

# Mild hypoxia-induced cardiomyocyte hypertrophy via up-regulation of HIF-1 $\alpha$ -mediated TRPC signalling

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Received: August 4, 2011; Accepted: November 23, 2011

## Abstract

Hypoxia-inducible factor-1 alpha (HIF-1 $\alpha$ ) is a central transcriptional regulator of hypoxic response. The present study was designed to investigate the role of HIF-1 $\alpha$  in mild hypoxia-induced cardiomyocytes hypertrophy and its underlying mechanism. Mild hypoxia (MH, 10% O<sub>2</sub>) caused hypertrophy in cultured neonatal rat cardiac myocytes, which was accompanied with increase of HIF-1 $\alpha$  mRNA and accumulation of HIF-1 $\alpha$  protein in nuclei. Transient receptor potential canonical (TRPC) channels including TRPC3 and TRPC6, except for TRPC1, were increased, and Ca<sup>2+</sup>-calcineurin signals were also enhanced in a time-dependent manner under MH condition. MH-induced cardiomyocytes hypertrophy, TRPC up-regulation and enhanced Ca<sup>2+</sup>-calcineurin signals were inhibited by an HIF-1 $\alpha$  specific blocker, SC205346 (30  $\mu$ M), whereas promoted by HIF-1 $\alpha$  overexpression. Electrophysiological voltage-clamp demonstrated that DAG analogue, OAG (30  $\mu$ M), induced TRPC current by as much as 170% in neonatal rat cardiomyocytes overexpressing HIF-1 $\alpha$  compared to negative control. These results implicate that HIF-1 $\alpha$  plays a key role in development of cardiac hypertrophy in responses to hypoxic stress. Its mechanism is associated with up-regulating TRPC3, TRPC6 expression, activating TRPC current and subsequently leading to enhanced Ca<sup>2+</sup>-calcineurin signals.

**Keywords:** mild hypoxia • TRPC • HIF-1 $\alpha$  • hypertrophy • calcineurin

## Introduction

The lasting hypoxic exposure or vigorous exercise could lead to the adaptive cardiac hypertrophy [1, 2]. Unlike the toxic effect of severe hypoxia, mild hypoxia (MH, 10% oxygen) could not induce any cytotoxicity but trigger hypertrophic responses in cultured neonatal rat cardiac myocytes [3], and the mechanisms involved remain to be elusive.

Hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) is a heterodimeric subunit of the transcription factor HIF-1, which regulates the transcription of genes involved in adaptive responses to hypoxia [4]. So far, role of HIF-1 $\alpha$  in the development of cardiac hypertrophy has been sparsely documented. Silter *et al.* [5] have shown that

HIF-1 $\alpha$  is critically involved in the preservation of cardiac function without affecting cardiac hypertrophy by using HIF-1 $\alpha$  knockdown mice with transverse aortic constriction. Recently, Xue *et al.* [6] have demonstrated that cardiac-specific overexpression of HIF-1 $\alpha$  could prevent deterioration of glycolytic pathway and cardiac hypertrophy in streptozotocin-induced diabetic mice. More interestingly, carvedilol, a  $\beta$ -receptor blocker, has emerged as a beneficial treatment for cardiac hypertrophy and inhibited the overexpression of HIF-1 $\alpha$  in pressure-overloaded rat heart [7]. These studies regarding the role of HIF-1 $\alpha$  in cardiac hypertrophy were based upon pathologic situation, and their conclusions were under the controversial arguments. The previous investigations have shown that hypoxia is a critical factor which triggers cardiac hypertrophy *in vivo* [2, 8], therefore, the potential role of HIF-1 $\alpha$  in adaptive cardiac hypertrophy, *e.g.* MH-induced cardiomyocytes hypertrophy, needs to be clarified.

Transient receptor potential canonical (TRPC) channels are nonselective cation channels mediating Ca<sup>2+</sup> influx into several cell types including cardiac myocytes [9]. TRPC expression in the

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cardiac hypertrophy has been studied by several laboratories, with somewhat variable results. For example, the previous studies have shown that TRPC3 promotes cardiomyocytes hypertrophy in several animal models, including abdominal aortic-banded (AAB) rats and spontaneous hypertensive heart failure rats [10]. Kuwahara *et al.* [11] have proved that TRPC6 sequentially initiates a calcineurin signalling circuit during pathologic cardiac hypertrophy. However, Ohba *et al.* [12] have demonstrated that TRPs C1, C3, C5 and C6 are constitutively expressed, but only TRPC1 expression is significantly increased in hypertrophic hearts from AAB rats. These results regarding TRPC expression in hypertrophic models are in the dispute. Therefore, it is necessary to reexamine the changes of TRPC in hypoxic adaptive cardiac hypertrophy. Furthermore, understanding the initial molecule that regulates TRPC expression may facilitate the elucidation of new therapeutic approaches to prevent the development of cardiac hypertrophy.

Taken together, we hypothesize HIF-1 $\alpha$ -regulated TRPC signals have an etiological role in cardiac hypertrophy induced by MH. In order to test this hypothesis, hypertrophic model of neonatal rat cardiac myocytes induced by MH stimuli was used to explore whether HIF-1 $\alpha$ , as an initial molecule, up-regulates expression of some TRPCs, in turn elevates Ca<sup>2+</sup>-calcineurin signalling and finally leads to development of cardiac hypertrophy.

## Materials and methods

### Reagents

HIF-1 $\alpha$  specific blocker, SC205346, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

### Cell culture and MH

Cardiomyocytes from 1- or 2-day-old Wistar rats were isolated, subjected to Percoll gradient centrifugation and cultured as previously described [13]. Use of animals was in accordance with the regulations of the ethic committees of Harbin Medical University, and confirmed 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). The purified cardiomyocytes were plated on 35-mm dishes ( $1.6 \times 10^5$  cells per dish) in minimum essential medium (MEM) supplemented with 5% foetus bovine serum (FBS), penicillin (100 U/ml; GIBCO, Grand Island, NY, USA), and streptomycin (100  $\mu$ g/ml; GIBCO). When cardiomyocytes were exposed to MH, cells were placed in a hypoxic chamber, which was kept at 37°C, 90% humidity. The chamber was filled with gas mixture of 10% O<sub>2</sub>/85% N<sub>2</sub>/5% CO<sub>2</sub>.

### Cloning and transfection

pCEP4/HIF-1 $\alpha$  construct deriving from human HIF-1 $\alpha$  cDNA sequence was purchased from ATCC (Manassas, VA, USA). The cardiomyocytes were

incubated in serum-containing medium at 37°C for 24 hrs, then subjected to transfection. Before transfection, medium was changed to serum-free MEM for 2–4 hrs. Cardiomyocytes were transiently transfected with 1.6  $\mu$ g of vectors carrying HIF-1 $\alpha$  or empty vectors using FuGene 6 (Roche Diagnostics, Alameda, CA, USA) according to the manufacturer's instructions. The cardiomyocytes, 44 hrs after transfection, were subjected to MH (10% O<sub>2</sub>) for 0, 1, 3, 6 or 12 hrs. pcDNA3.1 empty vector transfected cells in every single experiment, acts as negative control (NC).

### RNA extraction, cDNA synthesis

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) with in-column DNase treatment (QIAGEN). The quantity of RNA was measured with NanoDrop 1000 and RNA integrity estimated with Bioanalyzer 2100. One microgram of RNA was reverse-transcribed using random hexamers for priming (3 min. at 70°C) followed by the first strand cDNA synthesis protocol with Superscript III (Invitrogen, Carlsbad, CA, USA) and RNasin (Promega, Madison, WI, USA) enzymes (10 min. at 25°C, 50 min. at 42°C, and 4 min. at 4°C).

### Quantitative real-time PCR

PCR primers for HIF-1 $\alpha$ , rTRPC1, rTRPC3, rTRPC6, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were designed based on published sequences [14–17]. For details on primer sequences and setting of the PCR reaction, see Supporting Information. 18S rRNA was used as endogenous control. PCR reactions took place in 96-well plates using SYBR Green detection. Relative gene expression was calculated to 18S rRNA expression.

### Immunofluorescence

Immunofluorescence was performed as described [15]. Briefly, cardiomyocytes were fixed with ice-cold methanol/acetone and incubated with antibodies against  $\alpha$ -actinin (1:500 dilution; Sigma-Aldrich) or HIF-1 $\alpha$  (1:100 dilution; Novus Biologicals, Littleton, CO, USA) for 1 hr. Secondary antibodies used were coupled to Alexa fluor 594 (Molecular Biology, Cambridge, MA, USA) or FITC 488 (Invitrogen).

### Nuclear extracts and Western blotting

Total proteins or nuclear proteins were extracted from cultured cardiomyocytes from neonatal rats. Nuclear extracts were prepared using a Transfactor Extraction Kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. The protein concentrations were determined by the Bradford assay using bovine serum albumin as a standard. Protein samples (100  $\mu$ g) were separated by 6–10% SDS-PAGE and immunoblotted with anti-TRPC1, anti-TRPC3, anti-TRPC6 antibodies (1:400 dilution; Alomone Labs, Jerusalem, Israel), anti-HIF-1 $\alpha$  (1:500 dilution; Novus), anti- $\beta$ -MHC (1:200 dilution; Sigma-Aldrich), anti-calcineurin (1:200 dilution; BD Biosciences), anti-ANP (1:200 dilution; Santa Cruz Biotechnology), anti-BNP (1:500 dilution; Santa Cruz Biotechnology), anti-NFAT (1:200 dilution; Santa Cruz Biotechnology), anti-p-NFAT (1:200 dilution; Santa Cruz Biotechnology), anti-GAPDH (1:1000 dilution; Santa Cruz Biotechnology) or anti- $\beta$ -actin (1:200 dilution; Santa Cruz Biotechnology).

## Measuring intracellular calcium concentrations by flow cytometry

The intracellular calcium was measured by flow cytometry using the calcium-sensitive dyes, Fluo-3 and Fura Red (Molecular Probes, Eugene, OR, USA). Fluo-3 fluorescence at 530 nm increases with increasing  $\text{Ca}^{2+}$  binding, whereas Fura-Red fluorescence at 670 nm decreases with increasing  $\text{Ca}^{2+}$  binding, allowing ratiometric measurement of calcium [18]. Myocytes were resuspended in medium supplemented with 1% FBS, then stained with 4  $\mu\text{M}$  Fluo-3 and 10  $\mu\text{M}$  Fura Red for 30 min. at 37°C. The Fluo-3/Fura Red fluorescence ratio was read on a FACSCalibur flow cytometer (BD Biosciences).

## Patch clamp

Patch clamp is done as previously described [11, 19, 20]. For TRPC current recording, we used the whole-cell voltage clamp technique with pipette resistances of 2–3 M $\Omega$  when filled with internal solution. The junctional potential was corrected by zeroing the potential before the pipette tip touched the cell membrane. After the cell membrane was broken by application of additional suction, cell capacitance and series resistance were electrically compensated. After access was gained in the whole-cell voltage-clamp configuration, cardiomyocytes were allowed to equilibrate for 5 min. with the internal solution before data were collected. For details on patch clamp, see Supporting Information.

## Data analysis

Data are presented as the mean  $\pm$  S.E.M. Differences were evaluated using the unpaired Student's *t*-test, and  $P < 0.05$  was considered to be statistically significant.

## Results

### MH induces hypertrophy in cultured cardiomyocytes of neonatal rats

Neonatal rat cardiomyocytes were exposed to MH for 0 (control), 1, 3, 6 or 12 hrs. Morphology changes for hypertrophy in cardiomyocytes were determined by  $\alpha$ -actinin staining. In order to confirm cardiac hypertrophy, three markers, ANP, BNP and  $\beta$ -MHC, were also detected by Western blotting and real-time PCR. Figure 1A showed typical morphology of hypertrophy in cardiomyocytes exposed to MH for 6 hrs; surface area of cardiomyocytes evaluated by Imagepro-Plus software in cardiomyocytes exposed to MH for 6 and 12 hrs was increased significantly by 1.6-fold and 1.9-fold, respectively, when compared to non-hypoxia control. Moreover, Figure 1B–D showed a time-dependent increase of ANP, BNP and  $\beta$ -MHC mRNA and protein expression induced by MH in neonatal rat cardiomyocytes. These results indicate that MH is able to induce hypertrophy in cardiac myocytes.

### HIF-1 $\alpha$ is increased in cultured cardiomyocytes exposed to MH

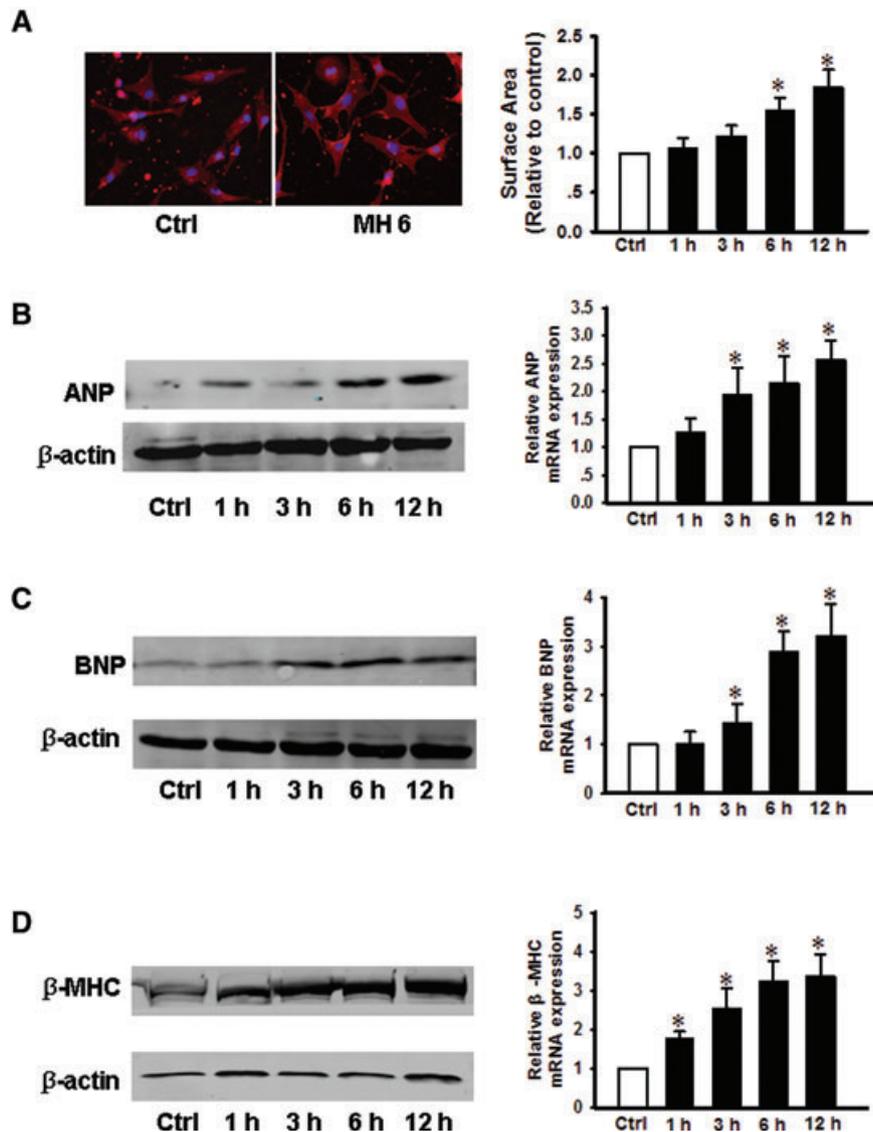
To investigate whether HIF-1 $\alpha$  correlates to the development of hypertrophy by MH in cultured cardiomyocytes, we detected the expression of HIF-1 $\alpha$  mRNA and protein. Immunofluorescence assay showed that an elevated HIF-1 $\alpha$  protein within nucleus area (Fig. 2A) in cardiac myocytes exposed to MH for 6 hrs. HIF-1 $\alpha$  mRNA level (Fig. 2B) and nucleus HIF-1 $\alpha$  protein (Fig. 2C) were elevated in a time-dependent manner in cardiomyocytes exposed to MH for 1, 3, 6 or 12 hrs. In order to determine reference control proteins are not altered by MH, two reference proteins, GAPDH and  $\beta$ -actin, were used as internal control, which did not reflect significant difference under MH in cardiomyocytes; data were shown in Supporting Information (Fig. S1).

### HIF-1 $\alpha$ controls development of hypertrophy induced by MH in cardiomyocytes

In order to investigate whether HIF-1 $\alpha$  participates in the development of hypertrophy, an HIF-1 $\alpha$  specific blocker, SC205346, for the loss-of-function and HIF-1 $\alpha$  transfection for the gain-of-function were used before cardiomyocytes exposed to MH. A successful transfection was demonstrated as shown in Figure 3A and B.  $\alpha$ -actinin (Fig. 3C) and a statistical graph were shown from cell surface area (Fig. 3D), Figure 3C showed that SC205346 (30  $\mu\text{M}$ ) prior to MH for 1 hr significantly inhibited cardiomyocytes hypertrophy, while HIF-1 $\alpha$  overexpression promoted this process. MH-induced  $\beta$ -MHC overexpression in cardiomyocytes was abolished by SC205346, but was enhanced by HIF-1 $\alpha$  transfection (Fig. 3E), and the data are consistent with  $\alpha$ -actinin staining assay in Figure 3C. Real-time PCR also showed that ANP, BNP and  $\beta$ -MHC mRNA up-regulation induced by MH were blocked by SC205346, but enhanced by HIF-1 $\alpha$  transfection (Fig. 3F). SC205346 as an HIF-1 $\alpha$  blocker inhibited nuclear HIF-1 $\alpha$  accumulation in hypoxic cardiomyocytes (Fig. S1). But SC205346 alone did not affect ANP, BNP and  $\beta$ -MHC expression when 30  $\mu\text{M}$  SC205346 was administered to neonatal cardiomyocytes for 6 hrs (Fig. S2). Interestingly, cardiomyocytes transfected with HIF-1 $\alpha$  without hypoxia exposure showed an increased expression of ANP, BNP and  $\beta$ -MHC (Fig. S3). Taken together, HIF-1 $\alpha$  controls the process of hypertrophy induced by MH in cardiac myocytes.

### TRPC-mediated calcineurin signalling is involved in hypertrophic cardiomyocytes induced by MH

Previous studies have demonstrated up-regulation of TRPC1, TRPC3 and TRPC6 is involved in the development of cardiac hypertrophy in various animal models [11, 12, 21]; therefore, we detect their expression levels of mRNA and protein by real-time PCR and Western blotting, respectively. Figure 4A and B showed

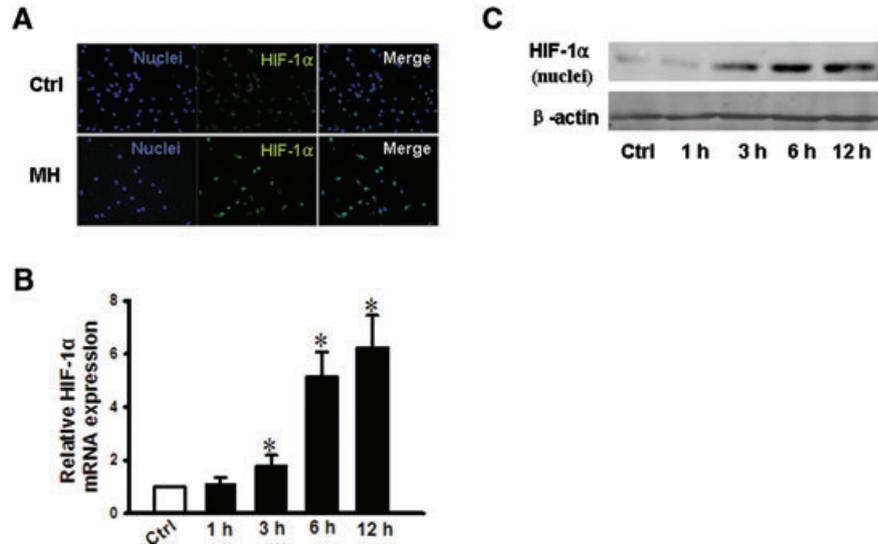


**Fig. 1** MH induces hypertrophy of rat neonatal cardiac myocytes. **(A)** Representative  $\alpha$ -actinin immunostaining images by confocal microscope (original magnification,  $\times 600$ ) in cardiac myocytes exposed to MH (10%  $O_2$ ) for 6 hrs, and statistical graph showed calculated surface area from 40 random cardiac myocytes exposed to MH for 1, 3, 6 and 12 hrs, respectively. ANP **(B)**, BNP **(C)** and  $\beta$ -MHC **(D)** protein and mRNA expression were also measured in cardiomyocytes exposed to MH for 1, 3, 6 or 12 hrs. \* $P < 0.05$  versus Ctrl;  $n = 5$  independent experiments for each condition.

an increased TRPC3 and TRPC6 expression, but not TRPC1 mRNA and proteins, in the hypertrophic cardiomyocytes induced by MH. These results suggest that up-regulated expression of TRPC3 or TRPC6 might regulate  $Ca^{2+}$ -calcineurin signalling which is indispensable for cardiac hypertrophy. Our current study showed that MH induced a mild intracellular calcium ( $[Ca^{2+}]_i$ ) elevation (Fig. S4A) and an increased calcineurin expression (Fig. S4B) in a time-dependent manner. More importantly,  $[Ca^{2+}]_i$  elevation induced by MH was blocked by two TRPC channel blockers, 2-APB and SK&F96365 (Fig. 4C). Calcineurin expression in hypertrophic cardiomyocytes was also inhibited by SK&F96365 (Fig. 4D). SK&F96365 also restored p-NFAT expression level, a substrate of calcineurin, induced by MH (Fig. 4E). The expression of modulatory calcineurin interacting protein 1 (MCIP1, also known as reg-

ulator of calcineurin) was up-regulated in response to calcineurin activation; therefore, we further measured MCIP1 mRNA as a reflection of endogenous calcineurin activity. As shown in Figure 4F, MCIP1 mRNA expression was significantly increased in MH exposed cardiomyocytes, and the increased MCIP1 mRNA expression was inhibited by SK&F96365 treatment. These results were consistent with the previous report [22]. In addition, SK&F96365 also prevented MH-induced hypertrophic morphology in cardiomyocytes (Fig. 4G) and blocked MH-induced hypertrophy-related genes expression, for instance, ANP, BNP and  $\beta$ -MHC (Fig. 4H). Together, it suggests that TRPC-mediated  $Ca^{2+}$ -calcineurin signalling participates in the cardiac hypertrophy induced by MH, but whether TRPC is regulated by HIF-1 $\alpha$  needs to be further investigated.

**Fig. 2** MH induces an increase of HIF-1 $\alpha$  mRNA and protein in rat neonatal cardiac myocytes. **(A)** Immunofluorescence images of HIF-1 $\alpha$  by confocal microscope (original magnification,  $\times 200$ ). Representative images of nuclear, HIF-1 $\alpha$  staining and overlay images in cardiac myocytes exposed to MH for 6 hrs. HIF-1 $\alpha$  mRNA **(B)** and nuclear HIF-1 $\alpha$  protein **(C)** accumulation at the respective time points was assessed by real-time PCR and Western blotting, respectively. Data are shown as mean  $\pm$  S.E.M. \* $P < 0.05$  versus Ctrl;  $n = 5$  independent batches of cells for each bar.



### SC205346, as specific HIF-1 $\alpha$ blocker, prevents increase of TRPC3 and TRPC6 expression and inhibits elevation of Ca<sup>2+</sup>-calcineurin signals under MH condition

In order to determine whether TRPC-mediated cardiomyocyte hypertrophy is through HIF-1 $\alpha$ , neonatal rat cardiac myocytes were treated with 30  $\mu$ M SC205346 for 1 hr prior to MH stimuli. Results showed that MH-induction could increase TRPC3 and TRPC6 mRNA (Fig. 5A) and protein expression levels (Fig. 5B and C), which could be completely suppressed by HIF-1 $\alpha$  blocker, SC205346. It also showed that the elevation of [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 5E) and enhanced expression of calcineurin (Fig. 5D) caused by MH in cardiomyocytes were also blocked by SC205346. But SC205346 alone did not affect TRPC3 and TRPC6 expression (Fig. S5A and S5B). Together, TRPC-mediated Ca<sup>2+</sup>-calcineurin signalings are down-stream events initiated by HIF-1 $\alpha$  in the cardiac hypertrophy induced by MH.

### Overexpression of HIF-1 $\alpha$ promotes expression of TRPC3 and TRPC6 and enhances Ca<sup>2+</sup>-calcineurin signals under MH condition

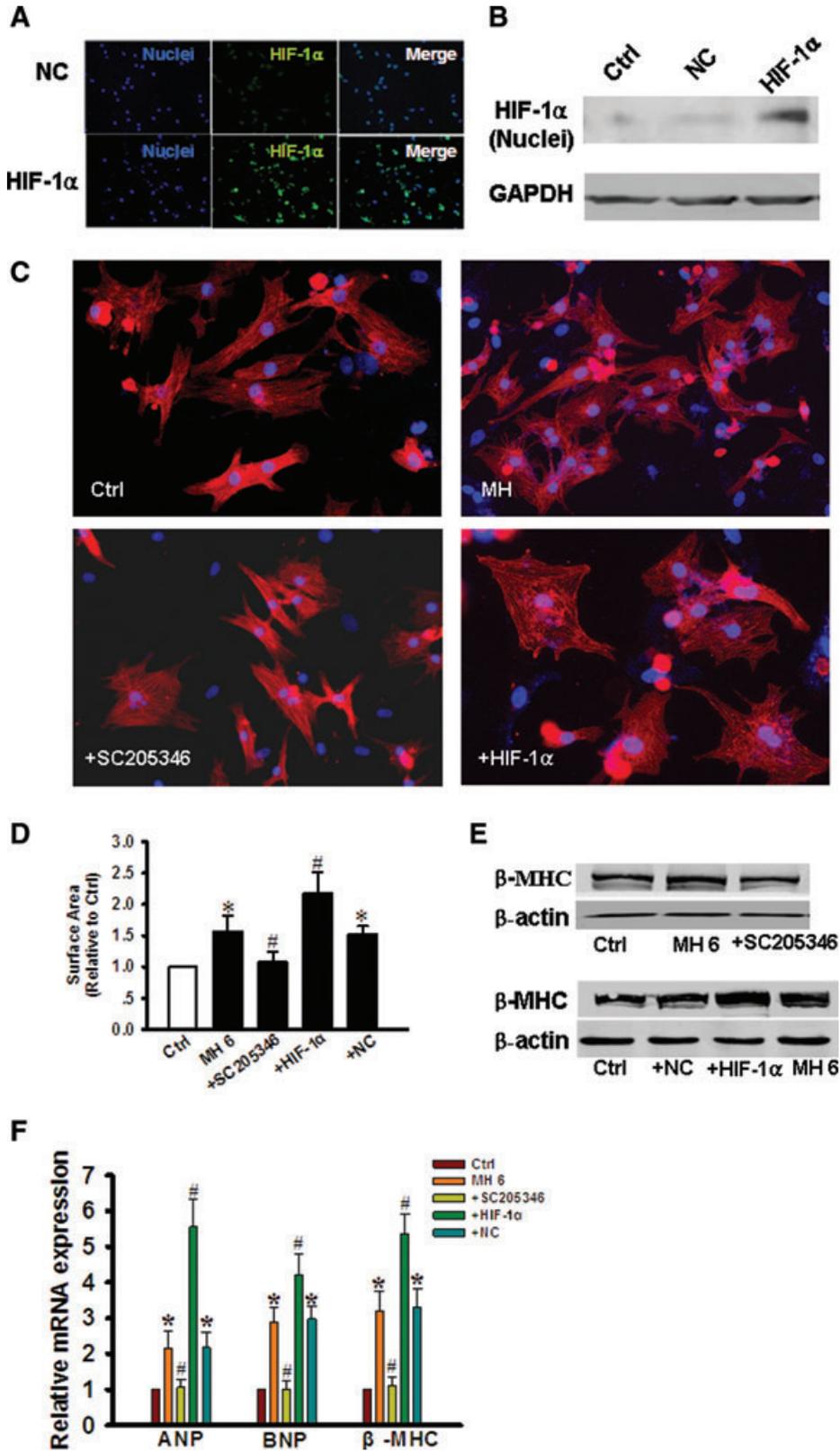
In order to further confirm the regulatory effect of HIF-1 $\alpha$  on TRPC-mediated Ca<sup>2+</sup>-calcineurin signalling in cardiac myocytes under MH, we used HIF-1 $\alpha$  construct to transfect cardiomyocytes and a mock plasmid as NC. Under MH condition, HIF-1 $\alpha$  transfected in cardiomyocytes resulted in enhanced TRPC3 and TRPC6 mRNA (Fig. 6A) and protein (Fig. 6B and C) level when compared to NC cells. HIF-1 $\alpha$  overexpression facilitated [Ca<sup>2+</sup>]<sub>i</sub> elevation

(Fig. 6E) and calcineurin up-regulation (Fig. 6D) by MH in cardiomyocytes. Interestingly, HIF-1 $\alpha$ -overexpressed cardiomyocytes under normal oxygen condition also caused an up-regulation of TRPC3 or TRPC6 protein (Fig. 7C), suggesting a tight regulatory role of HIF-1 $\alpha$  in TRPC expression.

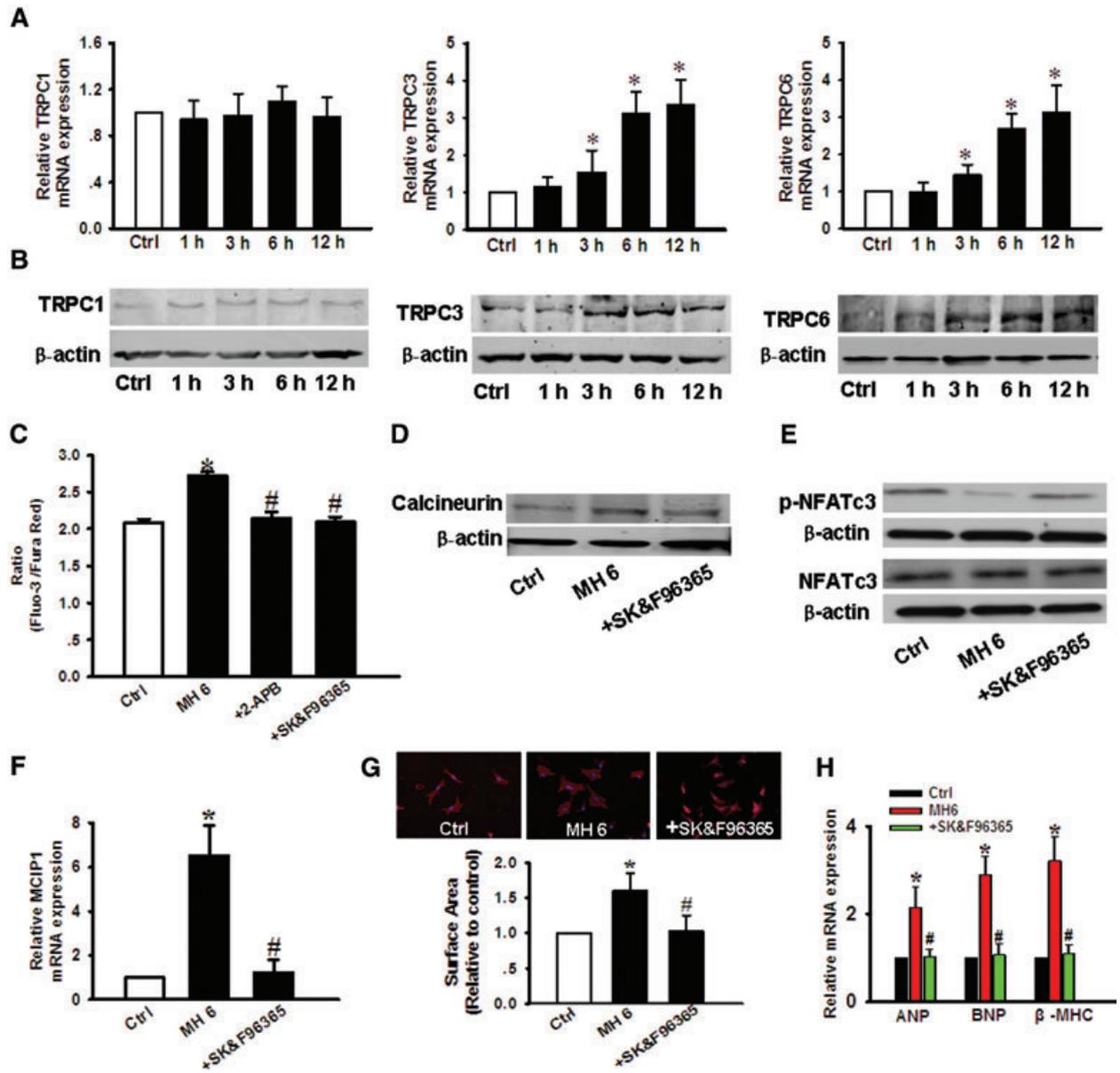
### HIF-1 $\alpha$ overexpression enhances TRPC current in cardiomyocytes

To further evaluate the stimulatory effect of HIF-1 $\alpha$  on TRPC3/6 channels, we conduct the Patch-Clamp experiments to measure the TRPC current by applying TRPC channel activator, membrane permeable nonmetabolizable DAG analogue,

To further evaluate the function of increased expression of TRPC3 and TRPC6 channels in cardiac myocytes over expressed HIF-1 $\alpha$ , we measured TRPC current across the membrane using electrophysiological techniques by applying TRPC channel activator, membrane permeable nonmetabolizable DAG analogue, OAG. In the presence of OAG (30  $\mu$ M), an outwardly rectifying current developed within minutes in both NC and HIF-1 $\alpha$  overexpressed cardiomyocytes (Fig. 7A). The maximum amplitude of the OAG-induced TRPC current was significantly increased by  $\sim 170\%$  in HIF-1 $\alpha$  overexpressing cardiomyocytes compared to NC cardiomyocytes. However, SC205346 significantly prevented HIF-1 $\alpha$  overexpression-induced TRPC currents (Fig. 7A) when it was added to the cardiomyocytes before HIF-1 $\alpha$  transfection. Figure 7A also showed that the reversal potential of TRPC current was not different among the groups:  $-7.9 \pm 1.8$  mV for NC ( $n = 12$ ),  $-8.2 \pm 1.9$  mV for HIF-1 $\alpha$  overexpressing cardiomyocytes ( $n = 10$ ) and  $-8.0 \pm 1.7$  mV for SC205346 + HIF-1 $\alpha$  ( $n = 5$ ). We next tested a battery of pharmacological tools frequently used



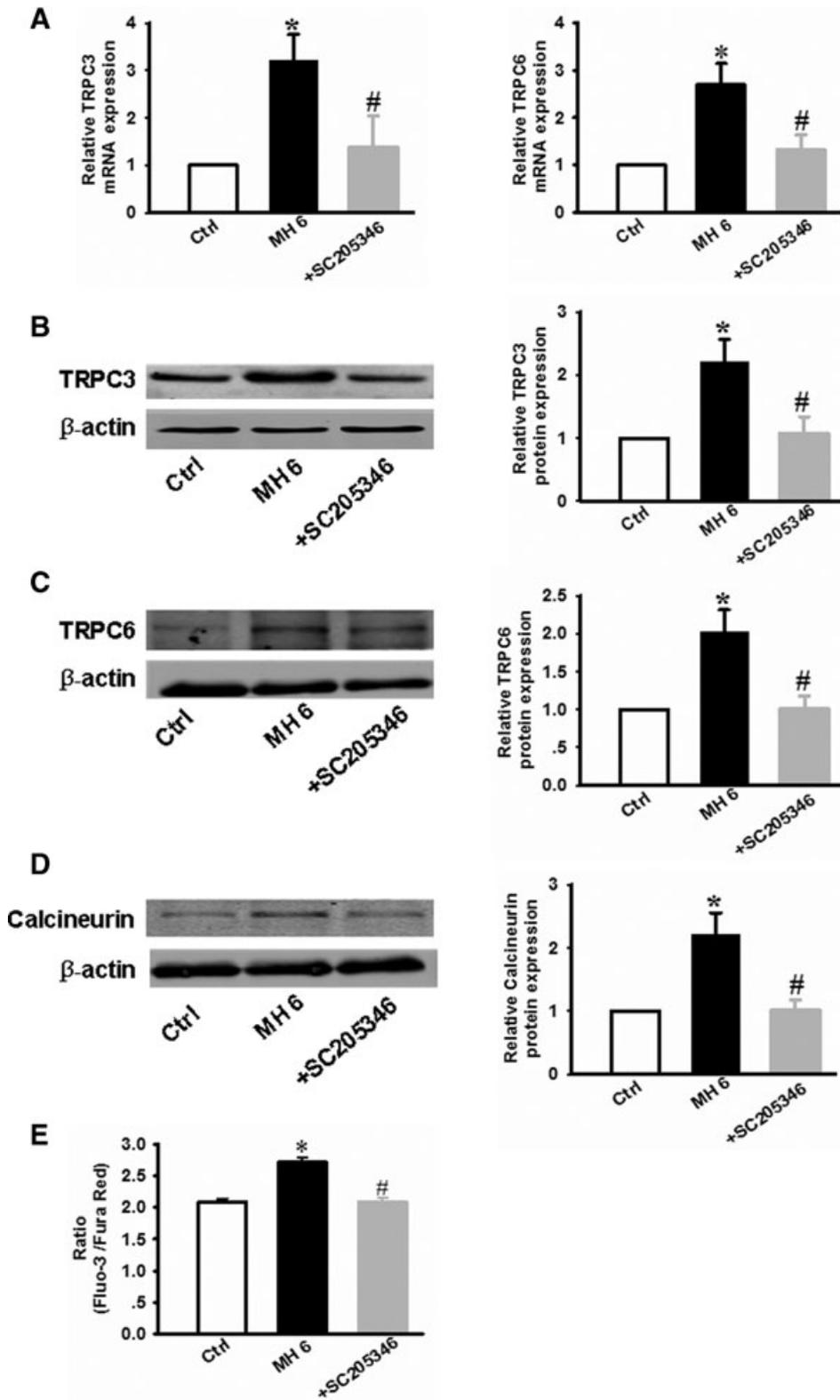
**Fig. 3** HIF-1 $\alpha$  controls development of hypertrophy in cardiac myocytes under MH. Representative immunofluorescence (A) and immunoblotting (B) for HIF-1 $\alpha$  showed overexpressed nuclear HIF-1 $\alpha$  in cardiomyocytes transfected with HIF-1 $\alpha$  compared to negative control (NC). Cardiomyocytes were transfected with HIF-1 $\alpha$  at a dose of 8.0  $\mu$ g for 48 hrs. (C) Representative  $\alpha$ -actinin immunostaining images were taken under different experimental conditions as indicated (original magnification:  $\times$ 600). (D) Statistical bar graph from (C) showed that hypertrophic cardiomyocytes under 6-hr MH were greatly prevented by 30  $\mu$ M SC205346 applied 1 hr prior to MH, but were promoted by overexpression of HIF-1 $\alpha$ . (E) Up-regulation of  $\beta$ -MHC expression induced by MH for 6 hrs in cardiomyocytes was attenuated by SC205346, but enhanced by HIF-1 $\alpha$  overexpression. Western blot band is a representative result from three independent experiments. (F) ANP, BNP and  $\beta$ -MHC mRNA up-regulation induced by MH were inhibited by SC205346, but promoted by HIF-1 $\alpha$  overexpression. \* $P$  < 0.05 versus Ctrl; <sup>#</sup> $P$  < 0.05 versus MH;  $n$  = 5 independent experiments for each bar; NC: empty vector pcDNA3.1 transfection serves as a negative control.



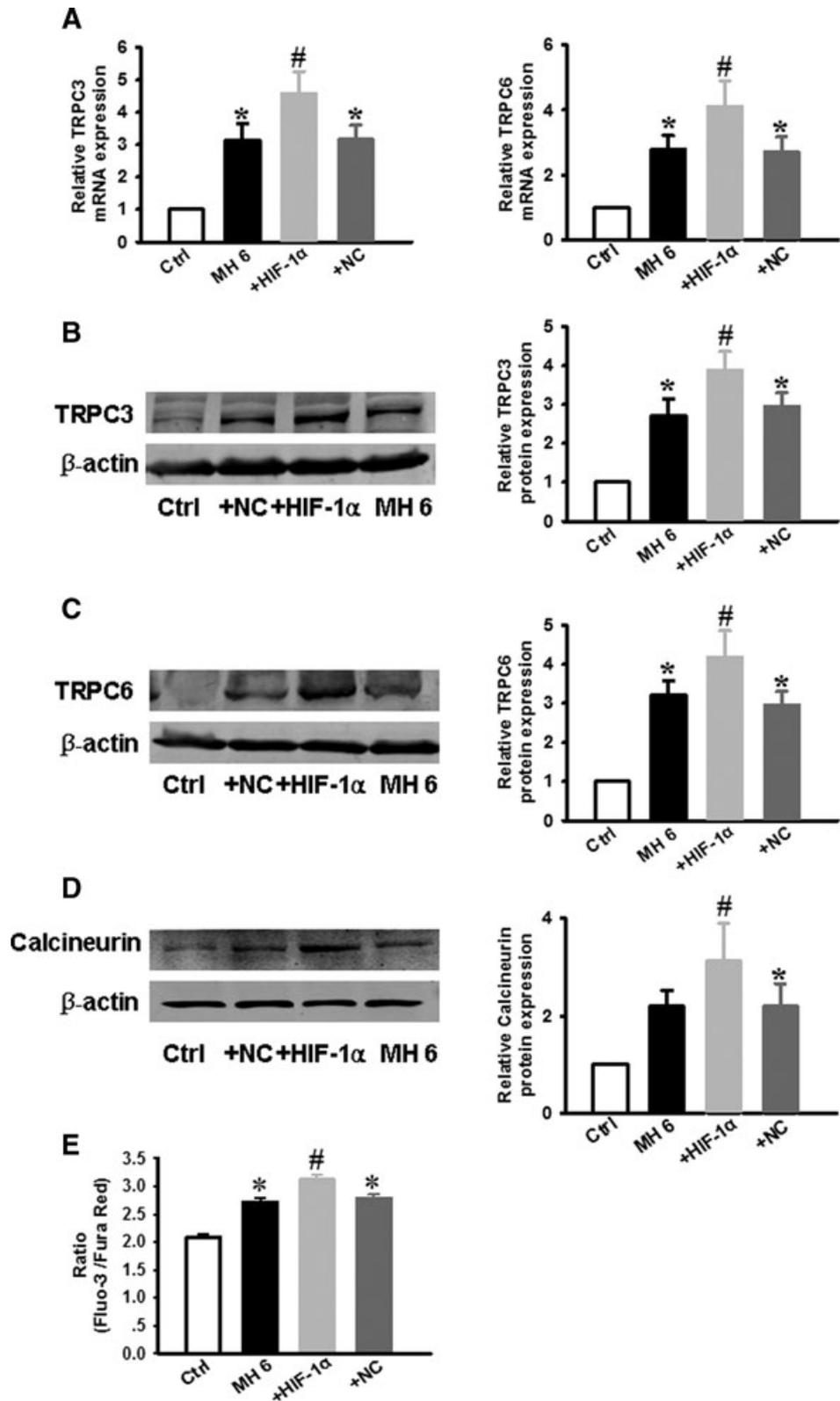
**Fig. 4** MH triggers elevation of TRPC-mediated  $Ca^{2+}$  signals. Bar graphs of TRPC1, TRPC3 and TRPC6 mRNA by real-time PCR (A) and protein expression by Western blotting (B) in cultured neonatal cardiac myocytes under MH condition at the indicated time points. (C) Elevation of  $[Ca^{2+}]_i$  in cardiomyocytes under MH conditions was inhibited by two TRPC channel blockers, 2-APB and SK&F3586. 2-APB (30  $\mu$ M) and SK&F3586 (10  $\mu$ M) were given to cardiomyocytes 1 hr before MH exposure. (D) Enhanced calcineurin in cardiac myocytes cultured under MH conditions was inhibited by SK&F3586. (E) TRPC channel blocker SK&F96365 blocked p-NFAT decrease induced by MH. Total NFAT was not changed under the indicated condition. (F) SK&F96365 blocked MCIP1 mRNA increase induced by MH. (G) SK&F96365 prevented MH-induced cardiomyocytes hypertrophy by  $\alpha$ -actinin immunostaining (H) SK&F96365 completely inhibited ANP, BNP and  $\beta$ -MHC mRNA up-regulation induced by MH. Western blot band is a representative result from three independent experiments. \* $P < 0.05$  versus Ctrl; # $P < 0.05$  versus MH;  $n = 3$  independent experiments for each condition.

to inhibit TRPC channel activity: 2-APB (30  $\mu$ M) and SK&F96365 (10  $\mu$ M). All these compounds significantly reduced TRPC current in NC and HIF-1 $\alpha$  overexpressed cardiomyocytes (Fig. 7B), suggesting that HIF-1 $\alpha$  not only regulates TRPC expression

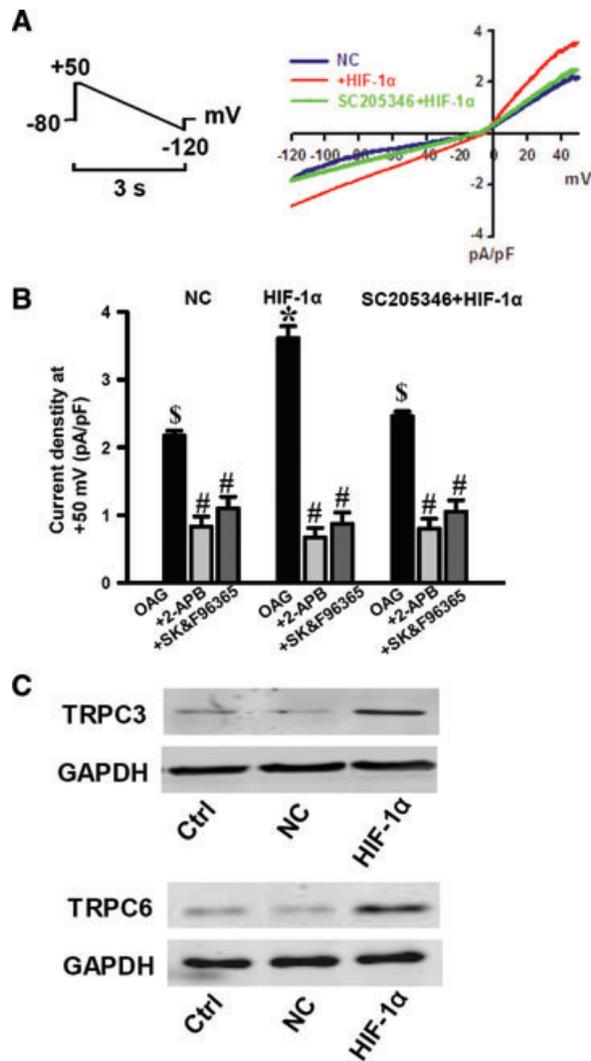
but also regulates TRPC function. HIF-1 $\alpha$  overexpression also increased TRPC3 and TRPC6 expression (Fig. 7C), which was consistent with TRPC current detection in HIF-1 $\alpha$  transfected cardiomyocytes.



**Fig. 5** SC205346 attenuates TRPC3, TRPC6 expression and  $\text{Ca}^{2+}$ -calcineurin signals in MH cardiomyocytes. Cardiac myocytes were treated with SC205346 (30  $\mu\text{M}$ ) 1 hr before MH. SC205346 attenuated the up-regulation of TRPC3 and TRPC6 mRNA (**A**), protein (**B** and **C**) expression, elevation of  $[\text{Ca}^{2+}]_i$  (**E**) and enhanced calcineurin (**D**) expression induced by MH. \* $P < 0.05$  versus Ctrl; # $P < 0.05$  versus MH;  $n = 3$  independent experiments for each condition.



**Fig. 6** HIF-1 $\alpha$  overexpression enhances TRPC3, TRPC6 expression and Ca<sup>2+</sup>-calcineurin signals under MH. Cardiac myocytes were transiently transfected with vectors of HIF-1 $\alpha$  or pcDNA3.1 (NC), then exposed to MH for 6 hrs. HIF-1 $\alpha$  overexpression promoted the up-regulation of TRPC3 and TRPC6 mRNA (A), protein (B and C) expression, elevation of [Ca<sup>2+</sup>]<sub>i</sub> (E) and enhanced calcineurin (D) expression induced by MH. \**P* < 0.05 versus Ctrl; #*P* < 0.05 versus MH; *n* = 3 independent experiments for each condition. Effect of HIF-1 $\alpha$  transfection on TRPC3 and TRPC6 expression was shown in Figure 7C.



**Fig. 7** Enlarged OAG-induced TRPC3/TRPC6 current in HIF-1 $\alpha$  overexpressing cardiomyocytes. **(A)** Representative I-V curves obtained after application of OAG (30  $\mu$ M) in NC (blue line), HIF-1 $\alpha$  overexpressing cardiomyocytes (red line) and SC205346 treatment 1 hr before cardiomyocytes transfected with HIF-1 $\alpha$  (green line). The stimulation protocol is displayed in the left panel. **(B)** Mean data ( $\pm$ S.E.M.;  $n = 10$  of NC,  $n = 9$  of HIF-1 $\alpha$  overexpressing cardiomyocytes, and  $n = 5$  of SC205346 + HIF-1 $\alpha$ ) of the OAG-induced TRPC density at +50 mV and the effect of pharmacological agents ( $n = 5$  in each condition). <sup>#</sup> $P < 0.05$  versus OAG-induced TRPC current; <sup>\*</sup> $P < 0.05$  versus NC; <sup>S</sup> $P < 0.05$  versus TRPC current in HIF-1 $\alpha$  overexpressing cardiomyocytes. **(C)** HIF-1 $\alpha$  overexpression enhanced the expression of TRPC3 and TRPC6 expression. NC: empty vector pcDNA3.1 transfection serves as a negative control.

## Discussion

Many efforts have been made to examine an important role of hypoxic stress in the development of cardiac hypertrophy, but the

initial molecule responded to oxygen still needs to be clarified. The established studies of HIF-1 $\alpha$  in oxygen-sensing responses to hypoxia set up the milestone in the field. A major advance in the understanding of oxygen-sensing processes and the mechanisms which indicates the cellular and tissular responses to hypoxia came with the discovery of HIF-1 $\alpha$ . Here, we demonstrate that MH can induce hypertrophy in cultured neonatal rat cardiac myocytes. MH stimulates an increase of HIF-1 $\alpha$  mRNA and HIF-1 $\alpha$  protein in nuclei. MH up-regulates TRPC3 and TRPC6, but not TRPC1 mRNA and protein expression, and enhances Ca<sup>2+</sup>-calcineurin signals. We further demonstrate HIF-1 $\alpha$  is involved in up-regulation of TRPC3 and TRPC6 expression and elevation of Ca<sup>2+</sup>-calcineurin signals under MH condition. These results suggest HIF-1 $\alpha$  plays a critical role in hypoxic adaptive cardiomyocytes hypertrophy, which gives a clue for future study *in vivo*.

Concerning the effect of hypoxia on HIF-1 $\alpha$  expression, it varies in different cell lines and the degree of hypoxia. Hypoxia at 1% O<sub>2</sub> induces HIF-1 $\alpha$  protein accumulation after 4 hrs followed by a strong desensitization (loss of the protein) after 24 hrs to 7 days in Hela cells [23]. Belaiba *et al.* [24] demonstrate that HIF-1 $\alpha$  mRNA level is increased in response to hypoxia (1% O<sub>2</sub>) within 0.5 hr, peaking at 1 hr and returning to basal levels after 4 hrs of hypoxic stimuli, and HIF-1 $\alpha$  protein levels are rapidly increased in response to hypoxia after 0.5 hr and remained elevated for up to 8 hrs in pulmonary artery smooth muscle cells. In Hela cells, MH (10% O<sub>2</sub>) for 30 min. is able to induce an increase of HIF-1 $\alpha$  protein [25]. In U87 MG glioblastoma cells, MH (10% O<sub>2</sub>) causes an increase of HIF-1 $\alpha$  expression at the time point of 1, 6 and 18 hrs [26]. These data are consistent with our results. In the present study, when neonatal cardiac myocytes are exposed to MH (10% O<sub>2</sub>) for 1, 3, 6 or 12 hrs, HIF-1 $\alpha$  mRNA and protein levels increase in a time-dependent manner, and reach the peak at 6 hrs. In addition, hypertrophy-related proteins, ANP, BNP and  $\beta$ -MHC, are also increased when the cardiomyocytes are exposed to MH for 6 hrs. With the observation of the persistent and significant changes of HIF-1 $\alpha$  and hypertrophy-related proteins at this time point, we apply 6-hr MH treatment through our hypoxia study.

We further observe that development of cardiac hypertrophy induced by MH could be inhibited by SC205346, a specific HIF-1 $\alpha$  inhibitor, but promoted by overexpression of HIF-1 $\alpha$ . HIF-1 $\alpha$  overexpression promotes the development of cardiac hypertrophy *via* up-regulation of Ca<sup>2+</sup>-calcineurin under MH condition. In fact, increase of [Ca<sup>2+</sup>]<sub>i</sub> is not only associated with hypertrophy, but also with apoptotic process under severe hypoxia situation [27, 28]. Some reports have shown that overexpression of HIF-1 $\alpha$  promotes apoptosis of cardiac myocytes in the presence of severe hypoxia, especially when other cellular energy substrates are lacking [29, 30]. However, HIF-1 $\alpha$  prevents H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis in HL-1 cardiomyocytes transfected with 1.6  $\mu$ g of vectors carrying HIF-1 $\alpha$ , suggesting a cardioprotective effect of HIF-1 $\alpha$  [15]. These studies suggest that effect of HIF-1 $\alpha$  on hypoxic cardiac myocytes, which result in hypertrophy or apoptosis, is related to hypoxia extent, hypoxia time and levels of HIF-1 $\alpha$  in the cells. The present study shows that MH causes a mild [Ca<sup>2+</sup>]<sub>i</sub> elevation of

130% compared to non-hypoxic cardiomyocytes, while many studies show that severe hypoxia elicits an ~5-fold  $[Ca^{2+}]_i$  elevation [31, 32]. Therefore, we speculate that variable hypoxic extent, stimuli time and HIF-1 $\alpha$  level result in the magnitude of  $[Ca^{2+}]_i$  elevation, and finally determine the fate for cardiomyocytes, hypertrophy or apoptosis.

We next explore the mechanism by which  $Ca^{2+}$ -calcineurin signals are elevated by HIF-1 $\alpha$ . As previously documented, TRPC1, TRPC3 and TRPC6 are the relatively high-level expressed TRPCs in heart and play critical roles in development of cardiac hypertrophy; thereafter, our hypothesis would emerge that HIF-1 $\alpha$  up-regulates expression of TRPC1, TRPC3 and TRPC6 and enhances  $Ca^{2+}$ -calcineurin signalling, and ultimately leads to cardiac hypertrophy. The increase of  $Ca^{2+}$ -calcineurin signalling and hypertrophy induced by MH in cardiomyocytes could be blocked by TRPC channel blocker, SK&F96365. SK&F96365, probably through inhibiting the phos-NFAT and MCIP1 expression. We also find the significant increase of TRPC3 and TRPC6 mRNA, protein levels under MH condition, other than TRPC1. Interestingly, some studies have indicated that increase of TRPC3 and TRPC6 channel activity may also contribute to apoptosis in cardiac myocytes. For example, overexpression of TRPC3 increases apoptosis but not necrosis in response to ischaemia-reperfusion in adult mouse cardiomyocytes [33, 34]. Calcium-sensing receptor activation leads to apoptosis by stimulating TRPC6 channel in rat neonatal ventricular myocytes [34]. These studies strongly support our conclusions: TRPC3 and TRPC6 are associated with the amplitude of  $[Ca^{2+}]_i$  increase which is responsible for the myocytes fate (hypertrophy or apoptosis) under hypoxic situation. Moreover, functional TRPC channels are comprised of homo- or heterotetramers between either TRPC1/4/5 or TRPC3/6/7 subfamily members [35]. Our results show that MH could not affect TRPC1 expression, but increase TRPC3 and TRPC6 in cardiomyocytes. And the enhanced expression of TRPC3 and TRPC6 induced by MH is regulated by HIF-1 $\alpha$  (will be discussed later). Taken together, it suggests that a TRPC3/6/7-formed channel may be involved in MH-induced cardiac hypertrophy.

The present study also shows that SC205346, a specific HIF-1 $\alpha$  blocker, could abolish up-regulation of TRPC3 and TRPC6 expression, but overexpression of HIF-1 $\alpha$  promotes up-regulation of TRPC3 and TRPC6 expression in cardiomyocytes exposed to MH. Most importantly, patch clamp data indicate that HIF-1 $\alpha$  up-regulated a functional channel formed by TRPC3 and TRPC6 occurred only in HIF-1 $\alpha$  overexpression cells. One important report has shown that HIF-1 mediates hypoxia-induced TRPC6 expression in pulmonary arterial smooth muscle cells [36], which is consistent with our data. These evidences suggest HIF-1 $\alpha$  is able to regulate TRPC protein expression and function, but the detailed mechanism has not been elucidated in this study. We predict the promoter regions of rat and mouse genes encoding TRPC3 and TRPC6 may contain HIF-1 $\alpha$  binding sites. It is possible that HIF-1 $\alpha$  regulated the expression of TRPC3 and TRPC6 through some intermediate factors, for instance, some evidence has shown that mild hypoxia induces endothelin-1 (ET-1) expression, which is able to activate TRPC3 and TRPC6 channel current [3, 37, 38].

In summary, when cardiomyocytes are exposed to MH, cardiac hypertrophy starts to occur, HIF-1 $\alpha$  plays a critical role in the development of cardiomyocytes hypertrophy. MH increases TRPC expression, leading to enhanced TRPC-mediated calcineurin signalling, which are both regulated by HIF-1 $\alpha$ . The present study has demonstrated that HIF-1 $\alpha$ -mediated TRPC signalling pathway plays a key role in hypoxia-induced cardiac hypertrophy. So far, some *in vivo* studies have shown that HIF-1 $\alpha$  overexpression relieves cardiac dysfunction in myocardial infarction animal model. For example, injection of naked DNA encoding for HIF-1 $\alpha$  directly into the left ventricle induces angiogenesis and reduces infarct size [39]. Mice with cardiac overexpression of HIF-1 $\alpha$  subjected to myocardial infarction develop reduced infarctions and attenuate progression of cardiac dysfunction 4 weeks after infarct induction [40]. Although these studies have suggest that HIF-1 $\alpha$  plays a cardioprotective role in pathological cardiac dysfunction. Its role in adaptive cardiac hypertrophy still needs to be elucidated by *in vivo* studies.

## Acknowledgements

This study was supported by the Major Program of National Natural Science Foundation of China grant 81130088; the Funds for Creative Research Groups of The National Natural Science Foundation of China grant 81121003; the Natural Science Foundation of China (30900575, 31100826); Key Laboratory of Bio-pharmaceutical-engineering (Harbin Medical University), Ministry of Education (2010-01); and Natural Science Foundation of Heilongjiang Province (QC2010097).

## Conflicts of interest

The authors confirm that there are no conflicts of interest.

## Supporting information

Additional Supporting Information may be found in the online version of this article.

**Table S1** Primers for real-time PCR.

**Fig. S1** 30  $\mu$ M SC205346 inhibited HIF-1 $\alpha$  nuclear accumulation in MH cardiac myocytes. GAPDH and  $\beta$ -actin serve as control to show there is no response to MH. Western blot band is a representative result from three independent experiments.

**Fig. S2** ANP (A), BNP (B) and  $\beta$ -MHC (C) protein expression in rat neonatal cardiac myocytes treated with 30  $\mu$ M SC205346 for 6 hrs. Western blot band is a representative result from three independent experiments.

**Fig. S3** ANP (A), BNP (B) and  $\beta$ -MHC (C) protein expression in rat neonatal cardiac myocytes transfected with HIF-1 $\alpha$ . Western blot band is a representative result from three independent experiments.

**Fig. S4** Effect of MH on Ca<sup>2+</sup>-calcineurin signals. MH causes a mild elevation of [Ca<sup>2+</sup>]<sub>i</sub> (A) and an enhanced calcineurin (B) expression in cardiomyocytes. \**P* < 0.05 versus Ctrl.

**Fig. S5** Effect of SC205346 on TRPC3 and TRPC6 expression. SC205346 did not affect TRPC3 (A) and TRPC6 (B) in cardiomyocytes treated with 30  $\mu$ M SC205346 for 6 hrs. Western blot band is a representative result from three independent experiments.

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