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Original Article

IP3R and RyR calcium channels are involved in neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-α

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Abstract: To investigate which calcium channels are involved in cardiac myocyte hypertrophy induced by TNF-α, cultured cardiomyocytes were treated with 100 μg/L TNF-α. In addition, three different calcium channel blockers (2-APB, ryanodine and nifedipine) were used, and the effects of each calcium channel blocker on cardiac hypertrophy induced by TNF-α were carefully observed. Measurements included cytosolic calcium transients ($[Ca^{2+}]i$), the level of intracellular calcium in individual cells, cell protein content, cell protein synthesis and cell volume. We found that the IP3R inhibitor (2-APB) and RyR inhibitor (ryanodine) both had significant suppressive effects on the level of $[Ca^{2+}]i$, calcium concentration, cell protein content, cell protein synthesis and cell volume of cardiomyocytes treated with TNF-α ($P<0.01$). Moreover, their combined effects were significantly enhanced compared with their single effects ($P<0.01$). However, the inhibitor of the L type Ca^{2+} channel nifedipine exhibited no significant suppressive effects on the increase in $[Ca^{2+}]i$, calcium concentration, cell protein content, cell protein synthesis and cell volume of cardiomyocytes induced by TNF-α ($P>0.05$). Our results suggest that TNF-α probably induces cardiac myocyte hypertrophy by activating IP3R and RyR calcium channels, which control the release of calcium ions from the sarcoplasmic reticulum (SR) in cardiomyocytes. On the other hand, extracellular calcium influx, which is mainly regulated by the L type Ca^{2+} channel, may not be involved in cardiac myocyte hypertrophy induced by TNF-α.

Keywords: Cardiac myocyte hypertrophy, tumor necrosis factor-α, calcium, calcium channel, inositol 1,4,5-triphosphate receptor (IP3R), ryanodine receptor (RyR)

Introduction

Myocardial hypertrophy is an adaptive response to stresses that increase cardiac work. During the hypertrophic response, cardiac myocytes increase in size without undergoing cell division [1]. However, prolonged hypertrophy is accompanied by fibrosis and myocyte dropout, and compensated hypertrophy generally progresses to decompensated cardiac failure with chamber dilatation and contractile dysfunction [2, 3]. Therefore, the elucidation of the mechanisms of cardiac hypertrophy is extremely important.

Tumor necrosis factor (TNF) derived its name from the observation that this protein was first identified as a substance that exerted profound antitumor effects *in vitro* and *in vivo* [4, 5]. However, since this original observation, it has become clear that TNF has a variety of different biological capacities [6]. For example, TNF influences growth, differentiation, and/or function of virtually every cell type investigated, including cardiac myocytes [7-9]. Since the observation that myocardial TNF-α expression is enhanced upon sustained hemodynamic overloading of the heart or ischemic injury [10], numerous studies investigating the correlation between TNF-α and cardiac diseases have been performed. Krown et al. found that TNF stimulation on isolated cardiac myocytes provoked progressive myocyte cell death through apoptosis [11]. Yokoyama et al. found that stimulation with TNF increased sarcomeric protein synthesis in cultured adult cardiac myocytes [7]. Currently, TNF-α is regarded as one of the important factors that can induce car-

diac hypertrophy, and the direct effect of TNF-α on cardiac hypertrophy has been demonstrated in cultured cardiac myocytes [7, 12] and animal models [13].

In addition to the well-known involvement of calcium in the regulation of cardiac contractility, recent studies have emphasized the importance of calcium in the activation of signal transduction pathways responsible for hypertrophic cardiac growth and heart failure [1]. A recent study by Defer et al. noted that in cardiac myocytes isolated from rats, TNF-a induced ROS production, exerted a dual positive and negative action on [Ca^{2+}] transient and altered cell survival [10]. Their results suggested that the change in [Ca^{2+}] transient may also play a central role in cardiac hypertrophy induced by TNF-α.

The apparent involvement of calcium in TNF-α-induced cardiac hypertrophy and heart failure provides numerous opportunities and challenges for genetic and pharmacologic modification of calcium-dependent events in the intervention of pathological cardiac hypertrophy [1]. However, numerous ion transport pathways are involved in the regulation of [Ca^{2+}]i, and it is not clear which calcium channel(s) is/are involved in the initiation and development of TNF-α induced cardiac hypertrophy [14]. In the current study, we used three different calcium channel blockers to determine which calcium channels are involved in cardiac myocyte hypertrophy induced by TNF-α.

Materials and methods

Animals and experimental reagents

One- to three-day-old neonatal SD rats were provided by the Laboratory Animal Center of Liaoning Medical University. All experimental protocols were approved by the Committee for the Use of Experimental Animals for Research and Teaching of China Medical University, China.

Trypsin, fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (USA). Recombinant human TNF-α was purchased from R&D (USA). [^{3}H] Leucine was provided by Shanghai Institute of Nuclear Research, Chinese Academy of Sciences (China). Bovine serum albumin (BSA), Fura-2/AM, Fluo-3/AM, sodium dodecyl sulfate (SDS) and 2-aminoethoxydiphenyl borate (2-APB) were obtained from Sigma Chemical Co. (USA). Ryanodine and nifedipine were purchased from Merck Drugs & Biotechnology (Germany).

Primary culture of neonatal rat ventricular myocytes

Primary cultures of cardiomyocytes were prepared from the ventricles of neonatal SD rats as described by Simpson [15]. The hearts of 1- to 3-day-old SD rats were harvested under sterile conditions. Then, the heart tissues were rinsed with Hanks' solution and sliced into 1-mm^3 cubes before 0.08% trypsin was added for digestion. After dissociation of the heart tissues with trypsin, cells were pre-plated for 1 h onto 100-mm culture dishes in DMEM containing 10% FBS to reduce the number of non-myocyte cells. Cells that were not attached to the pre-plated dishes were resuspended in DMEM containing 15% FBS and 1% penicillin-streptomycin, plated onto 24-well culture plates at a density of 1 × 10^6/ml, and maintained at 37°C in humidified air containing 5% CO_2. For the first 72 h, 0.1 mmol/L BrdU was applied to limit the growth of non-myocytes in the cultures. Then, cells were transferred into serum-free DMEM containing 5 μg/ml insulin, 5 μmol/L vitamin B12 and 10 μg/ml transferrin to be cultured for an additional 24 h.

Treatments

The myocytes were mainly divided into nine experimental groups: "① Control group: cells were incubated in DMEM containing 10% FBS; ② TNF-α group: cells were incubated in DMEM containing 10% FBS and 100 μg/L TNF-α; ③ IP3R inhibitor group: cells were incubated in DMEM containing 10% FBS and 100 μg/L TNF-α plus 30 μM 2-APB; ④ RyR inhibitor group: cells were incubated in DMEM containing 10% FBS and 100 μg/L TNF-α plus 50 μM ryanodine; ⑤ L type Ca^{2+} channel inhibitor group: cells were incubated in DMEM containing 10% FBS and 100 μg/L TNF-α plus 50 μM nifedipine; ⑥ IP3R inhibitor +RyR inhibitor group: cells were incubated in DMEM containing 10% FBS and 100 μg/L TNF-α plus 30 μM 2-APB and 50 μM ryanodine; ⑦ Pure IP3R inhibitor group: cells were incubated in DMEM containing 10% FBS and 30 μM 2-APB; ⑧ Pure

RyR inhibitor group: cells were incubated in DMEM containing 10% FBS and 50 μM ryanodine; ⑨ Pure L type Ca^{2+} channel inhibitor group: cells were incubated in DMEM containing 10% FBS and 50 μM nifedipine".

In groups ③, ④, ⑤ and ⑥, calcium inhibitors were added 30 min before the administration of TNF-α. In all of the groups, the indexes of cardiac hypertrophy including cell protein content, cell protein synthesis and cell sizes were measured 72 h after treatment.

Determination of cell protein content

Cell dishes were washed rapidly three times with Hanks' solution. Then, cells were adjusted to a density of 1×10^5/well and subsequently disrupted in 1% SDS. The protein content of the cells in each well was measured by the method of Lowry et al. as described in a previous study [16].

Determination of cell protein synthesis

[^{3}H]-Leucine uptake was used as the index of protein synthesis as described by Luo etc. [17]. The culture medium of myocardial cells was removed and replaced with 1 μCi [^{3}H]-leucine. Different concentrations of drugs were added, and incubation was continued for 72 h. The medium was then aspirated, and cells were washed rapidly three times with cold Hanks' solution. Then, the cells were lysed by the addition of 1 ml 1% SDS per well. Lysates were then collected and precipitated by the addition of 1 ml 5% trichloroacetic acid and applied to fiberglass GF/C filters. After three washes with 5 ml Hanks' solution, the filters were dried and transferred to vials containing 4 mL scintillation fluid, and their radioactivity was determined by liquid scintillation counting. The value of radioactivity, which represented the [^{3}H]-leucine incorporated into newly synthesized protein, was expressed as cpm per well.

Estimation of cell volume

Cardiomyocyte volume was calculated by the measurement of cell diameters [18]. Cells in each well were washed rapidly three times with D-Hanks' solution containing 8 g/L NaCl, 0.4 g/L KCl, 0.06 g/L $Na_2HPO_4 \cdot H_2O$, 0.06 g/L KH_2PO_4, 0.35 g/L $NaHCO_3$, and 0.02 g/L phenol red. Then, cells were subsequently treated with 0.3 ml 0.1% trypsin at 37°C for 10 min before digestion was stopped with 10% FBS (0.2 ml/well). Digested cells were collected and measured on an inverted microscope. For the measurements, four or five fields were randomly chosen and photographed at high power (400 ×), and 20 individual cells within each field were evaluated using the CIAS Daheng image analysis system (China).

Measurement of cytosolic calcium transients ($[Ca^{2+}]i$)

A spectrofluorimetric method [19] was used to measure cytosolic Ca^{2+} transients using Fura-2/AM as the indicator for Ca^{2+}. Cultured cardiomyocytes were incubated for 30 min with Fura-2/AM (3 μmol/L) in DMEM containing 0.2% albumin. To allow the de-esterification of Fura-2/AM into the cytosol, the loaded cells were maintained at 24 to 26°C for 60 min before $[Ca^{2+}]i$ was measured. Then, cardiomyocytes were rinsed with HEPES buffer and transferred to a superfusion chamber on the stage of an inverted microscope, which was coupled to a Till imaging system and DM3000 software (Germany). Then, cells were superfused with 37°C Hanks' buffer at the speed of 1 ml/min. The emitted light was filtered at 505 nm, and the sampling interval was set at 300 ms. Fluorescence signals at 340 nm (F340) and 380 nm (F380) were recorded in a personal computer for data processing and analysis. During each sampling, 5 to 10 cells were chosen from each group, and their fluorescence signals before and after treatments were measured. Then, $[Ca^{2+}]i$ values were calculated according to a previous study [20].

Fluorescent assessment of intracellular calcium in individual cells

To measure cytosolic calcium transients in a more precise manner, we performed confocal imaging to assess intracellular calcium in individual cells [21]. Cells were plated at a density of 1×10^4/ml in 35-mm plastic tissue culture dishes and incubated for 24 h in DMEM containing 10% FBS. After cell attachment, the cells were cultured in a serum-deprived environment for an additional 24 h. After aspirating the media from the dishes and rinsing the dishes three times with D-Hanks' solution, the cells were incubated for 30 min at 37°C in 5 μmol/L Fluo-3/AM working solution. After remo-

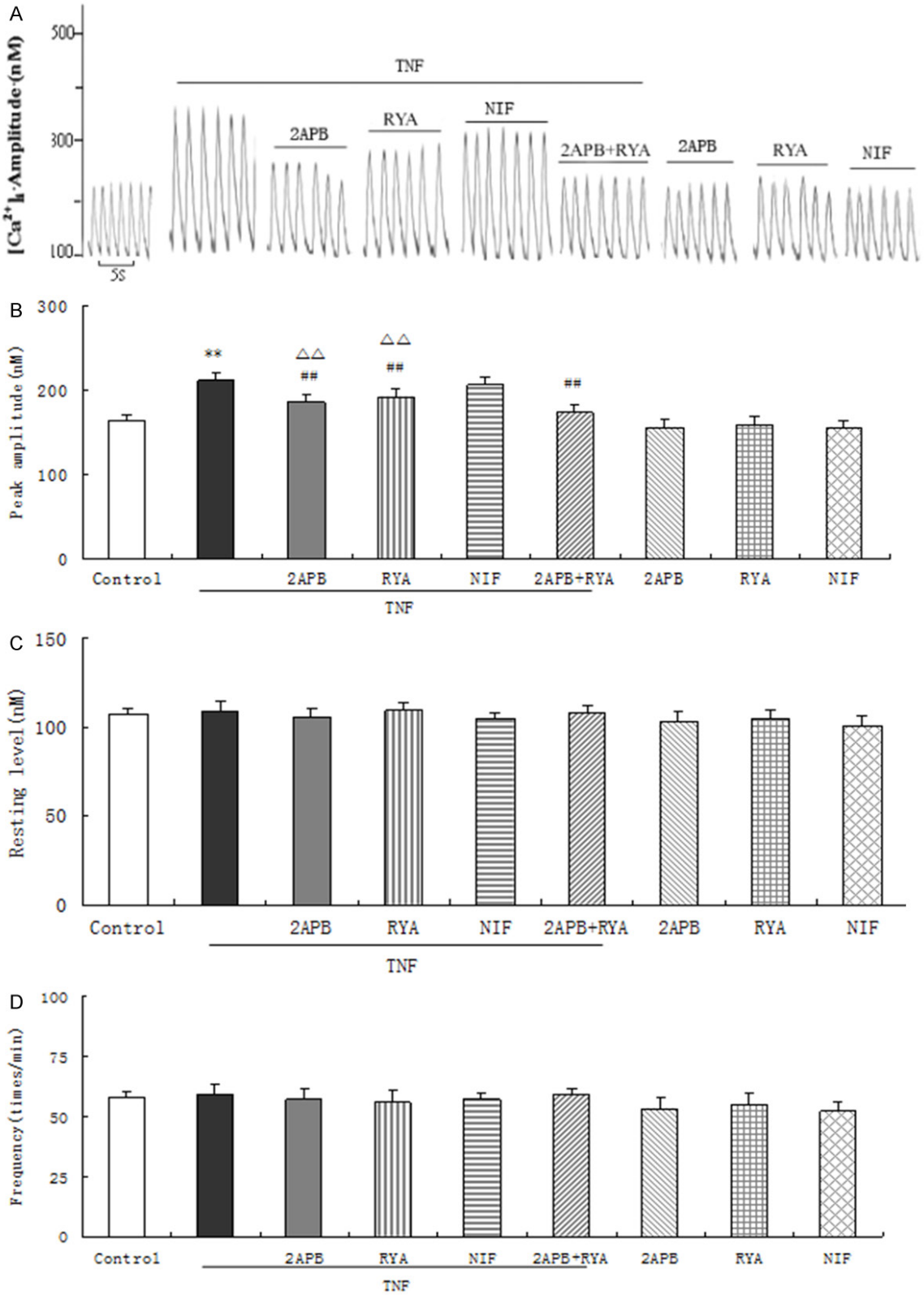

Figure 1. Effects of nifedipine, 2APB and ryanodine on TNF-α-induced calcium transients ($[Ca^{2+}]i$) in neonatal rat cardiomyocytes ($\bar{x} \pm s$, n=4). A: Shows the representative tracings. B: The peak amplitude of the spontaneous $[Ca^{2+}]i$ transient. C: The resting Ca^{2+} level. D: Frequency of the spontaneous $[Ca^{2+}]i$ transient in the cultured ventricular myocyte from the neonatal rat. TNF: TNF-α (100 μg/L); 2APB: 2-APB (30 μM); RYA: ryanodine (50 μM); NIF: nifedipine (50 μM). $^{**}P$<0.01 VS control group; $^{\#\#}P$<0.01 VS TNF-α group; $^{\Delta\Delta}P$<0.01 VS IP3R inhibitor +RyR inhibitor group.

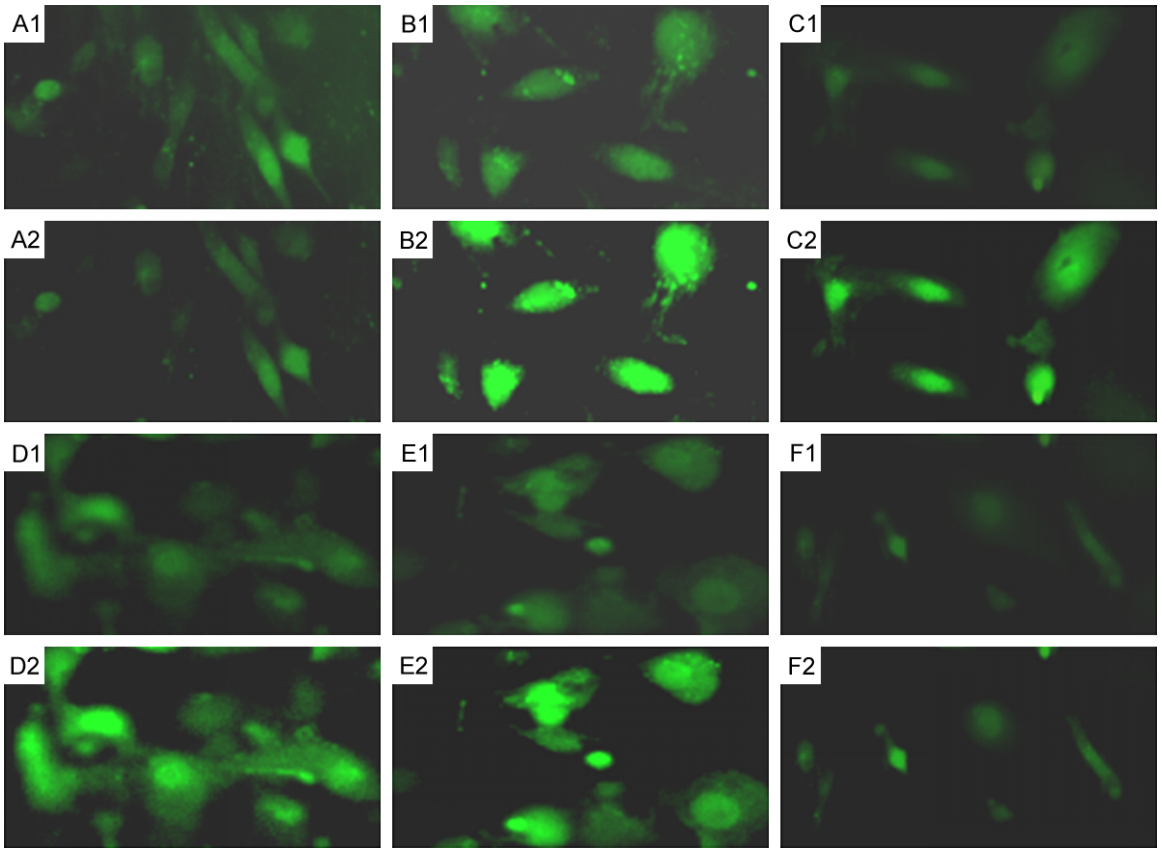

Figure 2. Confocal imaging before and after TNF-α stimulation of cardiomyocytes in each group. A1, A2: Were images of control group without TNF-α stimulation; B1-F1: Showed the fluorescence of cells before TNF-α stimulation; B2-F2: Showed the fluorescence of cells after TNF-α stimulation. B: TNF-α group; C: 2-APB+TNF-α; D: Ryanodine +TNF-α; E: Nifedipine +TNF-α; F: 2-APB+ ryanodine +TNF-α.

ving the dye labeling solution, the cells were rinsed with D-Hank's solution to ensure removal of any residual or unloaded dye that could influence background fluorescence. Then, 1 mL of Hanks' solution was added to the dishes, and the cells were left in this volume for the duration of the fluorescent measurements. After 30 min of incubation with calcium channel blockers, the dishes containing cells from each group were placed securely on the microscope platform and brought into focus using incandescent illumination. After adjusting the focus and setting an optimal fluorescence emission wavelength of 526 nm and an excitation wavelength of 488 nm, the PMT voltage was adjusted to appropriately set the baseline. Once the baseline [Ca^{2+}] was determined, stimuli including blank treatment, treatment with TNF-α, and treatment with different calcium channel blockers for calcium release were added manually in 1-ml volumes using a pipette. After data collection, LSM510 software was used for data analysis. A polygon selection tool was used to encompass an individual cell within the image field. The total fluorescence in each cell polygon area was measured by determining the integrated fluorescence intensity (FI value) of the selected cell for images collected at both wavelengths. The fluorescence ratio was calculated by dividing the Ca^{2+}-bound Fluo-3/AM-integrated FI by the unbound Fluo-3/AM-integrated FI. After compiling these ratio values, the absolute [Ca^{2+}]i and [Ca^{2+}]i variation were calculated using the following equations: [Ca^{2+}]i=kd × (F-Fmin)/(Fmax-F); Δ[Ca^{2+}]i= [Ca^{2+}]i after treatment-[Ca^{2+}]i before treatment. The Kd (equilibrium dissociation constant) was 320 nmol/L at room temperature. FI was the actual FI value measured during the experiments. Fmax (fully saturated FI value) was obtained by measuring [Ca^{2+}]i with the existence of Triton X-100 in the extracellular fluid. Fmin (minimal FI value) was obtained by measuring [Ca^{2+}]i after the administration of 10 μM EGTA.

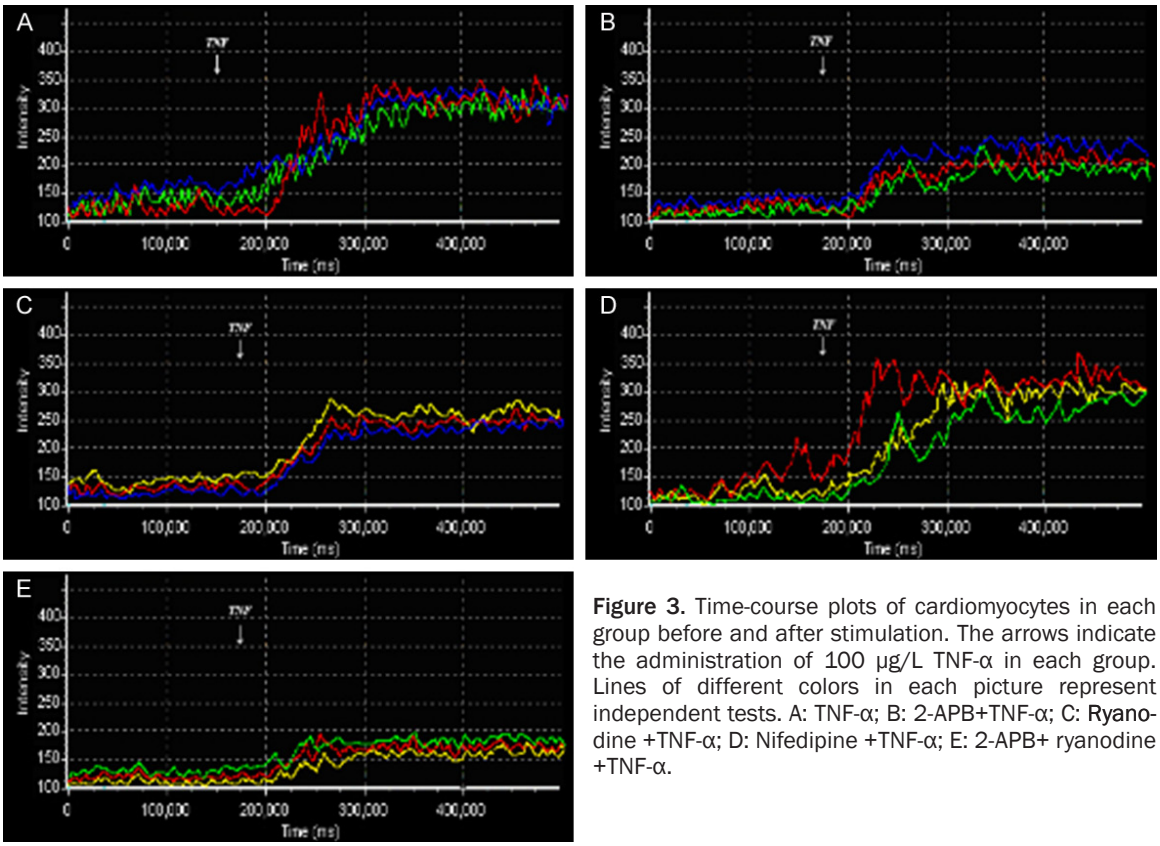

Figure 3. Time-course plots of cardiomyocytes in each group before and after stimulation. The arrows indicate the administration of 100 μg/L TNF-α in each group. Lines of different colors in each picture represent independent tests. A: TNF-α; B: 2-APB+TNF-α; C: Ryanodine +TNF-α; D: Nifedipine +TNF-α; E: 2-APB+ ryanodine +TNF-α.

There were two dishes for the data collection from each group, and the experiment was repeated three times using data collected from different cells.

Statistical analysis

Data are reported as means ± SD. Analysis of variance (one-way ANOVA) and factorial design analysis of variance were used for comparisons among the groups. SNK test was used to determine differences between two groups. $P<0.05$ was considered to be statistically significant.

Results

Effects of calcium inhibitors on TNF-α-induced calcium transients ($[Ca^{2+}]i$) in neonatal rat cardiomyocytes

We used the spectrofluorimetric method to investigate the effects of different calcium channel inhibitors on TNF-α-induced $[Ca^{2+}]i$ in cardiomyocytes. When 100 μg/L TNF-α was added to the culture medium of cardiomyocytes, the amplitude of cytosolic $[Ca^{2+}]i$ transients in the cells increased significantly (**Figure 1A**, **1B**, TNF-α group VS control group, $P<0.01$). However, TNF-α had no effect on the resting level or the frequency of spontaneous transients of these cells (**Figure 1C**, **1D**). In addition, 2-APB (inhibitor of IP3R) and ryanodine (inhibitor of RyR) both had significant suppressive effects on the increase in calcium transient induced by TNF-α (**Figure 1A**, **1B**, IP3R inhibitor group VS TNF-α group, $P<0.01$; RyR inhibitor group VS TNF-α group, $P<0.01$). Furthermore, when 2-APB and ryanodine were added to the medium simultaneously, their combined suppressive effect on the TNF-α-induced increase in calcium transient was even more obvious (**Figure 1A**, **1B**, IP3R inhibitor +RyR inhibitor group VS TNF-α group, $P<0.01$). On the other hand, as the blocker of L type Ca^{2+} channel, nifedipine exhibited no such suppressive effect on the increase in calcium transient induced by TNF-α (**Figure 1A**, **1B**, L type Ca^{2+} channel inhibitor group VS TNF-α group, $P>0.05$). In addition, all three calcium channel blockers exhibited no significant effect on the resting level or on the frequency of spontaneous transients of cardiomyocytes (**Figure 1C**, **1D**).

Effects of calcium inhibitors on the TNF-α-induced elevation of calcium concentration in individual cardiomyocytes

To verify our results obtained using the spectrofluorimetric method, we performed the fluorescent assessment of intracellular calcium in individual cells (**Figures 2**-**4**). When cells were loaded with the calcium sensitive fluorescent probe Fluo-3/AM and measured in baseline conditions, the fluorescence intensity (FI) values of cells in each group were comparable, with no significant differences among groups (**Figure 4**, P>0.05). When cells were stimulated with 100 μg/L TNF-α, their FI values were significantly increased (P<0.01). However, this increase in FI values induced by TNF-α was significantly suppressed in cells pre-treated with 2-APB and/or ryanodine (IP3R inhibitor group VS TNF-α group, P<0.01; RyR inhibitor group VS TNF-α group, P<0.01). Again, the combined effect of 2-APB and ryanodine was more obvious than their individual effects (IP3R inhibitor +RyR inhibitor group VS TNF-α group, P<0.01; IP3R inhibitor +RyR inhibitor group VS IP3R inhibitor group, P<0.01; IP3R inhibitor +RyR inhibitor group VS RyR inhibitor group, P<0.01). On the other hand, pre-treatment with nifedipine had no significant effect on the increase in FI in cardiomyocytes induced by TNF-α (L type Ca^{2+} channel inhibitor group VS TNF-α group, P>0.05).

Effects of calcium inhibitors on the TNF-α-induced increase in protein content in cultured cardiomyocytes

Using the Lowry method, we determined the protein content of cultured cardiomyocytes before and after the administration of TNF-α and calcium inhibitors, and the results are displayed in **Figure 5**.

We found that 100 μg/L TNF-α induced a significant increase in protein content compared with the control group (TNF-α group VS control group, increased by 47.62%, P<0.01). In addition, the administration of pure calcium channel inhibitors demonstrated no significant influence on the protein content of cultured cardiomyocytes (pure IP3R inhibitor group VS control group, P>0.05; pure RyR inhibitor group VS control group, P>0.05; pure L type Ca^{2+} channel inhibitor group VS control group, P>0.05).

The addition of 2-APB and/or ryanodine had significant suppressive effects on the increase in protein content of cultured cardiomyocytes induced by TNF-α (IP3R inhibitor group VS TNF-α group, decreased by 30.12%, P<0.01; RyR inhibitor group VS TNF-α group, decreased by 23.05%, P<0.01; IP3R inhibitor +RyR inhibitor group VS TNF-α group, decreased by 40.57%, P<0.01). Moreover, the combined effect of the IP3R inhibitor and RyR inhibitor was significantly stronger than the effect of either agent alone (IP3R inhibitor +RyR inhibitor group VS IP3R inhibitor group, P<0.01; IP3R inhibitor +RyR inhibitor group VS RyR inhibitor group, P<0.01). However, as the blocker of L type Ca^{2+} channels, nifedipine exhibited no significant suppressive effect on the increase in protein content induced by TNF-α (L type Ca^{2+} channel inhibitor group VS TNF-α group, P> 0.05).

Effects of calcium inhibitors on the TNF-α-induced enhancement of protein synthesis in cardiomyocytes

To investigate the effects of calcium inhibitors on the TNF-α-induced enhancement of protein synthesis in cultured cardiomyocytes, we used the [^{3}H]-leucine incorporation method to reflect the protein synthesis status of cardiomyocytes before and after different treatments (**Figure 6**). We found that 100 μg/L TNF-α induced a significant increase in protein synthesis compared with the control group (TNF-α group VS control group, increased by 43.62%, P<0.01). However, the addition of pure calcium channel blockers had no significant effect on the protein synthesis status of cultured cardiomyocytes (pure IP3R inhibitor group VS control group, P>0.05; pure RyR inhibitor group VS control group, P>0.05; pure L type Ca^{2+} channel inhibitor group VS control group, P>0.05).

Furthermore, the addition of 2-APB and/or ryanodine had significant suppressive effects on the enhancement of protein synthesis in cardiomyocytes, which was induced by TNF-α (IP3R inhibitor group VS TNF-α group, decreased by 30.08%, P<0.01; RyR inhibitor group VS TNF-α group, decreased by 26.27%, P<0.01; IP3R inhibitor +RyR inhibitor group VS TNF-α group, decreased by 38.56%, P<0.01). In addition, the combined effect of the IP3R inhibitor and RyR inhibitor was significantly stronger

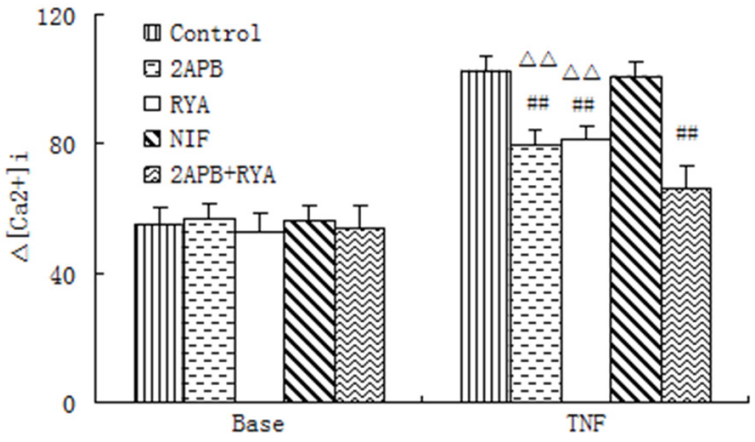

Figure 4. Effects of nifedipine, 2APB and ryanodine on the TNF-α-induced elevation of calcium concentration in individual cardiomyocytes ($\bar{x}$±s, n=30). Bars on the left showed the level of calcium concentration in baseline conditions; bars on the right showed the TNF-α-induced elevation of calcium concentration in each group. TNF: stimulated with 100 μg/L TNF-α; 2APB: pre-treated with 30 μM 2-APB; RYA: pre-treated with 50 μM ryanodine; NIF: pre-treated with 50 μM nifedipine; 2APB+RYA: pre-treated with 30 μM 2-APB and 50 μM ryanodine. $^{\#\#}P$<0.01 VS TNF-α group; $^{\Delta\Delta}P$<0.01 VS IP3R inhibitor +RyR inhibitor group.

than either agent alone (IP3R inhibitor +RyR inhibitor group VS IP3R inhibitor group, P< 0.01; IP3R inhibitor +RyR inhibitor group VS RyR inhibitor group, P<0.01). On the other hand, nifedipine, a blocker of L type Ca^{2+} channels, exhibited no suppressive effect on the enhancement of protein synthesis induced by TNF-α (L type Ca^{2+} channel inhibitor group VS TNF-α group, P>0.05).

Effects of calcium inhibitors on the increase in cell size of cardiomyocytes induced by TNF-α

We also measured the cell size of cardiomyocytes before and after different treatments to determine the effects of calcium blockers on the increase in cell size of cardiomyocytes induced by TNF-α (results shown in **Figure 7**). After treatment with 100 μg/L TNF-α, the average size of cardiomyocytes increased by 51.05% compared with cells in the control group (P<0.01). However, the addition of pure calcium channel blockers caused no significant change in the size of cultured cardiomyocytes (pure IP3R inhibitor group VS control group, P>0.05; pure RyR inhibitor group VS control group, P>0.05; pure L type Ca^{2+} channel inhibitor group VS control group, P>0.05).

Furthermore, the addition of 2-APB and/or ryanodine significantly reduced the increased cell sizes induced by TNF-α (IP3R inhibitor group VS TNF-α group, reduced by 20.12%, P<0.01; RyR inhibitor group VS TNF-α group, reduced by 17.01%, P<0.01; IP3R inhibitor +RyR inhibitor group VS TNF-α group, reduced by 26.22, P<0.01). In addition, the combined effect of the IP3R inhibitor and RyR inhibitor was significantly stronger than their individual effects (IP3R inhibitor +RyR inhibitor group VS IP3R inhibitor group, P<0.01; IP3R inhibitor +RyR inhibitor group VS RyR inhibitor group, P< 0.01). On the other hand, as the blocker of L type Ca^{2+} channels, nifedipine exhibited no significant effect on the TNF-α-induced change in cell size of cardiomyocytes (L type Ca^{2+} channel inhibitor group VS TNF-α group, P>0.05).

Discussion

As one of the most important features of many cardiac diseases, cardiac myocyte hypertrophy is associated with poor prognosis because it often causes an increased risk of arrhythmia and the development of congestive heart failure [3, 22]. Currently, TNF-α is regarded as an important factor that can induce cardiac myocyte hypertrophy, and evidence has been raised that TNF-α may induce cardiac hypertrophy by affecting calcium ion movement and regulating the intracellular free calcium concentration of cardiomyocytes [10, 23]. However, numerous calcium channels are involved in the regulation of $[Ca^{2+}]i$, and it is not clear yet which channels participate in the cardiac hypertrophy induced by TNF-α. In the current study, we investigated the effects of three different calcium inhibitors (2-APB as the inhibitor of IP3R, ryanodine as the inhibitor of RyR, and nifedipine as the inhibitor of L type calcium channels) on the TNF-α-induced change in intracellular calcium, cell protein and cell size of cultured cardiomyocytes.

First, we observed an increase in calcium transients and calcium concentration in cardiomyocytes stimulated by TNF-α. These results from the current study confirmed the results of previous studies that TNF-α could affect calcium regulation [24]. However, in our study, TNF-α

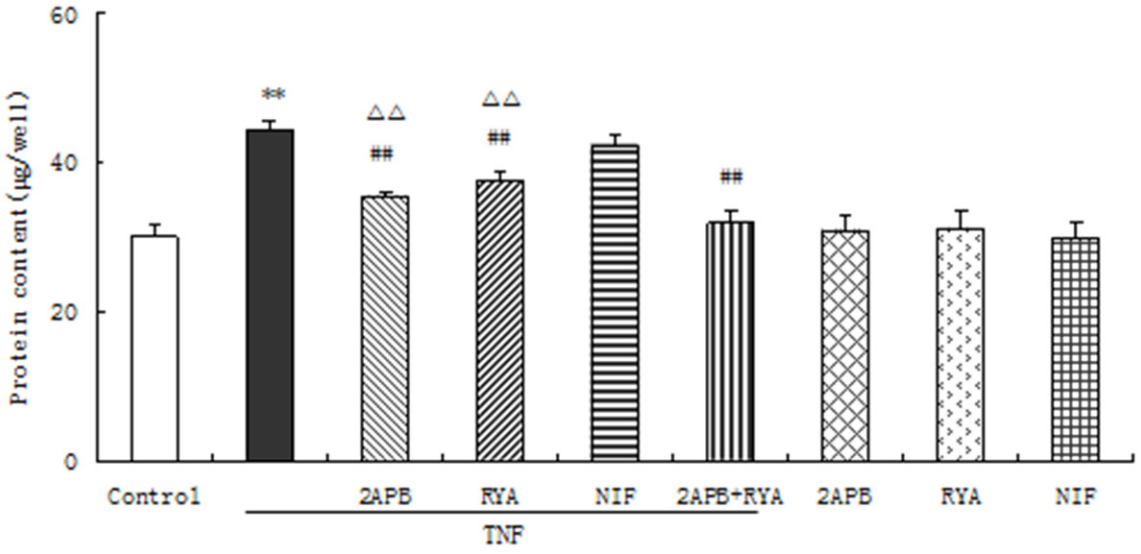

Figure 5. Effects of nifedipine, 2APB and ryanodine on the protein content of cultured ventricular myocytes from neonatal rats treated with TNF-α. ($\bar{x}$±s, n=8). Bars from left to right represented the different treatments in each group: blank control, TNF-α treated group, TNF-α plus 30 μM 2-APB, TNF-α plus 50 μM ryanodine, TNF-α plus 50 μM nifedipine, TNF-α plus 30 μM 2-APB and 50 μM ryanodine, group treated with 30 μM 2-APB, group treated with 50 μM ryanodine, and group treated with 50 μM nifedipine; $^{**}P$<0.01 VS control group; $^{\#\#}P$<0.01 VS TNF-α group; $^{\Delta\Delta}P$<0.01 VS IP3R inhibitor +RyR inhibitor group.

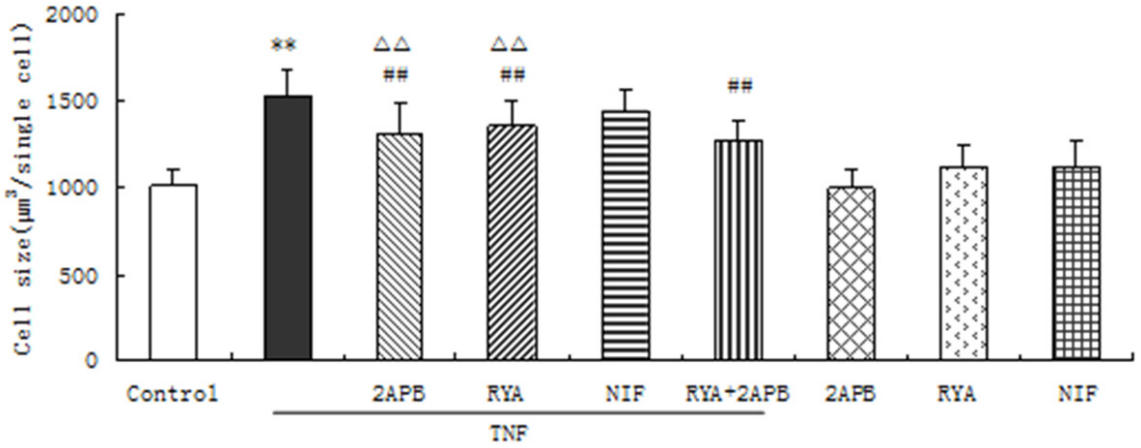

Figure 6. Effects of nifedipine, 2APB and ryanodine on [^{3}H] leucine incorporation of cultured ventricular myocytes treated with TNF-α. ($\bar{x}$±s, n=8). Bars from left to right represented the different treatments in each group: blank control, TNF-α treated group, TNF-α plus 30 μM 2-APB, TNF-α plus 50 μM ryanodine, TNF-α plus 50 μM nifedipine, TNF-α plus 30 μM 2-APB and 50 μM ryanodine, group treated with 30 μM 2-APB, group treated with 50 μM ryanodine, and group treated with 50 μM nifedipine; **P<0.01 VS control group; $^{\#\#}$P<0.01 VS TNF-αgroup; $^{\Delta\Delta}$P<0.01 VS IP3R inhibitor +RyR inhibitor group.

exhibited no significant effect on the resting level or the frequency of spontaneous transients of these cultured cardiomyocytes. However, when different calcium channel inhibitors were added to the culture system, interesting results were observed. Specifically, 2-APB (inhibitor of IP3R) and ryanodine (inhibitor of RyR) significantly suppressed the increase in calcium transient induced by TNF-α, and their combined effect was even stronger. On the other hand, nifedipine (inhibitor of L type Ca^{2+} channels) exhibited no such suppressive effect on the increase in calcium transient induced by TNF-α. Our results from the fluorescent assess-

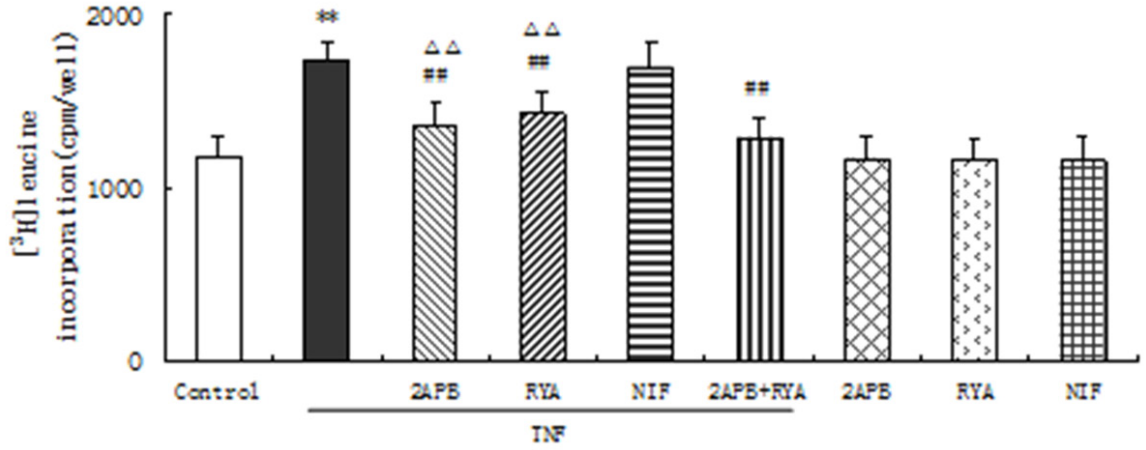

Figure 7. Effects of nifedipine, 2APB and ryanodine on the cell size of cultured ventricular myocytes from neonatal rats treated with TNF-α. ($\bar{x}$±s, n=80). Bars from left to right represented the different treatments in each group: blank control, TNF-α treated group, TNF-α plus 30 μM 2-APB, TNF-α plus 50 μM ryanodine, TNF-α plus 50 μM nifedipine, TNF-α plus 30 μM 2-APB and 50 μM ryanodine, group treated with 30 μM 2-APB, group treated with 50 μM ryanodine, and group treated with 50 μM nifedipine; $^{**}P$<0.01 VS control group; $^{\#\#}P$<0.01 VS TNF-α; $^{\Delta\Delta}P$<0.01 VS IP3R inhibitor +RyR inhibitor group.

ment of intracellular calcium in individual cells further strengthened our observations above that were obtained using the spectrofluorimetric method.

We also observed increases in the protein content, [^{3}H]-leucine uptake and in the average cell size of TNF-α-stimulated cardiomyocytes, confirming the previous results that TNF-α can induce myocardial hypertrophy [7]. However, when different calcium channel inhibitors were added, the increases in protein content, protein synthesis and cell size of cardiomyocytes, which were induced by TNF-α, were suppressed by 2-APB, ryanodine and 2-APB+ ryanodine but not by nifedipine (inhibitor of L type Ca^{2+} channels). The differential influences of these three calcium channel inhibitors on the TNF-α-induced changes in cell protein and cell size were consistent with their differential influences on the TNF-α-induced changes in calcium transients and concentrations. Together, these results indicate that the IP3R inhibitor and RyR inhibitor have significant suppressive effects on cardiac myocyte hypertrophy induced by TNF-α, whereas the inhibitor of L type Ca^{2+} channels does not. Thus, IP3R and RyR are calcium channels that participate in cardiac myocyte hypertrophy induced by TNF-α, whereas L type Ca^{2+} channels may not play a role in the relevant mechanisms. As IP3R and RyR are both involved in the release of Ca^{2+} from intracellular Ca^{2+} stores in the sarcoplasmic reticulum (SR) of cardiomyocytes [25], our results can be interpreted as follows. Instead of affecting the L type Ca^{2+} channel, TNF-α probably induces cardiac myocyte hypertrophy by activating the intracellular release of Ca^{2+} from the SR in cardiomyocytes.

Our study has laid the foundation for the possibility of highly selective management strategies for cardiac myocyte hypertrophy. However, further studies are needed to clarify the specific genes and pathways that participate in cardiac myocyte hypertrophy induced by TNF-α.

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Disclosure of conflict of interest

None.

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