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Biochemistry

Nobiletin Induced Ischemic Preconditioning in PC-12 Cells by Transient Activation of AMP-Kinase

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Ischemic preconditioning is a phenomenon in which brief episodes of sublethal ischemia protect the brain against sustained ischemia-reperfusion injury. Polyphenolic compounds such as nobiletin (NOB), have gained interest as potential candidates for pharmacologic preconditioning due to their neuroprotective effects. In this study, we investigated the impact of NOB on differentiated PC-12 cell survival, mitochondrial membrane potential, ROS production and AMP-dependent protein kinase (AMPK) under hypoxic conditions. Our findings revealed that preincubation with NOB led to increased cell viability and initiated a transient hyperpolarization of the mitochondrial membrane, succeeded by a mild depolarization. Furthermore, we observed that the transient hyperpolarization of mitochondrial membranes correlated with a temporal boost in AMP-dependent protein kinase (AMPK) activity. Based on our results, we propose that the short-term hyperpolarization of the mitochondrial membrane, coupled with subsequent activation of AMPK triggered by NOB, represents a crucial mechanism of chemical preconditioning. This mechanism facilitates adaptive processes within both the mitochondria and the entire cell. *© 2024 Bull. Georg. Natl. Acad. Sci.*

nobiletin, flavonoids, ischemia, ischemic preconditioning, stroke

The brain is particularly susceptible to hypoxia or ischemia, which can lead to a lack of oxygen and energy substrate supply. Different approaches can also artificially induce these mechanisms, resulting in a protective state known as ischemic tolerance [1]. Among potential chemical preconditioning strategies, a key target is mitochondrion, crucial in inducing ischemic precondition (IPC) in neurons [2]. Dysfunctional or damaged mitochondria leads to an overproduction of reactive oxygen species (ROS) which ultimately causes cell death [3]. Therefore, chemical agents targeted at mitochondria, including those derived from plants, such as Nobiletin (NOB) hold promise for chemical preconditioning. Our previous studies found that NOB alters oxygen consumption by bovine brain mitochondria, depending on the type of substrate used and induces a transient hyperpolarization followed by a mild mitochondrial membrane depolarization [4]. This suggests that NOB may act as a mild „uncoupler," indirectly activating AMP-activated protein kinase (AMPK) by increasing the AMP/ADP: ATP ratio, a critical energy sensor [5]. Therefore, we propose that the NOB-induced transient alterations in mitochon-

drial activity potentially involve AMPK-dependent signaling. Nevertheless, the precise mechanism of NOB-mediated metabolic protection in IPC remains to be fully elucidated. Given these findings, we hypothesized that NOB induces ischemic tolerance through transitory changes in mitochondrial bioenergetics, mitochondrial-derived ROS formation, and activation of AMPK. To test this, we examined the effect of NOB on hypoxia-induced injury in neuron-like rat pheochromocytoma (PC-12) cells. Our results indicate that NOB-induced transient hyperpolarization of mitochondria leads to a short-term increase in AMPK activity, which may be a pivotal mechanism in chemical preconditioning.

Materials and Methods

Cell culture and differentiation. Pheochromocytoma cells (PC-12, ATCC® CRL-1721™) were cultured in T25 flasks in a humidified atmosphere containing 5% CO_2 at 37°C in a high-glucose RPMI 1640 medium (Thermofisher cat.no.72400047) supplemented with 10% heat-inactivated horse serum (Gibco, cat no. 26050088), 5% fetal bovine serum (Sigma-Aldrich, cat.no.F2442), and 100 unit/mL penicillin as well as 50 µg/mL gentamicin sulfate according to [6]. To induce differentiation, cells were seeded on poly-D-lysine (Santa Cruz, sc-136156) coated flasks and incubated in low serum-containing high-glucose RPMI 1640 medium (1% HS and 1% FBS) supplemented with 100 ng/mL nerve growth factor (Alomone Labs, N-245) in T25 flasks for 5 days. NGF-containing medium was changed every 48 h. The cells were scored as differentiated if one or more neurites were longer than the cell body diameter. Cell viability was measured by staining the cells with trypan blue dye (Bio-Rad, cat no. 145-0013) and using an Automated Cell Counter TC 20TM (Bio-Rad, USA) for the live cells counting.

Normoxia study: Differentiated PC-12 cells were pre-treated with 10 mM NOB/100 mM NOB for 1 h, treatments containing medium were replaced with low serum-containing high-glucose RPMI 1640 medium and replaced with fresh medium to stop the action of agents. Next day, the culture medium was changed with fresh medium exactly as samples exposed to hypoxia and was used as normoxia samples.

Hypoxic study (Oxygen deprivation): Differentiated PC-12 cells (2 × 106 cells per sample) were pre-treated with 10mM NOB/100mM NOB for 1h, treatments containing medium were replaced with fresh low serum-containing high-glucose RPMI 1640 medium to stop the action of agents. Next day, the culture medium was changed with fresh low serum-containing high-glucose RPMI 1640 medium followed by exposure to hypoxic conditions for 1h without any treatment. Hypoxic conditions (0-1% oxygen) were maintained using nitrogen gas in a BioSpherix C-Chamber placed in a CO_2 incubator and controlled by a ProOx Model P110 controller (BioSpherix, USA).

The MTT viability test. This colorimetric assay is based on the reduction of a yellow tetrazolium salt (MTT) to purple formazan crystals by metabolically active cells. The viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT to formazan. Incubation with MTT (0.5 mg/ml) was carried out for 4 h at 37°C. The insoluble formazan crystals were dissolved using 200 µl solubilization solution (DMSO), and the resulting-colored solution was quantified by measuring absorbance at 600 nm wavelength using a microplate reader. All experiments were repeated at least three times; the obtained results (OD) were compared with respective control in each series and presented as % versus control (100%).

ROS production. Cellular Reactive Oxygen Species (ROS) generation was assessed using the DCFDA/H2DCFDA Cellular ROS Assay Kit (ab113851, Abcam). Adherent differentiated PC-12 cells were plated at a concentration of 1.3*105 cells/per well 24 hours before the addition

of NOB. 24 hours after hypoxia, the media were removed, cells were washed by 100 µL/well of 1X Buffer and stained by adding 100 µL/well of the diluted DCFDA solution. After 45 minutes of incubation with DCFDA solution at 37°C in the dark, DCFDA solution was removed, and 100 µL/well in 1X Buffer and 1X Supplemented Buffer were added. The plate was measured immediately on a fluorescence plate reader at Ex/Em = 485/535 nm. All experiments were repeated thrice and expressed as the mean of arbitrary fluorescence units.

Mitochondrial membrane potential. According to the manufacturer's protocol, the mitochondrial membrane potential (in cell culture) was measured using the JC-10 Mitochondrial Membrane Potential Assay Kit (ab112134, Abcam). Adherent differentiated PC-12 cells were plated at concentration 1*10 5 cells/per well 24 hours before the addition of NOB. The mitochondrial potential was estimated time-dependent immediately after adding agents then after 20 and 40 minutes. In all samples, the media were removed, and cells were treated by adding 10 µL of 10 X test compounds and incubated at room temperature for 30 minutes. 50 µL/well of JC-10 dye-loading solution was added, and the dye-loading plate was incubated at room temperature for 1 hour, protected from light. 50 µL/well of Assay Buffer B was added to the dye-loading plate before reading the fluorescence intensity at Ex/Em = 490/525 nm. All experiments were repeated thrice and expressed as a mean of arbitrary fluorescence units.

AMP-activated protein kinase (AMPK) activity. According to the manufacturer's protocol, AMP-activated protein kinase activity was assessed using the AMPK Phosphorylation Assay Kit (KA3789, Abnova). Briefly, adherent differentiated PC-12 cells were plated at concentration 3*10 4 cells/per well 24 hours before the addition of NOB. AMPK activity was estimated in a time-dependent manner immediately after adding agents, then after 20, 40, 60, and 90 minutes in normoxic conditions, and also after 24 hours in normoxic and hypoxic conditions. The media was removed; cells were fixed by adding 100 µL of 4% formaldehyde to the cell pellet. The plate was incubated for 20 minutes at room temperature after that, the formaldehyde solution was removed, and the cells were washed three times. After removing the Wash Buffer, 100 µL Quench Buffer was added to each assay well. The plate was incubated for 20 minutes at room temperature and then washed thrice. After removing the Wash Buffer, 100 µL of Blocking Buffer was added, and the plate was incubated for 1hr at room temperature. Following removing the Blocking Buffer, 50 µL of diluted pAMPK-Ab1 into the Blocking Buffer was added to each well, and the plate was incubated for another 90 min at room temperature. Then, the plate was washed three times. 50 µL of diluted secondary antibody was added and incubated for 90 min at room temperature with gentle shaking. After washing five times, 50 µL of mixed HRP Substrate was added to each well, and the plate was incubated for 30 min at room temperature in the dark. After that, 50 µL of Protein Stain was added to each well, incubated for 5 min at room temperature in the dark, and measured at Ex/Em = 530/585 nm. All experiments were repeated two times and expressed as a mean of arbitrary fluorescence units.

Protein total amount was assessed in lysates using Pierce™ BCA Protein Assay Kit (Thermo).

Statistical analysis. Significant differences were determined using two-way ANOVA by GraphPad Prism. Differences with p-values smaller than 0.05 were considered significant, *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

Results

Cell differentiation was observed under an inverted light microscope and was scored as differentiated if one or more neurites were longer than the cell body diameter. In the first phase of our study, we deter-

mined an effective dose of NOB and used two different concentrations, 10^{-5} and 10^{-4} and pretreatment time on PC-12 differentiated cell culture and cell survival rate after hypoxia. Our result shows that NOB at 10^{-5} and 10^{-4} M concentrations effectively increases cell survival under hypoxic conditions compared to its corresponding control (Fig. 1). Accordingly, further experiments were carried out using these doses.

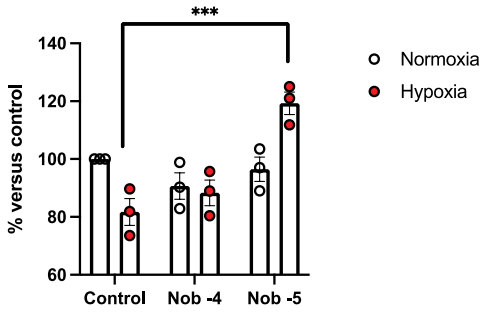

Fig. 1. NOB's two different concentrations (10^{-4} M and 10^{-5} M) on PC-12 cell culture under normoxic and hypoxic conditions.

It is well known that ischemia/reperfusion injury has been linked to generating free radicals like ROS [7]. Therefore, we assessed the intracellular production of reactive oxygen species. Elevated production of ROS was found only in the hypoxic cells, and pretreatments of cells by NOB in two different concentrations significantly decreased the production of ROS, especially at NOB^{-5} M concentration (Fig. 2). Changes in mΔΨ are essential in regulating cell death due to its effects on ROS production in the setting of ischemic preconditioning [8, 9].

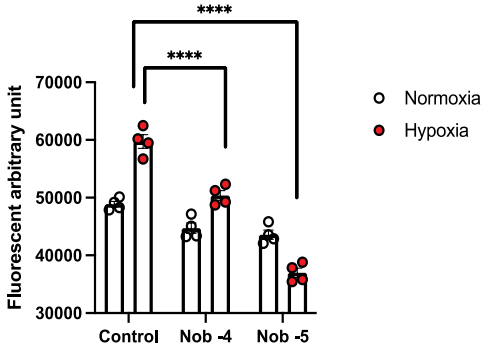

Fig. 2. Effect of NOB on the reactive oxygen species (ROS) production in differentiated PC-12 cells under normoxic and hypoxic conditions.

Since mitochondrial membrane hyperpolarization and depolarization could serve as triggers for cell survival and death, respectively, in the next series of experiments, we determined the time-dependent effect of NOB on the mitochondrial membrane potential. Our results have shown that treating cells with NOB induces the transient hyperpolarization of the mitochondrial membrane followed by mild depolarization. This effect was seen only at 10^{-5} M concentration of NOB (Fig. 3).

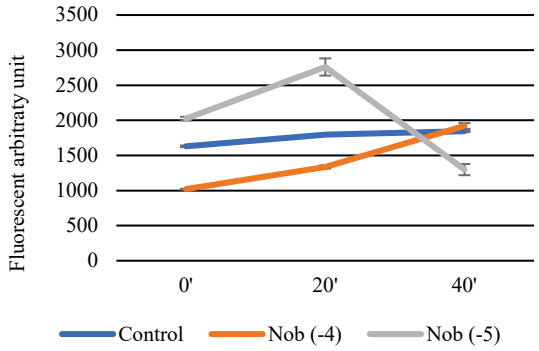

Fig. 3. Time-dependent alterations of mitochondrial membrane potential after 10^{-4} and 10^{-5} M NOB pretreatment.

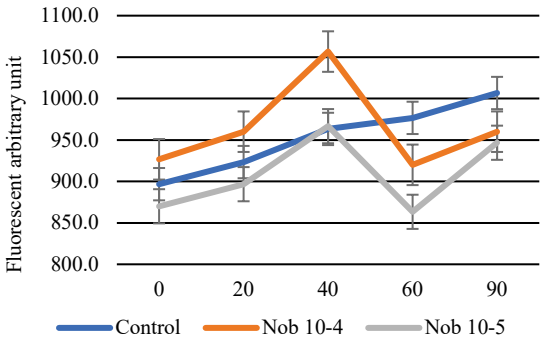

Fig. 4. Time-dependent alterations of AMPK activity after 10^{-4} and 10^{-5} M NOB pretreatment.

The 5-AMP-activated protein kinase (AMPK) is a key sensor of intracellular energy balance. AMPK can be activated by ischemic preconditioning as well [10]. Hence, in the next experiments, we determined AMPK activity in the same time intervals. We have found a similar transient increase in AMPK activity. However, the peak of activi-

ty, in this case, was shown only 40 minutes after addition of NOB (Fig. 4). Consequently, at 10^{-4} M concentration, NOB alters mitochondrial metabolism and triggers cell reprogramming to prepare them for hypoxic conditions, thus contributing to the development of the chemical preconditioning phenomenon.

Discussion

Understanding the mechanisms of metabolic adaptation during ischemic preconditioning has emerged as a major research focus, with the potential for pharmacological interventions to mitigate neuronal damage [11]. Polyphenols, especially flavonoids, exhibit protective effects against ischemia-reperfusion injury and can serve as pre-conditioning agents. Our recent findings demonstrated that NOB accelerates succinate-driven oxygen consumption by activating α-ketoglutarate dehydrogenase and matrix substrate-level phosphorylation [4].

This study investigated the impact of NOB pretreatment on differentiated PC-12 cell survival, mitochondrial membrane potential, ROS production and AMPK activities under hypoxic conditions. Our results revealed that preincubation with NOB enhances cell viability and effectively prevents the rise of ROS production during hypoxia. Additionally, our findings demonstrated that NOB induces a transient hyperpolarization followed by a mild depolarization of the mitochondrial membrane. Notably, this transient hyperpolarization of mitochondrial membranes is associated with a time-delayed stimulation of AMPK activities. ROS may potentially mediate the NOB-induced increase in AMPK activity stemming from transient hyperpolarization [4]. We conclude that NOB mimics short-term hypoxic hyperpolarization, triggering an AMPK-dependent shift towards a compensatory metabolic cascade. This adaptive signaling involves a brief surge in ROS production, likely originating from succinate-driven mitochondrial systems, resulting in a short-lived burst in ROS production through reverse electron transport [4, 12]. These processes lead to robust AMPK-dependent energetic adaptations that shield neurons from oxidative stress. This assumption is supported by our data, which showed that simultaneous glucose deprivation leads to a substantial decrease in ATP levels compared to hypoxia alone, and pretreatment of cells with NOB does not provide a protective effect. These findings suggest that the protective effects of nobiletin may result from compensatory activation of anaerobic glycolysis, which is the primary target of the compensatory action of AMPK [13].

Our results indicate that the transient hyperpolarization in mitochondria activates AMP-dependent protein kinase (AMPK), temporarily increasing the cell's energetic potential. We suggest that these temporary alterations may involve adaptive processes in the early response to ischemia. Activating these stress-response mechanisms after the action of NOB involves an AMP-dependent signaling pathway with subsequent induction of antioxidant and neuroprotective cascades that could prevent post-ischemic complications. In conclusion, this study proves that NOB induces chemical preconditioning and enhances cell survival following hypoxia. Additionally, the short-term hyperpolarization of the mitochondrial membrane and subsequent activation of AMPK caused by NOB can be considered one of the critical mechanisms of chemical preconditioning, enabling adaptive processes in both the mitochondria and the entire cell.

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ბიოქიმია

AMP-კინაზას გარდამავალი აქტივაციის გააქტიურებით ფლავონოიდ ნობილეტინის მიერ გამოწვეული იშემიური პრეკონდიცია PC-12 უჯრედებში

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იშემიური პრეკონდიცია წარმოადგენს ფენომენს, რომლის დროსაც მცირე სუბლეტალური იშემიური ეპიზოდები შემდგომში უჯრედებს იცავს უფრო ძლიერი იშემია-რეპერფუზიული დაზიანებებისაგან. ფარმაკოლოგიური აგენტებით გამოწვეული პრეკონდიცია წარმოადგენს პერსპექტიულ მიდგომას თავის ტვინის ინსულტის მკურნალობაში. მცენარეული წარმოშობის პოლიფენოლური აგენტები, როგორიცაა, მაგალითად, ნობილეტინი, წარმოადგენს პერსპექტიულ თერაპიულ საშუალებას იშემიური ინსულტის სამკურნალოდ, მათი ნეიროპროტექტორული ეფექტის გამო. წარმოდგენილ ნაშრომში შესწავლილია ნობილეტინის პრეინკუბაციის ეფექტი დიფერენცირებულ PC-12 უჯრედების სიცოცხლისუნარიანობაზე მწვავე ჰიპოქსიის პირობებში, აგრეთვე შევისწავლეთ სხვადასხვა უჯრედული პარამეტრები, მათ შორის, მიტოქონდრიული მემბრანის პოტენციალი, ROS წარმოება და AMP-დამოკიდებული პროტეინკინაზას (AMPK) აქტივობა. კვლევის საფუძველზე გამოვლინდა, რომ NOB-ით პრეინკუბაციამ გაზარდა უჯრედების სიცოცხლისუნარიანობა, გამოიწვია მიტოქონდრიული მემბრანის გარდამავალი ჰიპერპოლარიზაცია, რასაც მოჰყვა სუსტი დეპოლარიზაცია, რამაც არსებითად შეამცირა ჰიპოქსიით გამოწვეული რეაქტიული ჟანგბადის ფორმების გაძლიერებული წარმოქმნა. კვლევის საფუძველზე აგრეთვე გამოვლინდა, რომ მიტოქონდრიული მემბრანების გარდამავალი ჰიპერპოლარიზაცია დაკავშირებულია AMP-დამოკიდებული პროტეინკინაზას (AMPK) აქტივობის დროებით გაძლიერებასთან. კვლევის შედეგად მიღებულ შედეგებზე დაყრდნობით ვვარაუდობთ, რომ მიტოქონდრიული მემბრანის მოკლევადიანი ჰიპერპოლარიზაცია, AMPK-ის შემდგომ გააქტიურებასთან ერთად, რომელიც გამოწვეულია NOB-ით, წარმოადგენს ქიმიური პრეკონდიციის ძირითად მექანიზმს. ეს მექანიზმი კი, ხელს უწყობს ადაპტაციურ პროცესებს, როგორც მიტოქონდრიაში, ასევე მთლიან უჯრედში.

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