to prepare a standard curve for the final estimation of protein content. The absorbance of the final solution was then measured at a wavelength of 750 nm.

Incorporation of [^{3}H]-leucine. To measure newly synthesized protein of cardiomyocytes (25), [^{3}H]-leucine incorporation method was conducted as previously described by Luo *et al* (26). Firstly, 1 μCi [^{3}H]-leucine was added to cell culture medium with corresponding levels of glucose for cardiomyocytes treatment. β-blockers and inhibitors were then co-incubated with the cells for 72 h. Following incubation, cells were quickly washed with cold HBSS three times. A total of 1 ml 1% SDS was added to each well to lyse cells and the lysates were collected. Subsequently, 1 ml 5% trichloroacetic acid was added to the lysates at 4°C for 1 h before the lysates were precipitated and transferred to fiberglass filters. Finally, lysates were washed with HBSS before the precipitates were dried and moved to scintillation fluid. Radioactivity was detected and expressed as cpm/well by liquid scintillation counting.

Nucleus extraction. To detect the translocation of NF-κB and p-NF-κB in the nucleus by the glucose stimulation, nucleus extraction was conducted using Nuclear Extraction kit (cat. no. SN0020) according to the manufacture's protocol (Beijing Solarbio Life Science & Technology Co., Ltd.). Cells in the cultured plates with a density of 5x10^5/ml were firstly digested with EDTA buffer and washed with PBS. Those cells were centrifuged at the speed of 800 x g for 5 min at 4°C and resuspended with 1 ml cold lysis buffer with PMSF and 50 μl Reagent A provided by the kit. Then, the 1.05 ml cell suspension was transferred to a small glass homogenizer, and the cells were grinded 20-30 times in an ice bath. Then, the cell homogenates were centrifuged at the speed of 700 x g at 4°C for 5 min to collect the sediments. After resuspension with 0.5 ml cold lysis buffer, the same amount of medium buffer were mixed and centrifuged at the speed of 700 x g at 4°C for 5 min. The nucleus was resuspended with lysis buffer and centrifuged at the speed of 1,000 x g at 4°C for 10 min. The final sediments were resuspended by the store buffer provided by the kit.

RT-qPCR. Measurement of atrial natriuretic peptide (ANP), α-myosin heavy chain (α-MHC) and β-myosin heavy chain (β-MHC) and tumor necrosis factor-α (TNF-α) mRNA transcripts was performed using RT-qPCR. Total RNA from cardiomyocytes was extracted using TRIzon reagent and reverse transcribed into cDNA using a PrimeScript RT-PCR kit (Takara Bio, Inc.) according to the manufacturer's instructions. mRNA quantification was conducted using Nanodrop 2000 (Applied Biosystems; Thermo Fisher Scientific, Inc.) (27). The thermcycling condition used were as follows: Initial denaturation at 95°C for 10 min; followed by 40 cycles, each cycle included denaturation at 95°C for 15 sec and an extension at 60°C for 1 min. RT-qPCR was performed with UltraSYBR Mixture on the Viia 7 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The primers used in the current study are listed in Table I.

RT-qPCR and Southern blot. The mRNA expresssion level of TNF-α was measured by RT-qPCR and Southern blot. RT-qPCR was performed to convert the mRNA into cDNA as described above, followed by PCR using the primers listed in Table I to amplify the target fragments. The resulting products were then separated on 1.2% agarose gel and stained with 1 μg/ml ethidium bromide for 30 min at room temperature. The bands were then exposed and quantified using Gel DOC XR image system (Bio-Rad Laboratories, Inc.). The intensity of the TNF-α band was normalized by GAPDH. Densitometry was analyzed using ImageJ software (version 2.1.4.7; National Institutes of Health).

Western blot analysis of cultured cardiomyocytes. Cells were washed with cold PBS twice and lysed with RIPA buffer and PMSF (Beijing Solarbio Science and Technology Co., Ltd.; 100:1) for 15 min. Lysates were centrifuged at 8,049.6 x g for 15 min at 4°C. After quantification by bicinchoninic acid assay, the collected supernatant was added to 5X loading buffer (4:1) and incubated at 100°C for 5 min. Protein separation was conducted using 10% SDS-PAGE gels. A total of 20 μg of protein from each group and 5 μl of marker were loaded into each lane. The separated protein was then transferred to PVDF membranes and blocked with 5% non-fat milk for 1 h at room temperature. The membranes were incubated with primary

Table I. List of primers used for reverse transcription-quantitative PCR.

| Gene | Primer sequence 5'-3' | |
	Forward	Reverse
ANP	GCTCGAGCAGATCGCAAAAG	CACCACCTCTCAGTGGCAAT
α-MHC	GCCGAGTCCCAGGTCAACA	TATTGGCCACAGCGAGGGTCT
β-MHC	CACCCGCGAGTACAACCTTC	CCCATACCCACCATCACACC
TNF-α	GAACAACCCTACGAGCACCT	GGGTAGTTTGGCTGGGATAA
GAPDH	ACCCACTTCTCCACCTTTGAC	TGTTGCTGTAGCCAAATTCG

ANP, atrial natriuretic peptide; MHC, myosin heavy chain.

Table II. Effects of metoprolol, bisoprolol, Ro-31-8220 and BAY11-7082 on pulsatile frequency and cellular diameter of cardiomyocytes cultured in high glucose.

Treatment	Pulsatile frequency (bpm)	Diameter (μm)
Low glucose (5.5 mmol/l)	57.63±1.82	18.50±0.67
High glucose (25.5 mmol/l)	69.42±1.66[a]	24.81±0.78[a]
High glucose + metoprolol (0.5 μmol/l)	64.25±1.64[b]	21.82±0.60[b]
High glucose + metoprolol (10 μmol/l)	61.74±2.11[b,c]	20.12±0.57[b,c]
High glucose + bisoprolol (50 nmol/l)	63.83±1.65[b]	21.54±0.48[b]
High glucose + bisoprolol (200 nmol/l)	61.12±1.88[b,d]	19.76±0.6[b,d]
High glucose + Ro-31-8220 (50 nmol/l)	63.90±1.38[b]	19.98±0.55[b]
High glucose + BAY11-7082 (5 μmol/l)	62.31±1.70[b]	19.70±0.62[b]

Data are presented as the mean ± SD (n=100 cardiomyocytes for measurement of each group). [a]P<0.05 vs. LG group, [b]P<0.05 vs. HG group, [c]P<0.05 vs. HG group + metoprolol (0.5 μmol/l) group, [d]P<0.05 vs. HG + bisoprolol (50 nmol/l) group.

antibodies and 5% BSA (1:1,000) overnight at 4°C. Following primary antibody incubation, membranes were incubated with secondary antibodies (1:5,000) for 2 h at room temperature. Protein bands were visualized using the HRP-ECL kit (Bio-Rad Laboratores, Inc.), and optical density was measured using the Amersham Imager 600 System (GE Healthcare Life Sciences). The intensity of bands were normalized to β-actin. Densitometry of western blot bands was analyzed using ImageJ software (version 2.1.4.7; National Institutes of Health).

Statistical analysis. All experimental data are presented as the mean ± SD for at least three individual experiments. Statistical analysis of multiple comparisons was performed by one-way ANOVA followed by a Tukey's post-hoc test using GraphPad Prism software (Version 8.0; GraphPad Software, Inc.). P<0.05 indicated statistically significant differences.

Results

Metoprolol and bisoprolol decrease the pulsatile frequency, cellular diameter and cell surface area of HG-treated cardiomyocytes. Cardiomyocytes treated with HG showed increased pulsatile frequency and cellular diameter compared with LG-treated samples (69.42±1.66 vs. 57.63±1.82 bpm, P<0.05; and 24.81±0.78 vs. 18.50±0.67 μm, P<0.05). The cell

surface area of HG-treated cardiomyocytes was also increased by 2.6-fold compared with the LG-treated cardiomyocytes. After adding metoprolol, bisoprolol, Ro-31-8220 and BAY11-7082, the pulsatile frequency, cellular diameter and cell surface area of cardiomyocytes decreased significantly compared with the HG group, as shown in Table II and Fig. 1A.

Metoprolol and bisoprolol decrease the total protein content and [³H]-leucine incorporation of HG-treated cardiomyocytes. Cardiomyocytes treated with HG presented increased total protein content and [³H]-leucine incorporation compared with LG-treated cardiomyocytes (57.21±5.29 vs. 31.22±2.30 μg/well, P<0.05; and 1,510.64±82.31 vs. 1,033.21±60.33 cpm/well, P<0.05). Following addition of metoprolol, bisoprolol, Ro-31-8220 and BAY11-7082, total protein content and [³H]-leucine incorporation of cardiomyocytes was significantly reduced compared with the HG group (P<0.05), as shown in Table III.

Metoprolol and bisoprolol regulate ANP, α-MHC and β-MHC mRNA levels of HG-treated cardiomyocytes. Cardiomyocytes treated with HG showed increased mRNA levels of ANP and β-MHC and decreased mRNA levels of α-MHC compared with LG-treated cardiomyocytes (all P<0.05). Following addition of metoprolol, bisoprolol, Ro-31-8220 and BAY11-7082, mRNA

Table III. Effects of metoprolol, bisoprolol, Ro-31-8220 and BAY11-7082 on total protein content and [³H]-leucine incorporation of cardiomyocytes cultured in high glucose.

Treatment	Protein (μg/well)	[³H]-leucine incorporation (cpm/well)
Low glucose (5.5 mmol/l)	31.22±2.30	1,033.21±60.33
High glucose (25.5 mmol/l)	57.21±5.29[a]	1,510.64±82.31[a]
High glucose + metoprolol (0.5 μmol/l)	45.73±4.97[b]	1,318.15±63.91[b]
High glucose + metoprolol (10 μmol/l)	39.94±5.20[b,c]	1,152.82±87.84[b,c]
High glucose + bisoprolol (50 nmol/l)	42.14±4.89[b]	1,140.53±76.26[b]
High glucose + bisoprolol (200 nmol/l)	41.76±3.71[b]	1,130.29±73.05[b]
High glucose + Ro-31-8220 (50 nmol/l)	47.88±4.76[b]	1,330.27±74.84[b]
High glucose + BAY11-7082 (5 μmol/l)	40.60±3.66[b]	1,158.76±87.43[b]

Data are presented as the mean ± SD (n=100 cardiomyocytes for measurement of each group). [a]$P<0.05$ vs. LG group, [b]$P<0.05$ vs. HG group, [c]$P<0.05$ vs. HG group + metoprolol (0.5 μmol/l) group.
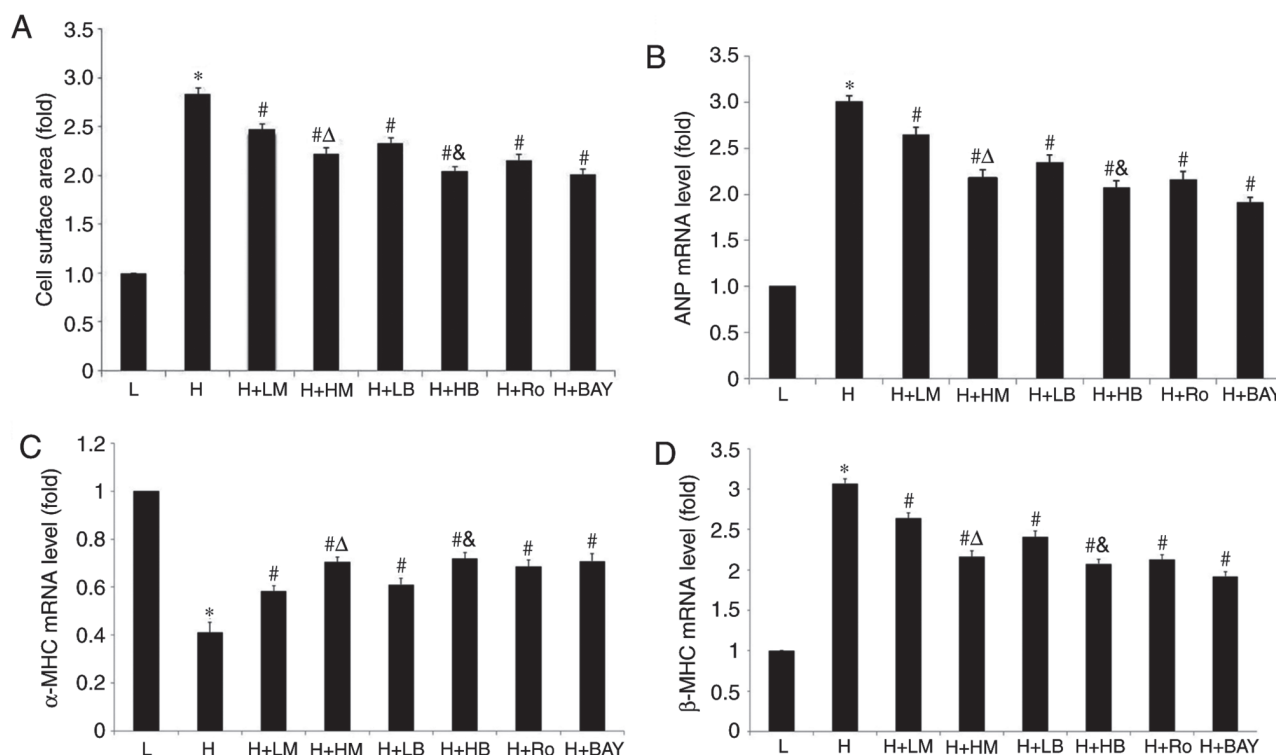

Figure 1. Metoprolol and bisoprolol decrease cell surface area and ANP and β-MHC mRNA levels and increase α-MHC mRNA levels of cardiomyocytes cultured in high glucose. (A) Cell surface area. mRNA expression levels of (B) ANP, (C) α-MHC and (D) β-MHC. n=100 cardiomyocytes for measurement of each group. *$P<0.05$ vs. L group, #$P<0.05$ vs. H group, Δ$P<0.05$ vs. H + LM group, &$P<0.05$ vs. H + LB group. L, low glucose; H, high glucose; LM, low metoprolol dose; HM, high metoprolol dose; LB, low bisoprolol dose; HB, high bisoprolol dose; Ro, Ro-31-8220; BAY, BAY11-7082; ANP, atrial natriuretic peptide; MHC, myosin heavy chain.

expression levels of ANP and β-MHC in cardiomyocytes were significantly reduced compared with HG-treated samples (P<0.05). Meanwhile, α-MHC mRNA levels of cardiomyocytes following treatment with of metoprolol, bisoprolol, Ro-31-8220 and BAY11-7082 were upregulated compared with HG-treated samples (all P<0.05), as shown in Fig. 1B-D.

Metoprolol and bisoprolol attenuate the expression and activity of PKC-α and PKC-β₂ in HG-treated cardiomyocytes. Cardiomyocytes treated with HG showed increased expression

and activity of PKC-α and PKCβ₂, reflected by the increased expression of PKC-α, p-PKC-α, PKC-β₂, p-PKC-β₂, as well as the ratios of p-PKC-α/PKC-α and p-PKC-β₂/PKC-β₂, compared with LG-treated samples (P<0.05). Following addition of Ro-31-8220 or either dose of metoprolol and bisoprolol, the expression levels of PKC-α and p-PKC-α were significantly decreased compared with HG-treated samples. In addition, the ratio of p-PKC-α/PKC-α was decreased in the Ro-31-8220 group. However, only high doses of metoprolol and bisoprolol could significantly attenute the
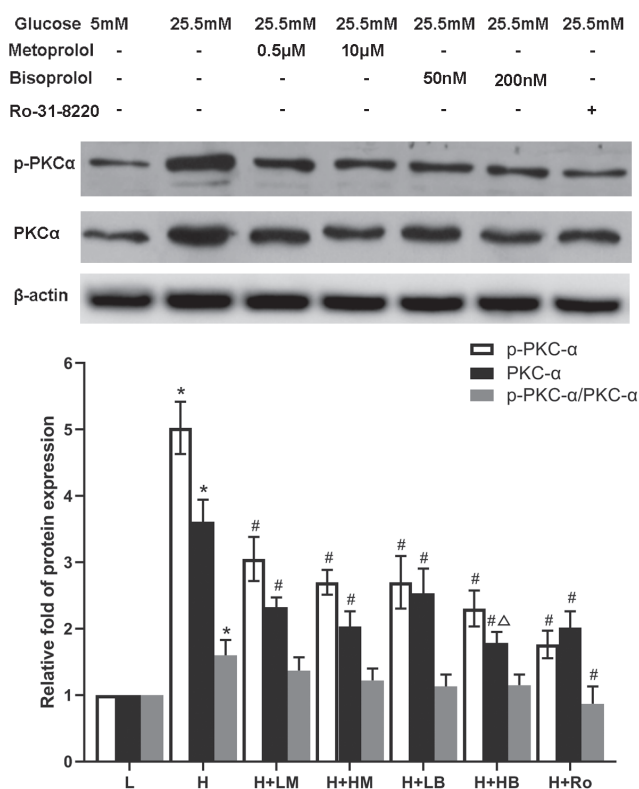
Figure 2. High doses of metoprolol and bisoprolol decreases the expression and activity of PKC-α in cardiomyocytes cultured in high glucose. p-PKC-α and total PKCα protein levels were detected in cells. n=4-5. *P<0.05 vs. L group, #P<0.05 vs. H group, ΔP<0.05 vs. corresponding low dose group of metoprolol or bisoprolol. L, low glucose; H, high glucose; LM, low metoprolol dose; HM, high metoprolol dose; LB, low bisoprolol dose; HB, high bisoprolol dose; Ro, Ro-31-8220.

Figure 3. High dose of metoprolol decrease the expression and activity of PKC-β$_2$ and high dose of bisoprolol decrease the activation of PKC-β$_2$ in cardiomyocytes cultured in high glucose. p-PKC-β and total PKCβ protein levels were detected in cells. n=4-5. *P<0.05 vs. L group, #P<0.05 vs. H group, ΔP<0.05 vs. corresponding low dose group of metoprolol or bisoprolol. L, low glucose; H, high glucose; LM, low metoprolol dose; HM, high metoprolol dose; LB, low bisoprolol dose; HB, high bisoprolol dose; Ro, Ro-31-8220.

increased PKC-β$_2$ and p-PKC-β$_2$ protein levels, and the ratio of p-PKC-β$_2$/PKC-β$_2$, except for the PKC-β$_2$ level treated by high dose of bisoprolol, compared with HG-treated samples (P<0.05; Figs. 2 and 3).

Metoprolol and bisoprolol reduce the expression of phospho-NF-κB, NF-κB, TNF-α and c-fos in HG-treated cardiomyocytes. The relative expression of p-NF-κB, NF-κB, TNF-α and c-fos significantly increased in cardiomyocytes treated with HG compared with LG-treated samples (P<0.05). In addition, the ratio of p-NF-κB/NF-κB was also increased. Following addition of metoprolol, bisoprolol, Ro-31-8220 and BAY11-7082, the relative expression of NF-κB was significantly reduced in those groups compared with HG-treated samples. However, only the high metoprolol dose group, Ro-31-8220 group and BAY11-7082 group decreased the expression level of p-NF-κB. The ratio of p-NF-κB/NF-κB was not significantly decreased following these treatments. Treatments of metoprolol, bisoprolol, Ro-31-8220 and BAY11-7082 reduced the mRNA expression levels of TNF-α and protein expression levels of c-fos compared with HG-treated samples (P<0.05; Figs. 4-6). In addition, high dose of bisoprolol further suppressed the mRNA expression levels of TNF-α and protein expression levels of c-fos compared with the low bisoprolol dose group. A similar dose-dependent suppressive effect was also found in high metoprolol dose group on the expression of c-fos protein levels.
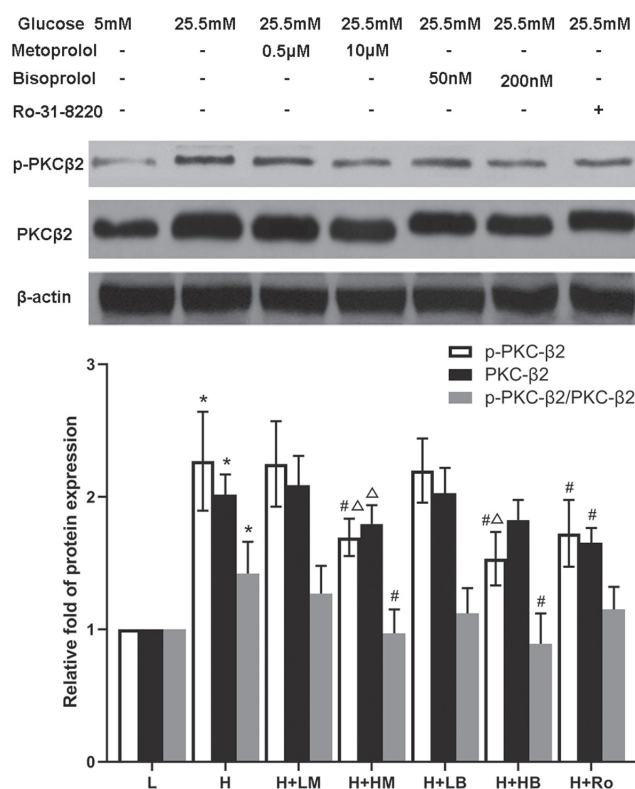
Combined use of PKC inhibitor, NF-κB inhibitor with metoprolol or bisoprolol further decreases the pulsatile frequency, cellular diameter, cell surface area and regulates ANP, α-MHC and β-MHC mRNA levels of HG-treated cardiomyocytes. Metoprolol reduced the pulsatile frequency, cellular diameter and cell surface area of cardiomyocytes treated with HG (Table IV; Fig. 7A). The mRNA levels of ANP and β-MHC of cardiomyocytes were significantly reduced, while α-MHC mRNA levels of cardiomyocytes showed a significant increase compared with HG-treated samples (Fig. 7B-D). Combined use of Ro-31-8220 or BAY11-7082 with metoprolol further decreased the pulsatile frequency, cellular diameter and cell surface area of cardiomyocytes treated with HG (Table IV; Figs. 7A and 8), as well as ANP and β-MHC mRNA expression levels (Fig. 7B and D) compared with the HG-treated samples. In addition, increased α-MHC mRNA expression levels were also observed in these treatment groups compared with the HG-treated samples. Similar results were observed when bisoprolol was used in combination with Ro-31-8220 or BAY11-7082 (P<0.05; Table IV; Fig. 7).

Combined use of PKC inhibitor and NF-κB inhibitor with metoprolol or bisoprolol further decreases total protein content and [³H]-leucine incorporation of HG-treated cardiomyocytes. In the HG-treated cardiomyocytes, the protein content and [³H]-leucine incorporation were significantly upregulated compared with LG-treated samples. The present
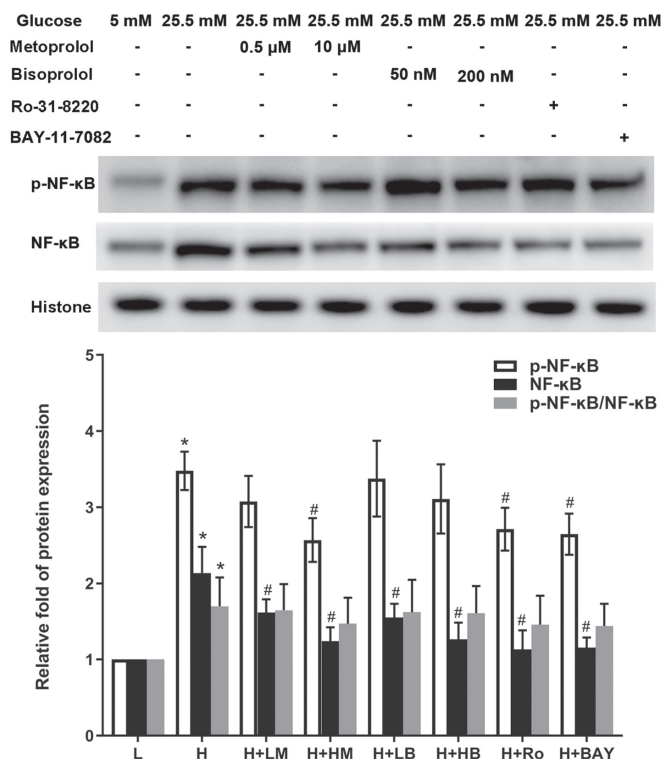
Figure 4. Effects of metoprolol, bisoprolol, PKC inhibitor Ro-31-8220 and NF-κB inhibitor BAY11-7082 on the expression and activity of NF-κB in cardiomyocytes cultured in high glucose. Cardiomyocytes cultured in HG showed higher expression and activity of NF-κB compared with LG-cultured cardiomyocytes. n=4-5. *P<0.05 vs. L group, #P<0.05 vs. H group, L, low glucose; H, high glucose; LM, low metoprolol dose; HM, high metoprolol dose; LB, low bisoprolol dose; HB, high bisoprolol dose; Ro, Ro-31-8220; BAY, BAY11-7082.
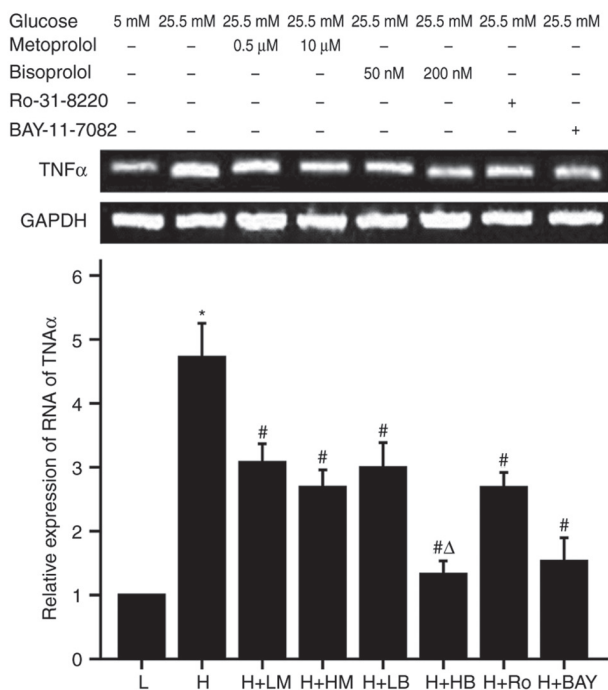

Figure 5. Effects of metoprolol, bisoprolol, PKC inhibitor Ro-31-8220 and NF-κB inhibitor BAY11-7082 on TNF-α expression in cardiomyocytes cultured in HG. n=4-5. *P<0.05 vs. L group, #P<0.05 vs. H group, ΔP<0.05 vs. HG+LB group. L, low glucose; H, high glucose; LM, low metoprolol dose; HM, high metoprolol dose; LB, low bisoprolol dose; HB, high bisoprolol dose; Ro, Ro-31-8220; BAY, BAY11-7082; TNF-α, tumor necrosis factor-α.
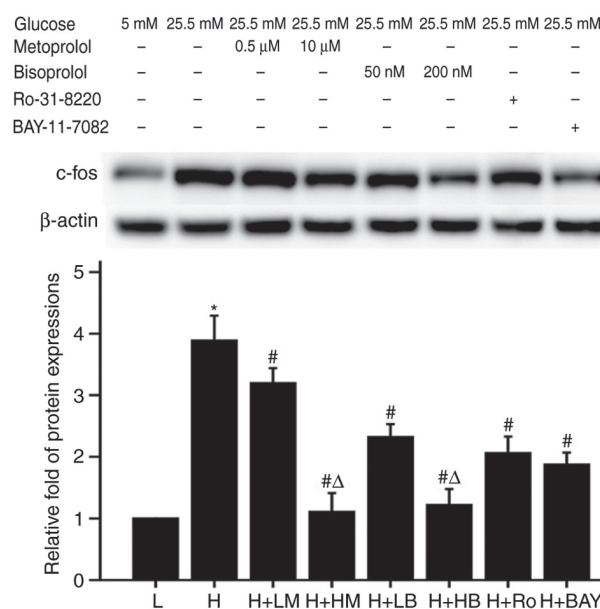

Figure 6. Effects of metoprolol, bisoprolol, PKC inhibitor Ro-31-8220 and NF-κB inhibitor BAY11-7082 on c-fos expression in cardiomyocytes cultured in HG. n=4-5. *P<0.05 vs. L group, #P<0.05 vs. H group, ΔP<0.05 vs. corresponding low dose group of metoprolol or bisoprolol. L, low glucose; H, high glucose; LM, low metoprolol dose; HM, high metoprolol dose; LB, low bisoprolol dose; HB, high bisoprolol dose; Ro, Ro-31-8220; BAY, BAY11-7082.

results suggested that reatments with metoprolol or bisoprolol could inhibit this upregulation in HG-treated samples. Moreover, when combining the use of the PKC inhibitor and NF-κB inhibitor with metoprolol or bisoprolol, the protein content and [³H]-leucine incorporation could be further downregulated compared with metoprolol or bisoprolol treatment alone (P<0.05; Table V).

Discussion

Patients with diabetic cardiomyopathy generally display progressive development of impaired systolic and diastolic dysfunction and heart failure (2). However, the exact underlying pathological mechanism remains to be further elucidated. The DAG-PKC signaling pathway plays an important role in the initiation and development of diabetic cardiomyopathy. A number of studies investigated the impact of HG on the structural and functional alterations in cardiac myocytes. The classical phenotype in diabetic cardiomyopathy is characterized by increased pulsatile frequency, myocardial size and protein content and synthesis (28). The present study mainly explored the protective effects of two types of beta-blockers, metoprolol and bisoprolol, on such hypertrophic changes and the associated signaling pathway.

Previous studies found that diabetic rats showed a significantly increased probability of developing cardiac hypertrophy compared with normal rats (28,29), which was regulated by the PKC/NF-κB/c-fos signaling pathway. In addition, another previous study found that overexpression of PKC activated NF-κB and promoted the expression of c-fos, resulting in cardiac hypertrophy and decreased contractility of cardiomyocytes, consequently leading to heart failure (30). However, upon treatment with pharmacological
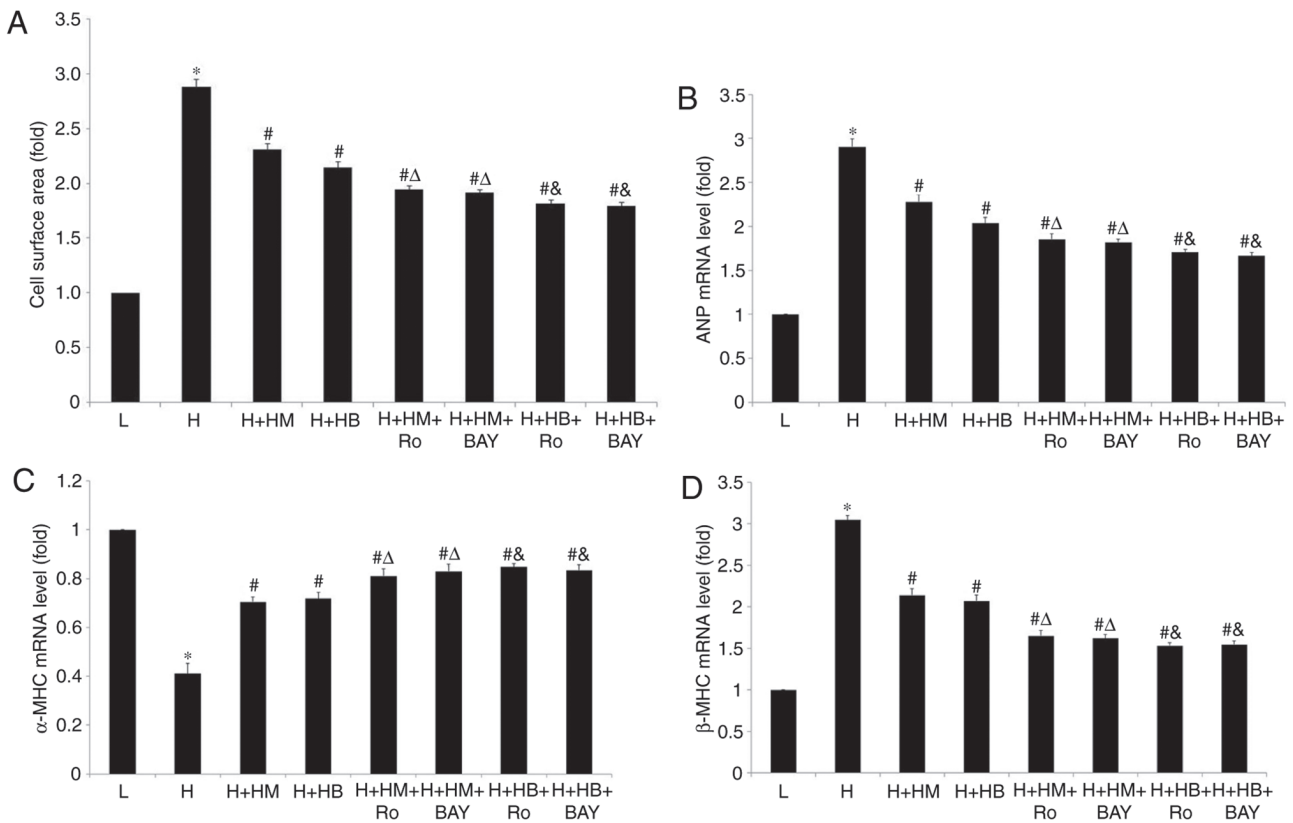
Figure 7. Combined use of PKC inhibitor Ro-31-8220 or NF-κB inhibitor BAY11-7082 with metoprolol or bisoprolol further decreases cell surface area, ANP and β-MHC mRNA levels and increases α-MHC mRNA levels of cardiomyocytes cultured in high glucose. (A) Cell surface area. mRNA expression levels of (B) ANP, (C) α-MHC and (D) β-MHC. n=100 cardiomyocytes for measurement of each group. *P<0.05 vs. L group, #P<0.05 vs. H group, ΔP<0.05 vs. HG + HM group, &P<0.05 vs. HG + HB group. L, low glucose; H, high glucose; LM, low metoprolol dose; HM, high metoprolol dose; LB, low bisoprolol dose; HB, high bisoprolol dose; Ro, Ro-31-8220; BAY, BAY11-7082; ANP, atrial natriuretic peptide; MHC, myosin heavy chain; PKC, protein kinase C.
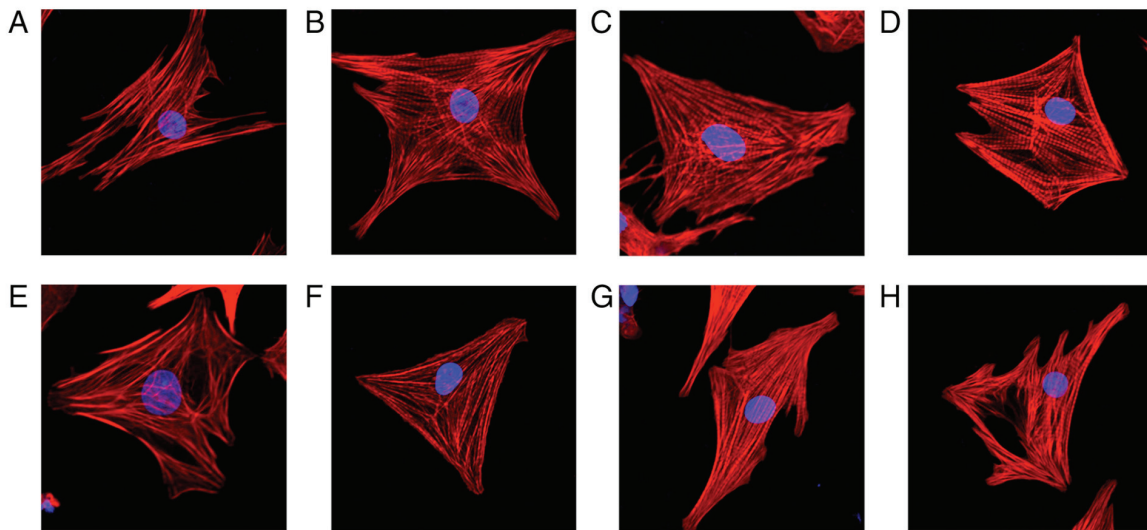

Figure 8. Representative image of the effects of metoprolol, bisoprolol, PKC inhibitor Ro-31-8220 and NF-κB inhibitor BAY11-7082 on the cell surface area of cardiomyocytes cultured in High glucose. The cell surface area of cardiomyocytes cultured (A) low glucose was significantly smaller compared with those cultured in (B) high glucose. High dose of (C) metoprolol and (D) bisoprolol treatment reduced the cell surface area of cardiomyocytes cultured in high glucose. Combined use of metoprolol and (E) PKC inhibitor Ro-31-8220 or (F) NF-κB inhibitor BAY11-7082 decreased the cell surface area compared with cardiomyocytes cultured in high glucose and metoprolol. Combined use of bisoprolol and (G) PKC inhibitor Ro-31-8220 or (H) NF-κB inhibitor BAY11-7082 further decreased the cell surface area compared with cardiomyocytes cultured in high glucose and bisoprolol. PKC, protein kinase C.

PKC inhibitors, such hypertrophic alteration in cardio-myocytes were reversed. In addition, cardiac hypertrophy in ischemic and heart failure models usually resulted in the activation of NF-κB and TNF-α (31,32). Hence, these factors may play a role in the pathological development of diabetic cardiomyopathy.

Table IV. Combined use of protein kinase C inhibitor Ro-31-8220 or NF-κB inhibitor BAY11-7082 with metoprolol or bisoprolol further decreased the pulsatile frequency and cellular diameter of cardiomyocytes cultured in high glucose.

Treatment	Pulsatile frequency (bpm)	Diameter (μm)
Low glucose (5.5 mmol/l)	57.22±1.69	18.16±0.78
High glucose (25.5 mmol/l)	69.85±1.87[a]	25.02±0.19[a]
High glucose + metoprolol (10 μmol/l)	62.23±1.58[b]	20.71±0.17[b]
High glucose + bisoprolol (200 nmol/l)	61.75±1.63[b]	20.44±0.36[b]
High glucose + metoprolol+Ro-31-8220	60.29±1.16[b,c]	19.03±0.55[b,c]
High glucose + metoprolol+BAY11-7082	60.13±1.79[b,c]	19.14±0.41[b,c]
High glucose + bisoprolol+Ro-31-8220	59.68±1.82[b,d]	18.76±0.63[b,d]
High glucose + bisoprolol+BAY11-7082	59.56±1.67[b,d]	18.62±0.38[b,d]

Data are presented as the mean ± SD (n=100 cardiomyocytes for measurement of each group). [a]P<0.05 vs. LG glucose, [b]P<0.05 vs. HG group, [c]P<0.05 vs. HG + metoprolol (10 μmol/l) group, [d]P<0.05 vs. HG + bisoprolol (200 nmol/l) group.

Table V. Combined use of protein kinase C inhibitor Ro-31-8220 or NF-κB inhibitor BAY11-7082 with metoprolol or bisoprolol further decreased the total protein content and [³H]-leucine incorporation of cardiomyocytes cultured in high glucose.

Treatment	Protein (μg/well)	[³H]-leucine incorporation (cpm/well)
Low glucose (5.5 mmol/l)	30.74±3.25	984.52±74.48
High glucose (25.5 mmol/l)	58.54±4.87[a]	1,578.93±69.23[a]
High glucose + metoprolol (10 μmol/l)	40.35±4.66[b]	1,201.67±76.53[b]
High glucose + bisoprolol (200 nmol/l)	40.16±3.32[b]	1,189.38±68.39[b]
High glucose + metoprolol+Ro-31-8220	37.61±3.91[b,c]	1,065.36±54.64[b,c]
High glucose + metoprolol+BAY11-7082	36.82±3.58[b,c]	1,052.81±62.71[b,c]
High glucose + bisoprolol+Ro-31-8220	37.45±3.34[b,d]	1,071.27±66.42[b,d]
High glucose + bisoprolol+BAY11-7082	36.78±3.79[b,d]	1,045.65±59.16[b,d]

Data are presented as the mean ± SD (n=100 cardiomyocytes for measurement of each group). [a]P<0.05 vs. LG group, [b]P<0.05 vs. HG group, [c]P<0.05 vs. HG + metoprolol (10 μmol/l) group, [d]P<0.05 vs. HG + bisoprolol (200 nmol/l) group.

In the present study, the expression levels of PKC-α, PKC-β₂, NF-κB, TNF-α and c-fos of cardiomyocytes cultured in HG were all upregulated compared with LG. The increased pulsatile frequency and hypertrophic changes, including cellular diameter, cell surface area, protein content and synthesis and mRNA levels of ANP, α-MHC, β-MHC of cardiomyocytes treated with HG were reversed upon treatment with the PKC inhibitor Ro-31-8220. The expression and activity of PKC-α and PKC-β₂ significantly decreased, alongside reduced expression of NF-κB, TNF-α, and c-fos levels. Although it was reported that Ro-31-8220 inhibited glycogen synthase kinase-3 (33), there was no doubt that PKC inhibition was its principal effect as RO-31-8220 is widely accepted as a PKC inhibitor (34). The present results suggested that the priciple effect of Ro-31-8220 may be mediated by PKC inhibition rather than GSK-3 inhibition, as shown by the suppression of the total and phosphrylated PKC-α and PKCβ₂. Likewise, following treatment with the NF-κB inhibitor BAY11-7082, the HG-induced increase in pulsatile frequency and cardiac hypertrophy was reversed, and a significant decrease in expression of NF-κB, TNF-α, and c-fos levels was observed.

NF-κB is a key transcription factor regulating inflammatory responses and the expression of hyperglycemic stress related immediate early genes (35). Previous studies showed that NF-κB regulated several signal transduction pathways in cardiomyocytes under stimulation with HG (36,37). Hence, blocking NF-κB might attenuate HG-induced cardiac hypertrophy (38) in cardiac hypertrophy models. TNF-α is a cytokine associated with NF-κB. As a contributor of cardiac dysfunction, elevated TNF-α level could trigger NF-κB translocation to the nucleus, allowing NF-κB to promote transcription of TNF-α (39,40).

c-fos is a proto-oncogene, whose activation could induce the enlargement of cardiomyocytes and increased protein content. TNF-α stimulates the expression of c-fos as an adaptive response to cardiac dysfunction, such as cardiac hypertrophy and ischemic heart disease (41,42). Moreover, previous studies demonstrated that c-fos is also regulated by PKC to participate in inendothelin-1-induced proliferation of neonatal cardiomyocytes (43,44). A previous study also revealed that the PKC/c-fos pathway was involved in HG-induced cardiac hypertrophy (11).

Beta-blockers are well studied and play a cardioprotective role in ameliorating cardiac dysfunction in rats with diabetic cardiomyopathy (45-47). Since numerous types of beta-blockers with different effects on glucose control are available at present, it would be beneficial for DM patients to select the appropriate beta-blocker to prevent or delay cardiovascular complication (48). According to evidence-based medicine, metoprolol and bisoprolol are two types of highly selective beta-blockers widely used in clinical practice (49). Autoantibodies against the β1 receptor could be a predictor for left ventricular hypertrophy for T2DM patients with hypertension (50,51). Treatment with metoprolol could inhibit intrinsic damage to the heart during diabetic cardiomyopathy (52). Several studies indicated that use of beta-blockers, such as bisoprolol, did not aggravate glycemic control, lipid profile or albuminuria status in T2DM patients with SHF (53,54).

To explore whether the protective effects of metoprolol and bisoprolol were associated with the PKC signal transduction pathway, expression and activation of two PKC isoforms (PKC-α and PKC-β$_2$) were detected in cardiomyocytes cultured with HG. Metoprolol and bisoprolol were found to decrease cellular pulsatile frequency and improve cardiac hypertrophy, and decrease expression and activity of PKC-α. These changes were more significant when cultures were treated with a higher dose of metoprolol and bisoprolol. Meanwhile, the expression of p-PKC-β$_2$ decreased when cells were treated with higher doses of metoprolol and bisoprolol compared with cells treated with HG. However, when treated with a lower dose of metoprolol and bisoprolol, the expression of p-PKC-β$_2$ in cardiomyocytes resulted in no significant change when compared with HG-treated samples, while the expression and activation of PKC-α of HG cultured cardiomyocytes were significantly inhibited by treatment with metoprolol or bisoprolol compared with those cultured with HG alone. Therefore, the protective role of metoprolol and bisoprolol on HG-induced hypertrophy was mainly conducted by inhibition of PKC-α. Expression of NF-κB, TNF-α and c-fos also significantly decreased when treated with metoprolol and bisoprolol. The present results suggested that high dose of bisoprolol could further inhibit the decrease in TNF-α expression leve in the low bisoprolol dose group. In addition, higher doses of metoprolol and bisoprolol showed a significant suppressive effect on c-fos expression compared with low doses. Collectively, metoprolol and bisoprolol provided a protective role against diabetic cardiomyopathy via the PKC-induced NF-κB/TNF-α/c-fos signal transduction pathway.

In the current study, high dose of metoprolol and bisoprolol inhibited c-fos expression to a lower degree compared with PKC or NF-κB inhibitor, although the differences were not significant. The present data suggested that beta-blockers may have other targets other than PKC and NF-κB. One explanation for the results is the involvement of another important cardiac hypertrophic pathway in cultured cardiomyocytes, the PKC-δ/protein kinase D (PKD)/histone deacetylases (HDAC) pathway. PKD, which can be phosphorylated by PKC-δ, regulates the ability of cardiomyocyte growth and contractility through phosphorylation of proteins such as class IIa HDACs (55). Metoprolol and bisoprolol may inhibit the expression of c-fos through regulating the PKC-δ signaling pathways. Although there is little evidence of the effects of signal transduction pathways of beta-blockers on the expression of c-fos, one study showed that bisoprolol, especially at high doses, could increase the survival rate of hypertensive diastolic heart failure model of rats, at least partly through the alleviation of inflammatory changes (interleukin-1β, transforming growth factor-β1 and monocyte chemoattractant protein-1) and oxidative stress (reactive oxygen species production and nicotinamide adenine dinucleotide phosphate oxidase activity) (52). Moreover, isoproterenol (ISO)-induced heart failure could be inhibited by a Rho-associated protein kinase (ROCK) inhibitor, fasudil, which suppressed isoproterenol-induced JNK activation, translocation of ERK to the nucleus, and increased expression of c-fos and c-jun through RhoA/ROCK. Consequently, those changes could suppress ISO-induced cardiac hypertrophy and ventricular remodeling in rat models (56). Since ISO-induced heart dysfunction was associated with RhoA/ROCK and its downstream signaling pathways, beta-blockers may also have protective effects on cardiomyocytes through similar signaling pathways, although this warrants further studies.

Activation of PI3K-Akt and ERK pathways may also contribute to the cardiac protective effects of metoprolol (57). Therefore, beta-blockers may have other targets than PKC and NF-κB, and ERK and inflammatory cytokines could also cause cardiomyocyte hypertrophy. Fujioka *et al* (58) proposed that the use of another beta-blocker, propranolol, could promote post-hypoxic contractile and metabolic recovery via non-beta-adrenoreceptors in ischemia-reperfusion rat hearts. Hence, the role of such non-beta-adrenorecepors activated by beta-blockers could be further investigated in diabetic cardiomyopathy. In the present study, combined use of PKC inhibitor Ro-31-8220 or NF-κB inhibitor BAY11-7082 with metoprolol further decreased the cellular pulsatile frequency, cellular diameter, cell surface area, total protein content and [^{3}H]-leucine incorporation of cardiomyocytes cultured with HG. The same result was observed when bisoprolol was combined with Ro-31-8220 or BAY11-7082. Combined use of beta-blockers with PKC or NF-κB inhibitor attenuated cardiac hypertrophy caused by HG. Beta-blockers may have targets other than PKC and NF-κB that could also inhibit the expression of c-fos. This hypothesis supports the observation that high doses of metoprolol and bisoprolol may inhibit c-fos expression to a lower degree than PKC or NF-κB inhibitors, although the differences were not statistically significant in the present study.

In conclusion, the data presented depicted that hyperglycemia could activate the PKC/NF-κB/TNF-α/c-fos signal transduction pathway in diabetic cardiomyopathy. The protective role of metoprolol and bisoprolol could significantly reverse cardiac dysfunction and hypertrophy. Future studies can be performed to identify other targets of beta-blockers and non-beta receptor-mediated effects of beta-blockers in diabetic cardiomyocytes.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MW, GF and WZ designed the research. MW, QL, YW and ZL performed the research. WZ contributed new reagents or analytic tools. MW, LZ, YL and WZ analyzed the data. MW, QL and YL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were approved by the Ethics Committee for the Use of Experimental Animals in Zhejiang University. All the experiments followed the instructions for animal care and usage provided by Zheijiang University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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