BK channel opener protects cell viability by regulating reactive oxygen levels in astrocyte cells

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Abstract. Astrocytes are greatly impacted by oxidative stress, which can also be related to neurodegenerative diseases. Therefore, preventing the production of reactive oxygen species (ROS) is crucial for maintaining healthy cells. Large conductance Ca^{2+}-activated big potassium (BK) channel openers are effective in eliminating the effects of oxidative stress. The present study aims to determine if NS11021, a BK channel opener, protects the astrocytes from harmful effects of hydrogen peroxide (H_2O_2), which is an oxidative stress inducer. For this purpose, primary astrocyte cultures were incubated with H_2O_2, NS11021, and Iberiotoxin both separately and together. H_2O_2 decreased cell viability by approximately 50% and increased the number of ROS-positive astrocytes. However, NS11021, but not Iberiotoxin, reversed the deleterious effects of H_2O_2 on cell viability and decreased ROS production. Moreover, dysregulations in Cyclin D1/CDK6/p21 gene expressions under conditions of oxidative stress were regulated again by the opener. To the best of our knowledge, this study has been the first to reveal that NS11021 reversed the deleterious effects of H_2O_2 on cell viability by regulating ROS production in astrocytes. Its effect may also be related to the regulation of cell cycle at the transcriptional level. NS11021 may also be used as an agent for the treatment of oxidative-stress related dysfunction of astrocytes.

Key words: Astrocytes — BK channels — NS11021 — Oxidative stress

Introduction

Astrocytes are one of the main and most abundant class of glial cells in the central nervous system (CNS). They play critical roles in many physiological processes, such as providing neuronal support, regulating synaptic signaling or plasticity, and mediating CNS homeostasis (Takuma et al. 2004; Kandel et al. 2013; Blanco-Suarez et al. 2017). Because of the astrocytic endfeet structure, they also notably control the cerebrovascular tone and cerebral blood flow as well as contribute to the formation and maintenance of a healthy blood–brain barrier (Abbott et al. 2006; Gordon et al. 2007,

2011; Howarth 2014). Given the importance of astrocytes in CNS, it is not surprising that dysfunction or impairment of astrocytes cause neurological diseases such as Alzheimer's disease (Talantova et al. 2013), Parkinson's disease (Bosson et al. 2015), Huntington's disease (Tong et al. 2014), and epilepsy (Coulter and Steinhauser 2015; Blanco-Suarez et al. 2017).

Reactive oxygen species (ROS) is normally produced during metabolic processes. However, an imbalance between defensive antioxidant mechanism and production of ROS may lead to oxidative stress, which has deleterious effects on many cells and functional defects (Dringen et al. 2005). High energy and oxygen consumption rates (i.e., approximately 20% of the total) indicate that the brain is greatly affected by oxidative stress (Chen et al. 2020). Moreover, many neurological diseases are linked to oxidative stress (Vieira de Almeida et al. 2008). Astrocytes have a more outstanding role in protecting the brain from oxidative stress damage owing

to the secretion of antioxidant enzymes and neurotrophic factors (Takuma et al. 2004; Cabezas et al. 2019; Chen et al. 2020). Nevertheless, they are also rapidly and greatly affected by ROS (Liddell et al. 2009; Hamdi et al. 2015). Therefore, protecting astrocytes from harmful effects of oxidative stress is important to prevent dysfunctional astrocyte-related diseases as well as maintain the antioxidative mechanism of astrocytes and an optimal number of astrocytes (Amri et al. 2017).

Astrocytes contain many ion channels, exchangers, and transporters that regulate homeostasis, osmosis, and dynamic function of the CNS and blood-brain barrier (Abbott et al. 2006). The main type of channel in astrocytes is the large-conductance big potassium (BK) channel activated by either intracellular calcium concentration or membrane voltage (Seifert et al. 2018). Activation of the channel initiates a large K^+ efflux and leads to close voltage-gated calcium channels; it also prevents the influx of more calcium into the cells (Vetri et al. 2014). Therefore, BK channel regulates many physiological processes such as calcium and neural signaling, ion homeostasis, or hormone secretion (Ghatta et al. 2006; Yang et al. 2015). Together with the Kir (inward rectifier potassium) channels, BK channel is responsible for buffering potassium in astrocytes. Moreover, astrocytic BK channels play a bidirectional role in the regulation of cerebrovascular tone because a calcium concentration of approximately 300 nm in the astrocytic endfeet leads to vasodilation, whereas a higher concentration mediates vasoconstrictive movements (Kim et al. 2019). Therefore, BK channels have become a target for the therapy of diseases based on their essential roles (Kshatri et al. 2018). Moreover, the efficiency of the channel openers in eliminating oxidative stress from skeletal muscles and neurons is also demonstrated in previous studies (Yan et al. 2015; Coskun et al. 2020). However, the antioxidative role of the channel opener has not yet been studied in astrocytes. Given the importance of eliminating the effects of ROS on astrocytes, the present study aimed to determine whether NS11021, a BK channel opener, protects astrocytes against cell death caused by oxidative stress. The effect of the opener was also evaluated at transcriptional levels of the cell cycle to achieve a better resolution. Moreover, Iberiotoxin, a BK channel inhibitor, was used as an internal control to evaluate the effect of the channel on ROS-induced mechanisms in astrocyte cells.

Material and Methods

Primary culture of astrocytes

The Local Ethical Committee approved this study for Experimental Research at Pamukkale University. Five-day-old Wistar rat's brains were used for the preparation of astrocyte cell cultures by varying previously reported methods (Cole and de Vellis 2001; Becerir et al. 2013). Dissected brains were mechanically minced, separated and then passed through a nylon sieve (pore size, 70 µm) followed by centrifugation for 5 min at 1500 rpm. After cell collection, pellets were suspended in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) (Biochrom, Germany), containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Thermo Scientific, UK), 500 µl gentamicin (10 mg/10 ml, Sigma-Aldrich, USA), and 5 ml fungizone (25 µg/ml amphotericin B, Gibco Invitrogen, New York, USA). After the resuspended cells were plated on poly-d-lysine-coated 75-cm^2 flasks, they were incubated in steady conditions at 37°C with 5% CO_2; the media was changed every 3 days. After a week of culturing, other cells were detracted from the astrocyte monolayer by shaking the culture in an orbital shaker for 1 h. Astrocyte cells were collected after 8–10 days, and cell pellets were resuspended in the astrocyte medium [DMEM/F12-containing 500 µl gentamicin, 5 ml fungizone, 15% FBS, 5 ml L-glutamine (200 mM, Gibco, Invitrogen, New York, USA), and 500 µl insulin (0.5 mg/ml, Biochrom, Berlin, Germany)]. The method was estimated to given ~95% astrocyte purity in cultures, and these astrocyte cell cultures were used for further experiments.

Experimental design

After preparation, cells were exposed to certain chemicals to achieve the goals of this study. 1 mM hydrogen peroxide (H_2O_2) was used as an oxidative stress inducer. While the dose of H_2O_2 was chosen by considering the previous studies measuring similar experimental parameters (Terashvili et al. 2012), the exposure time of the chemical was determined by analyzing the effect of H_2O_2 on cell viability at different time points of 30, 60, and 120 min.

After determining the application dose and time period for H_2O_2 exposure, the cells were separately treated for 30, 60, and 120 min with 3 µM NS11021 (NS11021 group), 3 µM NS11021 following 1 mM H_2O_2 (NS11021+H_2O_2 group), 50 nM Iberiotoxin (IbTx group), and 50 nM Iberiotoxin following 1 mM H_2O_2 (IbTx+H_2O_2 group). In the present study, Iberiotoxin was also used to evaluate if H_2O_2 affects BK channels.

Evaluation of cytotoxic effect of chemicals

To determine the toxicity of H_2O_2, we subjected the cells to H_2O_2 treatment for different time periods (30, 60, and 120 min). After the incubation period, the medium was removed and cells were incubated with a fresh medium. To evaluate the possible proliferative effects of NS11021 and Iberiotoxin, we treated the cells with NS11021 (3 µM) and Iberiotoxin (50 nM) for 60 and 120 min, respectively, after

H_2O_2 treatment (60 min). Additionally, we treated the cells separately with NS11021 and Iberiotoxin for 60 and 120 min. For all cell viability experiments, 3×10^4 cells were seeded in each 96-well plate. Cell survival was analyzed using Cell Counting Kit-8 (Boster Bio, USA). The absorbance was measured at 450 nm using a Promega Glomax multi-detection microplate reader (Promega, USA). The absorbance values obtained were used to calculate the percentage of cell viability using the formula $100\times A1/A0$, where A1 (incubation hour) and A0 (0th hour) are the absorbance values of treated and control cells, respectively.

ROS detection

According to the manufacturer protocol, intracellular ROS levels were evaluated using the Cellular ROS/RNS Detection Assay Kit (Abcam, USA). Briefly, cells were plated onto a 24-well plate, and following the treatments, cells were captured under a fluorescence microscope at 490–650 nm. The fluorescence-positive cells were measured using the ImageJ program.

Real-time PCR

In the present study, the effects of H_2O_2, BK channel activator, and inhibitor on the cell cycle of astrocyte cells, which are critical for maintaining cell viability were also investigated at the transcriptional level to obtain detailed molecular information. For this purpose, the gene expression levels of Cyclin D1, CDK6, p21, which are some of the most critical regulators at the G1 checkpoint, in the treated cells were determined by performing real-time PCR experiments. For this purpose, astrocyte cell lines were seeded into 6-well plates. At the end of the incubation period, total RNA was extracted using 500 µl Trizol reagent (Qiagen, Germany). After RNA was harvested from the cells, cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The qRT-PCR reaction was performed on the Bio-Rad CFX-96 device using 2×SYBR green (QuantiTect, Qaigen) master mix. Primers used in real-time PCR experiments are shown in Table 1.

Table 1. Primers used in the Real-time PCR experiments

	Sequences (5'->3')
CDK6 Forward	TTGTGACAGACATCGACGAG
CDK6 Reverse	GACAGGTGAGAATGCAGGTT
Cyclin D1 Forward	CAGACCAGCCTAACAGATTTC
Cyclin D1 Reverse	TGACCCACAGCAGAAGAAG
p21 Forward	AGTATGCCGTCGTCTGTTCG
p21 Reverse	GAGTGCAAGACAGCGACAAG
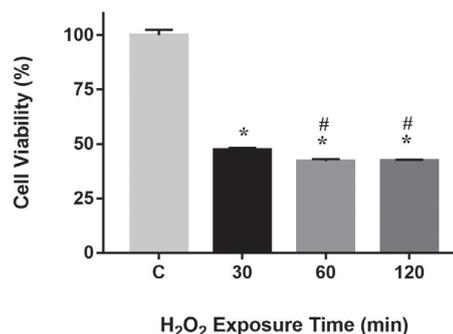
Figure 1. The cell viability (%) of astrocytes after treatment with H_2O_2 for 30, 60, and 120 min. Data (mean ± SD, $n = 3$) are provided by normalizing the cell numbers with the control cell numbers. * $p < 0.05$ *vs.* Control (C) group, $^{\#} p < 0.05$ *vs.* 30 min H_2O_2-treated group (one-way ANOVA followed by *post hoc* Tukey's test).

Statistical analysis

Here, one-way ANOVA and post-hoc Tukey's tests were performed to determine any difference between groups. Statistical analyses were conducted using GraphPad Prism (Graphpad Software, San Diego, CA, USA).

Results

In the present study, the cell viability of astrocytes in the presence of 1 mM H_2O_2 is demonstrated in Figure 1. At each exposure time, H_2O_2-induced cell death was >50% in comparison with the control (47.22 ± 1.06%; 42.13 ± 0.94%; 42.34 ± 0.45% for 30, 60, and 120 min, respectively). When the groups were compared using one-way ANOVA, astrocyte cell viability after 30-min H_2O_2 exposure was found to be statistically different from that after 60- and 120-min H_2O_2 exposure. However, there were no significant differences between the cell viability of astrocytes subjected to 60- and 120-min H_2O_2 exposure. To avoid more harmful effects of H_2O_2 at 120 min, which could be lethal, we chose to treat cells with H_2O_2 for 60 min in further experiments of this study; this decision was also reinforced by the fact that a 60-min H_2O_2 exposure is more effective in causing cell death than a 30-min H_2O_2 exposure.

Figure 2 shows the effects of BK channel activator or inhibitor on cell viability of astrocyte cells in the presence or absence of H_2O_2. The viability of cells treated with NS11021 for 60 and 120 min (100.7 ± 0.59% and 98.71 ± 1.08%, respectively) which was similar to that of control cells, was significantly different from the viability of cells treated with H_2O_2 for 60 min (Fig. 2A). Most notably, the viability of H_2O_2-treated cells with BK channel activator as an additive (NS11021+H_2O_2 groups) for both 60 and 120 min (66.08
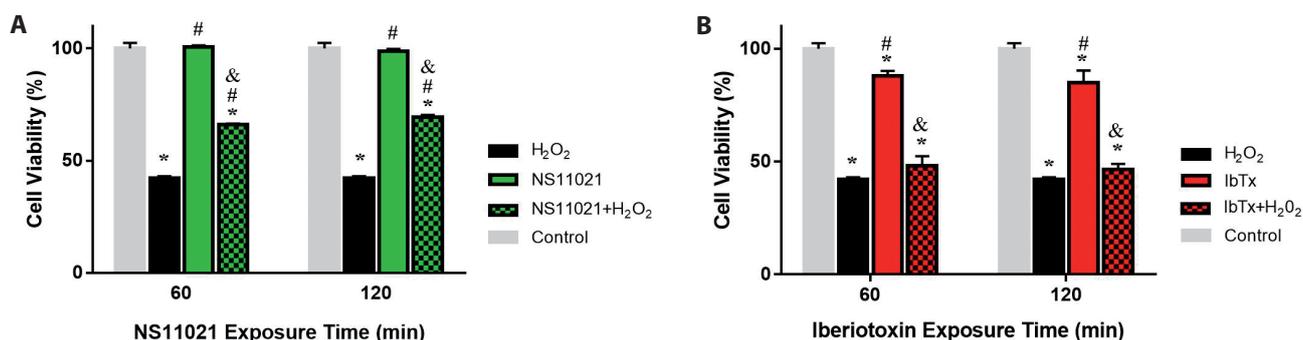
Figure 2. The viability (%) of cells after treatment with NS11021 (**A**) and Iberiotoxin (**B**) at different exposure times. Data (mean ± SD, $n = 3$) are given by normalizing the cell numbers with the control cell numbers. * $p < 0.05$ *vs.* Control group, # $p < 0.05$ *vs.* H_2O_2-treated group, & $p < 0.05$ *vs.* NS11021 or IbTx group (one-way ANOVA followed by *post hoc* Tukey's test). Control and H_2O_2 groups were not treated with NS11021 or Iberiotoxin. IbTx, Iberiotoxin.

± 0.43% and 69.34 ± 1.02%, respectively) was statistically higher than that of H_2O_2-treated and control groups. The viability of untreated cells significantly decreased after the addition of Iberiotoxin for 60 and 120 min (87.92 ± 2.26% and 85.05 ± 5.27%, respectively) in a dose-dependent manner (Fig. 2B). Nevertheless, the cell viability of these groups was still significantly higher than that of the H_2O_2-treated groups. Most importantly, the viability of H_2O_2-treated cells with Iberiotoxin additive (IbTx+H_2O_2 groups) for both 60 and 120 min (48.15 ± 4.25% and 46.47 ± 2.54%, respectively) was not statistically different from that of the H_2O_2-treated groups.

Considering that the treatment with NS11021 alone for 120 min is more effective in protecting cell viability than NS11021 treated with H_2O_2, and that present study aimed to investigate the efficiency of the BK channel opener, subsequent gene expression experiments were conducted using the cells subjected to chemical exposure (NS11021 or Iberiotoxin) for 120 min.

ROS-positive cells belonging to each group are shown in Figure 3. As expected, the control group has minor ROS

activity (19.33 ± 3.21%). With the addition of H_2O_2, about a third of four of the cell has ROS level (75.33 ± 7.02%) and its ROS activity were significantly different from that of control's (Fig. 3). Although the ROS activity of cells treated with NS11021 alone (20 ± 4.4%) was same as that seen in control cells (Fig. 3A), in comparison with the number of ROS-positive cells in the H_2O_2 group, it was found that after NS11021 addition in the NS11021+H_2O_2 group, the number of ROS-positive cells significantly reduced by half of what it was when treated with H_2O_2 alone (44.33 ± 6.11%). The ROS activity of Iberiotoxin-treated cells (44 ± 7%) was significantly higher than that of the control, but not as much as that of the H_2O_2-treated cells (Fig. 3B). In particular, ROS activity was the highest in the IbTx+H_2O_2 group (86.33 ± 4.93%) and the combined effect of Iberiotoxin and H_2O_2 on ROS activity was significant from the effect of Iberiotoxin alone but not from that of H_2O_2 treatment. Demonstrative illustrations of microscopic captions of cell viability and ROS-positive cells are provided in the Supplementary material (Fig. S1, S2 and S3).

Figure 4 demonstrates the targeted gene expression analysis of each group in the present study. Cyclin D1 gene
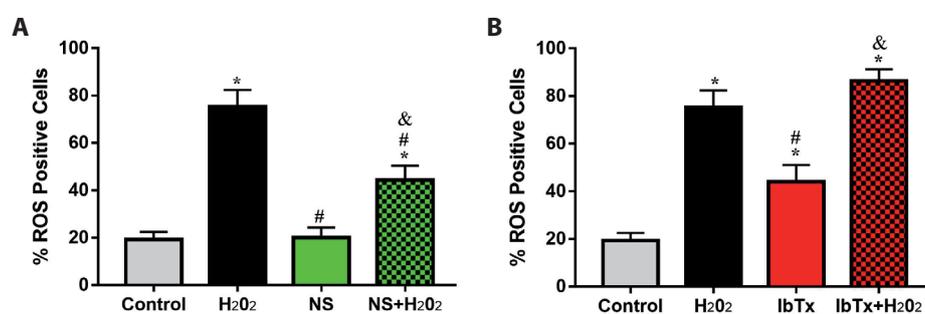
Figure 3. Percentage of ROS-positive cells after NS11021 addition (**A**) and Iberiotoxin addition (**B**). Data are the mean ± SD, $n = 3$. * $p < 0.05$ *vs.* Control group, # $p < 0.05$ *vs.* H_2O_2-treated group, & $p < 0.05$ *vs.* NS11021 or IbTx group (one-way ANOVA followed by *post hoc* Tukey's test). Control and H_2O_2 groups were not treated with either NS11021 or Iberiotoxin. NS, NS11021; IbTx, Iberiotoxin.

expression levels in the H_2O_2-treated group (0.44 ± 0.08) were significantly lower than those in the control group. The gene expression level in the NS11021-treated group (0.91 ± 0.02) significantly differed from the H_2O_2 group (Fig. 4A) but not with the control group. Cyclin D1 expression level in the NS11021+H_2O_2 group (0.78 ± 0.15) was significantly higher than that in the H_2O_2 group. The addition of Iberiotoxin significantly reduced the gene expression level (0.59 ± 0.12) as compared to the control's level (Fig. 4B). Moreover, both Iberiotoxin and H_2O_2-induced cells had the lowest

Cyclin D1 expression level in the IbTx+H_2O_2 group (0.10 ± 0.07). Similar results were obtained for CDK6 gene expression levels but data were not statistically significant because of a high standard error of the mean (Fig. 4C,D). The expression level of CDK6 was reduced in H_2O_2-treated cells (0.59 ± 0.15) when compared with that in the controls. Moreover, while NS11021 increased CDK expression (1.72 ± 1.09), Iberiotoxin reduced it (0.70 ± 0.45). When NS11021 was added to H_2O_2-treated cells, CDK6 expression increased to 0.70 ± 0.19 compared to H_2O_2. However, IbTx+H_2O_2 dramatically
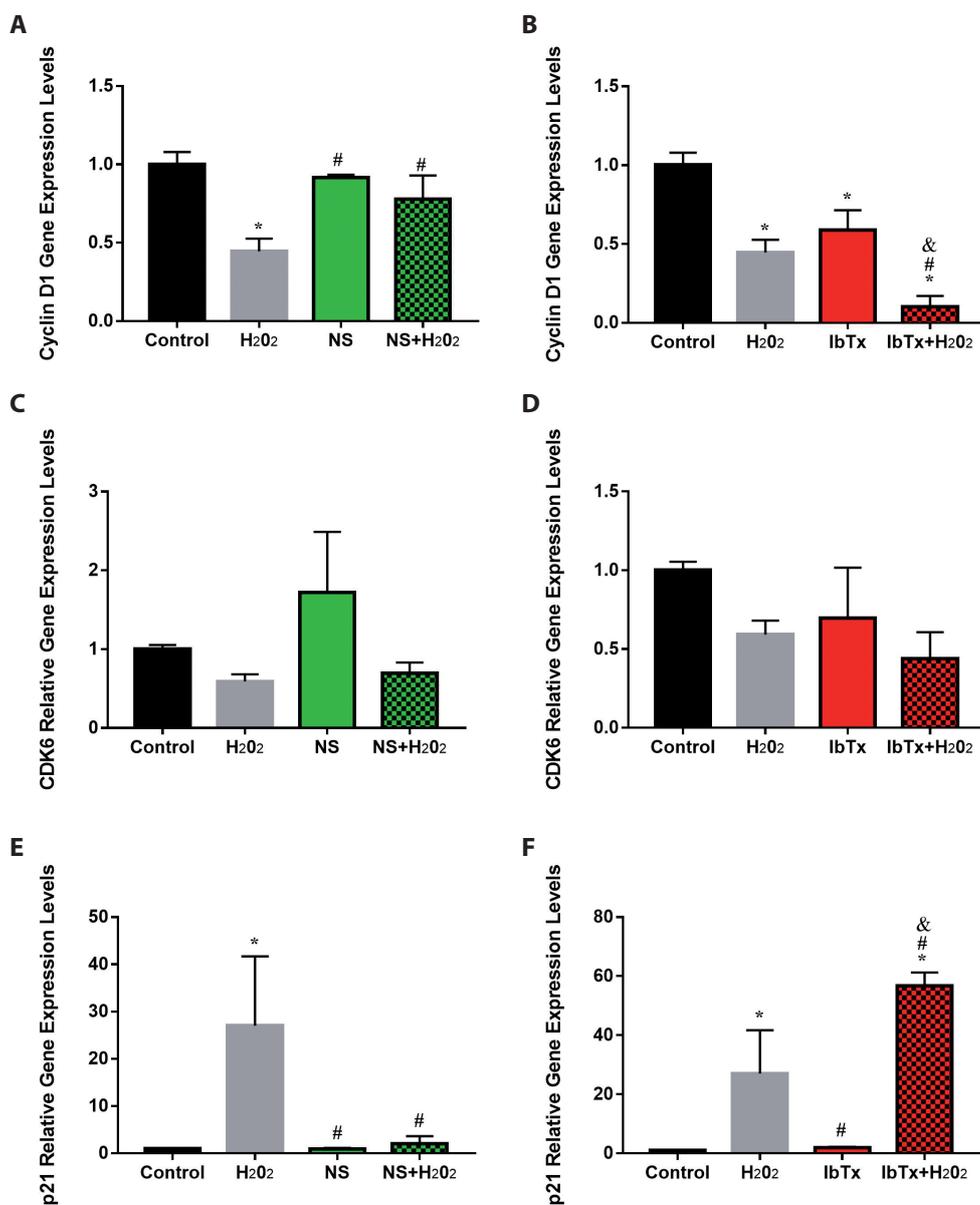
Figure 4. Gene expression levels of the targeted genes Cyclin D1 (**A, B**), CDK6 (**C, D**) and p21 (**E, F**) in the groups treated with H_2O_2, NS11021 (left) or Iberiotoxin (right). Data are the mean ± SD, $n = 3$. * $p < 0.05$ vs. Control group, # $p < 0.05$ vs. H_2O_2-treated group, & $p < 0.05$ vs. IbTx group (one-way ANOVA followed by post hoc Tukey's test). NS, NS11021; IbTx, Iberiotoxin.

decreased its expression to 0.44 ± 0.24. On evaluating p21 gene expression levels between groups, it was found that the gene transcriptional level significantly increased in the H_2O_2 group (27.04 ± 14.65) in comparison with the gene transcriptional level in the control (Fig. 4E,F). Although p21 levels in the NS11021 and NS11021+H_2O_2 groups (0.91 ± 0.23 and 2.07 ± 1.56, respectively) were not significantly different from the p21 level in the control, they significantly differed from the p21 level in the H_2O_2 group. Iberiotoxin-induced cells had a slightly increased p21 gene expression level (1.87 ± 0.34); however, this increase was not statistically significant. Notably, both Iberiotoxin and H_2O_2 mostly and significantly increased the p21 level (56.68 ± 4.58).

Discussion

With regard to oxygen consumption, the brain is one of the tissues most affected by ROS (Chen et al. 2020). Astrocyte cells, which possess defensive mechanisms against ROS, can also be easily affected by ROS. Additionally, since many neurological diseases are known to be associated with astrocytes, it is crucial to eliminate excessive ROS production to maintain a healthy antioxidative defense mechanism and prevent dysfunctional astrocyte-related disorders that can be caused by excess ROS (Amri et al. 2017; Chen et al. 2020). BK ion channels play an important role in regulating and homeostasis among many cells, including astrocytes (Yang et al. 2015; Seifert et al. 2018). Moreover, channel openers that activate the channels have been previously reported to prevent excessive ROS production in muscles and neurons (Yan et al. 2015; Coskun et al. 2020). However, to date, their effects on excessive ROS activity in astrocyte cells have not yet been demonstrated. The present study evaluated the action of NS11021, an effective BK channel opener, on ROS production in astrocytes; to our knowledge, the present study is the first to report that NS11021 reversed the deleterious effect of H_2O_2 on astrocyte cell viability in a time-dependent manner.

H_2O_2 is an ROS as well as a cell death inducer, and its effect at different concentrations and different exposure times has been frequently studied among various cells, including astrocytes. H_2O_2 has previously been used to induce astrocyte cell death at a concentration range between 0.1–1200 μM (Gorina et al. 2005; Kim et al. 2005; Choi et al. 2007; Terashvili et al. 2012). Based on a previous study (Gorina et al. 2005), it appears that the dose and application time of H_2O_2 which causes >50% cell death usually tends to be chosen. A similar strategy was also followed in this study, wherein the effects of 1 mM H_2O_2 were evaluated at different exposure times and cell death was analyzed. Even though each exposure time showed >50% cell viability, we chose to expose cells to H_2O_2 for 60 min as there

was no difference between the effects induced by 60- and 120-min exposure. The cell viability observed in this study was more or less compatible with that reported in a previous study when the same concentration of H_2O_2 was used for 60 min; the slight difference in the percentage of cell viability reported by Gorina et al. (2005) could be owing to measurement of cell viability 24 h after 60-min H_2O_2 exposure, and this prolonged incubation period could have increased the cell death induced by an irreparable ROS-induced damage. However, in the present study, we investigated the acute effect of H_2O_2. The results of H_2O_2 incubation on ROS production in astrocyte cells strongly demonstrated that H_2O_2 induced cell death by producing ROS; here, it was seen that as the ROS level increases, cell death in astrocyte also increases.

The effects of BK channels on cell viability were reported in previous studies. Dysfunction of the channel caused by mutations or inhibitors reduced cell viability in many cells, including neurons (Du et al. 2020). On the other hand, activation of the channel helped control cell death in various diseases and inhibited the proliferation of cancer cells (Borchert et al. 2013; Wojtovich et al. 2013; Sizemore et al. 2020). Our result was compatible with the result from a previous study (Hermann et al. 2015) that NS11021-treated astrocyte cells showed no significant difference from controls. In addition, activation of the channel by NS11021 also exhibited no difference in terms of ROS levels. These results might be important because the channel activators are targeted in various tissues, especially for muscle diseases such as periodic paralysis (Tricarico and Camerino 2011). It is also crucial that the use of channel activators to treat other tissue diseases does not cause any adverse effects on astrocytes with regard to cell death.

The most noteworthy result of this study was that NS11021 prevents H_2O_2-induced cell death in astrocytes. Moreover, the effect was dependent on the dose, i.e., a 120-min exposure of NS11021 was more effective in preventing cell death than a 60-min exposure. Even some previous studies showed the cryoprotective effects of NS11021 on pathological processes such as ischemia/reperfusion models (Borchert et al. 2013; Wojtovich et al. 2013), and a few studies showed how this channel opener could protect neuronal cells from oxidative stress conditions during the cell cycle (Gaspar et al. 2008; Yan et al. 2015); to the best of our knowledge, this study reported for the first time that NS11021 protects the viability of astrocytes under H_2O_2-induced oxidative stress conditions. On the other hand, when NS11021 was added to the cells that had already been exposed to H_2O_2, the number of ROS-positive cells was significantly lesser in the NS11021+H_2O_2 group than that in the H_2O_2 group. When these two results are evaluated together, it can be clearly elucidated that NS11021 exerts its protective effect on cells against oxidative stress by reducing ROS production. This

finding is also compatible with that of a previous study in which NS11021 was observed to exert its protective effect on neurons by decreasing ROS and exhibiting an antioxidative defense mechanism (Yan et al. 2015). On the other hand, because ROS is produced during normal physiological processes, it was expected to be produced in control cells (Forrester et al. 2018).

The correlation between cell death caused by Iberiotoxin-induced inhibition of BK channels and the increase in ROS level in the cells incubated with Iberiotoxin demonstrated that BK channels play a role in ROS-induced cell death of astrocytes. The result is also demonstrating the importance of targeting BK channels for eliminating the deleterious effects of oxidative stress conditions on astrocytes.

The cell cycle is controlled at certain checkpoints which determine if genome synthesis should continue or be stopped to begin cell division (Reece et al. 2014). Cyclin-dependent kinases, inhibitors, or growth factors play key roles in the regulation of these processes. One of the main checkpoints is at the G1 phase which determines whether the cell would enter the cycling processes or return to G0. Cyclin D1 and its dependent kinases, CDK6 and CDK4, are responsible for regulating the cell cycle and for controlling progression to the G1 checkpoint. On the other hand, cyclin-dependent kinase inhibitors (CDKi) regulate the cell cycle by binding specific kinases and suppressing its dependent kinase activity. Furthermore, p21 is one of the main CDKi which is usually active in response to DNA damage (Alberts et al. 2008). Exposure to external stimuli such as reactive oxygen species or radiation causes disruption of the cell cycle which in turn dysregulates the expression of Cyclin D1, CDK6, and p21 expression similar to other cyclin-dependent complexes (Kaneto et al. 1999; Masgras et al. 2012; Pyo et al. 2013). Therefore, re-regulation of the cyclic elements is essential for maintaining a healthy cell. Previous studies have shown that oxidative stress induced by H_2O_2 reduced and downregulated Cyclin D1 level in mammalian cells (Alao 2007; Pyo et al. 2013). In a similar manner, p21 expression increased in oxidative stress-induced pancreatic cells (Kaneto et al. 1999). In accordance with the data available in the literature, in the present study, we found that Cyclin D1 and CDK6 were downregulated, whereas p21 was upregulated after peroxide induction. Potassium ion flux throughout K^+ ion channels plays a prominent role in the proliferation of cells and regulation of the cell cycle, although its mechanism of action remains unclear (Urrego et al. 2014). The channels hyperpolarize the membrane potential at G1 phase of the cell (Urrego et al. 2014). Moreover, potassium channel blockers inhibit cell cycle progression (Abdul Kadir et al. 2018). The clear correlation between the channels and cell cycle previously indicated that the inhibition of potassium channels increases p21 expression and arrests the glial cells in the G1 phase (Ghiani et al. 1999). Here, inhibition of the BK channel

by Iberiotoxin decreased Cyclin D1 and CDK6 levels and increased p21 level. Therefore, this result, which is similar to the findings reported in the literature, may demonstrate that inhibition of the channel blocks cell cycle in the G1/S phase, induces apoptotic pathway *via* p21 accumulation, and decreases cell viability. These results clearly demonstrated that BK channels play an important role in cell cycle regulation and proliferation mediated by Cyclin D1/CDK/p21 pathway. NS11021, but not Iberiotoxin, reversed the effects of H_2O_2 on the expression of Cyclin D1/CDK6/p21 and re-regulated the transcriptional levels of these genes to resemble their expression in control cells. Therefore, it can be said that NS11021 may show its protective role against oxidative stress on cell viability by regulating the cell cycle process at the transcriptional level in the G1/S phase; however, this finding needs to be further validated by performing experiments at protein levels to elucidate an exact mechanism.

In conclusion, all results were evaluated together, and the importance and role of BK channels as well as theirs openers in astrocyte cells under oxidative stress conditions was demonstrated for the first time in the present study. Moreover, the BK channel opener protects cells from oxidative stress by reducing ROS production. The effects of the opener may also be a result of the regulation of crucial elements at the transcriptional level during G1/S transition. Therefore, it can be concluded that BK channel openers can be used as a therapeutic agent for maintaining cell viability of astrocytes and exerting antioxidative mechanism in order to manage oxidative stress-related diseases.

Conflict of interest. The authors have no conflicts of interest to declare.

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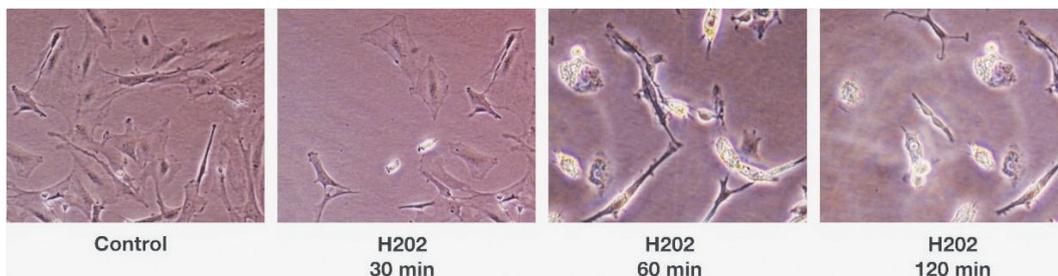https://doi.org/10.3389/fphys.2015.00029

BK channel opener protects cell viability by regulating reactive oxygen levels in astrocyte cells
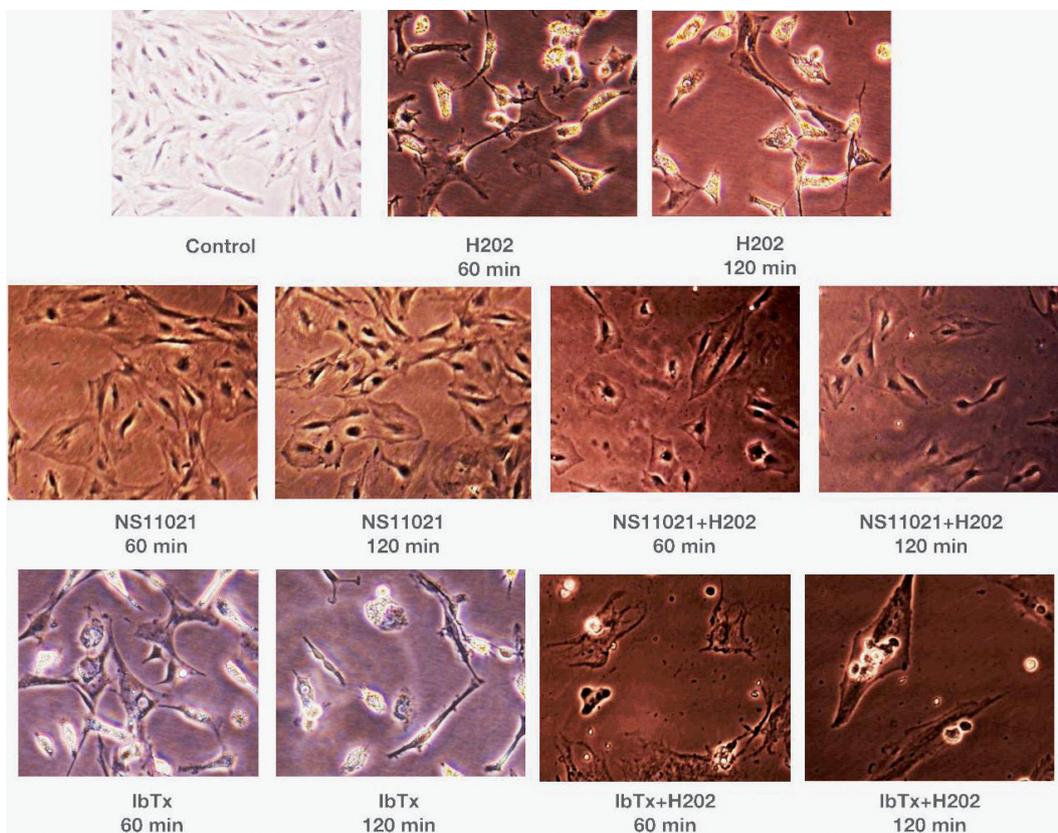
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Supplementary Figures

Figure S1. Representative microscopic captions (10× magnification) of astrocytes in the groups.

Figure S2. Representative cellular morphologies of treated and control astrocyte cells under the inverted microscope (10× magnification).

Control   H202   NS11021   NS11021+H202

Control   H202   IbTx   IbTx+H202
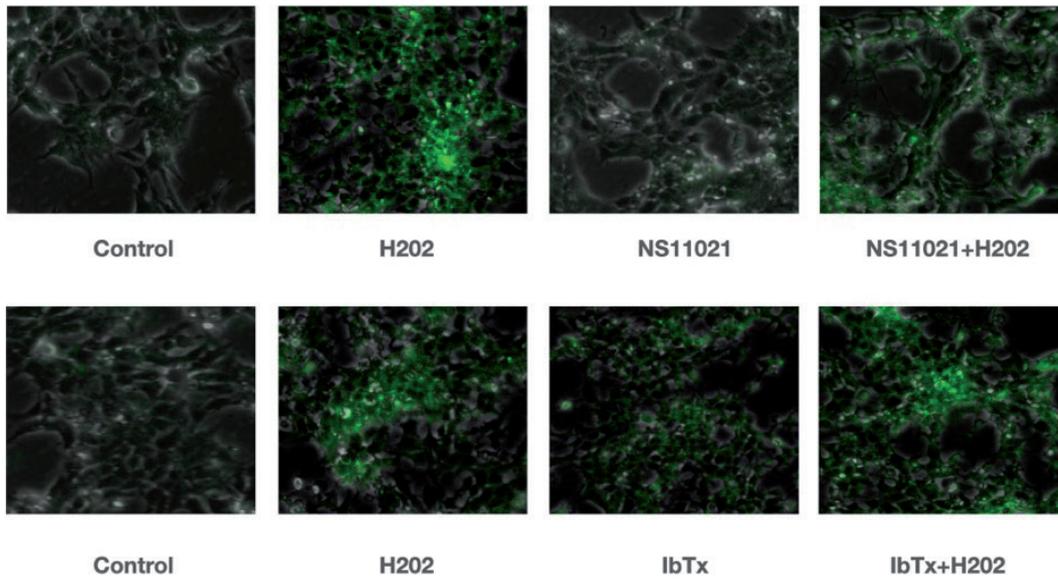
Figure S3. Representative flourescent images (20× magnification) of astrocytes in the groups.