EXPERIMENTAL STUDY

The effect of melatonin on digoxin-induced cardiac damage in cardiomyocytes

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ABSTRACT

OBJECTIVES: Digoxin is a cardiac glycoside which is widely used in cardiovascular medicine. Oxidative stress, as well as intracellular Ca2+ overload, plays an important role in digoxin toxicity. Transient receptor potential vanilloid 1 (TRPV1) channels are found in cardiomyocyte cells and they are activated by reactive oxygen species. We investigated the effects of digoxin toxicity and alterations in Ca2+ influx, oxidative stress and apoptosis through TRPV1 channels and modulator role of melatonin in cardiomyocytes.

METHODS: The cells were divided into seven main groups as control, digoxin, digoxin+capsazepine, digoxin+melatonin, digoxin+melatonin+capsazepine, melatonin and melatonin+capsazepine groups. Cells in the groups were stimulated with capsaicin and inhibited with capsazepine in related experiments for activation and inactivation of TRPV1 channels, respectively. We measured cytosolic calcium, intracellular reactive oxygen, mitochondrial depolarization, caspase 9 and caspase 3 levels.

RESULTS: The apoptosis values were significantly lower in the melatonin and digoxin+melatonin groups than in the digoxin group of cardiomyocytes (p < 0.001). The cell viability values were higher in the digoxin+capsazepine (p < 0.001), digoxin+melatonin (p < 0.001) and digoxin+melatonin+capsazepine (p < 0.001) groups than in the digoxin group.

CONCLUSION: TRPV1 channels are overactivated during digoxin toxicity and melatonin could show a cardioprotective effect through TRPV1 channel modulation (Fig. 5, Ref. 56). Text in PDF www.elis.sk.

KEY WORDS: digoxin, oxidative stress, cardiomyocyte.

Introduction

Digoxin is a cardiac glycoside which is widely used in heart failure and atrial fibrillation patients as it increases inotropy and decreases chronotropy. It has multiple direct and indirect cardiovascular effects (1, 2). The inhibition of sarcolemma-bound Na+/K+ ATPase by digoxin leads to an increase in sodium (Na+) ion concentration. This cellular Na+ overload causes an increase in free calcium (Ca2+) concentration mediated by the Na+/Ca2+ exchanger. When cytoplasmic Ca2+ increases above the storage capacity of the sarcoplasmic reticulum, digoxin toxicity occurs (3, 4). Toxic concentrations of digoxin are associated with oxidation of membrane phospholipids and increase in lytic enzymes due to increase in intracellular Ca2+ (5–7).

Melatonin is an amphiphilic molecule which protects mitochondria against oxidative stress. It has been demonstrated that melatonin is a potent antioxidant, anti-apoptotic and anti-inflamma-
(Thermo-Fischer). Cardiomyocyte cells were evenly distributed as $1 \times 10^5$ cells in each of 8–10 flasks (filter cap+sterile+ 5 ml; 25 cm²). A humidified incubator was used to incubate cardiomyocytes at 37 °C at 5 % CO₂. After cells had reached 75–85 % confluence, they were incubated with the chemical compounds described in groups section. Cells were examined daily for the evidence of contamination. After treatments, the cells were detached with 0.25 % Trypsin–EDTA for analysis and split into the sterile falcon tubes for analyses.

Cell viability (MTT) dye was purchased from Thermo Fischer (Massachusetts, USA). Dihydrorhodamine-123 (DHR123) obtained from Molecular Probes (OR, USA). Caspase 3 and Caspase 9 substrates were purchased from Biovision (San Francisco, USA). Dihydrorhodamine-123 (DHR123) was purchased from Calbiochem (Darmstadt, Germany).

**Groups**

The study was planned with 7 main groups as follows,

Group 1  (Control): None of the study drugs were used and cardiomyocytes were kept in a flask containing the same cell culture condition.

Group 2  (DGX): Cardiomyocytes were incubated with 0.03 μM digoxin for 30 min (12).

Group 3  (DGX+CAPZ): Cardiomyocytes were incubated with 0.03 μM digoxin for 30 min and then incubated with capsazepin (CAPZ, 0.1 mM, 30 min).

Group 4  (DGX+Mel): Cardiomyocytes were incubated with 0.03 μM digoxin for 30 min and then incubated with 300 μM melatonin for 2 h.

Group 5  (DGX+Mel+CAPZ): Cardiomyocytes were incubated with 0.03 μM Digoxin for 30 min and then incubated with 300 μM melatonin for 2 h and then incubated with capsazepin (CAPZ, 0.1 mM, 30 min).

Group 6  (Mel): Cardiomyocytes were incubated with 300 μM melatonin for periods of 2 h (13).

Group 7  (Mel+CAPZ): Cardiomyocytes were incubated with 300 μM melatonin for periods of 2 h and then incubated with capsazepin (CAPZ, 0.1 mM, 30 min).

In CAPZ incubated groups, cardiomyocytes were also blocked by TRPV1 blocker CAPZ (0.1 mM, 30 min) prior to the related analysis in the existence of 1.2 mM calcium in extracellular environment. For all experiments (except for calcium signaling), the cells were further treated with capsaicin (CPSN, 0.1 mM, 10 min) for activation of TRPV1 channel prior to the related analysis. During calcium signaling analysis (Fura-2/AM), the cells were stimulated on 20th cycles with 0.1 mM CPSN in the existence of 1.2 mM calcium in extracellular environment.

**Measurements of intracellular calcium concentration**

UV light excitable Fura 2 AM (acetoxymethyl ester) dye was used for measuring intracellular calcium level in cardiomyocytes. The relevant experimental procedures were carried out in accordance with the experimental procedure of Uğuz et al, which included 4 μM Fura 2 AM fluorescent dye for final staining period (14). Fluorescence emission intensity at 510 nm was determined in individual wells using a plate reader equipped with an automated injection system (Synergy™ H1, Biotek, USA) at alternating excitation wavelengths of 340 and 380 nm every 3 s for 50 acquisition cycles. During the measurement of intracellular calcium signaling, TRPV1 channels were stimulated by automatic injector with capsaicin (0.1 mM) on 20th cycle. Measurement of Ca²⁺ analysis was performed as modified by Uğuz et al, and Martinez et al, in previous studies (14, 15).

**Intracellular ROS production measurement**

DHR123 is a non-charged and non-fluorescent dye which easily goes through the cell membrane. The relevant experimental procedures were carried out in accordance with the experimental procedure of Espino et al (16). Inside the cardiomyocyte cell, DHR123 is oxidized to cationic rhodamine 123 (Rh 123) which localizes in the mitochondria and exhibits green fluorescence. Synergy™ H1 automatic microplate reader device was used for determining Rh 123 fluorescent intensities. The analyses were performed at 488 nm (excitation) and 543 nm (emission) wavelengths. We presented the data as fold changes as from their levels before treatment.

**Apoptosis assay**

The APOPercentage™ cell apoptosis assay was used for the detection and quantification of apoptosis. The APOPercentage dye is actively bound to phosphatidyl-serine lipids and transferred into the cells, which stains apoptotic cells red. The apoptosis analyzes procedure was performed according to manufacturer’s instruction and Özdemir et al (11). The cardiomyocytes were analyzed for apoptotic cells detection by spectrophotometry (multiplate reader) at 550 nm (Synergy™ H1, Biotek, USA).

**Caspase 9 and caspase 3 activity assays**

Caspase 9 and Caspase 3 activity evaluation methods were based on previously reported studies (17, 18). Caspase 9 (AC-LEHD-AMC) and Caspase 3 (ACDEVD-AMC) substrates cleavages were calculated with Synergy™ H1 microplate reader (Biotek, USA) with 360 nm and 460 nm wavelengths (excitation/emission). The values were evaluated as fluorescent units/mg protein and shown as fold changes as from their levels before treatment (experimental/control).

**Mitochondrial membrane potential (JC1) analyses**

JC1 (1 μM), which is a mitochondrial membrane potential fluorescence dye, was evaluated by 485 nm (green) excitation wavelength and emission wavelength of 535 nm, red signal at 540 nm (excitation) and 590 nm (emission) wavelengths (Synergy™ H1, Biotek, USA). Data are presented as emission ratios (590/535). Mitochondrial membrane potential changes were quantified as the integral of the decrease in JC1 fluorescence ratio of experimental/control.
Cell viability (MTT) assay

Cell viability was evaluated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. After treatments with chemical compounds as described in group section, the cardiomyocytes were washed and then incubated with fresh DMEM containing MTT (0.5 mg/ml) at 37 °C for 90 min (19). Then, the supernatants were removed, and Dimethyl-sulfoxide was added to dissolve the formazan crystals. Optical density was estimated by Synergy™ H1 automatic microplate reader device (Biotek, USA) at a test wavelength of 490 nm and reference wavelength of 650 nm to nullify the effect of cell debris. The obtained data are shown as fold changes as from their levels before treatment (experimental/control).

Statistical analysis

The values are presented as means±standard deviations (SD). To compare the different treatments, statistical significance was calculated by one-way analysis of variance (ANOVA) and Mann-Whitney U test. All data were analyzed by SPSS statistical program (version 9.05 software, SPSS Inc. Chicago, Illinois, USA) and p < 0.05 was considered significant.

Results

Effects of digoxin toxicity and melatonin on cytosolic calcium levels in cardiomyocytes

The effect of digoxin toxicity and melatonin administrations on cytosolic calcium levels in cardiomyocyte cells are shown in Figure 1a, b. The TRP Vanilloid 1 channel blocker capsazepine was used to evaluate intracellular Ca²⁺ increase through TRPV1 channels in digoxin toxicity model of cardiomyocytes. As shown in Figure 1b, the Ca²⁺ concentration in cardiomyocytes was greater in the digoxin group when compared with the control (p < 0.001). The Ca²⁺ level was lower in melatonin and melatonin+capsazepine groups than in the control (p < 0.001). Also, cytosolic Ca²⁺ concentration was lower in the digoxin+capsazepine, digoxin+melatonin and digoxin+melatonin+capsazepine groups than in the digoxin group (p < 0.001).

In addition, cytosolic Ca²⁺ concentration in the cardiomyocytes was markedly lower in the digoxin+melatonin+capsazepine group, compared to the digoxin+melatonin group (p < 0.001).
Effects of digoxin toxicity and melatonin on apoptosis levels in cardiomyocytes

Effects of digoxin toxicity and melatonin administrations on apoptosis levels are shown in Figure 2. The apoptosis values were higher in the digoxin group than in the control. The apoptosis values were lower in melatonin and digoxin+melatonin group than in the digoxin group of cardiomyocytes (p < 0.001). Also, the values were lower in the digoxin+melatonin+capsazepine group.
Digital glycosides are used for patients with heart failure and arrhythmia worldwide, although excessive doses could cause cardiac adverse effects (22). Intracellular Ca\(^{2+}\) overload and oxidative stress play an important role in digoxin toxicity. It has been shown that digitalis-induced Na\(^+\) accumulation causes an increase in Ca\(^{2+}\) through the Na\(^+\)/Ca\(^{2+}\) exchanger. As a result of this internal Ca\(^{2+}\) overload, the net increase in intracellular Ca\(^{2+}\) activates further calcium release from the sarcoplasmic reticulum. The toxic effects have been seen when the sarcoplasmic reticulum storage capacity is exceeded. Intracellular Ca\(^{2+}\) overload is related to persistent inhibition of the Na\(^+\)/K\(^+\) ATPase and is associated with a rise in cell automaticity, impaired homeostasis and cell death. Adverse toxic effects are characterized by arrhythmia as well as apoptosis. Digitalis-induced arrhythmogenic effects are evaluated in previous studies but digitalis-induced apoptosis is not yet well understood (23, 24). In a study by Qubaassine et al, it has been shown that digoxin induced cell death in adult rat cardiomyocytes (25). Also, it has been described that toxic concentrations of digitalis cause a sustained elevation of internal Ca\(^{2+}\) levels that provoke apoptotic cell death (7).

Cardiac glycosides bind to Na\(^+\)/K\(^+\) ATPase and it opens the mitochondrial ATP-sensitive K\(^+\) channel which leads to an increase in ROS. The reactive oxygen species are associated with inhibition of mitochondrial permeability transition which decreases myocyte viability. Cytochrome c release from mitochondria is a critical apoptotic event and this important event was induced by digoxin. Consistent with cytochrome c release induced by digoxin, is the activation of caspase 9 and caspase 3. It is followed by proteolytic process with suggested oxidative stress-induced apoptosis (26–28).

Thus, we hypothesized that TRPV1 channels could be involved in glycoside-induced apoptosis. We think that the mechanism of digoxin to induce apoptotic cell death either in cultured cells or in live organism could follow a common pathway, in which Ca\(^{2+}\) plays a principal role. This intra cellular Ca\(^{2+}\) overload has been linked to mitochondrial dysfunction. We also think that digitalis-induced cell death is mainly apoptotic although a mixed mechanism of cell death cannot be ruled out.

In the current study, we showed that TRPV1 channels in cardiomyocytes are activated by digoxin administration. TRP channels are a family of unique ion channels which affect important cellular functions and signaling pathways. Also, they are primary targets for several potential drugs. They are known to play critical roles in cells’ survival as well as their development. TRP channels can be divided into seven subfamilies as TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin), and TRPN (NOMPC) family (29, 30). They are activated by intracellular and extracellular messengers, chemical and mechanical stimuli and osmotic stress. Their main difference from voltage-dependent channels is that they are weakly sensitive to membrane potential. Some channels are activated by intracellular Ca\(^{2+}\) load whereas others are constitutively open (31, 32). Calcium has an important role as a second messenger in cardiac function including cardiac energy homeostasis, as well as
cell death. Transient receptor potential (TRP) proteins are responsible for Na\(^+\) and Ca\(^{2+}\) conducting channels which cause changes in the Ca\(^{2+}\) homeostasis and mediate longer lasting modulation of Ca\(^{2+}\) levels (33, 34). TRPV1, a member of vanilloid TRP family, is expressed in many organs including heart, kidney, brain, dorsal root ganglions and sensory neurons. It is a ligand-gated, homotetrameric, non-selective cation channel which is activated by heat (over 42 °C), capsaicin (pungent substance from chili peppers) and low extracellular PH (35–37). Capsazepine is a competitive antagonist of capsaicin and it specifically blocks the TRPV1 channel (38). It has been demonstrated that TRPV1 channels are found in the ventricles of heart in the cardiovascular system and have an important role in modulating cardiovascular diseases including atherosclerosis, congestive heart failure and systemic hypertension (39–42). Authors have reported that activation of TRPV1 channels could aggravate heart failure (43, 44). Also, it has been shown that the toxicity of digoxin was amplified in congestive heart failure as a consequence of Na\(^+\)/Ca\(^{2+}\) exchanger upregulation (45). Furthermore, previous studies have demonstrated that activation of TRPV1 channels have a dual role in the progression of heart failure (42).

Although oxidative stress has an important role in digoxin toxicity, its role in TRPV1 channel activation has not been investigated yet. Dai et al, showed that extracellular ROS activated TRPV1 channels in apoptosis which is induced by hypoxia in hippocampal neurons (46). Also, authors of previous studies evaluated the role of TRPV1 channel activation in oxidative stress, apoptotic cell injury, and increased cytosolic free Ca\(^{2+}\). Chuang et al demonstrated that TRPV1 channel activation by capsaicin was increased in oxidative stress (47). Similarly, Hu et al, showed that increased intracellular Ca\(^{2+}\) entry and activation of NADPH oxidase was associated with TRPV1-induced oxidative stress. On the other hand, oxidative stress causes activation of ion channels by phosphorylation and this process is involved in Ca\(^{2+}\) influx through TRPV1 channels (48). We observed that digoxin toxicity increased oxidative stress, intracellular Ca\(^{2+}\) entry and apoptosis in cardiomyocytes. In the current study we showed that intracellular ROS production and Ca\(^{2+}\) levels were increased through TRPV1 channel activation by digoxin toxicity and capsaicin stimulation in cardiomyocytes.

Also, we demonstrated that melatonin reduced ROS, apoptosis, and Ca\(^{2+}\) entry through modulation of TRPV1. Melatonin is a pineal secretory product, strong antioxidant and an anti-apoptotic molecule which can pass through all cellular compartments easily (49, 50). It participates in the antioxidant and anti-apoptotic processes directly or indirectly. Melatonin has protective effects in many organs including heart, brain and liver (51–53). Furthermore, protective effects of melatonin during oxidative stress and apoptosis have been shown before in previous studies (8). Moreover, it has been demonstrated that it decreased the effectiveness of voltage-operated Ca\(^{2+}\) channels and induced the Ca\(^{2+}\)-dependent ATPase in cardiac sarcolemma (54). In addition, the protective effect of melatonin in reperfusion arrhythmias has been reported previously and melatonin was suggested to be used as an adjunct therapy in myocardial infarction (55). In a study by Kahya et al., the effect of melatonin on oxidative stress parameters, apoptosis levels and intracellular calcium entry via TRPV1 channels in diabetic rats has been evaluated. They found that the modulation of this channel by melatonin involves neuroprotective activities in diabetic rats (56). In the present study, we observed that melatonin could influence intracellular homeostasis during digoxin toxicity. We found that melatonin administration has beneficial effects on apoptosis levels and intracellular ROS production during digoxin toxicity in cardiomyocytes.

Study limitations

Mainly, we did not evaluate the concentration response to distinguish effects of therapeutic versus toxic levels of digoxin on the molecular mechanism studied in the present study. Also, we could not perform an electrophysiological study and evaluate whether digoxin toxicity changes also the expression of TRPV1 channels in cardiomyocytes.

Conclusion

In conclusion, we showed that TRPV1 channels are over-activated by ROS and mediate the lethal cytosolic Ca\(^{2+}\) increase in digoxin toxicity. Melatonin could show a cardioprotective effect through TRPV1 channel modulation in cardiomyocytes.

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