Regulatory role of TRPM7 cation channels on neuronal hypoxia model

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Received 31 July 2023; revised 25 September 2023

Low levels of oxygen have harmful effects on cells especially in neurons because of their vulnerable status for oxygen consumption. Intracellular calcium concentration ([Ca$^{2+}$]$_i$) is accumulated by several kinds of calcium-permeable channels including the Transient Receptor Potential (TRP) channels. The TRPM7 cation channels are calcium ion (Ca$^{2+}$) permeable non-selective cation channels belonging to TRP superfamily. The TRPM7 is expressed in different organs of the human body including nervous system components especially in the brain. Some of the TRP channel subtypes are related to oxidative stress and increased oxidative stress triggers channel activity. Recently, TRPM7 cation channels involved in hypoxia. Hence, alterations of [Ca$^{2+}$]$_i$ may be a key factor in TRPM7 activity in hypoxia and preventing hypoxic injury of neurons TRPM7 could be a therapeutic target. Therefore, it has been investigated effects of downregulation of TRPM7 cation channels by siRNA applications to induce getting TRPM7 activity down. The role of silencing of TRPM7 cation channels on apoptosis and cell morphology, production of intracellular reactive oxygen species (iROS), mitochondrial membrane depolarization (MMD) levels, enzymatic activity values of caspase 3, 8 and 9 in SH-SY5Y neuronal cells investigated in this study. It has been shown that the downregulation of TRPM7 cation channels may prevent cell death and protect cellular morphology and viability in neuronal cells after chemical hypoxia induction. Decreasing TRPM7 channel activity may also decrease calcium overload and it is a key regulatory function of TRPM7 channels in hypoxic conditions. In conclusion, TRPM7 cation channel antagonists or suppression of the channel expression by genetic manipulations can be a useful and potential therapeutic approach against neuronal hypoxia-related cell death.

Keywords: Apoptosis, Calcium signaling, Hypoxia, SH-SY5Y cells, TRPM7 channels

The oxygen consumption rate of brain tissue is about twenty percent of all human body in rest, and neuronal cells are vulnerable to reactive oxygen species (ROS) production because of oxidative phosphorylation. The second cause of the elevation of intracellular ROS production (iROS) is cellular hypoxia. Mitochondria are the main source of ROS production and dysfunction of mitochondria also trigger oxidative stress. Excessive oxidative stress may cause activation of oxidative stress-sensitive on channels and leads to cationic influx from the out of the cell into cytosol. Excessive increase of calcium concentration in the cytosol may also triggers caspase-mediated apoptotic cell death. Hence, it is very important that extracellular and intracellular concentration balance of calcium need to be kept under physiological limits. The calcium balance of a cell is controlled by several types of calcium-permeable ion channels including the Transient Receptor Potential Cation (TRP) Channels Superfamily subtypes. These non-selective cation channels were discovered by the beginning of the millennium and there are six subfamilies and 28 subtypes in mammals of this channel superfamily, and some of them show oxidative stress related activity. In neurological tissues, TRP cation channels have several activities for physio-pathological functions such as external signal transduction, intracellular calcium signaling, pain relief, neuronal cell death, neurodegeneration, and oxidative stress-related pathways. Until recently, several studies have investigated that TRP channels could be important in some diseases including neurological diseases and cancers. The TRP Melastatin (TRPM) subfamily consist of 8 subtypes (TRPM1-8) and TRPM7 is a unique member of the group. The TRPM7 channels show channel kinase molecular structure and behave as both enzyme and channel for the permeation of ions through the cell membrane. TRPM7 cation
channels are activated by different chemicals such as cholesterol and naltriben, but cationic flow can be gated by carvacrol, and novel agonists and antagonists have been added to the literature day by day. TRPM7 channels are expressed in many neurological tissues in central and peripheral nervous systems. The SH-SY5Y neuronal cells are also expressed in those channels and are well-known in vitro model for studies in neurodegenerative diseases, apoptosis, oxidative stress, and hypoxia. Cobalt chloride is a hypoxia-mimicking agent, and in studies, have shown that CoCl₂ incubation increases expression of hypoxia-inducible factor 1 (HIF1), a key regulator of hypoxic conditions in cell. In several research, TRPM7 cation channels have shown to be related to Parkinson’s disease, cell differentiation, apoptosis, and oxidative stress-mediated activity. In a study, carvacrol is effective agent for recovery of TRPM7-related ischemic cell death. However, there are a limited number of studies focusing on the possible role of downregulation of TRPM7 channels in the pathological state in hypoxia. Hence, this study aimed to investigate the possible effects of downregulation of TRPM7 channel expression on cell viability, caspase (3, 8 and 9) enzyme activity, apoptosis levels, mitochondrial membrane depolarization and ROS production levels in CoCl₂-induced hypoxia as well as bioinformatical approaches.

Materials and Methods

Reagents, cell culture and transfection

All chemicals, reagents and probes in this research were provided from Sigma-Aldrich, USA unless otherwise stated. The scrambled Control siRNA (sc-36869) and the TRPM7 siRNA (sc-42662) were purchased from Santa Cruz Biotechnology, USA. Sequences of siRNAs are listed in (Table 1). The SH-SY5Yneuronal cells were cultured in DMEM/F12 basal medium mixture (1:1) containing 10% fetal bovine serum (Cegrogen, Germany) and 1% Penicillin-Streptomycin antibiotics solution (Cegrogen, Germany) at 37°C in 5% CO₂ humidified incubator (Heal Force HF90, Japan). When cells reached the ~80% confluence, each flask was trypsinized and split into new flasks or studied in the analyses, and 10-15 passages of the cells were used in the experiments. The LipoFectMax™ Transfection Reagent (FP 310) was purchased from ABP Biosciences, USA. The Opti-MEM™ I Reduced Serum Medium for transfections was purchased from Termo Fisher Scientific, USA. The transfection process was carried out as described elsewhere. After completion of the transfection process, cells were incubated with a CoCl₂-containing growth medium for 24 h. For analyses, cells were trypsinized and detached, after centrifugation (125 g for 5 min) the pellets resuspended in a 1x phosphate buffered saline (PBS), then cells were divided into eppendorf tubes for plate reader analysis.

Bioinformatics analyze

Bioinformatic databases are useful for analyzing complex data and better way to investigate former studies in terms of chemical-gene interactions, protein-protein interactions, and gene network biology. To investigate how CoCl₂ incubation affects gene expression profile The Comparative Toxico genomics Database (https://ctdbase.org/) is used for queries of chemical-gene interactions for CoCl₂. After the query, the top ten genes were chosen for protein-protein network analysis by using String Database (https://string-db.org/) and also investigated with Enrichr (https://maayanlab.cloud/Enrichr/) for pathway analysis.

Western blotting

For the efficacy of transfection and the role of CoCl₂-induced hypoxia on TRPM7 channel expression western blotting was applied. The expression level of TRPM7 cation channel protein was assayed with western blotting after siRNA transfection and CoCl₂ incubation procedure. For the protein extraction step, the SH-SY5Y neuronal cells from the study groups were resuspended in 1x RIPA buffer (Biobasic, Canada) centrifuged at 13.000 g for 18 min. Cell supernatants were collected in an

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<th>siRNA Seq</th>
<th>Sense</th>
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<tr>
<td>A</td>
<td>GAGAUGUGGUUGCUCCUUA</td>
<td>UAAGGAGCAACCACAUCC</td>
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<tr>
<td>B</td>
<td>CCAUAUUGGGUGAGAUUA</td>
<td>UUCAUGUCACCCCAAUAUG</td>
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<td>C</td>
<td>GCAUUAUGUUGCCUUAAG</td>
<td>UCUUACAGGCAACUAUGC</td>
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The total amount of lysates was measured with a Bradford assay at 595 nm by a microplate reader (Tecan Infinite M200 Pro, Austria). For the blotting step, equal amounts of proteins (30 μg) were loaded into 8% sodium dodecyl sulfate-polyacrylamide gel, after the gel electrophoresis in Mini-Protean System (Bio-Rad, USA) and with 130 mV for 1.5-2 h, gels were transferred to a nitrocellulose membrane. The blots were blocked for 1 h at room temperature with a 5% non-fat dry milk powder (Havancızade, Türkiye) solution in Tris Buffered Saline with 0.05% Tween 20. Then, the membrane was incubated with TRPM7 primary antibody (BT-AP09233, BT Lab, China). The β-actin and secondary antibody were from GE Healthcare, Amersham, UK. Band signals were visualized by using ECL Western HRP Substrate (Millipore Luminate Forte, USA) and images were captured by (G:Box, Syngene, UK) and normalized against the β-actin protein as formerly described.

Calcium signaling
To investigate the role of the downregulation of TRPM7 cation channels on CoCl₂-induced hypoxia calcium signaling analysis was performed. The Fura-2 AM probe (Invitrogen, Carlsbad, USA) was used for the assay. Changes in calcium levels after cholesterol (1 µM) stimulation were shown by using the Fura-2 340/380 nm fluorescence ratio and calibrated according to a former method. The TRPM7 channel-mediated cytosolic free calcium concentration ([Ca²⁺]j) was expressed as nM, taking a sample every second as previously described.

Apoptosis assay
The APO Percentage™ apoptosis assay kit was used for(Biocolor Ltd, Northern Ireland)cell death analysis. After the incubation of the probe, color changes were read by using a microplate reader (Tecan Infinite M200 Pro, Austria) as described elsewhere.

Caspase activities
The caspase 3, 8 and 9 activities were evaluated by the cleavage of the specific fluorogenic substrates. Enzymatic activity of caspases 3, 8 and 9 was measured by using a microplate reader (Tecan Infinite M200 Pro, Austria) with 360 nm excitation and 460 nm emission wavelengths. The data were calculated as fluorescence units per mg/protein.

Mitochondrial membrane depolarization (MMD) and Intracellular ROS production (iROS) analyses
The MMD levels were measured by using the JC-1 probe (Santa Cruz Biotechnology, USA). The SH-SY5Y cells were incubated with the JC-1 dye (1 µM) at 37°C for 30 min. Fluorescence changes were analyzed by using a microplate reader (Tecan Infinite M200 Pro, Austria) as described elsewhere. The dihydrodorhodamine-123 was used for the detection of iROS (DHR-123, Molecular Probes, USA). SH-SY5Y cells were loaded with the probe (2 µM) and incubated at 37°C for 30 min. The intensity of fluorescence rhodamine-123 was measured by using a microplate reader (Tecan Infinite M200 Pro, Austria) as described elsewhere.

Statistical analyses
All acquired data were expressed as mean±standard deviation. The significance of difference among the groups was assessed one-way analysis of variance. Data were analyzed by using the SPSS, version 9.05 (SPSS, Inc., USA). P <0.05 was considered as significant.

Results
Bioinformatic findings
The bioinformatic findings were given in (Fig. 1A-C). It was found that CoCl₂ administration affects more than six thousand gene expression levels and mostly affected ten genes listed as HIF1A, VEGFA, HMOX1, IL6, CASP3, EPO, TP53, PTGS2, SLC2A1, and CXCL8 and chemical-gene interaction results given as bar graph in (Fig. 1A). After then, The String Database used for gene-gene interaction analysis and given in (Fig. 1B). It has been shown that these top ten genes generally interact with each other and has several connections (edges). In Figure 1B, by using Enrichr (MSigDB) it has also shown that these top ten genes interacting with the CoCl₂, contribute to different kind of pathways but especially resulted in hypoxia.

Western blotting results
The TRPM7 cation channel protein expression levels were evaluated by western blotting and after β-actin normalization given in (Fig. 2). Relative TRPM7 protein expression levels in the control group were very higher than TRPM7 siRNA group but lower than in only CoCl₂ induced group. So, it was concluded that CoCl₂ incubation increases the TRPM7 channel expression levels. On the other hand,
in the TRPM7 siRNA+CoCl2 group, the protein level was also lower than in only CoCl2 induced group. Hence, it was concluded that the downregulation of TRPM7 channels was decreased to channel protein levels as compared to only the CoCl2 group.

**Calcium signal findings**

The TRPM7 cation channels-mediated elevation of [Ca^{2+}]_i was measured by Fura-2 probed calcium signaling were given in (Fig. 3). The line graph (Fig. 2A) is shown that real-time calcium signaling results (F340/F380) while in the bar graph (Fig. 2B) has shown the cytosolic concentration of calcium as nM. In only CoCl2 group, [Ca^{2+}]_i levels were high which compared to TRPM7 siRNA and Control groups. However, calcium levels were very low in TRPM7 siRNA + CoCl2 group as compared to only CoCl2 group. It is concluded that the downregulation of TRPM7 channel was also decreased TRPM7-related activity-mediated [Ca^{2+}]_i levels by reducing the effects of CoCl2.
Apoptosis and cellular viability levels of the study groups were measured, and the results were given as bar graph in (Fig. 4A & B). In CoCl₂ group, apoptosis levels were higher than in the control and TRPM7 siRNA group. Moreover, TRPM7 siRNA + CoCl₂ group’s cell viability levels were higher than only CoCl₂ incubated hypoxia group. It was observed that the downregulation of TRPM7 channels decreased apoptosis, and decreasing TRPM7-related calcium trafficking could have positive effect on cell viability in SH-SY5Y neuronal cells after CoCl₂ incubation.

**Caspase 3, 8 and 9 enzymatic activity results**

The caspase 3, 8 and 9 enzymatic activities among the groups were given in (Fig. 5A & B). In the CoCl₂ group, caspase activities were higher than in the control and TRPM7 siRNA group. While enzyme activities in only the TRPM7 downregulated group was very lower than in control group. It is observed that the downregulation of TRPM7 cation channels decreased apoptosis via caspase-mediated pathways against CoCl₂ application.

**iROS production and MMD results**

Measurements of the iROS production and MMD levels were given in (Fig. 6A-C). In the CoCl₂ group, iROS production and MMD levels were lower than TRPM7 cation channel downregulated group, while levels in the downregulated group were much lower.

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**Fig. 4 — Measurement of iROS production (A) and MMD; and (B) of the study groups.** a: $P < 0.001$ vs. the Control group, b: $P < 0.001$ vs. the siM7 group, c: $P < 0.001$ vs. the CoCl₂ group (mean ± SD; n=6)

**Fig. 5 — Measurement of apoptosis and microscopic images of the study groups.** Apoptosis results of study groups a: $P < 0.001$ vs. the Control group, b: $P < 0.001$ vs. the siM7 group, c: $P < 0.001$ vs. the CoCl₂ group (mean ± SD; n=6). Inverted microscopy images (200x) have shown relative cellular density of study groups

**Fig. 6 — The (A) caspase 3; (B) caspase 8; and (C) caspase 9, enzyme activity findings among the study groups.** a: $P < 0.001$ vs. the Control group, b: $P < 0.001$ vs. the siM7 group, c: $P < 0.001$ vs. the CoCl₂ group (mean ± SD; n=6)
than in the control group. It is observed that TRPM7 cation channel downregulation decreased iROS and MMD values of CoCl₂ incubation.

Discussion

Oxygen consumption is very important for body cells in metabolic processes. Due to their high oxygen necessities, neurons are vulnerable to oxidative stress caused by the electron transport chain in mitochondria, however decreasing oxygen levels and in hypoxic conditions (~1% O₂) reactive oxygen species production is also triggered because of mitochondrial dysfunction⁴³. Recent studies have shown that some TRP channel superfamily subtypes including TRPM7 are sensitive to oxidative stress products and the activation pattern of the channels can be regulated by several antioxidants⁹,³⁴. TRPM7 channels have also unique structures because of both cations conducting channel and enzyme characteristics⁵⁵. It is also known that calcium ion has an important role in ischemia progression, calcium overload in the cells trigger apoptotic mechanisms, mitochondrial membrane depolarization, intracellular ROS production, caspase enzyme activities and finally cell death⁶. Since TRPM7 cation channels are essential for embryonic development, cellular death is triggered when they are genetically deleted. Hence, in TRPM7-related pathologies, it seems that antagonist usage for inhibition of TRPM7 channel activation and suppression of TRPM7 channel expression by siRNA applications are only two ways for controlling of TRPM7 channel gating³⁶,³⁷. Therefore, in the current study, it was aimed to investigate the role of TRPM7 channel downregulation on hypoxia induced by CoCl₂ in neuronal cells. With the bioinformatics approach, when the top ten genes were investigated it was found that these genes were intervened in hypoxia-related pathways and gene expression levels alters by CoCl₂ administration, as a hypoxia-mimicking agent. These findings also show the chemical hypoxia model’s efficiency and representing of real/physical hypoxia by using CoCl₂. Several research have also shown that TRPM7 channel may contribute to ischemia and hypoxia, and moreover its harmful effects may be regulated by its potent antagonist carvacrol²¹,³⁸,³⁹. Yang et al. have investigated the inhibition of TRPM7 channel activity in a myocardial in vitro ischemia model. They have observed that increasing of TRPM7 channel expression by hypoxic conditions also results in high expression of HIF1A and elevation of intracellular calcium levels and downregulation of TRPM7 channel expression by miR-22-3p inhibits injury on cardiomyocytes⁴⁰. In another study, it was reported that cobalt administration increased TRPM7 channel expression levels in rat hearts⁴¹. In a novel study, Turlova et al. also investigated the role of TRPM7 on neurite outgrowth under hypoxic conditions induced by low oxygen levels (5% O₂) in embryonic primary hippocampal neurons. They have reported that short-term hypoxia reduced TRPM7 channel activity and induced neurite outgrowth, however, long-term hypoxia enhanced TRPM7 channel activity but caused axonal retraction⁴². In a former study, Aarts et al. also reported that TRPM7 currents increased in oxygen-glucose deprivation model in mouse cortical neurons and led to anoxic neuronal death⁴³. In the current study, it was also observed that TRPM7 channel expression elevates by the incubation of CoCl₂, and this is a novel finding also for the current literature for human neuronal cells. Similarly, calcium signaling results have shown the channel activity also increases by the chemical hypoxia. However, the downregulation of TRPM7 channels by siRNA application contributes to decreasing calcium signals and increasing cellular viability in neuronal cells. As a well-known cellular pathway, excessive increase of intracellular calcium levels triggers mitochondrial membrane depolarization, and it causes to iROS production. Elevation of iROS production could cause to oxidative stress-related cell death⁴⁵. Hence, in the study, it was also measured to elucidate how TRPM7 silencing affect mitochondrial membrane depolarization, iROS production and caspase activities in SH-SY5Y neuronal cells. It has been demonstrated that CoCl₂ administration increases MMD and iROS production levels and silencing of TRPM7 channels could potentiate the levels to control. The results were very similar in caspase 3, 8 and 9 enzymatic activities, it has also been shown that prevention of TRPM7 channel activity by its down regulation could reverse increased caspase activity and protect cells from apoptosis. In conclusion, in this study, it was concluded that TRPM7 channels could be a potential therapeutic target for the treatment of neuronal hypoxia and have a protective role in neuronal damage after hypoxia. New TRPM7 channel blockers, which are systemically non-toxic and had fewer side effects for usage, and downregulation strategies would be future curative aspects for hypoxia.

Conflict of interest

The author declares no conflicts of interest.

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