Decreases of voltage-dependent K^+ currents densities in ventricular myocytes of guinea pigs by chronic oxidant stress[1]

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KEY WORDS hydrogen peroxide; potassium; apoptosis; cardiac myocytes

ABSTRACT

AIM: To determine the changes of delayed rectifier K^+ currents (I_k) and inward rectifier K^+ currents (I_{k1}) in the ventricular myocytes of guinea pigs during the gradual apoptotic process by the chronic oxidant stress treatment. **METHODS:** H_2O_2 50 μmol/L (24 h) was used for inducing apoptosis in the cardiomyocytes culture of neonatal rats and to treat the isolated ventricular myocytes of adult guinea pigs *in vitro* for 24 h. Apoptosis was evaluated by TUNEL methods and voltage-dependent K^+ currents were recorded by patch-clamp techniques. **RESULTS:** H_2O_2 50 μmol/L (24 h) induced cell apoptosis in the cardiomyocytes culture of neonatal rats. This concentration was used to treat the isolated ventricular myocytes of adult guinea pigs *in vitro* for 24 h and the voltage-dependent K^+ currents densities (I_k, I_{k1}) were down-regulated. The densities of the delayed rectifier K^+ currents (I_k) in 50 μmol/L H_2O_2 group were 2.52±0.57 pA/pF *vs* 5.73±1.84 pA/pF in the control group at +50 mV (n=8, P<0.01). The densities of the inward rectifier K^+ currents (I_{k1}) in 50 μmol/L H_2O_2 group were -13.9± 2.70 pA/pF, 2.52±0.57 pA/pF *vs* -59.7±11.9 pA/pF, 5.73±1.84 pA/pF in the control group at -120 mV (n=8, P< 0.01) and -40 mV (n=8, P<0.05), respectively. The extent of inward rectifier property of I_{k1} was weakened by 50 μmol/L H_2O_2 treatment. **CONCLUSION:** The densities of I_k, I_{k1} in the cardiomyocytes of guinea pigs were down-regulated and the inward rectifier property of I_{k1} was weakened during the gradual apoptotic process after 50 μmol/L H_2O_2 treatment for 24 h.

INTRODUCTION

Intracellular K^+ concentration plays an important role in the regulation of apoptosis process[1]. Excessive K^+ efflux and intracellular K^+ depletion may be responsible for the cell shrinkage and apoptotic death[2,3]. Cellular K^+ homeostasis is maintained by K^+ efflux and K^+ uptake mechanism. Voltage-dependent K^+ currents are the major pathways for the K^+ efflux, which indicates that voltage-dependent K^+ currents are involved in the apoptosis. Several studies reported that the enhancement of K^+ currents was related to the apoptosis in rat liver cell line[4] or neurons[3,5], providing convincing evidence that decreased intracellular K^+ content attributable to K^+ efflux through K^+ channels results in activation of apoptotic signalling pathways. Loss of intracellular K^+ can lead to cell shrinkage because of intracellu-

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lar osmolarity and withdrawal of inhibitory factor for caspase activation. Since the voltage-dependent K^+ channels are abundantly expressed in the heart, suggesting these channels may be modulated during the ongoing apoptotic process. However, little is known about the alteration of these K^+ currents in the cardiomyocytes during the gradual process by the apoptotic stimuli.

Hydrogen peroxide (H_2O_2) is the typical apoptosis inducer which suppresses the growth of human leukaemia cell line[6], induces expression of phosphor-p38 and phosphor-p42/p44 Ca^{2+}-calmodulin dependent protein kinases in bovine aortic endothelial cells[7], and increases content of p53 and Bad protein[8]. The acute effects (reperfusion for 5 min[9] or 15-25 min[10]) of reactive oxygen species(ROS) on the voltage-dependent K^+ channels in the cardiac myocytes have been reported, but the chronic effects of H_2O_2 have not been mentioned. The apoptotic stimuli by H_2O_2 chronic treatment was more closely mimicking the pathological process *in vivo* environment, so the aim of the present study was to characterize the alterations of voltage-dependent K^+ currents in the ventricular myocytes of guinea pigs during the gradual apoptotic process by the chronic H_2O_2 treatment.

MATERIALS AND METHODS

Chemicals RPMI-1640 and fetal calf serum were Hyclone products. TUNEL kit was provided by Wuhan Boster Biological Technology Co; 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), taurine, Na_2ATP, K aspartate, collagenase (II-type), and trypan blue were Sigma products.

Animals Wistar rats and adult guinea pigs were provided by the Animal Center of Harbin Medical University (Certificate No 0921, Grade II).

Cell cultures Hearts were taken from 1-day-old Wistar rats , then were cut into pieces of about 1 mm^3 that were digested into cell suspension by 0.2 % collagenase. After centrifugation, cells were resuspended in RPMI-1640 containing 20 % fetal calf serum. Cardiomyocytes were accounted for about 90 % after repeated purification. The cells were diluted to 5×10^8 cells/L, plated in 6-well plates for 48 h in CO_2 incubator, and cultured for 24 h with saline or H_2O_2 (50 μmol/L) alone in serum-free medium before measurement.

Determination of cell death Cardiomyocytes death was assessed by cell counts after staining with 0.4 % trypan blue dye. Two hundred cells were counted for 8 times.

Determination of apoptosis Apoptosis of cardiomyocytes was investigated by TUNEL method for the percentage of apoptotic cells. After that, the cardiomyocytes were stained with hematoxyline for the microscopic observation.

Single ventricular myocyte isolation Hearts from guinea pigs were perfused retrogradely with Tyrode's solution containing 1 mmol/L $CaCl_2$ (pH 7.4) 37 ºC for 2 min, then perfused with a Ca^{2+}-free Tyrode's solution for 5 min, and finally, with a Ca^{2+}-free Tyrode's solution containing II-type collagenase for 10 min[11]. The free wall of the ventricle was cut and triturated for 2 min and then incubated in KB solution consisting of (mmol/L): KCl 25, KH_2PO_4 10, $MgCl_2$ 3.0, potassium glutamate 70, egtazic acid 0.5, glucose 10, HEPES 10, taurine 20, and KOH (pH adjusted to 7.4) at 4 ºC . Half of this storage KB solution was exposed to H_2O_2 (50 μmol/L) for 24 h and another half as control group.

Whole-cell patch-clamp recording Macroscopic K^+ currents in cardiomyocytes of guinea pigs were recorded at room temperature using previously reported methods[12]. Currents were elicited by 200 B amplifier and pCLAMP 8.2 software (Axon Instruments,USA) and digitized by using 1322 interface. Electrodes had tip resistances of 2.8-3.5 MΩ when filled with an internal solution containing in mmol/L: K aspartate 110; $MgCl_2$ 4; Na_2ATP 4.2; $CaCl_2$ 1; NaCl 8; HEPES 5; egtazic acid 10; pH 7.2 adjusted with KOH. A liquid-junction potential of approximately 10 mV (pipette negative) was not corrected. Myocytes were superfused at 2 mL per min with an HEPES-buffered Tyrode's solution containing in mmol/L: NaCl 140; KCl 4; $MgCl_2$ 1; $CaCl_2$ 1; glucose 10; HEPES 10; pH 7.4 adjusted with NaOH. The L-type Ca^{2+} current was blocked by $CdCl_2$ (0.3 mmol/L). Cell capacitance and series resistance were read from the front panel of amplifier. The series resistance was electrically compensated to minimize the duration of the capacitive transient and was checked regularly to ensure no variations with time in this study.

Statistical analysis Data were analyzed using Clampfit software and were presented as mean±SD. Changes were identified as significant if *P* value in *t*-test was less than 0.05.

RESULTS

H_2O_2-induced cardiomyocytes death of neonatal rats Under the reversed optical microscope, the

cells of control group were in the spindle or elliptic shape. After an incubation with 50 μmol/L H_2O_2 for 24 h, some cardiomyocytes changed into round shape with refractivity and lost the connection with surrounding cells. Survival ratio of cardiomyocytes in H_2O_2 incubation group (68.7 %±5.5 %) decreased markedly compared with the control group (92.6 %±6.6 %, n=8, P <0.05), indicating 50 μmol/L H_2O_2 inhibited the cell survival.

After hematoxyline staining, the color of normal cardiomyocytes was light blue (TUNEL-negative) and apoptotic cells (TUNEL-positive) was light brown under the microscope. At the present condition, no apoptotic cell was found in control group. Apoptosis ratio in H_2O_2 incubation group was 13.8 %±1.3 % *vs* 0.9 %±0.5 % in control group (n=8, P<0.01). H_2O_2 50 μmol/L treatment for 24 h markedly increased the number of TUNEL-positive cells.

Decreases of delayed rectifier K^+ currents densities by H_2O_2 treatment Representative examples of I_k in control group and 50 μmol/L H_2O_2 treatment group were illustrated in Fig 1A and B with the protocol shown in the inset. Since there existed contamination of I_{k1} when the test potential was less than 0 mV, the deporlarizing pulse was set from 0 mV to +50 mV. Compared with the control group, 50 μmol/L H_2O_2 treatment for 24 h significantly decreased the steady-state current densities of I_k (Fig 1C). For example, at +50 mV, densities of I_k in control group were 5.73±1.84 pA/pF *vs* 2.52±0.57 pA/pF in the H_2O_2 treatment group (P<0.01). Not only the densities of I_k were decreased, the pattern of I_k was also changed. The amplitude of tail current of I_k was markedly larger than that of steady-state current in the H_2O_2-treated group, which was different from the pattern of the control group.

Decreases of inward rectifier K^+ currents densities by H_2O_2 treatment Representative examples of I_{k1} in control group and 50 μmol/L H_2O_2 treatment group were illustrated in Fig 2A and B with the protocol shown in the inset. Compared with the control group, 50 μmol/L H_2O_2 treatment for 24 h significantly decreased the inward and outward current densities of I_{k1} (Fig 2C). For example, the densities of I_{k1} in 50 μmol/L H_2O_2 group were -13.9±2.70 pA/pF, 2.52±0.57 pA/pF *vs* -59.7±11.94 pA/pF, 5.73±1.84 pA/pF in the control group at -120 mV (n=8, P<0.01) and -40 mV(n=8, P<0.05) respectively. More importantly, the inward rectifier property of I_{k1} were weaker in the H_2O_2 chronic treatment group than that in the control group, meaning the activity of cardiomyocytes to keep the rest potential at the K^+ equilibrium potential was weakened by the H_2O_2 chronic treatment.

No change of I_k and I_{k1} by H_2O_2 acute treatment Since the chronic treatment of H_2O_2 50 μmol/L significantly decreased the current densities of I_k and I_{k1}, we wondered the effects were secondary to the direct action or not, so the acute effects of H_2O_2 50 μmol/L reperfusion for 10 min were observed. I_k was elicited

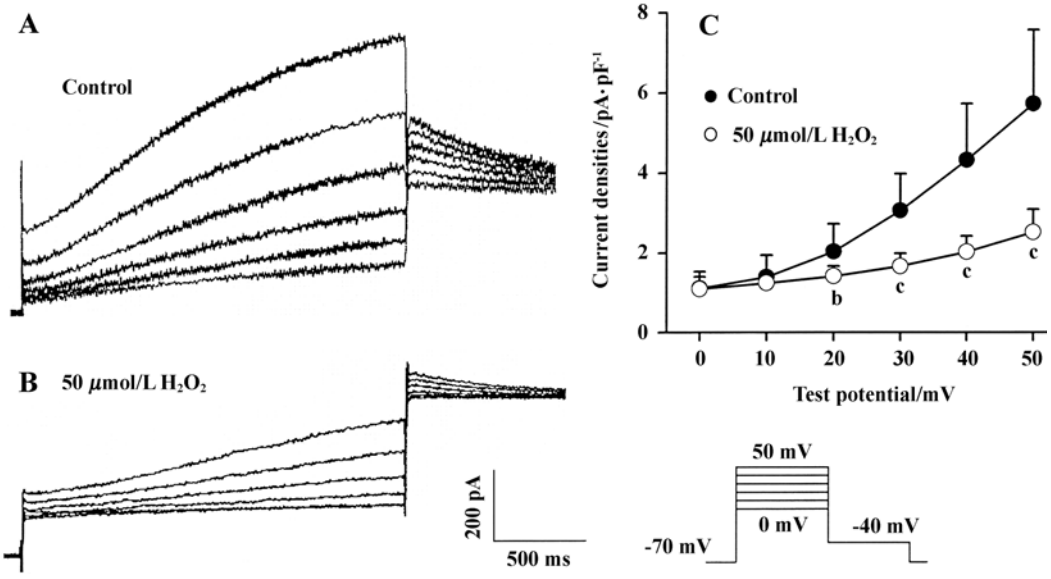

Fig 1. Decreases of I_k densities by 50 μmol/L H_2O_2 treatment for 24 h. A) Representative I_k traces from the control group. B) Representative I_k traces from the group of 50 μmol/L H_2O_2 treatment for 24 h. C) Current-voltage relations for I_k under control conditions and 50 μmol/L H_2O_2 treatment for 24 h. n=8 from two animals per group. ^bP<0.05, ^cP<0.01 *vs* control.

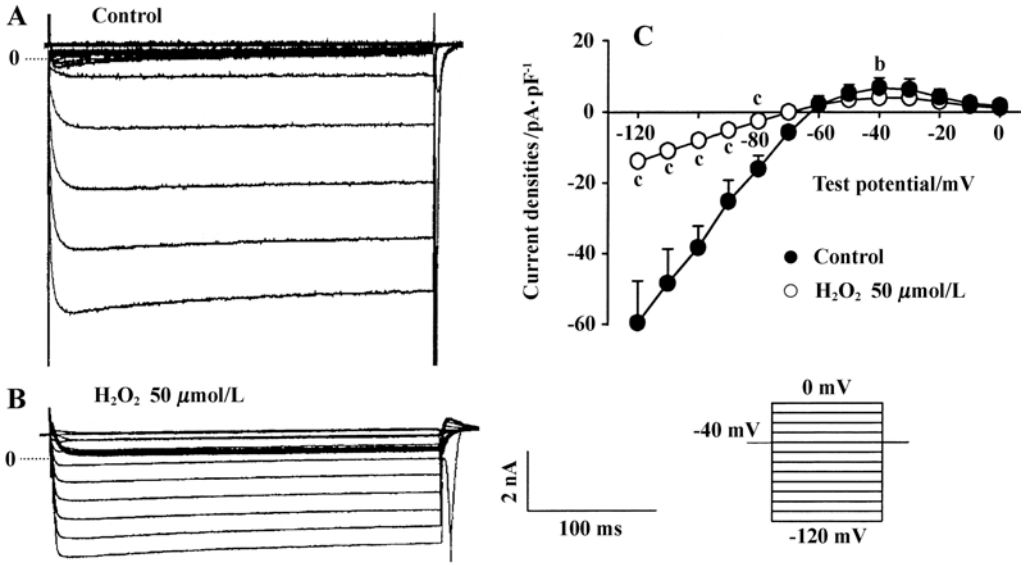

Fig 2. Decreases of I_{k1} densities by 50 μmol/L H_2O_2 treatment for 24 h. A) Representative I_{k1} traces from the control group. B) Representative I_{k1} traces from the group of 50 μmol/L H_2O_2 treatment for 24 h. C) Current-voltage relations for I_{k1} under control conditions and 50 μmol/L H_2O_2 treatment for 24 h. n=8 from two animals per group. ^bP<0.05, ^cP<0.01 *vs* control.

by +50 mV depolarizing pulse from the holding potential of -70 mV. I_{k1} was elicited by -120 mV hyperpolarizing pulse from the holding potential of -70 mV. Both protocols were performed at a 10-s interval for 15 min. No change of I_k and I_{k1} was seen during a 10-min reperfusion of H_2O_2 50 μmol/L in 5 cells.

DISCUSSION

Although several studies had reported the effects of H_2O_2 on the voltage-dependent K^+ currents in cardiomyocytes, they mainly focused on the acute effects and the dose of H_2O_2 was high, eg, reperfusion of H_2O_2 at the concentrations of 100 μmol/L for 15-25 min[10]. Apoptosis is a gradual process, during which K^+ currents alterations is also the ongoing course, and changes by such a strong damage factor could not reflect the changes of K^+ currents in the apoptotic process. The dose of H_2O_2 should both induce occurrence of apoptosis and keep enough cells alive for electric recording. For the concentration of H_2O_2 required to reach the same degree of apoptosis was variable in different cell lines[15]. We examined H_2O_2 at different concentrations firstly and found H_2O_2 50 μmol/L could both induce apoptosis and keep enough cells in normal state.

The cells used for currents record in the present study had rod shape and clear striation, showing no apoptotic features. In this normal state, I_k, the major repolarizing K^+ currents, was decreased by the apoptotic oxidant stress treatment, which suggested that the decrease of outward K^+ currents might be a self-protective mechanism in cardiac myocytes to reduce the K^+ loss by apoptotic stimuli. This kind of self-protective adaptation is different from the enhancement of K^+ currents related to the apoptosis in rat liver cell line[3] or neurons[4,5], so increases of voltage-dependent K^+ can not be simply extrapolated from liver cell line or neurons to cardiomyocytes during the apoptosis process. We and others also found the transient outward K^+ currents (I_{to}) responsible for cardiac repolarization in rats and mouse decreased when heart hypertrophy happened[11,13], and heart hypertrophy usually accompanied apoptosis[14], all the information supported the hypothesis.

The acute effects of ROS on I_k had been reported in other studies. Satoh and Matsui *et al*[10] found H_2O_2 100 μmol/L enhanced I_k for 15-25 min treatment. However, Cerbai *et al*[9] found oxygen radicals generated by dihydroxyfumarate (5 mmol/L) within 5 min markedly decreased I_k and I_{Ca}, but I_{k1} was unaffected. Compared with these reports, we found both I_k and I_{k1} were reduced by H_2O_2 50 μmol/L treatment for 24 h. This discrepancy might be due to the differences of the ROS and the incubation duration.

We observed that both the densities and the inward rectifier property of I_{k1} was decreased by the chronic H_2O_2 treatment, suggesting the activity of cardiomyocytes to keep the rest potential at the K^+ equi-

librium potential was weakened, thus the electrical disorders would happen more easily. This could partially explain the arrhythmogenesis of ROS.

The limitation of the present study was that it only investigated the changes of K^+ currents in cardiomyocytes of adult guinea pigs by chronic 50 μmol/L H_2O_2 treatment. For the expression of the K^+ channels was species-dependent, so it deserved to study the changes of I_{to} in cardiomyocytes of rats or mice with the same treatment of H_2O_2 to explore the common law of K^+ current alteration during the gradual apoptotic process. And it was also important to elucidate that the decreased of densities of I_k and I_{k1} were secondary to functional modulation or protein expression changes. H_2O_2 has multiple actions including apoptosis of cells, induction of membrane phospholipids peroxidation, activation of intracellular signal cascades, oxidation of channel proteins, *etc*. Apoptosis was the putative result of H_2O_2 stimulation and the present study only discussed the relationship between K^+ current changes and cell apoptosis, the K^+ current changes related to other actions of H_2O_2 were still unexplored.

The new information provided in this study is that chronic oxidant stress by H_2O_2 50 μmol/L treatment for 24 h decreases the voltage-dependent K^+ currents and alters the inward rectifier property of I_{k1} in cardiac myocytes of guinea pigs.

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