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Effect of Hyperglycemia on the Changes of Intracellular $[Ca^{2+}]_i$ in Heart Myoblast

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Abstract

A rise in cytosolic free Ca^{2+} is the immediate trigger for contraction in heart muscle. In the present study, we investigated changes of intracellular Ca^{2+} increased by potassium chloride (KCl) and phenylephrine (PE) under hyperglycemia in rat heart myoblast H9c2 cells (BCRC 60096), respectively. We employed the fluorescent Ca^{2+}-indicator, fura-2, and digital imaging microscopy to measure $[Ca^{2+}]_i$ in H9c2 cells. Cells were cultured in hyperglycemic (30 mM glucose) Dulbecco's Modified Eagle's Medium. The variation of $[Ca^{2+}]_i$ induced by KCl and PE in hyperglycemia was examined, respectively. Moreover, tiron, one of the antioxidants, was pretreated in hyperglycemia-treated H9c2 cells to measure the role of free radicals in the changes of intracellular $[Ca^{2+}]_i$. An influx in intracellular Ca^{2+} induced by KCl or PE was observed in a dose-dependent manner and reached the highest concentration of 434 $\pm$ 42.3 nM and 443 $\pm$ 42.8 nM (n = 24 cells), respectively. Moreover, this increase of intracellular $[Ca^{2+}]_i$ induced by KCl or PE was markedly reduced in cells exposed to hyperglycemia (434 $\pm$ 42.3 *vs*. 1.26 $\pm$ 0.21 nM and 443 $\pm$ 42.8 *vs*. 2.54 $\pm$ 0.25 nM, n = 24 cells, $P < 0.001$, respectively). Similar changes were not observed in cells received mannitol showing same osmolarity. However, the reduction of intracellular $[Ca^{2+}]_i$ induced by hyperglycemia was abolished significantly in the presence of tiron. Our results suggest that an increase of intracellular Ca^{2+} by KCl or PE in heart cell was markedly reduced by hyperglycemic treatment; mediation of free radicals in this action can be considered because it was reversed in the presence of tiron.

Key Words: Ca^{2+}, H9c2 cells, potassium chloride, phenylephrine, hyperglycemia

Introduction

The physiological mobilization of free intracellular calcium or Ca^{2+} signaling is needed for all cells to function, and is involved in numerous biological processes such as muscle contraction, cell death, cell section, and neurotransmission (9). A rise in intracellular free Ca^{2+} concentration is an important second messenger in most types of cells (8). Therefore, it is necessary to gain a more complete understanding of the intracellular $[Ca^{2+}]_i$. The Ca^{2+}-sensitive fluorochrome, fura-2, is now widely employed to monitor intracellular Ca^{2+} in a variety of tissues and preparations (6). Moreover, digital analysis of images of fura-2 fluorescence has also been used to study the free Ca^{2+} in isolated cells at rest and during activation (4, 12, 13).

Hyperglycemia is a major factor in the development of diabetic cardiomyopathy (1, 14). Hyperglycemia-induced oxidative stress has been

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implicated in the onset and progression of diabetic cardiomyopathy (1, 5). However, the change of intracellular $[Ca^{2+}]_i$ due to hyperglycemia in heart muscle is not well known. Potassium chloride (KCl) is known to activate the voltage-operated Ca^{2+} entry (3), while phenylephrine (PE) is introduced to increase intracellular Ca^{2+} uptake *via* voltage-dependent Ca^{2+} channels and to stimulate Ca^{2+} release from sarcoplasmic reticulum (11). In this experiment, we utilized KCl and PE to increase the intracellular $[Ca^{2+}]_i$ in cells through the measurement with a digital imaging microscopy and fura-2. Rat heart myoblast H9c2 cells (BCRC 60096) were cultured in hyperglycemic Dulbecco's Modified Eagle's Medium (DMEM) to know the changes of intracellular $[Ca^{2+}]_i$ induced by KCl and PE, respectively. Then, role of free radicals in the changes of intracellular Ca^{2+} was also characterized by antioxidant named tiron. The present study aims to investigate the change of intracellular $[Ca^{2+}]_i$ in heart cell under hyperglycemia, relating to free radicals or not. As a result, we originally demonstrated that the reduction of intracellular $[Ca^{2+}]_i$ is due to hyperglycemia.

Materials and Methods

Materials

KCl, PE, glucose, mannitol, tiron and fura-2/AM were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO, USA). Rat heart myoblast derived H9c2 cells (BCRC 60096) were obtained from the Culture Collection and Research Center of the Food Industry Institute, Hsin-Chiu City, Taiwan, ROC. Other reagents used were of analytical grade.

Cell Culture and Treatment

Rat heart myoblast derived H9c2 cells were grown in 100 mm dishes and incubated at 37°C in a humidified atmosphere of air supplemented with 5% CO_2. The cells were maintained in growth medium composed of DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. Medium was changed every 2 day (10). In order to compare with H9c2 cells were exposed to hyperglycemic condition in a final concentration of 30 mM glucose alone, H9c2 cells were incubated with tiron in a final concentration of 100 μM in serum-free growth medium before exposure to glucose. After 1 h incubation, cells were treated with glucose in a final concentration of 30 mM for 24 h. In addition, cells were treated with 5.5 mM glucose and 30 mM mannitol as controls (10). Then, H9c2 cells were subjected to the measurement of intracellular $[Ca^{2+}]_i$ induced by KCl and PE, respectively.

Measurement of Intracellular $[Ca^{2+}]_i$

To determine the effects of KCl and PE on intracellular calcium rise, H9c2 cells were seeded onto 24 mm glass coverslips. 13 μl of 1 mM fura-2/AM (the membrane permeant acetoxymethyl ester derivative) in dimethylsulfoxide (DMSO) was added to serum-free DMEM (final fura-2/AM concentration = 2 μM). Cells grown on 24 mm coverslips were incubated in 1 ml fura-2/AM containing medium for 30 min at 37°C, under an atmosphere of 5% CO_2. Prior to the study of Ca^{2+} influx, coverslips were then transferred to a chamber mounted on microscope stage where they were superfused with physiologic saline solution (NaCl 140, KCl 5.9, NaH_2PO_4 1.2, $NaHCO_3$ 5, $MgCl_2$ 1.4, $CaCl_2$ 1.8, D-glucose 11.5, HEPES 10 mM at pH = 7.4) or a Ca^{2+} free solution from which Ca^{2+} had been omitted and containing 0.5 mM EGTA. In the studies for changes of intracellular Ca^{2+}, KCl (0.04 to 200 mM) or PE (1×10^{-8} to 5×10^{-5} M) was added into medium to incubate cells, respectively. The spectral characteristics of fura-2/AM are the same as those of unbound fura-2, but uncleaved fura-2/AM is insensitive to Ca^{2+}. The fluorescence of the cells from each coverslip was measured and recorded using an inverted Olympus microscope IX-70. $[Ca^{2+}]_i$ in H9c2 cells were monitored by alternating excitation wavelengths between 340 and 380 nm and an emission wavelength of 510 nm with a Delta Scan system (Photon Technology International, Princeton, NJ, USA). Fluorescent images of the cells were sent to the computer and saved. Imagemaster software (Photon Technology International, Princeton, NJ, USA) was used to analyze the images and extract the emission signal from individual cells. Data were then copied to the Felix software program (Photon Technology International, Princeton, NJ, USA), where the ratio was determined and Ca^{2+} concentration calculated (7).

Statistical Analysis

Data were expressed as means ± standard error of the mean (SEM) for the repetitions of cell culture experiments as indicated. Comparisons within and among groups were made using one-way analysis of variance (ANOVA) with repeated measures, followed by a post hoc comparison. Differences were considered to be statistically significant at P value of 0.05 or less.

Results

Effects of Hyperglycemia on Intracellular Ca^{2+} in KCl -Treated H9c2 Cells

Cultured monolayer cells on 24 mm glass cover-

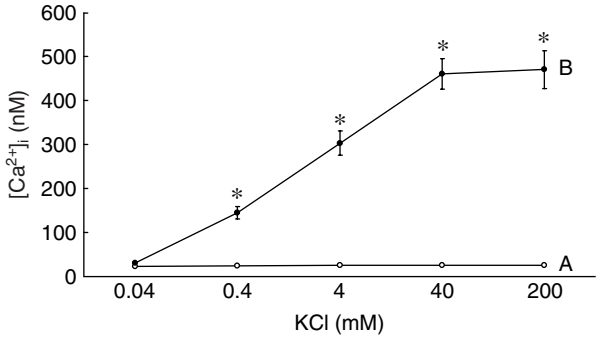

Fig. 1. Effects of KCl on $[Ca^{2+}]_i$ increase in H9c2 cells are shown. Cells were cultured with control medium (5.5 mM glucose) for 24 h. Cells were incubated in a Ca^{2+}-free (○) or containing Ca^{2+} (●) medium before KCl added. Effect of KCl on Ca^{2+} influx from extracellular medium is calculated by B nM (●) minus A nM (○) at each KCl sample utilized, also as shown in Fig. 2A (434 ± 42.3 nM, when induced by 40 mM KCl). All values are presented as means ± SEM (n = 24 cells). *P < 0.001 as compared with Ca^{2+}-free medium.

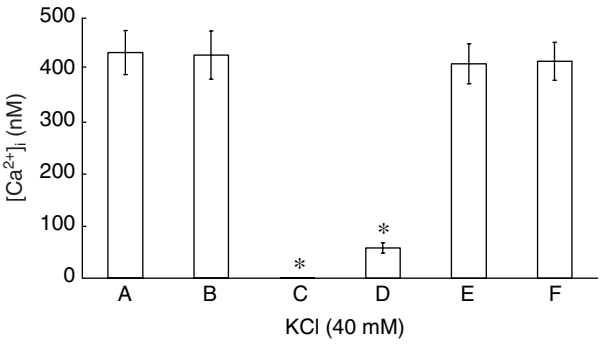

Fig. 2. Effects of KCl (40 mM) on Ca^{2+} influx in H9c2 cells are shown. Cells were cultured with control medium (A), 30 mM mannitol (B), 30 mM glucose (C), 15 mM glucose (D), 30 mM glucose by pretreatment with 100 μM tiron (E), and 15 mM glucose by pretreatment with 100 μM tiron (F). Effects of hyperglycemic treatment (C and D) and pretreatment with tiron (E and F) on intracellular $[Ca^{2+}]_i$ were investigated. Cells were cultured for 24 h (A to D). Cells were pretreated with tiron for 1 h, followed by treated with 30 or 15 mM glucose for 24 h (E and F). Cells were incubated in a Ca^{2+}-free or containing Ca^{2+} medium for the analysis of intracellular $[Ca^{2+}]_i$ induced by KCl (A to F). All values are presented as means ± SEM (n = 24 cells). *P < 0.001 as compared with medium control.

slips were incubated in Ca^{2+}-free medium, the intracellular $[Ca^{2+}]_i$ induced by KCl (0.04 to 200 mM) as shown in Fig. 1A. There was no change in intracellular $[Ca^{2+}]_i$. While in normal Ca^{2+} medium, KCl had an effect on intracellular $[Ca^{2+}]_i$ increase in a concentration-dependent manner and exhibited a maximal increase of intracellular $[Ca^{2+}]_i$ at 40 mM (Fig. 1B). An increase of Ca^{2+} influx by KCl can be considered. While KCl (40 mM) added into Ca^{2+}-free and Ca^{2+} medium, the intracellular $[Ca^{2+}]_i$ was 25.7 ± 3.71 nM in cell incubated in the absence of extracellular Ca^{2+} (Fig. 1A) and became 460 ± 34.6 nM in normal medium, also inclusive of Ca^{2+} influx (Fig. 1B). Thus, the effect of KCl (40 mM) on Ca^{2+} influx was about 434 ± 42.3 nM in H9c2 cells (B minus A).

In addition, H9c2 cells were treated with mannitol (30 mM), glucose (30 and 15 mM), or pretreated with a superoxide scavenger, tiron (100 μM), prior to the treatment with glucose. Intracellular $[Ca^{2+}]_i$ changed by KCl (40 mM) was then measured and indicated in Fig. 2. To exclude the hyperosmolar effect of hyperglycemia, 30 mM mannitol was added into medium to produce same osmolarity as described previously (2). However, Ca^{2+} content was not changed in cells treated with equivalent osmolar medium supplemented with mannitol compared to control condition (Fig. 2A *vs*. B: 434 ± 42.3 *vs*. 429 ± 46.8 nM). Then, we employed H9c2 cells to investigate the effect of hyperglycemia on Ca^{2+} influx induced by KCl. Cells were exposed to hyperglycemic condition (30 mM glucose) for 24 h to compared with that treated with 5.5 mM glucose as normal control (10). As shown in Fig. 2C, a marked reduction in intracellular $[Ca^{2+}]_i$ was observed in H9c2 cells exposed to glucose at 30 mM compared with 5.5 mM glucose control (1.26 ± 0.21 *vs*. 434 ± 42.3 nM, n = 24 cells, P < 0.001). As shown in Fig. 2D, a significant reduction in intracellular $[Ca^{2+}]_i$ was also observed in cells exposed to glucose at 15 mM (57.9 ± 9.97 *vs*. 434 ± 42.3 nM, n = 24 cells, P < 0.001) while a more marked reduction of intracellular $[Ca^{2+}]_i$ was observed between 30 and 15 mM glucose. Otherwise, the change of Ca^{2+} influx by hyperglycemia in KCl-treated cells was altered in the presence of tiron at the concentration (100 μM) sufficient to work as antioxidant according to previous report (10). The reduction of intracellular $[Ca^{2+}]_i$ due to hyperglycemic treatment was reversed by the pretreatment with tiron (Fig. 2, E and F).

Effects of Hyperglycemia on Changes of Intracellular Ca^{2+} by Phenylephrine in H9c2 Cells

Cells were incubated in Ca^{2+}-free medium, the intracellular $[Ca^{2+}]_i$ evoked by PE (1×10^{-8} to 5×10^{-5} M) as shown in Fig. 3A. PE had an effect on intracellular $[Ca^{2+}]_i$ augment in a dose-dependent manner and showed a maximal augment of intracellular $[Ca^{2+}]_i$ at 1×10^{-5} M (Fig. 3B). An augment of Ca^{2+} influx by PE can be thought. When PE (1×10^{-5} M) added into Ca^{2+}-free and Ca^{2+} medium, the intracellular $[Ca^{2+}]_i$ was 70.5 ± 6.74 nM in cell incubated in the lack of extracellular Ca^{2+} (Fig. 3A) and became 513 ± 48.1 nM in normal medium, also including

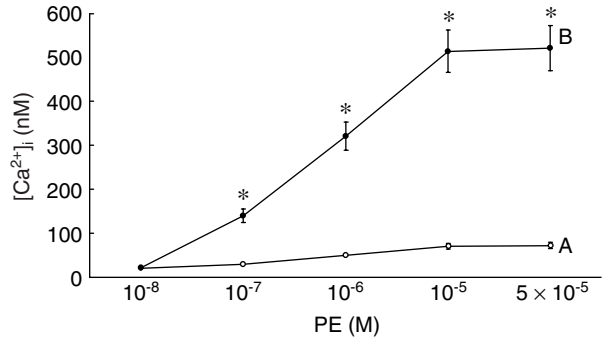

Fig. 3. Effects of PE on $[Ca^{2+}]_i$ increase in H9c2 cells are shown. Cells were cultured with control medium (5.5 mM glucose) for 24 h. Cells were incubated in a Ca^{2+}-free (○) or containing Ca^{2+} (●) medium before PE added. Effect of PE on Ca^{2+} influx from extracellular medium is calculated by B nM (●) minus A nM (○) at each PE sample utilized, also as shown in Fig. 4A (443 ± 42.8 nM, when induced by 1×10^{-5} M PE). All values are presented as means ± SEM (n = 24 cells). *P < 0.001 as compared with Ca^{2+}-free medium.

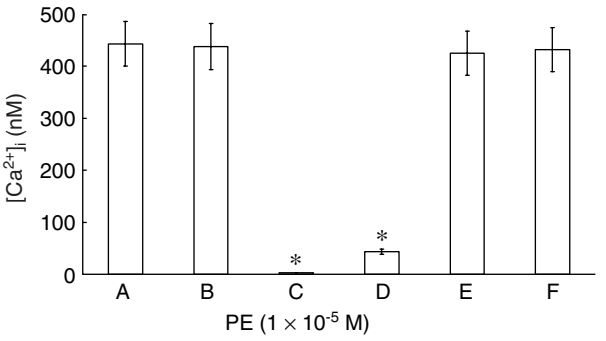

Fig. 4. Effects of PE (1×10^{-5} M) on Ca^{2+} influx in H9c2 cells are shown. Cells were cultured with control medium (A), 30 mM mannitol (B), 30 mM glucose (C), 15 mM glucose (D), 30 mM glucose by pretreatment with 100 μM tiron (E), and 15 mM glucose by pretreatment with 100 μM tiron (F). Effects of hyperglycemic treatment (C and D) and pretreatment with tiron (E and F) on intracellular $[Ca^{2+}]_i$ were investigated. Cells were cultured for 24 h (A to D). Cells were pretreated with tiron for 1 h, followed by treated with 30 or 15 mM glucose for 24 h (E and F). Cells were incubated in a Ca^{2+}-free or containing Ca^{2+} medium for the analysis of intracellular $[Ca^{2+}]_i$ induced by PE (A to F). All values are presented as means ± SEM (n = 24 cells). *P < 0.001 as compared with medium control.

Ca^{2+} influx (Fig. 3B). Therefore, the effect of PE (1×10^{-5} M) on Ca^{2+} influx was nearly 443 ± 42.8 nM in H9c2 cells (B minus A).

Besides, H9c2 cells were treated with mannitol (30 mM), glucose (30 and 15 mM), or pretreatment with a superoxide scavenger, tiron (100 μM) prior to the treatment with glucose. Intracellular $[Ca^{2+}]_i$ varied by PE (1×10^{-5} M) was then calculated and exhibited in Fig. 4. Moreover, Ca^{2+} content was not altered in cells treated with equivalent osmolar medium provided with mannitol compared to control condition (Fig. 4A *vs*. B: 443 ± 42.8 *vs*. 438 ± 44.6 nM). Then, H9c2 cells were utilized to study the effect of hyperglycemia on Ca^{2+} influx evoked by PE. Cells were exposed to hyperglycemic status (30 mM glucose) for 24 h to contrast with that treated with 5.5 mM glucose as normal control (10). As shown in Fig. 4C, a obvious decrease in intracellular $[Ca^{2+}]_i$ was observed in H9c2 cells exposed to glucose at 30 mM contrasted with 5.5 mM glucose control (2.54 ± 0.25 *vs*. 443 ± 42.8 nM, n = 24 cells, P < 0.001). As shown in Fig. 4D, a noteworthy decrease in intracellular $[Ca^{2+}]_i$ was also observed in cells exposed to glucose at 15 mM (43.1 ± 5.08 *vs*. 443 ± 42.8 nM, n = 24 cells, P < 0.001) while a more significant decrease of intracellular $[Ca^{2+}]_i$ was observed between 30 and 15 mM glucose. Furthermore, the variation of Ca^{2+} influx by hyperglycemia in PE-treated cells was changed in the existence of tiron at the concentration (100 μM) qualified to work as antioxidant accordance with previous report (10). The decrease of intracellular $[Ca^{2+}]_i$ due to hyperglycemic treatment was returned by the pretreatment with tiron (Fig. 4, E and F).

Discussion

To exclude the hyperosmolar effect of hyperglycemia, mannitol was added into medium to produce same osmolarity as described previously (2). However, Ca^{2+} content was not changed in cells treated with equivalent osmolar medium supplemented with mannitol compared to control condition. Thus, mediation of osmolarity can thus be ruled out. In this experiment, we utilized two stimulants (KCl and PE) to compare the changes of intracellular $[Ca^{2+}]_i$ due to KCl and PE by different modes of intracellular $[Ca^{2+}]_i$ increase reported previously (3, 11) as described in introduction section. H9c2 cells were employed to investigate the effect of hyperglycemia on Ca^{2+} influx induced by KCl and PE, respectively. Cells were exposed to hyperglycemic condition to compare with that treated with glucose as normal control (10). A marked reduction in intracellular $[Ca^{2+}]_i$ was observed in H9c2 cells exposed to hyperglycemic condition compared with glucose control. Then, the change of Ca^{2+} influx by hyperglycemia in KCl and PE-treated cells, respectively, was altered in the presence of tiron sufficient to work as antioxidant according to previous report (10). The reduction of intracellular $[Ca^{2+}]_i$ due to hyperglycemic treatment was reversed by the pretreatment with tiron. Therefore, mediation of free radicals in the decrease of intracellular $[Ca^{2+}]_i$ during hyperglycemia can be con-

sidered. This view is consistent with the previous report (15) in heart.

In conclusion, the reduction of intracellular $[Ca^{2+}]_i$ is associated with hyperglycemia in H9c2 cells that has not been mentioned. This change is related to the free radicals because the reduction was reversed in the presence of tiron which is known as antioxidant.

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