ORIGINAL ARTICLE

The Effects of Lung Ischaemia/Reperfusion on TRPM Gene Expression

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ABSTRACT

Objective: *Transient receptor potential melastatin (TRPM) are integral membrane proteins that have broad range of cellular functions. Roles of TRPM2, TRPM3, TRPM4 and TRPM7 among these channels are very important, and their roles in lung ischaemia/reperfusion injury have not been evaluated yet. The aim of this study is to investigate the contribution of these genes in lung ischaemia/reperfusion injury and evaluate histopathology of tissues.*

Methods: *A total of 40 Wistar albino rats were enrolled for the study. Ischaemia was performed by the application of an atramvatic clamp to pulmonary artery. Gene expressions were determined by the semi-quantitative reverse transcription-polymerase chain reaction method. Histopatholical evaluations were held by a standard haematoxyline–eosin staining.*

Results: *The major histopathological tissue damage was observed in ischaemia performed groups, and expression of TRPM channels was found to be obviously downregulated. Substantial changes were determined between TRPM2, TRPM3, TRPM4 and TRPM7 and lung ischaemia/reperfusion injury. In particular, expression of TRPM2 and TRPM7 was reversibly downregulated in ischaemia. Yet, the expression of TRPM3 and TRPM4 was irreversibly downregulated after ischaemia.*

Conclusion: *Consequently, these results indicate that TRPM family of cation channels may have significant roles in the lung ischaemia/reperfusion injury.*

Keywords: Lung ischaemia reperfusion, transient receptor potential channels, TRPM2/3/4/7.

INTRODUCTION

Ischaemia is a pathophysiological process in which sufficient levels of oxygen and other metabolites are not provided to tissues. In ischaemia-related tissue injury, accumulation of toxic metabolites and depletion of energy stores leads to cell deaths. However, reperfusion is the process of resupplying blood flow to ischaemic tissues (1). If the cell or tissue is not irreversibly damaged, energy storages and cellular homeostasis will be ameliorated. However, cells may have irreversible damage while making new blood supply to ischaemic organ (2). Therefore, reperfusion can result in more serious complications than complications observed in ischaemia (3).

Previously, it is believed that lungs are resistant to ischaemia because of its dual circulation and alveolar oxygenation. However, ischaemic injuries may occur as a result of loss of blood flow as well as reduction of alveolar ventilation. Furthermore, reperfusion of the ischaemic lung is a mandatory event to keep up the viability of the lung. Yet, restoring viability of the ischaemic lung by perfusion can lead to pulmonary ischaemia/reperfusion (I/R) injury (4). Also, lung I/R injury is one of the severe complications that is commonly observed after cardiopulmonary bypass and lung transplantation operations (5).

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The transient receptor potential (TRP) channels are integral membrane proteins that have broad range of cellular functions. These channels are subdivided into six subfamilies including transient receptor potential melastatin (TRPM) subfamily. Transient receptor potential melastatin channel proteins consist of eight members designated as TRPM1–8 (6). The functions and structures of TRPM channels have been reviewed by many investigators (7, 8). Roles of TRPM2, TRPM3, TRPM4 and TRPM7 among these channels are very important. TRPM2 is a non-selective cation channel commonly expressed in mammalian tissues such as neutrophils, bone marrow, spleen, heart, kidney, liver, and lungs (4, 9, 10). This channel plays an important role in stress responses and get activated with H_2O_2 and other free oxygen radicals (8). TRPM3 is the least studied member of the TRPM family. In literature, information about TRPM3 is very limited. Its function has not been defined clearly. TRPM4 is expressed in lymphoid tissue (11), and in a Ca^{2+}-dependent manner, it induces membrane depolarization (12). TRPM7 is known to express in many tissues, especially in lymphoid (11) and brain tissue (13). TRPM7 expression is regulated by free oxygen radicals (15, 16), and it is upregulated with increasing levels of free oxygen radicals (17).

Taken together, the role of TRPM family of ion channels is well studied in many health disorders, yet molecular pathways causing lung I/R injury are not clear enough and determining molecular contributors of lung I/R injury is still high priority. In particular, the role of TRPM 2, TRPM3, TRPM4 and TRPM 7 in lung I/R injury is yet to be elucidated. Hence, the present work was aimed to determine expression levels of TRPM 2, TRPM3, TRPM4 and TRPM 7 and histopathology associated with the ischaemic lung tissue.

MATERIALS AND METHODS

Study design and lung I/R model

For the study, a total of 40 Wistar albino rats were included in the present study. The study was ethically approved by local Animal Ethical Committee of Gaziantep University (Ethics Board Number: 26.12.2011/57). Animals were 200 to 250 g in weight. For the study, four separate groups were created and each group consisted of 10 rats. Regular biological rhythms of rats were maintained in separate cages at the following conditions: 12-hour light period, 12-hour dark photoperiod and 24°C–26°C. Feeding of animals was carried out by standard pellet feed and tap water. Feeding was stopped 18 hours prior to experiment. Experimental study groups were designated and treated as presented in the Table. Subsequently, lung tissue samples were collected from all animals. Collected lung tissue samples were divided into two parts by longitudinal incision. One of the parts was placed into 10% formalin solution for histopathology. Other parts were stored at –80ºC for expression studies. Animals were then sacrificed by intracardiac blood withdrawal.

Table: Experimental study groups

Groups (n = 10)	Application
Group 1	The group 1 was the control group and I/R was not performed in this group
Group 2	In this group ischaemia was performed for 1 hour by using an atramvatic clamp in pulmonary artery
Group 3	In this group ischaemia was performed for 1 hour by using an atramvatic clamp in pulmonary artery followed by the 2 hours of reperfusion
Group 4	In this group ischaemia was performed for 1 hour by using an atramvatic clamp in pulmonary artery followed by the 4 hours of reperfusion

Histopathological evaluations

Histopathological assessments of tissue samples were evaluated as previously described (18). Briefly, after I/R, lungs were removed and fixed in formalin. Subsequently, the lung tissue samples were embedded in paraffin blocks according to the standard protocol. Sections of 5-µm thickness were obtained and subjected to haematoxylin–eosin staining and subsequently observed under light microscope for semi-quantitative histopathological scoring.

Isolation of RNA and semi-quantitative reverse transcriptase polymerase chain reaction RNA samples of lung tissues were isolated by using Qiagen RNeasy Mini Kit (QIAGEN, Sample & Assay Technologies, Germany). RNA samples were then reverse transcribed by Transcriptor First Strand cDNA Synthesis Kit according to the manufacturer's supplied protocol. Semi-quantitative polymerase chain reaction (PCR) reactions were held in ABI Thermal Cycler (Applied Biosystems Inc., Foster City, CA, USA). The following primer pairs were used to assess regarding gene expression levels: TRPM2: forward TGGGAGCTCTACCTGAAGGA and reverse CAGAAACTCTGCCTCCCAAG; TRPM4: forward CAGCGACCTCTACTGGAAGG and reverse TCACGAGCTTGTGCCAATAG; TRPM3: forward GGGACTACGGCCTGAAACTC and reverse ACGGGGCATTAAGGTTGGAG and TRPM7: forward CTAGCCTTCAGCCACTGGAC and reverse

CCCTGAAAGGAAAAACGTCA. Polymerase chain reaction products were further subjected to electrophoresis in 3% agarose gel under 140 V for 30 minutes, and banding patterns were visualized with ethidium bromide staining. Corresponding band densities were quantified by ImageJ v1.46r program.

Statistical analyses

Statistical analyses of data were conducted by using Graphpad Prism 5 program (GraphPad Software, La Jolla, CA, USA), and non-parametric Wilcoxon rank test and Mann-Whitney *U* test were applied. All values were reported as mean ± SD. All statistical analyses were two-tailed, and $p < 0.05$ accepted as significant.

RESULTS

Histopathology of lung tissues

All lungs were subjected to histopathological evaluations. In group 1, lung parenchyma was observed as histologically normal (Fig. 1A). In group 2, in which 1-hour ischaemia is performed, severe congestion in the pulmonary veins and inter-alveolar septum were observed together with the sporadic erythrocyte accumulations in the alveolar lumen as shown in Fig. 1B. Also, a marked thickening of inter-alveolar septa was observed (Fig. 1B).

Mild perivascular infiltration of neutrophils was also observed (Fig. 1B). Desquamation was observed in the respiratory mucosal epithelium. In the right lobe, a mild thickening in the inter-alveolar septa and perivascular sporadic neutrophil infiltration were observed. In group 3 in which 1 hour of ischaemia is followed by 2 hours of reperfusion application, in addition to congestive symptoms, severe perivascular neutrophil and leucocyte infiltrations and intra-alveolar oedema were obviously present in this group as presented in Fig. 1C. Lastly, in group 4, varying degrees of congestive atelectasis were observed in all animals in the group (Fig. 1D). Along with the severe perivascular alveolar oedema and leucocyte and neutrophil infiltrations, prominent macrophage hyperplasia in alveolar lumens was observed. The presence of severe necrotic changes in the alveolar epithelium was noticed. In the bronchi and bronchiolar epithelia, clear desquamation was observed.

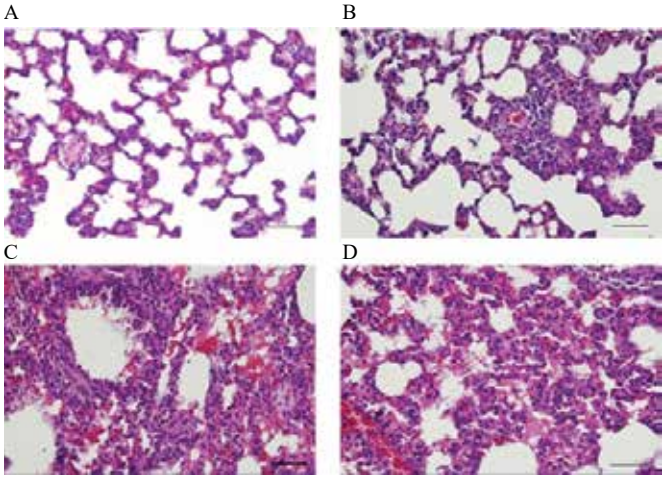

Fig. 1: Histopathology of lung tissues under light microscope. Tissue samples were subjected to haematoxylin–eosin staining and subsequently observed under light microscope (H&E, × 100).

Gene expression analysis of TRPM channel proteins

To investigate the role of TRPM family of cation channels in lung I/R model study animals were divided into four groups as control group, 1-hour ischaemia group (group 2), 1-hour ischaemia + 2-hour reperfusion group (group 3) and 1-hour ischaemia + 4-hour reperfusion group (group 4). Each experimental group was consisted of 10 study animals. Later, RNA samples were isolated from lung tissue samples and reverse transcribed into cDNA and quantified by RT-PCR to evaluate relative expression levels of TRPM2, TRPM3, TRPM4 and TRPM7 genes. β-Actin was used as internal normalization control to evaluate relative expression levels.

Relative band densities were calculated by the help of ImageJ v1.46r program, and images are presented in Fig. 2. As a result, while expression of TRMP2 was found to be downregulated in ischaemia group, its expression was restored in group 3 and then dramatically reduced in group 4 as compared to control group (Fig. 3). Similar results were also observed in TRPM4. While TRPM4 expression was decreased in ischaemia group, it was restored to normal levels in group 3 and reduced in group 4 as compared to control group. These expression changes were found to be statistically significant ($p < 0.05$). Also, changes in the expression of TRPM3 and TRPM7 were similar.

A gradual reduction was observed in ischaemia and reperfusion groups as presented in Fig. 3. Expression levels of TRPM3 and TRPM7 were decreased in group 2, group 3 and group 4 as compared to controls. Expression of these two channels was gradually decreased from group 1 to group 4, least expressed in group 4 and results were found to be statistically significant ($p < 0.05$).

DISCUSSION

In the present study, to reveal the role of TRPM family of proteins, the relative expressions of TRPM2, TRPM3,

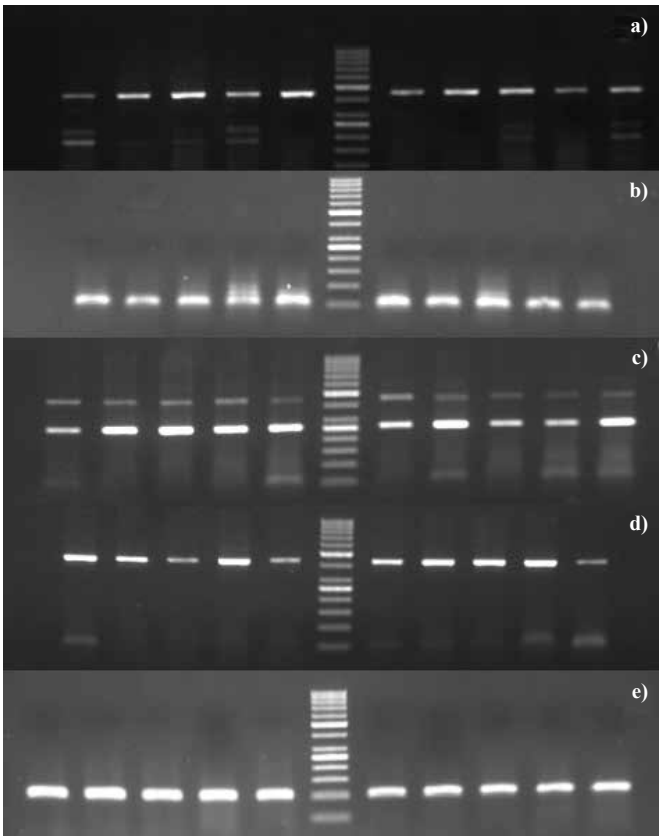

Fig. 2: Semi-quantitative PCR products were resolved using agarose gel electrophoresis and visualized with ethidium bromide staining. (a) TRPM2, (b) TRPM3, (c), TRPM4 (d), TRPM7, (e) β-actin.

TRPM4 and TRPM7 were evaluated by semi-quantitative RT-PCR method. As a result of the current study, significant alterations were detected in the expression of TRPM family of genes. In particular, TRPM3 and TRPM7 were found to be downregulated in ischaemia and I/R groups as compared to controls. Also, expression of TRPM2 and TRPM4 was also found to be downregulated in ischaemia group. Moreover, expression of these two was found to be restored to normal levels after 2 hours of reperfusion application as compared to control group. Again, a dramatical decrease was observed in group 4 in which 4 hours of reperfusion was performed. This reduction can be explained by the irreversible damage to lungs, thus strongly suggesting that these channel proteins may have important roles in the pathology of lung I/R injuries.

Furthermore, calcium has a major role in the intracellular signalling and immune response, and especially TRPM2 and TRPM7 are important channel proteins in the regulation of intracellular calcium homeostasis (12). Additionally, it has been known that TRPM2, TRPM4 and TRPM7 are modulated by oxidative stress and these channel proteins are the most important channel proteins among oxidative stress regulated channels (15, 19). These channel proteins play a variety of roles in the progression of diversity diseases. Reactive oxygen species have the ability to modulate the function of these channels; thus, various biological processes are regulated by this way (15).

In addition, variable expressions of these channels have been reported in several tissue damages and health manifestations (9, 10). As an example, accumulating body of evidence suggests that expression of TRPM2, 4 and 7 has increased in several types of cancer (15). In addition, in our previous work we investigated the expression levels of TRPM2, TRPM4 and TRPM7 in I/R models of cardiac tissue (9). Only expression of TRPM7 was found to be increased, and no expression changes were observed in TRPM2 and TRPM4. Also, in the present study, expression of TRPM2 and TRPM4 was found to be upregulated in ischaemia group, suggesting that expression of these channel proteins can vary depending on the type of tissue and experimental setup. In the present study, the major histopathological tissue damage was observed in group 2 and in this group expression of TRPM channels was found to be obviously downregulated. Then, at 2 hours of reperfusion (group 3), TRPM2 and 4 gene expressions were found to be elevated again, indicating that increased duration of oxygen exposure may increase the production of free oxygen radicals and this in turn activates the TRPM channels thus regulating calcium haemostasis and reducing tissue damage. Moreover, reperfusion did not affect expression of TRPM3 and TRPM7, showing that resupplying oxygen has not much effect to these channel proteins.

CONCLUSION

The role of TRPM family of protein channels is very important in I/R injuries as well as in variety of diseases. In our study, we evaluated the expressions of TRPM2, TRPM3, TRPM4 and TRPM7 channels and histopathology changes in lung tissues. Significant relations were detected between TRPM2, TRPM3, TRPM4 and TRPM7 and lung I/R injury. To better understand the role of these genes in lung I/R injury, protein levels will be great of interest in the upcoming research. Also, determination of intracellular and extracellular calcium contents and free oxygen radicals may provide more information. This report is unique in terms of providing information about expression of TRPM channel proteins and histopathological changes in lung tissues after I/R application. Lastly, further investigations are needed to

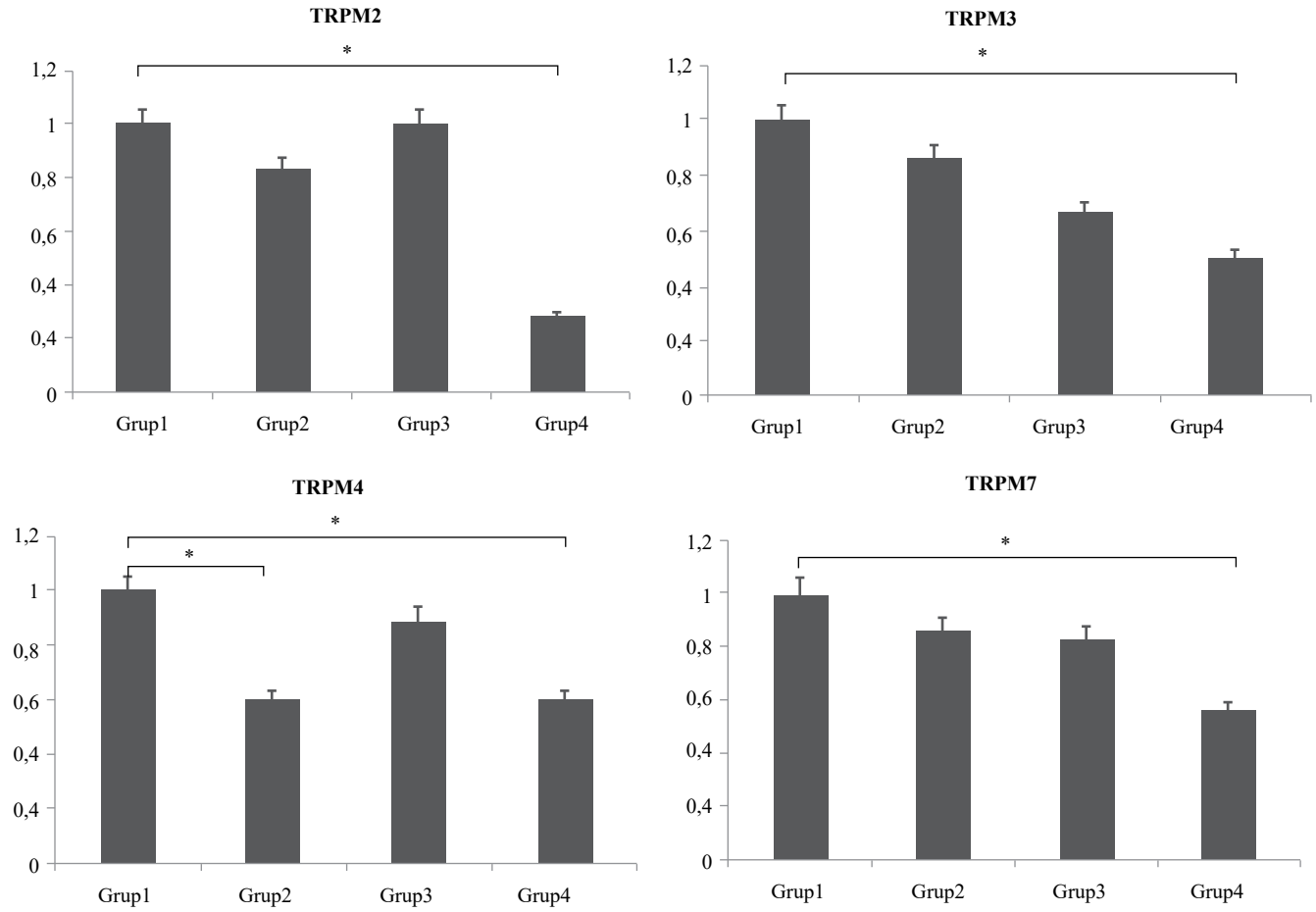

Fig. 3: Expression changes of TRPM family of genes in experimental groups (* $p < 0.05$).

clarify the role of these channel proteins in lung tissue damages and pulmonary diseases.

AUTHORS' NOTE

HDA conceived paper, oversaw data collection and conducted data analysis. TD participated in study design, wrote manuscript and approved final version. RD provided oversight to study, revision of manuscript and approved final version, OY participated in study design, interpretation of data and revision of manuscript. SO provided data analysis, and interpretation of data and revision of manuscript, AOC provided data analysis and interpretation. RB provided data analysis, and interpretation of data and revision of manuscript. BC provided data analysis, and interpretation of data and revision of manuscript, SD oversaw data collection and conducted data analysis. CB provided data analysis and interpretation, critically revised manuscript and approved final version. There are no known conflicts of interest associated with this publication and there has been no

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