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Original Research

The effect of Diazoxide on norepinephrine-induced cardiac hypertrophy, in vitro

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Abstract: Cardiac hypertrophy is associated with mitochondrial dysfunctions, which leads to heart failure if sustained. The aim of present study is to test hypothesis whether activation of mitochondrial K_{ATP} channel (mitoK_{ATP}) by diazoxide improve mitochondrial membrane potential (MMP) and oxidative stress in an *in vitro* model of cardiac hypertrophy. Rat cardiomyocytes cell line (H9c2) was used to create four groups as control, diazoxide, hypertrophy, hypertrophy and diazoxide. Norepinephrine was used to induce hypertrophy. Diazoxide and norepinephrine were simultaneously administered. After 24 hours treatment, total oxidant status (TOS), total antioxidant status (TAS) and superoxide dismutase (SOD) activities were measured. MMP and F-actin distribution were analyzed. Hypertrophy significantly elevated TOS level. In addition, diazoxide administration significantly increased TOS level in the normal cell line. There were no significant differences in SOD activity, TAS and oxidative stress index (OSI) between groups. Hypertrophy caused a decrease in MMP and destrupted F-actin. Diazoxide improved MMP and F-actin in mitochondria. Hypertrophy impaired the function and structure of mitochondria. The opening of mitoK_{ATP} by diazoxide failed to improve oxidative stress; however, it is effective against mitochondrial damage caused by hypertrophy.

Key words: Hypertrophy**;** Mitochondrial K_{ATP} channel; Diazoxide; Membrane potential; Oxidative stress.

Introduction

Cardiac hypertrophy occurs as a compensatory response of the heart to stresses, including increased pressure overload, hypertension, coronary disease and myocardial infarction to maintain adequate hemodynamic functions. It is characterized with abnormal fibrosis and enlarged cardiomyocytes. In addition, consistent hypertrophy is a major factor leading to heart failure, which remains to be one of the main reasons of high mortality and morbidity (1, 2). Cardiac hypertrophy is also associated with several cellular changes such as impairment of mitochondrial function, oxidative stress and imbalanced Ca^{2+} levels in cytosol.

Cardiomyocytes contain high amount of mitochondrial to maintain the energy need of the heart for contraction and relaxation activities. Approximately 8% of ATP is consumed by heart to pump the blood and 90 % of cellular energy is used by cardiomyocytes to sustain the contraction–relaxation cycle as well. Therefore, healthy mitochondria is indispensable for a normal cardiac physiology. In cardiac hypertrophy, mitochondrial abnormality is not only responsible for inadequate ATP generation but also it leads to cell death or injury because abnormality in the mitochondria leads to the generation of more reactive oxygen species (ROS). In addition to these, disruption of actin-myosin filament, Ca^{2+} homeostasis, metabolic dysfunction, inflammation, apoptosis and fibrosis all caused by abnormal mitochondria, which is one of the leading causes for cardiovascular diseases such as cardiac hypertrophy and heart failure (3, 4).

Mitochondrial ATP dependent potassium channel (mitoK_{ATP}) is proposed to be important in the protection and pro-survival against hypertrophy induced mitochondrial dysfunction (5, 6). MitoK_{ATP} plays an important role in the regulation of oxidative stress. Oxidative stress underlines the occurrence of cardiac hypertrophy and heart failure later. Excessive production and accumulation of ROS (such as hydrogen peroxide and superoxide) in mitochondria induces apoptosis, DNA damage and mutation (7). ROS production in the mitochondria may be related with attenuated or collapsed mitochondrial membrane potential (MMP) (3). Therefore, targeting mitoK_{ATP} by an agonist or an antagonist might be effective way to manage cardiac hypertrophy. For example, opening of the channel is protective against ischemia/reperfusion-induced cell death by decreasing the Ca^{2+} uptake and the generation of ROS via inhibiting mitochondrial permeability transition (mPTP) (8). In addition, mitoK_{ATP} activation improved cardiac hypertrophy and attenuated heart failure by preserving endothelial function (9). On the other hand, the relationship between the opening of mitoK_{ATP} and the protection against cardiac hypertrophy is still not exactly clarified. It has been proposed that opening of the channel diminishes excess Ca^{2+} uptake and decrease oxidative stress; by this way, the protective effects are observed; however, a previous study indicate that moderate production of ROS in mitochondria exerts cytoprotection by inducing the activation of pro-survival signaling pathways such as ERK or PI3K (10).

F-actin is an important partner of cell cytoskeleton system, helping to maintain cell shape and migration of cell. In addition to actin's role in cell shape, the association with apoptotic pathway and actin has been studied

in recent years. Caspase could degrade actin, so its fragmentation around 15 kDa is reported to initiate apoptosis (11). The interaction of actin assembling and mitoK_{ATP} channel opening under hypertrophic condition is still unclear. The aim of the present study is to investigate the effects of the opening of mitoK_{ATP} channel by diazoxide in cardiomyocytes treated with norepinephrine in the terms of F-actin structure and MMP *in vitro.*

Materials and Methods

Experimental Design

Rat cardiomyocytes cell line (H9c2, a kind gift from Prof. Dr. Belma TURAN, TURKEY) was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum to a final concentration of 10%, 100 U/ml penicillin and 100 µg/ml streptomycin under 95% oxygen; 5% carbon dioxide (CO_2) at 37°C. When reached 80% confluence, the cells were subcultured.

Cell treatments

The H9c2 cells were divided into four groups; (I) control (II) hypertrophy, (III) diazoxide, (IV) hypertrophy and diazoxide. Hypertrophy was induced by using norepinephrine (NE) at 2.5 µM dose for 24 hours. 100-µM dose of diazoxide, an ATP sensitive potassium channels (K_{ATP}) opener, was used at for 24 hours. Co-treatment of norepinephrine and diazoxide was simultaneously started to treatment.

Total oxidant status, Total antioxidant status and Oxidative stress index

Total oxidant status (TOS) and Total antioxidant status (TAS) were measured with a BioTek µQuant microplate spectrophotometer using Rel Assay kits (Cat No: RL0017 and Cat No: RL0017; Gaziantep, Turkey, respectively) according to the manufacturer's instructions.

Briefly, 9 µL samples or standart and 150 µL reagent1 were added each well of plate, before read at 660 nm by a using microplate reader to measure TOS. After 22.5 µL reagent2 was incubated for 5 min at 37 ºC by shaking, the second read was done at 660 nm. The results were expressed as a µmol H_2O_2 Equiv./L for TOS.

Briefly, 22.5 µL samples or standart and 150 µL reagent1 were added each well of plate, before read at 530 nm by a using microplate reader to measure TAS. After 7.5 µL reagent2 was incubated for 5 min at 37 ºC by shaking, the second read was done at 530 nm.mmol Trolox Equiv./L for TAS.

Oxidative stress index (OSI) is the ratio between TOS and TAS levels measured (eight samples per group).

SOD activity assay

SOD activity was determined by a commercial kit (Cat No: 19160, Sigma-Aldrich, St. Louis, MO, USA). According to the manufacturer's instructions, 20 µL samples and/or blank 2 and 200 µL WST working solution were added each well of plate, before 20 µL dilution buffer to each blank 2 and blank 3 were added. Then, the plate was incubated for 20 min at 37 ºC by shaking. Lastly, the SOD activity was measured at 450 nm and 37 °C with a microplate spectrophotometer (BioTek µQuant) and was expressed as inhibition rate (%).

Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was measured using a commercial kit (Sigma-Aldrich kit, Cat. No. CS0390) according the manufacturer's instructions. Briefly, the cells were incubated with JC-1 dye, specific for the mitochondrial membrane, with a 5% CO_2-95% O_2 air mixture at 37 °C for 20 min. After incubation, the cells were rinsed with staining buffer. The coverslips were directly observed under a fluorescence microscope (Olympus BX51 Microscope equipped with a DP72 camera, controlled by Olympus DP2-TWAIN software).

F-actin filaments

Cells were washed with PBS and fixed in 5% paraformaldehyde at room temperature for 60 min. Fixed cells were permeabilized in 0.1% (vol/vol) Triton X-100 in PBS at room temperature for 10 min and blocked in 5% BSA in PBS at room temperature for 60 min. Actin filaments were visualized using Alexa fluor 595 phalloidin, and nuclei with 4'',6-diamidino-2-phenylindole dichloride (DAPI) (Invitro Molecular Probes, OR, USA). All images were obtained on an Olympus BX51 microscope equipped with a DP72 camera controlled by Olympus DP2-TWAIN software.

Statistical analysis

Statistical analyses were performed by SPSS version 23.0. All results are expressed as mean ±SEM. One-way analysis of variance (One-Way ANOVA) with post doc Tukey for equal variance assumed and post hoc Dunnett'T3 for equal variance not assumed were used to compare mean between the groups. $p < 0.05$ was considered to be statistically significant.

Results

The effect of mitochondrial K_{ATP} opening on oxidative stress

In order to determine the role of mitoK_{ATP} opening by diazoxide on oxidative stress in NE-induced hypertrophy in cardiomyocyte H9c2 cells, we measured TAS and TOS levels, OSI index and SOD activity. TAS level and OSI index did not show a significant difference between groups (Fig. 1a and Fig. 1c). Hypertrophy significantly increased the TOS level compared to C group. Interestingly, diazoxide significantly increased TOS level in compared to C group (Fig. 1b). Diazoxide decreased OSI levels in NE-induced hypertrophy; however, the differences between H and HD groups were not statistically significant. In addition, there were no significant differences in SOD activity (inhibition rate %) between groups (Fig. 1d).

The effect of mitochondrial K_{ATP} opening on mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was measured by using cationic dye,named JC-1, special for mitochondria. Red fluorescent represented normal healthy mitochondria, although green fluorescent is unhealthy, depolarized mitochondria. The MMP at control was very high, the red fluorescent is returning yellow **(**Fig. 2Aa). MMP at diazoxide was a little lower compared to control but was not different from control (Fig. 2Bb). MMP from hypertrophy had green fluorescent, which

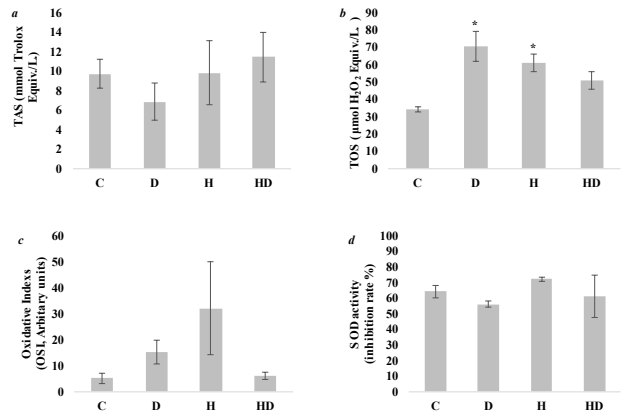

Figure 1. The effect of K_{ATP} opening on oxidative stress in hypertrophic cardiomyocytes-mediated with norepinephrine. TAS, total antioxidant status **(*a*)**; TOS, total oxidant status **(*b*)**; OSI, oxidative stress index **(*c*)**; SOD activity; superoxide dismutase activity **(*d*);** C, control; D, control+diazoxide; H, hypertrophy; HD, hypertrophy+diazoxide.* is significantly different from C. All data are expressed as mean ±SEM.

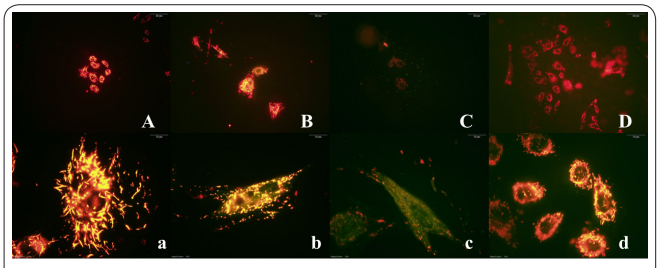

Figure 2. The effect of K_{ATP} opening on mitochondrial membrane potential at hypertrophic cardiomyocytes-mediated with norepinephrine. Aa; control, Bb; control+diazoxide, Cc; hypertrophy, Dd; hypertrophy+diazoxide. All big capital is 20x, small is 100x.

means that hypertrophy caused a decrease in MMP, resulting in depolarization (Fig. 2Cc). However, MMP from diazoxide+hypertrophy had almost normal, healthy mitochondria (Fig 2Dd).

The effect of mitochondrial K_{ATP} opening on F-actin distribution

It is well known that hypertrophy led to a distruption in filamentous actin (F-actin). That is why F-actin distribution was also evaluated. As accepted, the control had normal F-actin (Fig. 3A). Diazoxide had no effect on F-actin distribution (Fig. 3B). Therefore, diazoxide had also normal F-actin structure. However, hypertrophy gave rise to a distruption in F-actin, leading to many filopodia formations as well as had a thick peripheral ring which means that a loss of some F-actin structure at cytosol (Fig. 3C). Co-treatment of diazoxide and hypertrophy caused by norepinephrine exposed almost healed all filopedia formation (Fig. 3D).

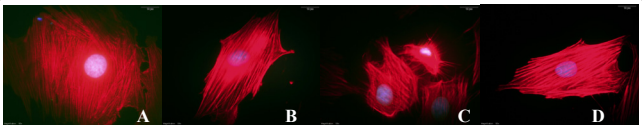

Figure 3. The effect of K_{ATP} opening on F-actin distribution. A; control, B; control+diazoxide, C; hypertrophy, D; hypertrophy+diazoxide.

Discussion

The main findings of the present study are (i) hypertrophy elevated TOS level in cardiomyocytes, (ii) diazoxide increased TOS levels in normal cells without effecting NE-induced hypertrophic cardiomyocytes, (iii) hypertrophy attenuated MMP and distrupted F-actin, (iv) diazoxide failed to modulate SOD activity, TAS and OSI while it improved MMP and F-actin in NE-induced hypertrophic cardiomyocytes.

mitoK_{ATP} regulates energy metabolism and bioelectrical activity of the organelle, including MMP, ROS production (12). It was showed for the first time in 2004 that opening of mitoK_{ATP} channel by diazoxide was effective against phenylephrine-induced hypertrophy in cultured neonatal cardiomyocytes via attenuating the upregulation of Na–H exchanger isoform I (13). Then, the protective effect of the drugs on some pathologies has been started to report. One of these is the opening of the channel is protective against ischemia/reperfusion-induced cell death by decreasing Ca^{2+} uptake and generation of ROS via inhibiting mitochondrial permeability transition (mPTP) (8). Opening of mitoK_{ATP} by diazoxide has been reported to have a neuroprotective effect against oxidative stress and cellular dysfunction as well (12). Moreover, the other K_{ATP} channel opener, iptakalim, has been demonstrated to attenuate cardiac hypertrophy and heart failure by preserving endothelial function (9). Actually, the relationship between the opening of mitoK_{ATP} and protection against cardiac hypertrophy is still not exactly clarified. (10). In the present study, NE was preferred to imitate hypertrophic condition based on the knowledge that the subunit of K_{ATP}, Kir6.2, has been reported to participate to adapt of β-adrenergic stimulation (14). So, sarcolemmmal K_{ATP} is probably involved in the respond of the drug. In addition, diazoxide is reported to enhance Mg-ATPase activity of the ion channel (14).

Moreover, diazoxide pretreatment has been shown to protect heart tissue against metabolic insults in myocardial ischemic preconditioning. Those protections were lost when actin structure disrupted. Therefore, both mitoK_{ATP} channels and actin have essential role in this kind of pathological situation (15). Somewhat F-actin participates to innate of apoptosis (16). F-actin has a pivotal role in cell survival due to association with Bim and Bmf that are member of BH_3 protein family playing a role in suppression of anti-apoptotic and activation of pro-apoptotic Bcl_2 as well as mechanical transformation. These two proteins could trigger apoptotic pathways. Although Bim connects with tubulin, Bmf relates to actin network under normal physiologic condition. Those proteins tend to translocate into mitochondria when interaction could be disrupted under pathophysiologic condition, such as hypertrophy. Therefore, dislocation could activate intrinsic apoptotic pathways. F-actin destruction causes to a translocation into mitochondria one pro-apoptotic factor that normally associates with intact F-actin. Moreover, this translocation has been reported to be elevated when MMP is dissipated. Apoptosis is initiated by the activation of caspase-3 relying on dissipation of MMP at smooth muscle cells. Therefore, the loss of actin polymerization and depolymerizing balance could trigger apoptotic cell lost (17). Thus,

hypertrophy induced by NE resulted in filopodia formation at cardiomyocytes in the present study. In parallel, hypertrophic effect of NE was not related to the elevation of oxidative stress based on TAS, TOS, OSI and SOD levels in the current study. However, the dissipation of MMP in NE treated cell is well obvious in the present study. Therefore, destruction of actin structure relies on loss of contraction function of cardiomyocytes under cardiac hypertrophic condition imitated with NE. How actin filopodia is formed might be related to Rho family proteins, e.g. RhoA having role in actin assembling. Therefore, RhoA activation causes the formation of actin filament branch (17). That is why we speculate that NE-treated causes to disrupt F-actin filament, resulting in decreasing MMP.

In a previous study, myocardial ischemia/reperfusion is reported to destroy F-actin structure. In addition, a previous study reported that myocardial K_{ATP} has a close interaction with actin. Moreover, Ap4A has crucial role in these interactions. Therefore, when actin filament is disturbed, Ap4A lost from these interactions, resulting in a decrease in K_{ATP} channel opening probability. It means that intact actin filament structure is required for Ap4A-mediated K_{ATP} channel. Therefore, actin filaments modulates the ability of Ap4A on blocking K_{ATP} channels. Moreover, K_{ATP} channel openers, such as diazoxide has protective effect against metabolic insult e.g. myocardial ischemia/reperfusion (18). Enhancing F-actin stabilization by using phalloidin show that ATP sensitivity of K_{ATP} decreased based on Mg-ADP. In line with, actin structure disruption, for example cytochalasin, caused to an elevation in the sensitivity, whereas actin stabilization by phalloidin caused to a decrease in the sensitivity (19). The actin and K_{ATP} channels interaction supported the knowledge that many ion channels and/or transporter are stabilized in the cell membrane by actin. Therefore, the alternation of F-actin gives rise to a change in the ion channels or transporters. It is suggested that F-actin disruption leads to the activation of K_{ATP} channels in heart. F-actin and K_{ATP} channel have functional interaction at heart tissue. Furthermore, pancreatic β-cell has same interaction between F-actin and KATP channels (20). The other explanation for protective effect of the drug might be related to the modulation of apoptotic cell death. In normal cardiac ventricular myocytes, the activation of mitoK_{ATP} by diazoxide might suppress apoptosis induced by oxidative stress via attenuating the release of cytochrome c (21), suppressing caspase 9 and caspase 3 activation (22), by this way; cardioprotection was observed (23).

Oxidative stress is defined as an imbalance between oxidant and antioxidant systems, which can lead to a damage on all components of the cell. In addition, oxidative stress plays a key role in the pathogenesis of many diseases (24-29). In normal cells, the results of present study indicates that diazoxide increased total oxidative stress without effecting membrane potential. These data are supported by a previous study demonstrated that the activation of mitoK_{ATP} channel resulted in the generation of ROS and mitochondrial swelling with no change in mitochondrial membrane potential in human atrial cardiocyte-derived cell line (30). The author proposed that changes in ion transporters and/or functional coupling might be a reason for free radical production. However, it is still unclear how the mitoK_{ATP} channel activation by diazoxide induces production of ROS species (31). Numerous studies indicate that ROS level decrease when K_{ATP} channel is opened (32-34). However, there are also studies showing that inactivation of K_{ATP} channel has opposite effects (33, 35). Nevertheless, it could be available opposite responds as well (36, 37). In addition, it should be mentioned that mitoK_{ATP} channel opening by diazoxide failed to modulate oxidative stress in the present study. Herein, the question is that why diazoxide increased the total oxidative stress in normal cell although it failed to modulate oxidative stress in norepinephrine-induced cardiac hypertrophy *in vitro*. One explanation may be related with increased intracellular iron levels. A recent study show that activation of K_{ATP} by diazoxide hyperpolarizes the membrane that leads to increased intracellular iron levels and following elevated ROS production in SK-N-SH cells *in vitro* (38). Another explanation is how diazoxide forms ROS also may be related to connexin 43 (37). It is also discussed that enhancing ROS might involve in the protective effect of diazoxide under some pathologic circumstances. For example, a previous study suggested that increased oxidative stress underlined the cardio protective mechanism exerted by diazoxide (39); on the other hand, the other study did not (40). Nevertheless, it is obvious that more studies are needed to clarify the question. Based on the current study, the ROS production might not participate in the protection.

In conclusion, myocardial hypertrophy induced by norepinephrine destroys F-actin assembling, elevate ROS formation and accompanies with depolarized MMP. However, diazoxide provides protective effect against to hypertrophy mediated by NE, correlated with restoration of F-actin deformation confirmed with the decrease of filopodia, resulting in modulation of MMP. These results suggest that hypertrophy induced mitochondrial damage might be controlled by the opening mitoK_{ATP} channel. On the other hand, it should be mentioned that further studies are necessary to clarify the possible signaling pathways underlying the protective effects of the channel activation in cardiac hypertrophy in both *in vivo* and *in vitro.*

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Interest conflict
The author state that there is no conflict of interest.

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