Chloroquine Decreased Kir6.2 Immunoreactivity in Chronic Hypoxic Heart

Klorokin Kronik Hipoksik Kalpte Kir6.2 İmmünoreaktivitesini Azaltır

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ABSTRACT

Objective: Experimental and clinical studies indicate that cardiovascular system diseases have the highest mortality rate. One of the major factors underlying this high mortality rate is hypoxia. The inward rectifying potassium channel 6.2 (Kir6.2) plays a key role in the adaptation and metabolic regulation of cardiac tissue to hypoxia. In this study, the effect of chloroquine on Kir6.2 expression in heart tissue exposed to moderate chronic hypoxia was investigated.

Methods: In this study, 32 8-12-week-old male Wistar albino rats weighing 200-300 g were used in 4 groups. Accordingly, the first group exposed to a normoxic (at 21% O2 concentration) environment, the second group was administered daily chloroquine (50 mg/kg intraperitoneal) injection in a normoxic environment, the third group exposed to a moderately hypoxic environment (10% O2 concentration), and the fourth group in the hypoxic environment had chloroquine (50 mg/kg intraperitoneal) injection. At the end of the 28-day period, the heart tissues were dissected with anesthesia. Immunohistochemical analysis was performed for Kir6.2 channels.

Results: In hypoxic heart tissue, a significant decrease in Kir6.2 immunoreactivity was observed due to chloroquine administration compared to the control group (p<0.05). In addition, in the hypoxia

ÖZ.


Yöntemler: Bu çalışmada 32 adet 8-12 haftalık, 200-300 g ağırlığında yetişkin Wistar albino cins erkek sıçanlar, 4 grupta incelenmek üzere kullanıldı. Bu gruptulara ilk grup normoksidik (%21 O2 konsantrasyonunda) ortama, ikinci grup normoksidik ortamda günlük klorokin (50 mg/kg intraperitoneal) ejeksiyonuna, üçüncü grup ortada moderate hypoksidik (10% O2 konsantrasyonunda) ortama, dördüncü grup ise hipoksik ortamda klorokin (50 mg/kg intraperitoneal) ejeksiyonuna maruz bırakıldı. Yırtmaç ve şafak sonunda anestezi altında kalp dokuları sarktifye edildikten sonra Kir6.2 için immünohistokimyasal analiz yapıldı.

Bulgular: Hipoksik kalp dokusunda, klorokin uygulamasına bağlı olarak kontrol grubuna kıyasla Kir6.2 immünoreaktivitesinde anlamlı azalma gözlemlendi (p<0.05). Ayrıca, yine klorokin
group, which was also treated with chloroquine, hemorrhagic areas were significantly reduced compared to the control group (p<0.001).

**Conclusion:** Data may demonstrate the potential protective and adaptive effects of chloroquine in hypoxic heart. Further molecular and functional studies are required to confirm the Kir6.2 related mechanism.

**Keywords:** Kir6.2, KATP channel, ischemia, heart

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**Introduction**

Oxygen is essential for normal aerobic metabolism in mammals. The key reaction occurs in the mitochondria of the cells. With this reaction, the energy need is provided by adenosine triphosphate (ATP) (1). Hypoxia is the presence of oxygen at low levels and at low partial pressure in the cell. Depending on cell types, metabolic demands, their ability to adapt to hypoxia, and response to various levels of hypoxia range from adaptation to cell death (2).

Hypoxic states of the cardiovascular system are caused by an imbalance between the amount of oxygen delivered to the cardiac cell and the amount required. The degree of hypoxic damage depends not only on the intensity and duration of the hypoxic stimulus, but also on the tolerance of the cardiac system to oxygen deprivation (3). The adaptation to metabolic stress and hypoxia in cardiac tissue can be provided by Kir6.1 and Kir6.2 channels, which are ATP-dependent and sub-members of inward rectifying potassium channels (Kir) (4). However, the adaptation mechanisms have still not been fully known (5).

The ATP sensitive K+ (K\(_{\text{ATP}}\)) channels include the Kir channel family and Sulfonylurea subunits. K\(_{\text{ATP}}\) channels are complex structures formed by subunits (Kir6.1 and Kir6.2 encoded by KCNJ8 and KCNJ11, respectively) and Sulfonylurea receptors that form 4 pores. In addition, K\(_{\text{ATP}}\) channels function in the physiological process depending on the cellular ATP/ADP balance (6). K\(_{\text{ATP}}\) channels play a protective role in cardiac myocytes during ischemia or hypoxia (7).

In cardiac muscle cells, cell protection mechanisms are associated with the activation of sarcolemma and mitochondrial K\(_{\text{ATP}}\) channels in conditions such as ischemia/hypoxia (8-11). In addition, K\(_{\text{ATP}}\) channels maintain the balance between oxygen delivery and demand during heart failure (12). The number of K\(_{\text{ATP}}\) channels is critical at determinant of myocardial membrane excitability. Optimization of cardiac energy expenditure may cause a stress in cardiac metabolism (13). K\(_{\text{ATP}}\) channels regulate the blood flow, especially in cardiomyocytes (14). Investigating the possible effects of various agents on mechanisms and especially the widespread use of chloroquine in recent years can be considered as an important goal.

Chloroquine has been used as a drug in the prophylaxis and treatment of malaria for years. It has also been used in the treatment of autoimmune inflammatory diseases such as rheumatoid arthritis (15). In addition, there are evidences that chloroquine may regulate vascular tone (16,17). The effects of chloroquine on cardiac tissue and its role in hypoxia adaptation are of critical importance.

In summary, with this study, we assessed the Kir6.2 expression in chloroquine-applied hypoxic heart tissue for the first time and we nominated for the relevant mechanism (Figure 1).

**Method**

Thirty two male adult Wistar albino rats, which were 8-12-week-old and weighing 200-300 g, were raised in Kayseri Erciyes University Experimental and Clinical Research Center (DEKAM). The rats kept in cages were provided with water and nutrients in the normal order of the day at 21 °C and 12 hours in light/dark environment. Experimental groups were formed by weighing the subjects and bringing those with similar weights together. At the end of the experiment, the animals were sacrificed under combining ketamine and xylazine anesthesia and their heart tissues were removed. The experimental protocol of this study was approved by the Animal Ethics Committee of Kayseri Erciyes University (ethics committee decision number: 2018/027).

Eight rats from each group to be used for the experimental groups were placed in normobaric rooms (BioSpherix, NY, USA) with their own cages. Groups to be placed in a normobaric room were exposed to 21% O\(_2\) for sham (normoxic) and 10% O\(_2\) for moderate hypoxia for 28 days and kept in a normobaric room where normoxic and continuous hypoxic conditions would be imitated.

**Normoxic Control (N_CON):** No drug was administered to the rats (n=8) to be kept in normal room air for 28 days.

**Normox Chloroquine (N_CLQ):** Daily 50 mg/kg Chloroquine was administered intraperitoneally to rats (n=8) which were kept in normal room air for 28 days.

**Moderate Chronic Hypoxia Control (mcHX_CON):** No medication was applied to rats (n=8) that were exposed to 10% O\(_2\) for 28 days in a normobaric room.

**Moderate Chronic Hypoxia + Chloroquine (mcHX_CON + CLQ):** Daily 50 mg/kg Chloroquine was administered intraperitoneally to rats (n=8) which were exposed to 10% O\(_2\) for 28 days in a normobaric room.

**Tissue Preparation**

Tissue samples taken from sacrificed rats were followed up for light microscopic and immunohistochemical examinations at
Yozgat Bozok University, Faculty of Medicine, Department of Histology and Embryology. Tissue samples taken were fixed for 1 day in 10% buffered neutral formaldehyde prepared in phosphate buffer (PBS). Detected tissue samples were dehydrated by passing through graded alcohols according to the routine light microscope tissue tracking method. After being transparent in xylol, they were embedded in paraffin. A fixed vacuum tissue tracking device was used to ensure optimization during tissue tracking. Thick sections of 3-4 µm were taken from the paraffin blocks with a rotary microtome on grinded slides covered with chrome alum gelatin. Heart sections of 5-6 µm from paraffin blocks were left in the oven for a certain period of time using histological methods, then paraffin was removed with xylene and passed through graduated alcohol series and diluted. To see the general histological structure, hematoxylin and eosin (HE) staining was performed. For hemorrhage, it was done counting under a microscope in 25 different regions. The length of 30 cells was measured with the Image J program.

**Hematoxylin-Eosin Staining**

Paraffin sections with a thickness of 3-4 µm taken on grinded slides were deparaffinised in the oven for 1 night and then staining was performed in the following order.

- Incubated 3 times 15 minutes in xylol.
- Rehydrated for 10 minutes in 96%, 96% and 80% alcohols, respectively.
- Washed in H$_2$O for 10 minutes.
- Incubated in filtered Hematoxylin for 15 minutes.
- Washed in H$_2$O for 10 minutes.
- Dipped in acid alcohol and removed.
- Washed in H$_2$O for 10 minutes.
- Immersed in ammonia for 2 times.
- Incubated in eosin for 60 seconds.
- Passed through 80%, 96%, 96% alcohols with glaze.

After drying and incubating in xylol for 45 minutes, the slides were covered with Canadian balsam. Kir6.2 expression was detected immunohistochemically using by streptavidin-biotin-peroxidase technique. The sections were deparaffinized in xylene, rehydrated through graded alcohols and washed in deionized water. Antigen retrieval was performed by microwave treatment in 0.01 M sodium citrate buffer, pH 6.0, at 270 °C for 5 min. The slides were cooled and held at room temperature for 10 min. Sections were washed with PBS. Endogenous peroxide activity was inhibited by immersion in 3% (w/v) H$_2$O$_2$ for 12 min. Lab Vision™ UltraVision™ Large Volume Detection System (TP-125-HL; Thermo Fisher Scientific, Waltham, MA) was used. All sections were washed with distilled water, then Ultra V block was applied for 10 min at room temperature to block background staining. Sections then were incubated overnight at 4 °C with a Kir6.2 (alomone labs: APC-020), and after washing with PBS, sections were incubated with biotinylated goat anti-Polyvalent secondary antibodies (TP-125-BN) (TP-125-HL; Thermo Fisher Scientific). The immunoreaction was amplified using the streptavidin-avidin-peroxidase complex and visualized using 3,3’ p-diaminobenzidine tetrahydrochloride (TA-060-HDX; Thermo Fisher Scientific). After counterstained with Gill’s
hematoxylin, sections were washed 3 times with deionized water. Then, the sections were dehydrated through rising alcohols, cleared in xylene, and mounted with Entellan. Images were taken using a light microscope (Olympus BX51; Olympus). In total, 25 different fields were evaluated for each department. At least 3 randomly chosen fields in each slide were counted at the original 20 magnification.

**Statistical Analysis**

The Kolmogorov-Smirnov test was used to identify the normal distribution of the data. One-way analysis of variance and post-hoc Tukey test were used to determine differences between groups. Results were presented as mean ± standard deviation. The SPSS/PC program (Version 20.0; SPSS, Chicago, IL) and Graph pad Prism 8.0 software were used for statistical analysis. A p value <0.05 was considered as statistically significant.

**Results**

The image of the heart section HE is shown in Figure 2. In the hypoxia (HX) group, increased hemorrhagic areas were detected in the heart tissue compared to the control group (p<0.001). It was observed that hemorrhage was significantly decreased in the HX+CLQ group compared to the HX group (p<0.001). At the same time, cardiomyocyte length was significantly decreased in the HX group compared to the control group (p<0.001).

Heart tissue Kir6.2 immunohistochemistry results and staining images are shown in Figure 3. Kir6.2 immunoreactivity showed a significant increase in HX group compared to the control group (p<0.05). Kir6.2 immunoreactivity in the HX+CLQ group showed a significant decrease compared to the HX group (p<0.005).

Cardiomyocyte length was significantly decreased in the HX group compared to the control group (p<0.001). However, there was no significant difference between the HX+CLQ group and the HX group. Cardiomyocyte length was significantly decreased in the CLQ and HX+CLQ groups compared to the control group (p<0.001, figure 3).
Discussion

The findings showed that the chloroquine applied to the hypoxic heart significantly reduced the Kir6.2 immunoreactivity and hemorrhagic areas. Chloroquine was reported to have a vasodilatory effect on coronary arteries (16). This agent also acts as a vasodilator in the pulmonary arteries in hypertension caused by hypoxia (17). Accordingly, in our study, reduced hemorrhage due to chloroquine administration in the hypoxic heart may be described as an adaptation or protection mechanism in terms of cardiac tissue and metabolism.

Atrial natriuretic peptide (ANP), an important cardiac peptide for cardiovascular homeostasis, significantly modulates the activity of ventricular $K_{ATP}$ channels by an intracellular signaling mechanism consisting of NPR-A, PKG, ROS, ERK1/2, CaMKII and RyR2. This novel mechanism may regulate cardiac excitability and contribute cytoprotecting by partially opening myocardial $K_{ATP}$ channels (18). Similarly, examining the effect of chloroquine on Kir6.2 channels in terms of possible ion current change and signal mechanisms involved in channel functioning may be shown as a target for future studies. On the other hand, our findings may indicate that the chloroquine administration, especially in hypoxic condition leads to decreased immunoreactivity of Kir6.2 channel. Data may need to be clarified in terms of hypoxia markers. This may be accepted as a limitation of the study. In order to understand the effects of changed Kir6.2 immunoreactivity and hemorrhagic areas on hypoxia adaptation mechanisms in more detail, functional and molecular studies can be done in the future. Investigation of potentially changing Kir6.2 protein levels and ion current measurements in tissue is essential in elucidating the mechanism.

The protective effect of phencyclidine hydrochloride substance against myocardial ischemia/reperfusion injury was investigated. Accordingly, the protective effects of phencyclidine hydrochloride against ischemic preconditioning and ischemia/reperfusion injury may be related to mitochondrial $K_{ATP}$ channels and Akt/ GSK-3β and Akt/mTOR signaling pathways (19). In this perspective, evaluation of chloroquine to reduce Kir6.2 immunoreactivity and hemorrhagic areas in hypoxic heart tissue in terms of cellular signaling pathways appears as a novel target. In addition, the therapeutically potential effect of chloroquine administration by providing inhibition of CD154-induced IL-6 production in acute kidney injury with hypoxia was shown (20). By investigating the effect of chloroquine on proinflammatory cytokines, especially under hypoxic conditions, more new therapeutic approaches can be developed.

Conclusion

Chloroquine may cause proarrhythmic or antiarrhythmic effects, particularly by affecting Na+, Ca2+ and voltage-gated $K^+$ channels. Furthermore, ischemia/reperfusion damage by inducing autophagy pathways may be increased (21). The findings of our study show that chloroquine has a significant effect on the adaptation of the heart in hypoxic condition and ischemia/reperfusion damage by affecting Kir6.2 channels. The effect should continue to be investigated in future studies with signal pathways and functional measurements.

Our findings suggest that chloroquine may be a potential target in terms of adaptation mechanisms to hypoxia and the development of possible therapeutic targets.

Ethics

Ethics Committee Approval: The experimental protocol of this study was approved by the Animal Ethics Committee of Kayseri Erciyes University (ethics committee decision number: 2018/027).

Peer-review: Externally peer reviewed.

Authorship Contributions


Conflict of Interest: No conflict of interest was declared by the authors.

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