The Effects of Lung Ischemia-Reperfusion on TRPM Gene Expression

HD Atabay¹, T Demir¹, R Dokuyucu², O Yumrutas³, S Oztuzcu⁴, AO Ceribasi⁵, R Bayraktar¹, B Cengiz⁶, S Demiryurek¹, C Bagci⁷

ABSTRACT

Objective: 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 ischemia/reperfusion injury have not been evaluated yet. The aim this study is to investigate the contribution of these genes in lung ischemia/reperfusion injury and evaluate histopathology of tissues.

Methods: Total number of 40 Wistar albino rats were enrolled for the study. Ischemia was performed by the application of an atramvatic clamp to pulmonary artery. Gene expressions were determined by semiquantitative RT-PCR method. Histopathological evaluations were held by a standard hematoxyline–eosin staining.

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

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

Keywords: Lung ischemia reperfusion, TRP channels, TRPM2/3/4/7

¹University of Gaziantep, Faculty of Medicine, Department of Physiology, Gaziantep, 27000, Turkey, ²University of Mustafa Kemal, School of Medicine, Department of Physiology, Hatay, 31100, Turkey, ³University of Adiyaman, Faculty of Medicine, Department of Medical Biology, Adiyaman, 02200, Turkey, ⁴University of Gaziantep, Faculty of Medicine, Department of Medical Biology, Gaziantep, 27000, Turkey, ⁵University of Firat, Faculty of Veterinary, Department of Pathology, Elazig, 23119, Turkey, ⁶University of Gazi, Faculty of Medicine, Department of Medical Genetics, Ankara, 06560, Turkey

Correspondence: Dr R Dokuyucu, Department of Physiology, School of Medicine University of Mustafa, Kemal Hatay, Turkey, Fax: +90 326 245 5305, e-mail: drecepfatih@gmail.com
INTRODUCTION

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

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

The transient receptor potential channels are integral membrane proteins that have broad range of cellular functions. TRP channels are subdivided into six subfamilies including TRPM subfamily. TRPM channel proteins are consisted 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$_2$O$_2$ 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), in a Ca+2-dependent manner, it induces membrane depolarization (12). TRPM7 is known to expressed in many tissues, especially in lymphoid (11) and brain tissue (13). TRPM7 expression regulated by free oxygen radicals (15, 16) and its expression is upregulated with increasing of 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 is not clear enough and determining molecular contributors of lung ischemia/reperfusion injury is still high priority. In particular, the role of TRPM 2, TRPM3, TRPM4 and TRPM 7 in lung ischemia/reperfusion 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 ischemic lung tissue.

**SUBJECTS AND METHODS**

**Study Design and Lung ischemia/reperfusion Model**

For the study, total numbers 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 24-26 Celsius. Feeding of animals was carried out by standard pellet feed and tap water. Feeding were stopped 18 hours prior to experiment. Experimental study groups were designed as presented in Table1. Subsequently, lung tissue
samples were collected from all animals. Collected lung tissues samples were divided into
two parts by longitudinal incision. One of the parts were placed into 10 % formalin solution
for histopathology. Other parts were stored at −80 ºC for expression studies. Animals were
then sacrificed by intracardiac blood withdrawal.

**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. 5-μm sections
were obtained and subjected to hematoxylin–eosin staining and subsequently observed under
light microscope for semiquantitative histopathological scoring.

Isolation of RNA and Semi-quantitative Reverse Transcriptase PCR

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 manufacturer’s supplied protocol.
Semi-quantitative PCR reactions were held in ABI Thermal Cycler (ABI Inc. CA, USA). The
following primer pairs were used to assess regarding gene expression levels: TRPM2:
Forward TGGGAGCTCTACCTGAAGGA and Reverse CAGAAACTCTGCTCCTCCAAG;
TRPM4: Forward CAGCGACCTCTACTGGAAGG and Reverse TCA
TCACGAGCTTGTGCCAATAG; TRPM3: Forward GGGACTACGGCCTGAAACTC and
Reverse ACGGGGCATTAAGGTTGGAG and TRPM7: Forward
CTAGCCTTCAGCCACTGGAC and Reverse CCCTGAAAGGAAAGCGTCA. PCR
products were further subjected to electrophoresis in 3% agarose gel under 140 voltage for 30
minutes and banding patterns visualized with ethidium bromide staining. Corresponding band densities were quantified by ImageJ v1.46r program.

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

RESULTS
Histopathology of lung tissues
All lungs were subjected to histopathological evaluations. In group1, lung parenchyma were observed as histologically normal (Figure1-A). In group2 in which 1 hour ischemia is performed, severe congestion in the pulmonary veins and interalveolar septum were observed together with the sporadic erythrocyte accumulations in the alveolar lumen as shown in Figure1-B. Also, a marked thickening of interalveolar septums was observed (Figure1-B).

Mild perivascular infiltration of neutrophils was also observed (Figure1-B). Desquamations was observed in the respiratory mucosal epithelium. In the right lobe a mild thickening in the interalveolar septums and perivascular sporadic neutrophil infiltration were observed (Figure1-B). In group3 in which 1 hour of ischemia is followed by the 2 hours of reperfusion application, in addition to congestive symptoms, severe perivascular neutrophil and leukocyte infiltrations and intraalveolar edema were obviously present in this group as presented in Figure1-C. Lastly, in group4, varying degrees of congestive atelectasis were
observed in all animals in the group (Figure1-D). Along with the severe perivascular alveolar edema and leukocyte and neutrophils infiltrations, prominent macrophages hyperplasia in alveolar lumens was observed (Figure1-D). The presence of severe necrotic changes in the alveolar epithelium was noticed. In the bronchi and bronchiolar epithelia clear desquamation was observed (Figure1-D).

**Gene expression analysis of TRPM channel proteins**

To investigate the role of TRPM family of cation channels in lung ischemia/reperfusion model study animals were divided into four groups as control group, 1 hour ischemia group (group2), 1 hour ischemia+2 hours reperfusion group (group3) and 1 hour ischemia+4 hours reperfusion group (group4). 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. B-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 Figure 2. As a result, while expression of TRMP2 was found to be down-regulated in ischemia group, its expression was restored in group3 and then dramatically reduced in group4 as compared to control group (Figure 3). Similar results were also observed in TRPM4. While TRPM4 expression was decreased in ischemia group, its expression was restored to normal levels in group3 and reduced in group4 as compared to control group (Figure 3). These expression changes were found to be statistically significant (p<0.05). Also, changes in the expression of TRPM3 and TRPM7 were also similar (Figure 3).

A gradual reduction was observed in ischemia and reperfusion groups as presented in Figure 3. Expression levels of TRPM3 and TRPM7 was decreased in group2, group3 and
group4 as compared to controls (Figure 3). Expression of these two channels was gradually decreased from group1 to group4, least expressed in group4 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, TRPM4, TRPM7 were evaluated by semiquantitative 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 ischemia and ischemia/reperfusion groups as compared to controls. Also, expression of TRPM2 and TRPM4 were found to be also downregulated in ischemia group. Moreover, expression of these two were found to be restored to normal levels after 2 hours of reperfusion application as compared to control group. Again a dramatical decreasing was observed in group4 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 ischemia-reperfusion injuries.

Furthermore, calcium has a major role in the intracellular signaling and immune response, 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 is modulated by oxidative stress and these channel proteins are the most important channel proteins among oxidative stress regulated channels (15, 19). These channel proteins plays variety of role in the progression of diversity diseases. Reactive oxygen species have 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 suggest that expression of TRPM2, 4 and 7 have increased in several types of cancer (15). In addition to, in our previous work we investigated the expression levels of TRPM2, TRPM4 and TRPM7 in ischemia-reperfusion 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 ischemia 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 down-regulated. Then, at 2 hours of reperfusion [Group 3], TRPM2 and 4 gene expressions was 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 hemostasis and reducing tissue damage. Moreover, reperfusion did not affected expression of TRPM3 and TRPM7. Showing that resupplying oxygen have not much affect to these channel proteins.

CONCLUSION

The role of TRPM family of protein channels is very important in ischemia/reperfusion 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 that in lung tissues. Significant relations were detected between TRPM2, TRPM3, TRPM4 and TRPM7 and lung ischemia reperfusion injury. To better understand the role of these genes in lung/ischemia reperfusion 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 is report is unique in terms of providing an information about expression of TRPM channel proteins and histopathological changes in lung tissue after ischemia-reperfusion application. Lastly, further investigations are needed to clarify the role of these channel proteins in lung tissue damages and pulmonary diseases.

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AUTHORS’ NOTE
HD Atabay conceived paper, oversaw data collection, conducted data analysis. T Demir participated in study design, wrote manuscript and approved final version. R Dokuyucu provided oversight to study, revision of manuscript, and approved final version. O Yumrutas participated in study design, interpretation of data and revision of manuscript. S Oztuzcu provided data analysis, and interpretation of data and revision of manuscript. AO Ceribasi provided data analysis and interpretation, R Bayraktar provided data analysis, and interpretation of data and revision of manuscript. B Cengiz provided data analysis, and interpretation of data and revision of manuscript. S Demiryurek oversaw data collection and conducted data analysis. C Bagci data analysis and interpretation, critically revised manuscript and approved final version.
REFERENCES


Table 1: Experimental study groups

<table>
<thead>
<tr>
<th>Groups (n=10)</th>
<th>Application</th>
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<tbody>
<tr>
<td>Group 1</td>
<td>The group 1 was the control group and I/R was not performed in this group.</td>
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<tr>
<td>Group 2</td>
<td>In this group ischemia was performed for 1 hour by using an atramvatic clamp in pulmonary artery.</td>
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<tr>
<td>Group 3</td>
<td>In this group ischemia was performed for 1 hour by using an atramvatic clamp in pulmonary artery followed by the 2 hours of reperfusion.</td>
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<tr>
<td>Group 4</td>
<td>In this group ischemia was performed for 1 hour by using an atramvatic clamp in pulmonary artery followed by the 4 hours of reperfusion.</td>
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Fig. 1: Histopathology of lung tissues under light microscope. Tissue samples were subjected to hematoxylin–eosin staining and subsequently observed under light microscope (H&E, X 100).

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