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The Ca^{2+}-activated K^+ (BK) Channel-opener NS 1619 Prevents Hydrogen Peroxide-induced Cell Death and Mitochondrial Dysfunction in Retinal Pigment Epithelial Cells

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Potassium channel openers (KCOs) produce physiological and pharmacological defense mechanisms against cell injuries caused by oxidative stress of diverse origins. Openings of mitochondrial and plasmalemmal K^+ channels are involved in the defense mechanisms. This study tested whether NS 1619, an opener of large-conductance BK channels, has a similar beneficial influence on the pigment epithelial cells of retinas. The human retinal pigment epithelial cell line ARPE-19 was exposed to H_2O_2-induced oxidative stress in the absence and presence of NS 1619. The degrees of the cells' injuries were assessed by analyzing the cells' trypan-blue exclusion abilities and TUNEL staining. NS 1619 produced remarkable protections against cell injuries caused by H_2O_2. It prevented apoptotic and necrotic cell deaths. The protective effect of NS 1619 was significantly diminished when the cells were treated with NS 1619 in combination with the BK channel-blocker paxilline. NS 1619 significantly ameliorated cellular ATP deprivations in H_2O_2-treated cells. It helped mitochondria preserve their functional integrity, which was estimated by their MTT reduction abilities and mitochondrial membrane potential. In conclusion, it was suggested that NS 1619 had a beneficial effect on mitochondria in regards to preserving their functional integrity under oxidative stress, and it produces defense mechanisms against oxidant-induced cell injuries in ARPE-19 cells.

Key words : Cell death, KCOs, mitochondria, oxidative stress, RPE cells

Introduction

The retinal pigment epithelium (RPE) supports overlying photoreceptor cells and regulates transport across the blood-retina barrier. The relationship between the RPE and photoreceptor cells is crucial to sight. It has been demonstrated that dysfunction of the RPE can result in the death of visual cells and blindness [26]. Oxidative stress plays an important role in diverse types of RPE pathophysiology including age-related macular degeneration [24].

Large conductance Ca^{2+}-activated (BK) channels show increased open provability by a decrease in membrane potential or rise in the cellular Ca^{2+} concentration ($[Ca^{2+}]_i$). These characteristics of the BK channels allow them to serve as an important physiological regulator of the membrane potential or Ca^{2+} concentration in cells and mitochondria [16]. Wimmers [31] have shown the RPE cell line ARPE-19 as well as freshly isolated RPE cells express functionally active BK channels.

Maintenance of appropriate level of intracellular ATP is essential for optimal cellular function. Intracellular ATP is supplied primarily by through oxidative phosphorylation in mitochondrial machinery. Conditions that result in decreased oxygen supply or accumulation of reactive oxygen species (ROS) can compromise mitochondrial energetic machinery and lead to cell injury. Moreover, mitochondria itself is a principal source of ROS during ischemic or ischemia/reperfusion challenges [12, 30]. Although a lot of trials to find pharmacological tools to improve cell protection mechanisms have been suggested, no effective pharmacological tools that help mitochondria to preserve their function or morphological integrity have been developed.

It is now well documented that potassium channel openers (KCOs) can provide effective defense mechanisms to protect myocardium against ischemic or oxidative stress-induced cell injuries. ATP-sensitive K^+ (K_{ATP}) channels [28] as well as BK channels [1] are believed to play important roles to provide the protection mechanism. Initially, the protective effects provided by KCOs were believed to be due to activation of BK or K_{ATP} channels in plasma membranes. However, later studies demonstrated that the KCOs-induced defense

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mechanism could not be entirely attributed to the plasmalemmal K^+ channels, suggesting that interaction with additional cellular targets could mediate the action of KCOs. The overriding candidate is mitochondrial membranes which also express BK and K_{ATP} channels [14].

NS 1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl) phenyl]-5-(trifluoromethyl)- 2H-benzimidazol-2-one) is a close chemical relative of NS 004 (5-trifluoromethyl-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2H-benzimidazole-2-one), a prototype of benzimidazole derivative. It was shown to hyperpolarize vascular smooth muscle cells by directly activating the BK channels [7]. Later studies have shown that it renders the ischemic heart resistant to the progression of necrosis and hence, facilitate survival of myocardial cells [1]. However, the pharmacological effects and action mechanism in other tissues are still to be elucidated. In this study, we examined the effect of NS 1619 on oxidative injuries in RPE cells.

Materials and Methods

Cell culture

ARPE-19 cells (ATCC, Manassas, VA, USA) were cultured on plastic flasks in DMEM/F12 supplemented with 10% FBS and antibiotics streptomycin (50 µg/ml) and penicillin G (50 IU/ml). When cells reached confluency (approximately 4-5 days after seeding) cells were detached using 0.05% trypsin solution containing 0.53 mM EDTA. The subsequent cell suspension was reseeded at one-sixth of the initial density.

Induction of oxidant-induced injury

Experiments were carried out with cells that were grown on 12-well plates for 3 to 4 days when cells form confluent monolayers. Culture media bathing the cell monolayer were washed out, and cells were incubated in serum-free media with hydrogen peroxide (H_2O_2), t-butylhydroperoxide (t-BHP), or menadione for 3 hr at 37°C, unless otherwise indicated.

Induction of chemical hypoxia

To induce chemical hypoxia, cells were deprived of glucose and treated with the inhibitor of mitochondrial electron transport, antimycin A (20 µM) as described by Hagar et al. [13].

Trypan blue exclusion assay

Dead cells become unable to exclude trypan blue and get stained by the dye. Accordingly, assessment of cellular ability to exclude trypan blue provides an excellent indicator for the measurement of cell viability [2]. After treatment with experimental agents, cells were further incubated in HBSS containing 4% trypan blue for 30 min. Trypan blue-stained cells were counted as dead cells under light microscopy.

Tunel assay

Cells were incubated for 3 hr with 0.1 mM H_2O_2 in serum-free media to initiate apoptotic signal. Cells were then transferred to fresh media and further incubated for 18 hr. Tunel staining analysis was carried out to assess the degree of apoptosis. With this procedure, necrotic cell death did not exceed 5% of the whole cell population when analyzed by trypan blue exclusion ability.

MTT reduction assay

Mitochondria in intact cells reduce 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl- 2H-tetrazolium bromide (MTT) to formazan [19]. Thus, production of formazan as a result of MTT reduction is an excellent indicator to assess the functional integrity of mitochondria. After treatment with experimental agents, MTT (62.5 µg/ml) was added to each culture well and cells were incubated for 30 min. After removal of supernatant by aspiration, crystalized formazan was dissolved in DMSO. Spectrophotometry (absorbance at 570 nm) was performed to determine the concentration of formazan.

Assay of ATP content

Luciferin-luciferase assay [18] was used to determine cellular ATP content. After treatment with experimental agents, cells were solubilized in a mixture of 0.5% Triton X-100 (500 µl) and 0.6 M perchloric acid (100 µl). The solubilized sample was diluted with potassium glutamate buffer which is composed of 10 mM potassium glutamate and 4 mM $MgSO_4$ (pH 7.4). Luciferin-luciferase (100 µl of 20 mg/ml) was added to each suspension, and light emission was determined with a luminometer. Biorad protein assay kit was used to determine the protein content in the cell preparations.

Measurement of mitochondrial membrane potential

Changes in mitochondrial membrane potential were measured by fluorocytometry using $DiOC_6(3)$. Cell-permeable $DiOC_6(3)$ incorporates into mitochondria depending upon the transmembrane voltage across the inner mitochondrial

membrane. When there is a disturbance in the transmembrane voltage across the inner mitochondrial membrane, it is reflected by decreased cellular $DiOC_6(3)$ fluorescence. Cells were loaded with $DiOC_6(3)$ by incubation for 20 min at 37°C in PBS containing 50 nM $DiOC_6(3)$. After wash-out three times cells were suspended in PBS, and then flurocytometeric analysis was carried out.

Chemicals

NS 1619, NS 1619, 5-hydroxydecanoic acid, and paxilline were obtained from Research Biochemicals International (Natick, MA). $DiOC_6(3)$ was obtained from Molecular Probes (Eugene, OR, USA). Other chemicals were purchased from Sigma-Aldrich Korea Co. (Seoul, Korea).

Data analysis

Data were presented as means ± SE. The data were analyzed by ANOVA. Duncans multiple comparison test was carried out when necessary. We considered the difference as statistically significant when *p* value was less than 0.05.

Results

Protective effect of NS 1619 on H_2O_2-induced cell death

Fig. 1 depicts effects of different concentrations of NS 1619 on cell viability assessed by trypan blue exclusion. After treatment for 3 hr with different concentrations of H_2O_2, cell death rate increased in a dose-dependent manner. At 0.5 mM, H_2O_2 resulted in cell death by 63.8%. In the presence of the BK channel opener NS 1619, H_2O_2-induced cell death was significantly reduced.

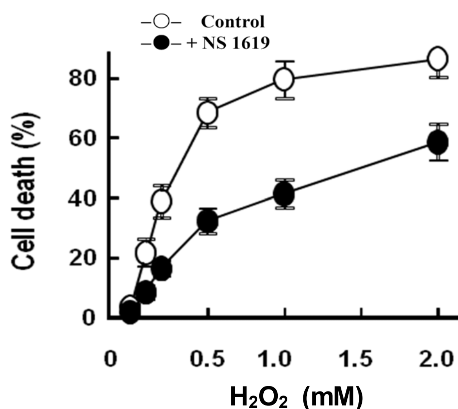

Fig. 1. Effect of NS 1619 on H_2O_2-induced cell injury. RPE cells were incubated for 3 hr at 37°C in serum-free media containing indicated concentrations of H_2O_2 with or without NS 1619 (10 µM). Trypan blue exclusion ability was determined to assess cell viability. Means ± S.E. (n=5).

Effect of NS 1619 on different types of oxidative cell injuries

In the results presented in Fig. 2, it was examined whether NS 1619 could prevent cell injuries induced by different types of oxidative stress. Menadione and t-butylhydroperoxide (t-BHP) were adopted as models for oxidative agents. In addition, chemical hypoxia was induced by deprivation of glucose combined with treatment with antimycin A, an inhibitor of mitochondrial respiratory chain. These maneuvers all caused cell death assessed by trypan blue exclusion assay. In the presence of NS 1619, cell death was significantly prevented regardless of the cause of oxidative injuries. These results suggest that NS 1619 might have protective effects against cell injuries caused by different types of oxidative stress.

Effects of KCOs and potassium channel blockers on H_2O_2-induced cell death

In Fig. 3, effects of NS 1619 and diazoxide were examined in combination with the BK and K_{ATP} channel blockers, paxilline and 5-hydroxydecanoate [22]. Diazoxide as well as NS 1619 was effective to ameliorate cell death determined by trypan blue exclusion ability. Paxilline antagonized the pro-

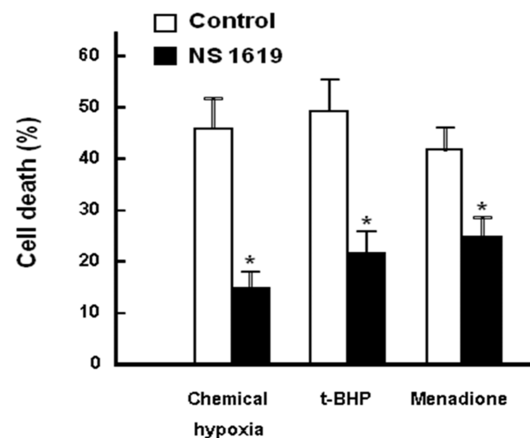

Fig. 2. Effect of NS 1619 on cell injuries caused by different types of oxidative stresses. RPE cells were treated with chemical hypoxia, 0.5 mM t-butylhydroperoxide (t-BHP) and menadione (2 mM) for 3 hr at 37°C with or without NS 1619 (10 µM). Trypan blue exclusion ability was determined to assess cell viability. Chemical hypoxia was induced by glucose deprivation in combination with antimycin A (20 µM). Means ±S.E. (n=5). *p<0.01 vs. control.

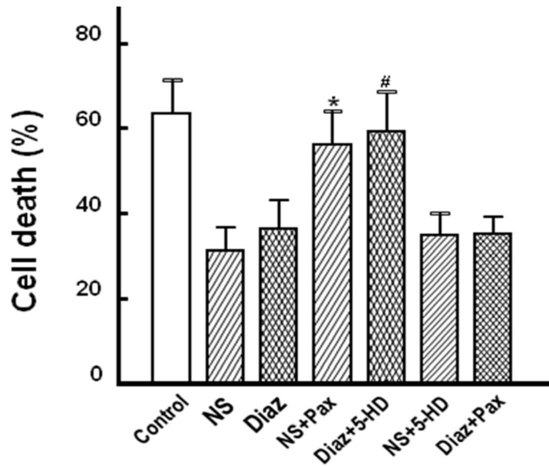

Fig. 3. Effects of different K^+ channel openers and blockers on RPE cell death caused by H_2O_2. RPE cells were incubated for 3 hr at 37°C in serum-free media containing 0.5 mM H_2O_2 with or without of NS 1619 (NS, 10 μM) and diazoxide (Diaz, 10 μM) in combination with paxilline (Pax, 10 μM) or 5-hydroxydecanoic acid (5-HD, 10 μM). Trypan blue exclusion ability was determined to assess cell viability. Means ± S.E. (n=4). *p<0.01 vs. NS alone, #p<0.01 vs. Diaz alone.

tective effect of NS 1619, whereas 5-hydroxydecanoate antagonized the protective effect of diazoxide, respectively.

Effect of NS 1619 on H_2O_2-induced apoptotic cell death

We examined whether NS 1619 has a protective effect to ameliorate apoptotic events. We treated RPE cells for 3 hr in HBSS containing 0.1 mM H_2O_2. In these cell populations, necrotic cell death estimated by trypan blue staining did not exceed 5% of the whole cell population. Dead cells floating in the media caused by necrotic injury were removed by aspiration. Cells were then transferred to fresh media and incubated further for 18 hr. Tunel staining was then performed for assay of apoptosis. Apoptotic cells could be discriminated by their condensed or fragmented nuclei in the micrographs of Tunel staining as shown in Fig. 4A. In H_2O_2-treated preparations 61.4.±6.8% of cells were counted as Tunel-positive (Fig. 4B). The number of apoptotic cells was remarkably reduced in cells with NS 1619. In the presence of paxilline, the protective effect of NS 1619 was significantly inhibited.

Effect of NS 1619 on H_2O_2-induced changes in MTT reduction

To examine whether NS 1619 could prevent H_2O_2-induced

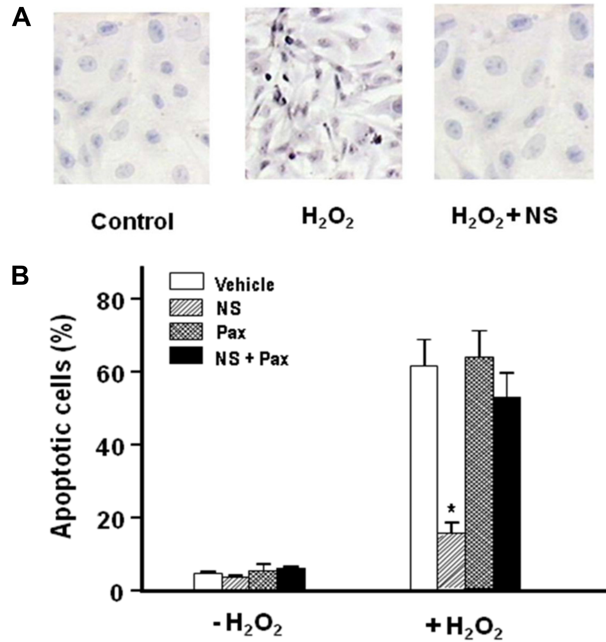

Fig. 4. Protection by NS 1619 against apoptotic cell death caused by H_2O_2. RPE cells were incubated for 3 hr at 37°C in serum-free media containing 0.1 mM H_2O_2 with or without NS 1619 (NS, 10 μM) and Paxilline(Pax, 10 μM), and were then transferred to the fresh media. After further incubation for 18 hr, Tunel staining was performed to assess apoptosis. Means ± S.E. (n=6). *p <0.01 vs. Vehicle.

mitochondrial dysfunction, changes in mitochondrial function was examined using a MTT reduction assay. When we treated cells with 0.5 mM H_2O_2, MTT reduction ability decreased to 43.2% of the control cells (Fig. 5). When we treated the cells with NS 1619 together, the H_2O_2-induced effect on MTT reduction was significantly attenuated. These results indicate that NS 1619 provide a defense mechanism to mitochondria to maintain their functional integrity under oxidative stress induced by H_2O_2.

Effect of NS 1619 on cellular ATP content

Deprivation of cellular ATP precedes irreversible injury process occurring upon ischemic- or oxidative stress-induced tissue damage [9]. Therefore, it was investigated whether NS 1619 could ameliorate H_2O_2-induced ATP deprivation. The results in Fig. 6 represent the effect of NS 1619 on changes in ATP concentration in H_2O_2-treated cells. In H_2O_2-treated cells, cellular ATP content was diminished to lower than 10% of the control level in 3 hr. When cells were pre-treated with NS 1619, ATP depletion by H_2O_2 was remarkably delayed. These results, together with those from MTT assay, suggest that NS 1619 exerts a beneficial influence

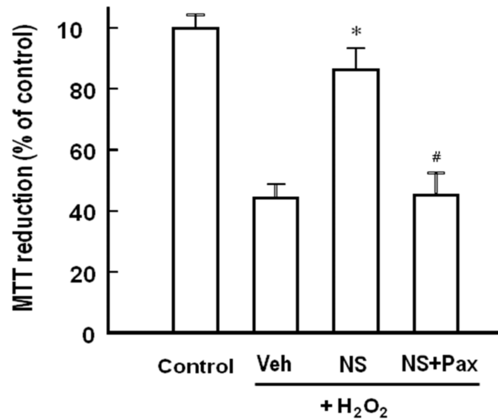

Fig. 5. Effects of NS 1619 and paxilline on H_2O_2-induced decrease in mitochondrial MTT reduction ability. RPE cells were incubated for 3 hr at 37°C in serum-free media containing 0.5 mM H_2O_2 for 3 hr with or without NS 1619 (NS, 10 µM) and Paxilline (Pax, 10 µM), and degree of MTT reduction was determined. Means ± S.E. (n=6. *p<0.01 vs. Vehicle (Veh), #p<0.01 vs. NS.

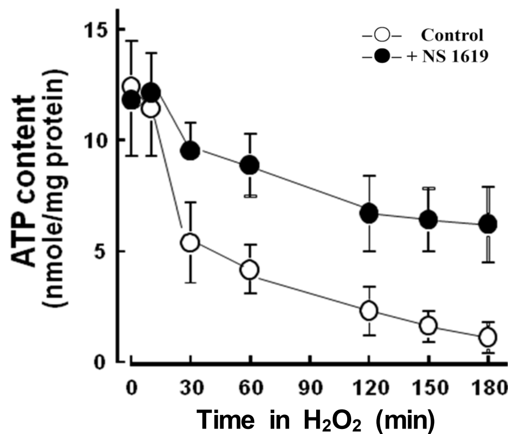

Fig. 6. Effect of NS 1619 on cellular ATP content. H_2O_2 (0.1 mM)-induced decrease in cellular ATP content was monitored in the presence and absence of NS 1619 (10 µM). Mean ± S.E. (n= 4).

on mitochondria to protect their energy production machinery against irreversible injuries under oxidative stress.

Effect of NS 1619 on mitochondrial membrane potential

In the results summarized in Fig. 7, effects of NS 1619 on mitochondrial membrane potential were presented. As shown in fluorocytometric analysis in Fig. 7A and Fig. 7B, NS 1619 effectively prevented H_2O_2-induced disruption of the mitochondrial membrane potential. These results demonstrate that NS 1619 ameliorates functional and structural deterioration of mitochondria and it could provide a protection mechanism against H_2O_2-induced cell death in the

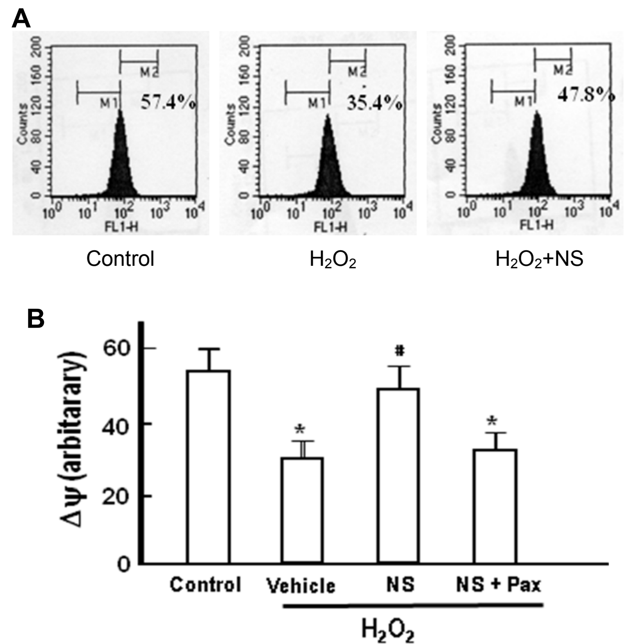

Fig. 7. Effect of NS 1619 on H_2O_2-induced changes in mitochondrial membrane potential. RPE cells were treated with H_2O_2 (0.1 mM) for 3 hr in the presence and absence of NS 1619 (NS, 10 µM) and paxilline (Pax, 10 µM), and analyzed by fluorocytometric analysis of $DiOC_6(3)$ fluorescence. A, Representative graphs of flurocytormetric analysis. B, Summarized data of 4 independent experiments. Mean ± S.E. (n=4). *p<0.01 vs. control, #p<0.01 vs. Vehicle.

RPE cells.

Discussion

Cell death processes appear to be distinguished as three morphologically distinct modes. They include necrotic cell death, apoptosis (programmed cell death) and autophagocytotic cell death [3, 23]. The present study suggested that H_2O_2 could trigger different modes of cell death processes depending on its concentration. In RPE cell preparations treated with relatively higher concentration (0.5 mM) of H_2O_2, necrotic cell death seemed to be dominant. On the other hand, in cell preparations treated with relatively lower concentration (0.1 mM) of H_2O_2 apoptotic cell death was observed after further overnight incubation in fresh media. With the later procedure, necrotic cell death estimated by trypan blue staining did not exceed 5% of the whole cell population.

This study suggested that NS 1619 might produce an effective protection mechanism against RPE cell injury induced by H_2O_2. It ameliorated RPE cell death induced by

different types of oxidative agents as well as H_2O_2 (Fig. 1 and Fig. 2). It was effective to prevent both the necrotic and apoptotic cell death. These results imply that the beneficial effect of NS 1619 to prevent RPE cell death should be explained in connection with the common pathways associated with both the apoptotic and necrotic cell death (Fig. 4). NS 1619 and diazoxide have been described as relatively specific openers of mitochondrial BK and K_{ATP} channels, respectively [29]. The present study demonstrated that diazoxide, an opener of mirochondrial K_{ATP} channels, also provided a similar protective effect against H_2O_2-induced RPE cell death (Fig. 3). It suggested that whether the K^+ channel type is BK and K_{ATP} does not have influence on the efficacy of the defense mechanism provided by the opening of mitochondrial K^+ channel. The protective effects NS 1619 and diazoxide disappeared almost completely when cells were pre-treated with their structurally related K^+ channel blockers paxilline and 5-hydroxydecanoate, respectively (Fig. 3 and Fig. 4). These blockers did not affect the protective effects provided by structurally unrelated openers, respectively, suggesting that the opening of mitochondrial K^+ channels is a crucial process in NS1619- as well as diaxoxide-induced protection mechanism.

KCOs were first introduced in the early 1980s. They produce their pharmacological effect by facilitation of ion flow through their target K^+ channels. It was followed by consecutive identification of many other drugs as a member of KCOs [6, 8]. Initially, KCOs which include pinacidil, cromakalim, diazoxide and nicorandil etc. were introduced as medications for hypertension (pinacidil and cromakalim) or angina (nicorandil). These actions are not irrational when we recognize that the increased opening provability of K^+ channels leads to hyperpolarization of smooth muscle membranes, inhibition of Ca^{2+} entry through voltage-gated Ca^{2+} channels, and hence promotion of vasodilation [5, 10, 20, 25].

Recently, accumulating evidence has demonstrated that the pharmacological actions of KCOs are not confined to vasodilatation of peripheral and coronary vessels. KCOs have been reported to provide a protection mechanism which mimics the endogenous protection mechanism described as 'ischemic preconditioning' [4, 15, 27, 28]. It is a paradoxical phenomenon in which repeated exposure to brief periods of ischemia provide the heart with a beneficial effect to become resistant to later lethal ischemia [21]. Recruitment of such defense mechanism pharmacologically leads to opening of new prospects for preventing or ameliorating cardiac damages as a consequence of coronary artery disease. Now it has become evident that protective action of KCOs is not confined to the heart but also functioning in other organs including the neuron [11].

In the heart, opening of plasmalemmal BK channels induces hyperpolarization of membrane potential, reduces cellular Ca^{2+} and thereby, alleviates myocardial work load [1]. In addition to this initially proposed protection mechanism, mitochondrial membrane which also possesses BK channels was suggested as an additional important cellular target that could mediate the defense mechanism provided by NS 1619. Protective effect of NS 1619 has also been described in neuron cells. NS 1619 has been shown to induce immediate and delayed preconditioning [11]. However, these neuroprotective effects were suggested to be independent of BK channels and rather to be the consequence of ROS generation, activation of the PI3K pathway, and inhibition of caspase activation [11]. In the present study, as RPE cells are non-excitable cells, the role of plasmalemmal BK channels are not considered to be importantly involved in the defense mechanism provided by NS 1619. We did not examine the role of ROS and PI3K in this study

Several evidence in this study indicates that prevention of mitochondrial dysfunction during oxidative stress is a key event essential for the protection mechanism provided by NS 1619 against H_2O_2-induced RPE cell injury. It provided mitochondria with beneficial effects to retain MTT reduction ability under H_2O_2-induced oxidative stress (Fig. 5). In addition, it remarkably prevented H_2O_2-induced ATP deprivation (Fig. 6). These results suggested that NS 1619 produces a beneficial effect on mitochondria to protect their metabolic machinery against irreversible injuries under oxidative stress.

Formation of mitochondrial permeability transition (MPT) pores and cytochrome c release through these MPT pores has been suggested to be importantly associated with oxidative stress-induced cell injury [17]. Cytochrome c released into cytosol triggers activation of caspase pathways that are essential events for the progression process of apoptosis. Formation of MPT pore results in loss of selective permeability in the inner mitochondrial membrane which leads to deterioration of mitochondrial membrane potential. The present study demonstrated that NS 1619 ameliorated the H_2O_2-induced disruption of mitochondrial membrane potential (Fig. 7). It also seems to be importantly related to the protection mechanism of NS 1619 against apoptotic cell

death. In conclusion, it is suggested that NS 1619 prevents functional and structural deterioration of mitochondria under oxidative stress and it could provide a defense mechanism that renders the RPE cells resistant to H_2O_2-induced cell injury.

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초록 : 망막 색소상피세포에서 산화성 세포 손상과 미토콘드리아기능 저해에 미치는 NS 1619의 보호 효과

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K^+ 통로 개방제들은 심근, 뇌, 골격근 등에서 세포막 혹은 미토콘드리아 내막에 존재하는 큰 전도성의 Ca^{2+}-의존성 K^+ (BK) 통로 및 ATP-조절성 K^+ 통로(ATP-sensitive K^+ channels, K_{ATP})에 작용하여 허혈성 혹은 산화성 세포 손상을 완화하는 효과가 있는 것으로 보고되어 있다. 본 연구에서는 망막 색소 상피세포주인 ARPE-19 세포를 실험 모델로 하여 큰 전도성의 BK 통로 개방제인 NS 1619가 유사한 보호 효과를 나타낼 수 있는지, 또한 그 작용기전이 무엇인지를 확인하고자 하였다. AREE-19 세포를 여러 형태의 산화 스트레스에 노출시켜 세포 손상을 유발하고 그 손상의 정도 및 이에 미치는 NS 1619의 효과를 trypan blue 배출능, Tunel 염색 분석을 통하여 측정하였다. NS 1619는 여러 형태의 산화 스트레스에 의한 괴사성 및 apoptosis에 의한 세포 손상을 효과적으로 방지하였으며 그 보호 효과는 BK 통로 봉쇄제인 paxilline 의해 차단되었다. NS 1619는 H_2O_2에 의한 세포내 ATP 고갈을 현저히 완화시켰으며, 또한 MTT 환원능으로 측정한 미토콘드리아의 기능을 보호하는 효과를 보였다. 유세포형광분석법을 이용한 실험에서 NS 1619는 H_2O_2에 의한 미토콘드리아 막전압의 소실을 유의하게 방지하였다. 이상의 결과들을 종합하면 NS 1619는 망막 색소 상피세포에서 산화성 세포 손상을 방지하는 효과를 나타내며 그 기전에 미토콘드리아 기능에 대한 보호 작용이 연관되어 있는 것으로 사료된다.