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Research Article

EXPRESSION PATTERN OF BK CHANNELS UNDER VARIOUS OXIDATIVE STRESS CONDITIONS IN SKELETAL MUSCLES

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Abstract: *BK (large conductance Ca^{2+}-activated potassium) channels are expressed in myocytes, but changes in their molecular levels in the presence of oxidative stress are not clear yet. Determination of the molecular effects of various oxidative stress conditions may reveal the possible mechanism and potential therapeutic effects. In the present study, isolated rat soleus muscle where KCNMA1 genes encoding BK channel protein expressed widely in skeletal muscle, were exposed to cyclopiazonic acid (CPA) and hydrogen peroxide (H_2O_2) as oxidative stress inducers. A streptozotocin-induced diabetes mellitus model was also used to demonstrate the effects of an endogenous source of oxidative stress. Moreover, NS1619, a BK channel opener, was used to determine whether activation of the channel re-regulated the channel expression. After the incubation periods, KCNMA1 gene expression levels in each group were determined by real-time PCR experiments. While CPA and H_2O_2 decreased KCNMA1 expression significantly, the expression did not change under the systemic diabetes mellitus condition. However, the transcriptional level significantly decreased in diabetes in the presence of H_2O_2. On the other hand, KCNMA1 expression was re-regulated back to the control level by the addition of NS1619 in only H_2O_2 groups. The results demonstrated for the first time that acute oxidative stress, rather than systemic conditions, affects the KCNMA1 gene expression level in skeletal muscles. The study also showed the effects of NS1619 on the regulation of the transcriptional levels of BK channel protein under hydrogen peroxide conditions.*

Keywords: *Skeletal muscle, BK channels, Oxidative Stress, KCNMA1, NS1619*

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1. Introduction

Large conductance Ca^{2+} activated K^+ (BK) channels, one of the diverse groups of potassium channels, have crucial roles in the regulation of membrane potentials. The channels are expressed in many cells, including muscles and nerves, and they are responsible for many physiological and pathophysiological processes due to their unique negative feedback mechanism in determining intracellular calcium concentration [1, 2]. Given their important role in vasodilation/vasoconstriction, functional changes in the channels in smooth muscles may result in arterial hypertension or hypoxia [3]. BK channels also control cardiac pacing and heart rhythm [4, 5]. Moreover, the channels located at presynaptic regions in the central nervous system (CNS) regulate neurotransmitter release and neuronal firing by modulating K influx and the intracellular Ca^{2+} concentration [6, 7]. Therefore, an imbalance or dysregulation in the function of the channel may lead to important CNS diseases, such as epilepsy and cerebral ischemia [6]. The BK channels are also expressed in skeletal muscles, besides smooth and cardiac muscles. Dysregulations in normal BK channel activity are often associated with hyper/hypokalemic periodic paralysis [8]. In addition, the alpha subunit of the BK channels which is the

functional unit including the pore-forming structure is encoded by *KCNMA1* gene and is located on chromosome 10 (10q22.3) in humans [2]. Alterations in *KCNMA1* gene expression have also been linked to some diseases including temporal lobe epilepsy [9] and breast cancer [10]. Moreover, more than 10 *KCNMA1* mutations that change the normal activity of the BK channel, have been identified [2]. Considering the striking relations between BK channels and such diseases, targeting the regulation of these channels through gene therapy and activating/blocking the channels are important treatment approaches that should be developed in the future [8, 11, 12].

Reactive oxygen species (ROS) are maintained at relatively low levels in healthy tissues, and they participate in many physiological processes, such as apoptosis and protein phosphorylation. However, their production is balanced with antioxidant defense systems otherwise, excessive amounts of ROS can result in cellular damage, which is called "oxidative stress". Moreover, there are many oxidative stress-related diseases, such as cancer, and cardiovascular or neurodegenerative diseases [13, 14]. ROS are generated in not only metabolic conditions but also in contraction activities in skeletal muscles [15]. However, an abnormal increase in the ROS level commonly leads to muscle fatigue associated with intense exercise. In an extreme situation, it can lead to muscle degeneration and muscle loss if oxidative stress increases further and is not prevented by antioxidant mechanisms [15, 16]. On the other hand, the modulatory effect of ROS on BK channels has been shown in many different cells and tissues [17]. Hydrogen peroxide (H_2O_2), which is a non-radical ROS, changes the opening probability and activities of the channels in HEK cells or the coronary artery [18, 19]. However, the effect of ROS on BK channels in skeletal muscles is not clear, even though it is well known that there is a significant expression of the channels in these muscles. It has been shown that BK channels are more expressed in slow-twitch rat fibers than in fast-twitch fibers [8, 12].

Oxidative stress can be induced endogenously or exogenously. One of the main associated diseases related to oxidative stress is diabetes. Excessive ROS production is linked to hyperglycemia and vice versa [20, 21]. While some chemicals are introduced exogenously, such diseases can be an endogenous source of oxidative stress. Therefore, the present study aimed to determine the effects of various endogenous and exogenous oxidative stress conditions on the *KCNMA1* gene in rat soleus muscles having slow-twitch fibers. On the other hand, NS1619, a BK channel activator, reverses the effects of oxidative stress on the channel in various conditions, while its effect at the gene level is unclear. Therefore, the present study also aimed to determine the effect of NS1619 on the channel gene expression.

2. Material and Methods

2.1. Animals

All experimental procedures involving animals in the study were approved by Cukurova University Local Ethics Committee on Animal Experiments (Decision Date: 24.08.2017; Decision Number: 7). In the present study, thirty-two adult male Wistar albino rats, weighing approximately 250-300 g, were kept in stable laboratory conditions with a 12h:12h photoperiod and 22±1 °C room temperature during all animal experimental procedures. They were given free access to standard food pellets and water. Rats were equally and randomly divided into groups of controls and diabetes, before incubation processes.

2.2. Induction of diabetes by streptozotocin (STZ) injection

In the present study, animals in the diabetes groups were injected with 45 mg/kg STZ (Sigma S0130) as described earlier [22]. Rats in the control groups were injected with equivalent physiological saline. Blood glucose levels should be >250 mg/ dL to define diabetes mellitus (DM) which is correlated

with hyperglycemia [23]. Hyperglycemic conditions in the diabetes groups were confirmed by measuring the blood glucose levels 3 days after the injection. The hyperglycemic indexes of the animals were checked every week for 4 weeks.

2.3. Exogenous incubations

After the rats were euthanized with an overdose of isoflurane, both soleus muscles of each rat were immediately dissected by sterile surgical techniques. Afterwards, the tissues were placed in an organ bath containing Krebs solution (112 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 11.5 mM glucose; pH 7.4) and were aerated with 95% O_2 and 5% CO_2 at 37°C. Chemicals used in the present study were added to the bath solution by an appropriate micropipette following a stabilization period for the tissues of about an hour. During the experiments, the tissues were incubated with 1 mM H_2O_2, 30 μM NS1619, or 20 μM cyclopiazonic acid (CPA), which produces oxidative stress [24].

After the end of the incubation period, the tissues were taken from the tissue bath, freeze shocked in liquid nitrogen, and stored at -80 °C.

2.4. mRNA expressions

In the present study, RNA isolation of the soleus tissues was performed by using a Nucleozol reagent (Macherey Nagel-MN). For this purpose, tissues were homogenized first with the help of a glass homogenizator containing 1 mL of the Nucleozol solution. RNA was isolated by following the steps of the kit instructor's manual, and cDNA samples were synthesized by using high-capacity cDNA reverse transcription kit (Applied Biosystems) in a total of 20 μL.

Expression levels of the *KCNMA1* gene in each group were determined by real-time PCR experiments. After the preparation of a 20 μL reaction mix containing 1 μL of cDNA sample, 10 μL of GoTaq qPCR master mix (Promega), 0,5 uL of each primer and 8 μL of RNase-free water, a conditional temperature program was performed (95 °C for 2 min for a cycle, 95 °C for 15s, and 60 °C for 60 s for 40 cycles) for the reaction mix in a Biorad CFX96 thermal cycler.

Expression levels in each group were determined by a relative quantification method known as the $2^{-\Delta\Delta Ct}$ method [25]. GAPDH was used as a housekeeping gene in the study.

KCNMA1 and GAPDH gene amplification primers used in the study are shown in Table 1.

Table 1. Primers used in the study

Primer Name	Genbank Accession Code	Primer sequences (5'→3')
KCNMA1-Forward KCNMA1-Reverse	NM_031828.2	AAGGGCTGTCAACATCAACC CTGTCCATTCCAGGAGGTGT
GAPDH-Forward GAPDH-Reverse	NM_017008.4	AAGATGGTGAAGGTCGGTGT TGACTGTGCCGTTGAACTTG

2.5. Statistics

In the study, one-way ANOVA (performed in GraphPad Prism 5.0) was used to indicate statistical significance between groups ($p < 0.05$). Data analysis results have been presented as mean ± SEM.

3. Results

3.1. The effects of muscle incubations with H_2O_2 and H_2O_2 +NS1619 on the KCNMA1 expression level

Incubation of the soleus muscles with H_2O_2 (1 mM) for 60 minutes led to a statistically significant reduction in the BK channel expression at the transcriptional level (Fig 1). Hydrogen peroxide decreased the *KCNMA1* expression by 70% (0.296 ± 0.06). In order to determine the effects of NS1619 on oxidative stress via BK channels, muscles were incubated with H_2O_2 (1 mM) and NS1619 (30 µM) (H_2O_2 + NS1619) in a tissue bath for an hour. The gene expression levels also decreased (0.747 ± 0.14) but the difference was not statistically significant when compared to that in the control group. More importantly, gene expression levels for H_2O_2 and H_2O_2 + NS1619 were significantly different.

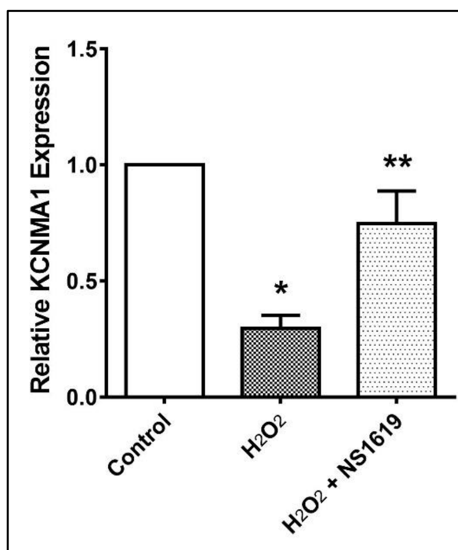

Figure 1. Effect of H_2O_2 and NS1619 on KCNMA1 expression. The soleus muscles were incubated with H_2O_2 (1 mM), and H_2O_2 (1 mM) + NS1619 (30 µM). Each group's data were normalized to control data. Bars indicate mean ± SEM, n = 6-8 (each measurement performed in duplicated). * demonstrate the difference between control and H_2O_2 ($p < 0.05$), and ** demonstrate the difference between H_2O_2 and H_2O_2 + NS1619 ($p < 0.05$).

3.2. The effects of CPA on the KCNMA1 gene expression level

Muscles were incubated for 1 hour with CPA, a SERCA (Sarco/Endoplasmic Reticulum CaATPase) inhibitor. The *KCNMA1* transcriptional level decreased with the presence of CPA (0.275 ± 0.07) (Fig 2). The difference was statistically significant when compared to that in the control group. In addition, the *KCNMA1* gene expression level was significantly different in the CPA + NS1619 group when compared to that in the control group, as the gene expression level was 0.473 ± 0.07 in the CPA + NS1619 group. In the meantime, there were no meaningful differences in terms of the BK channel transcriptional level between the CPA and CPA + NS1619 groups.

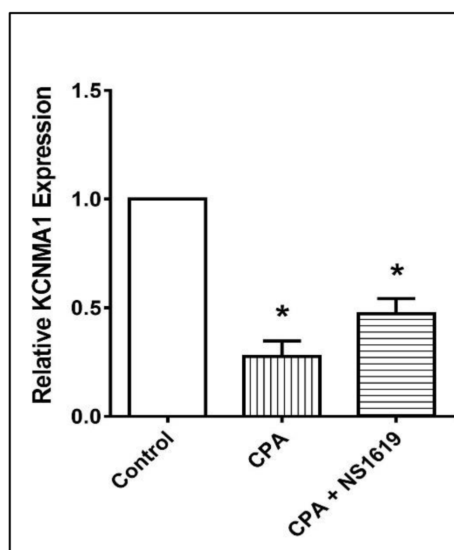

Figure 2. Effect of CPA and NS1619 on *KCNMA1* expression. The soleus muscles were incubated with CPA (20 µM), and CPA (20 µM) + NS1619 (30 µM). Each group's data were normalized to control data. Bars indicate mean ± SEM, n = 6-8 (each measurement performed duplicated). * demonstrate the difference between the control group and the other groups ($p < 0.05$).

3.3. KCNMA1 gene expression level under diabetic conditions.

In the study, at first, the *KCNMA1* gene expression level in the soleus muscles in the STZ-induced diabetes groups was compared to that in the control group (Fig 3). Although the gene expression decreased under diabetic conditions (DM group: 0.848 ± 0.18), the difference was not statistically significant when compared to that in the control group. Then, the soleus muscles in the DM group were divided into the DM + H_2O_2 and DM + H_2O_2 + NS1619 groups, and each group was incubated with the stated chemicals for an hour. The gene expression in the DM + H_2O_2 group significantly decreased when compared to that in the control group (0.311 ± 0.04). Moreover, *KCNMA1* gene expression did not change significantly in the DM + H_2O_2 + NS1619 group when compared to that in the control group (1.009 ± 0.11). However, the difference between the DM + H_2O_2 and DM + H_2O_2 + NS1619 groups was statistically significant.

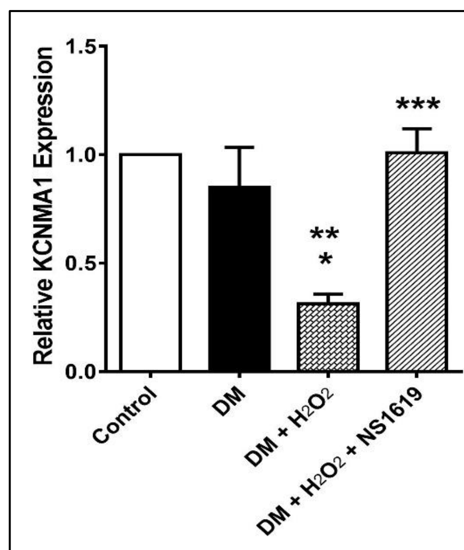

Figure 3. Relative *KCNMA1* expression under diabetic conditions. Each group's data were normalized to control data. DM soleus muscles were also incubated with H_2O_2 (1 mM), or H_2O_2 (1 mM) + NS1619 (30 µM). Bars indicate mean ± SEM, n = 6-8 (each measurement performed duplicated). * demonstrate the difference between the control and DM + H_2O_2 groups ($p < 0.05$),** demonstrate the difference between the DM and DM + H_2O_2 groups ($p < 0.05$), and *** demonstrate the difference between the DM + H_2O_2 and DM + H_2O_2 +NS1619 groups ($p < 0.05$). DM: diabetes mellitus.

4. Discussion

The present study demonstrated the transcriptional effects of various oxidative stress conditions on BK channels in skeletal muscles in terms of the changes in the *KCNMA1* gene that encodes the channel protein. In addition, the effect of channel activation on this stress was also examined through changes in the *KCNMA1* expression level.

Oxidative stress was induced by 3 methods in this study. Hydrogen peroxide is widely used as an oxidative stress agent in many studies [26]. Its production is increased during normal skeletal muscle exercise [27]. Moreover, H_2O_2 affects the release of Ca^{2+} from sarcoplasmic reticulum vesicles via ryanodine binding in skeletal muscles dose-dependently [28]. Therefore, we used H_2O_2, which has such a direct effect on the functionality of skeletal muscles, to generate oxidative stress in this study. In addition, previous studies have also shown the effects of H_2O_2 on BK channels. Byckhlow et al. showed that 1 mM H_2O_2 changed the channel activity in human endothelial cells [29]. Another study showed that hydrogen peroxide affected the membrane potentials via BK channels [30].

The present study demonstrated that incubation with H_2O_2 significantly decreased the *KCNMA1* gene expression level in skeletal muscles. To the best of our knowledge, the data are the first to show the effects of oxidative stress on BK channels at the transcriptional level. However, there were no meaningful expression changes in the presence of both H_2O_2 and a BK channel opener, NS1619. The results strongly indicated that NS1619 abolished the transcriptional effects of H_2O_2 on BK channels in skeletal muscles. Previous studies have demonstrated that NS1619 protects cells from H_2O_2-induced cytotoxicity, including neurons, epithelial cells, and astrocytes [31-33]. However, the present study demonstrated the reverse effects of a BK channel opener on H_2O_2-induced transcriptional dysregulation of *KCNMA1*.

Another factor that causes oxidative stress is the increase in Ca^{2+} concentration [34, 35]. Furthermore, CPA triggers a robust Ca^{2+} increase by blocking $Ca2^{+}$-ATPase [36], and such blocking induces oxidative stress [24]. Therefore, in the present study, CPA was used as another oxidative stress agent, which significantly inhibited the *KCNMA1* gene expression. Although the presence of NS1619 hindered the decrease in the gene expression level, the expression level did not return to the control level in the CPA+NS1619 group. BK channels are regulated in various signal pathways in different cells [37-39]. Considering the fact that NS1619 incubation did not make any difference to the transcriptional levels of *KCNMA1* in the presence of CPA, unlike in the case of H_2O_2, it could be claimed that NS1619 may show its transcriptional effects on BK channels via the mechanisms where H_2O_2 is active.

Previous studies have shown cells in systemic pathologies, such as diabetes mellitus, are exposed to oxidative stress at varying levels compared with healthy cells. In addition, many stress pathways are also activated in diabetes [40, 41]. Therefore, *KCNMA1* gene expression in the soleus muscles of diabetic rats was investigated, since diabetes was considered an oxidative stress inducer in the present study. Although the gene expression level decreased in the DM group, the change was not statistically significant. It is argued that various signaling pathways for a cell adapt to different stress conditions caused by DM or excessive ROS production [42]. Moreover, the findings of the present study may correlate with previous findings that the function of the BK channels may have been impaired, although the protein expression did not change [43]. In addition, a significant decrease in *KCNMA1* gene expression in diabetes with hydrogen peroxide may demonstrate that the transcriptional changes of the BK channel may result from the acute effect of hydrogen peroxide application. On the contrary, the metabolic system may have adapted the gene expression levels through feedback mechanisms in long-term diabetes conditions. In the present study, NS1619 reversed the gene expression level back to that in the control group in dual oxidative stress conditions (H_2O_2 plus diabetes). The results may again demonstrate that BK channel activation shows its regulatory effect via H_2O_2 active pathways. In previous studies, it was stated that NS1619 exerts its protective effect against cytotoxic agents through other pathways that are independent of the activation of the channel [44].

In conclusion, the present study showed that *KCNMA1* gene expression levels differed under various oxidative stress conditions. Moreover, the change in the expression could be dependent on the acute effects of oxidative stress. NS1619 re-regulated the channel expression back to levels seen in the control's under only hydrogen peroxide conditions. The results may suggest that this activator could be a useful potential therapeutic agent against diseases resulting from hydrogen-peroxide-induced oxidative stress. However, the topic requires further molecular research, since hydrogen peroxide could also have harmful effects as a DNA damager and apoptosis inducer for cells in addition to oxidative stress [45, 46]. Therefore, more experiments should be carried out in which the other effects of H_2O_2 are taken into account and evaluated.

Conflict of interest: The authors declare that there are no conflicts of interest
Compliance with Research and Publication Ethics: This study was carried out by obeying research and ethics rules.
Ethical statement: This study was approved by Cukurova University Local Ethics Committee on Animal Experiments. (Decision Date: 24.08.2017; Decision Number: 7)
Authors' Contributions:
C.C: Conceptualization, Methodology, Formal analysis, Data Curation, Investigation, Resources, Writing - Original draft preparation
F.A.C.: Conceptualization, Methodology, Formal analysis, Data Curation, Investigation, Writing - Original draft preparation

O.T.: Methodology, Resources, Formal analysis, Data Curation, Investigation, Writing - Original draft preparation,
I.O.: Methodology, Writing - Original draft preparation
All authors read and approved the final manuscript.

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