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Electrophysiological alterations in diaphragm muscle caused by abdominal ischemia-reperfusion

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ABSTRACT

Ischemia-reperfusion injury is the major complication of abdominal aortic surgery, and it mainly affects the lower extremities and remote organs. In the present study, the electrophysiological alterations in diaphragm that underlie the post-operative respiratory dysfunction were investigated.

Wistar Albino rats were randomly divided into two groups: SHAM (only laparotomy was performed) and IR (abdominal aorta was clamped for 30 min and reperfused for 2 h). Following the operational period diaphragm muscles were isolated and electrophysiological experiments were carried out in-vitro. 3 nM Ryanodine application, Na^+ and K^+ current blockage (0.3 mM 4-Aminopyridine and 127 mM *N*-methyl-D-glukamine) experiments were also conducted to further reveal any alterations.

Twitch and tetanic force were decreased significantly. Action potential overshoot, amplitude and area were increased while diaphragm muscle cells were found to be hyperpolarized significantly.

Mechanical alterations were shown to be caused by deterioration of Ca^{++} homeostasis. At resting state, a decrease in persistent Na^+ current was found. The reshaping of action potential, on the other hand, was shown to be due to altered kinetics of Na^+ channels and delayed activation of voltage dependent K^+ channels.

1. Introduction

Lower torso ischemia-reperfusion is essential for most of the surgeries related to some complications and/or injuries below the waist. Cessation of blood flow below kidneys is a necessity for lower torso surgical applications and aortic surgeries. Abdominal ishemia-reperfusion injury is related to lower torso surgical applications and aortic surgeries. Especially in repair procedure of Abdominal Aorta Aneurysm (AAA) that has high prevalence rates estimated in between 1.3–8.9% in men and 1.0–2.2% in women (Sakalihasan et al., 2005). The main reason of high morbidity and mortality rates after ischemia-reperfusion of abdominal aorta is respiratory complications. The incidence of respiratory complications after abdominal aortic surgery is 60%, which is explained by mechanical dysfunction attributed to cellular level alterations (Montgomery et al., 1985). Most of these alterations are associated with disruption by neutrophil-derivated proteases and oxidants in tissues (Barry et al., 1997a). Respiratory dysfunction has two main components: the first component is injury in alveolar gas exchange mechanism and the second component is muscle dysfunction in mechanical way of respiration. Even though previous studies were predominantly about parenchymal damage (Barry et al., 1997b), more recent experimental studies have shown the relation of diaphragm muscle dysfunction with systemic ischemia-reperfusion similar to clinical findings (McLaughlin et al., 2000).

Diaphragm is a skeletal muscle that has major role in respiration. Pleural pressure is decreased by contraction of diaphragm, making the inspiration step of ventilation possible. Consequently, the efficiency of respiration is mostly determined by diaphragm muscles' contractile performance. Contraction of a skeletal muscle is controlled by the muscle cell membrane potential changes determined by ions and their concentrations across the cell membrane. The mechanical and electrophysiological alterations and their mechanisms caused by abdominal ischemia-reperfusion are not clarified yet.

Therefore, the aim of this study is to determine whether respiratory dysfunction secondary to major surgery caused by abdominal ischemia-reperfusion has any influence on the electrophysiological properties of diaphragm muscle cells, and to investigate for a possible remodeling of ion channels responsible for action potential (AP) after ischemia-reperfusion injury.

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2. Materials and methods

2.1. Animals and experimental ischemia-reperfusion model

This study was approved by Necmettin Erbakan University Experimental Medicine Research and Application Center (Approval no. 2010-85). Adult (14–16 weeks old) male Wistar albino rats weighing 250–350 g were used for the study. After birth, rats were housed as 5 per cage at ambient temperature and humidity on a 12/12 light/dark cycle and all animals received food and water ad libitum. Rats were randomized into two groups: the IR group had occlusion of abdominal aorta for half an hour followed by two hours reperfusion, and SHAM (i.e. the control) group underwent the same procedures as the IR group, except for the occlusion of abdominal aorta. The number of animals used were 10 for each experimental group.

Rats were anaesthetized by using ketamine (8 mg per 100 g of animal) combined with xylazine (1 mg per 100 g of animal), and body temperature was maintained at 37 °C using a heating pad (MAY RTC9404-A Animal Rectal Temperature Controller, Commat Ltd., Turkey) with a feedback of rectal probe during the experiments. Abdominal region was prepared for surgery and a ~3 cm median incision was made. To achieve the experimental ischemia-reperfusion injury model, abdominal aorta was made free in the infrarenal region from connective tissue by help of a retractor, and a microvascular clamp was placed to occlude blood flow for 30 min. After this ischemic period, the clamp was removed and the incision was sutured with 4-0 silk. For the SHAM group, exactly the same procedure was followed except that microvascular clamp was placed. After this stage, the rats were put in to their cages again and leaved as before the operation for 2 h of reperfusion period. As final step, they were anaesthetized again for dissection of whole diaphragm as described in the Bülbring isolation method (Bülbring, 1946). Diaphragm muscles were then quickly transferred to our experimental setup for mechanical (contraction) and electrophysiological measurements.

2.2. Contraction experiments

Muscle strips were prepared by cropping diaphragm to have dimensions of 20 × 5 mm, and mounted longitudinally within the isolated tissue bath, as their costal side attached upward to the transducer (FT03 Force Displacement Transducer, Grass Instruments) and tendonous side downward to a micromanipulator to adjust the strip for optimal length. Resting tension was adjusted to 2 g at optimal length and maintained throughout the experiments. Tissue bath was perfused with modified Krebs solution (in mM: 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 15 $NaHCO_3$, 1 Na_2HPO_4, 11 glucose at pH 7.4, gassed with %95 O_2 and %5 CO_2 mixture) and temperature was fixed at 37 °C via a water bath circulator connected to heat jacket. After an equilibration period of 30 min, rectangular shaped pulses of 1 ms duration were delivered using an electrophysiological stimulator (S48, Grass Instruments) through an isolation unit (SIU5, Grass Instruments) to achieve electrical field stimulation. Stimulus strength was gradually increased to supramaximal level to obtain maximal twitch responses. Pulse frequency was adjusted to 1 Hz for recording single twitch responses and to 50 Hz for tetanic responses. Tetanic force-frequency relationship was evaluated by applying pulses with frequency gradually increased from 20 to 70 Hz. Isometric contractions and pulses were simultaneously acquired using a data acquisition unit (MP45, Biopac) via a software (BSL Pro 3.7.5, Biopac) for further analysis. Muscle strips were then blotted and weighed to normalize the force per muscle cross sectional area, which were calculated according to the formula $F' = (FxdxL)/m$, where F is the applied force in Newtons, and d, L, and m respectively correspond to specific density of muscle (1.06 g/cm^3), length (cm) and mass (g) of the strip (Close, 1972).

Twitch force (F), contraction time (CT) and half relaxation time (HR) were calculated from single twitch data, while tetanic force (TF) was calculated from the 50 Hz recordings. Tetanus frequency (Tf) was defined as the stimulation pulse frequency at which tetanus was observed while increasing pulse rate.

2.3. Electrophysiological experiments

In order to perform resting membrane potential and action potential related measurements, dissected hemidiaphragms were pinned to bottom of Sylgarg-lined tissue chamber. Tissue chamber was perfused with modified Krebs solution (in mM: 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 15 $NaHCO_3$, 1 Na_2HPO_4, 11 glucose at pH 7.4, gassed with %95 O_2 and %5 CO_2 mixture) and temperature was fixed at 37 °C using a water bath circulator. Costal side of hemidiaphragm was horizontally attached to a transducer (FT03 Force Displacement Transducer, Grass Instruments) for recording contraction and AP simultaneously. Direct stimulations were made to achieve AP recordings. Hemidiaphragms were stimulated using a pair of tungsten electrodes, with 0.2 ms duration supramaximal pulses having maximum frequency of 2 Hz, generated with a stimulator (S48, Grass Instruments) and applied through an isolator (SIU5, Grass Instruments). Intracellular recordings were performed via borosilicate microelectrodes fabricated using a micropipette puller (PN-31, Narishige Japan). The electrodes had 10–20 MΩ resistance when filled with 3 M KCl solution. Intracellular potentials were recorded using an intracellular amplifier (IE-251A, Warner Instruments) and data acquisition unit (MP100, Biopac) at 25 kHz sampling rate.

Hemidiaphragms were hypothetically divided into four areas, and for each, the AP was recorded from ten individual cells randomly entered by microelectrode. The reason for using separate cells was due to any structural changes potentially caused by dislodging of electrode from the cell during muscle contraction. APs were recorded only from cells having a stable resting membrane potential.

In order to block K^+ currents, 4-Aminopyridine (4AP) was used at a concentration of 4 mM and N-methyl-D-glucamine (NMDG)-replaced extracellular medium (in mM: 127 NMDG, 40 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 15 $NaHCO_3$, 1 Na_2HPO_4, 11 glucose) was used to decrease Na^+ currents. In simultaneous recordings of contraction and AP, Ryanodine (RyR) was used at a concentration of 3 nM and latent period (LP, i.e. the interval between onsets of AP and contraction) was measured. Properties of the APs were characterized with some measured and calculated parameters, given as follows: amplitude (difference between resting membrane potential and peak voltage), overshoot (difference between 0 mV and the peak voltage), area (integral of AP measured relative to resting membrane potential), time to peak (time required for an AP to reach peak voltage from resting), maximal rate of rise (maximum derivative of an AP), maximal rate of fall (minimum derivative of an AP), time to maximal rate (time required for an AP to reach its maximal rate from resting), and 25%, 50%, 75% and 90% decay times (APD25, APD50, APD75, APD90; times required for an AP to repolarize to 25%, 50%, 75% and 90% of peak voltage level, respectively). Additionally, change of the derivative value of an AP with membrane potential during depolarization was plotted and skewness of this curve was calculated.

2.4. Statistics

Data are represented as mean ± SEM throughout the text. Sample sizes are indicated in figure legends. Comparison between groups were done with unpaired Student's t-test and comparison between more than two groups were done with one-way analysis