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Hypoxia induces Internalization of Kappa Opioid Receptor

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Abstract

Background—It has been demonstrated that kappa opioid receptor (KOR) agonists can reduce hypoxia–ischemia (HI) brain injury in animal models. However, it is unclear how KOR responds to HI. In the current study, we used *in vitro* model of oxygen-glucose deprivation (OGD) and re-oxygenation to explore how KORs respond to hypoxia and re-oxygenation.

Methods—Mouse neuroblastoma Neuro2A cells stably transfected with mKOR–tdTomato fusion protein (N2A–mKOR–tdT) or Flag-tagged mKOR (N2A–mKOR–FLAG) and divided into several groups (n=6 to 12), and were used to investigate the KOR movement. Observations were performed under normal oxygen, at 30 min to 1 h after OGD, and at 1 h after re-oxygenation using high resolution imaging techniques including immunoelectronmicroscopy in the presence and absence of KOR antagonist, dynamin inhibitors, potassium channel blockers, and dopamine receptor inhibitor.

Results—Hypoxic conditions caused KOR to be internalized into the cells. Inhibition of dynamin by Dyngo-4a prevented the receptor internalization. Interestingly, a specific KOR antagonist norbinaltorphimine blocked internalization, suggesting a specific KOR activation involvement. KOR internalization appears to be reversed by re-oxygenation. Quantities of intracellular KOR-associated gold particles as demonstrated by immunoelectron microscopy were increased from 37 % to 85% ($P < 0.01$) after OGD. Potassium channel blockers and dopamine receptor inhibitor failed to block hypoxia induced KOR internalization.

Conclusion—Hypoxia induces reversible KOR internalization, which was inhibited by selective KOR antagonists or dynamin inhibitor, and can be reversed by re-oxygenation in neuroblastoma

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cells, indicating the modulating effects between KOR and hypoxia via KOR activation and dynamin dependent mechanism.

Introduction

The opioid receptors belong to the G-protein-coupled receptor (GPCR) family with seven transmembrane domains.¹ The opioid system is composed of three major receptor subtypes, mu (MOR), kappa (KOR), and delta (DOR) opioid receptors, which is essentially an inhibitory neurotransmitter system. They are expressed not only within the central nervous system^{2–5} but also on peripheral sensory nerve terminals.^{6,7} Opioid system is involved in the modulation of various physiological and pathological activities, including nociceptive/analgesic signal transduction, neurotransmitter secretion, respiration, immune responses as well as ion channel functions.

GPCR internalization is one of the important processes in response to agonist exposure, which occurs over a time scale ranging from minutes to hours.^{8–10} Agonist-induced KOR internalization has been extensively studied.¹¹ It was demonstrated that agonist-induced internalization of the human KOR is required for down-regulation¹². Previous *in vitro* studies have shown that internalization of KOR is agonist specific and occurs in dose and time dependent manner.^{13–18} For example, KOR is internalized by the agonists U50,488 and dynorphin 1–17, but not other agonists such as etorphine or levorphanol.¹⁷ KOR antagonist norbinaltorphimine, GPCR phosphorylation inhibitor phosducin, as well as endocytosis blocking agents strongly inhibited selective KOR agonist U50,488H-induced internalization of KOR.^{14,18,19} Cells have been shown to mediate agonist-induced internalization of KOR through GPCR kinase 2, dynamin and beta-arrestin dependent mechanisms.^{14,20,21} Although internalization of GPCRs is generally described as rapid agonist-induced motion of the receptor into the cytoplasm from the cell membrane,¹⁴ long-term agonist exposure leads to the down-regulation of the receptors trafficked to the lysosomes and proteasomes for degradation.¹²

Recently, it has been shown that KOR agonists exhibit neuroprotective effects in animal models of hypoxic–ischemic (HI) injury.^{22–24} Considering the involvement of KOR in HI, it is of great importance to investigate the effect of hypoxic conditions on the cellular translocation of KOR. In this study, we explored whether hypoxic conditions induce KOR internalization in agonistic fashion. We used mouse Neuro 2A cells stably expressing mKOR–tdTomato fusion protein or Flag-tagged mKOR (mKOR-FLAG) to simulate HI injury *in vitro* and track the KOR under oxygen-glucose deprivation (OGD) conditions.

Material and methods

M1 mouse anti-FLAG monoclonal antibody, normal goat serum, poly-D-lysine, triton X-100, norbinaltorphimine (nor-BNI), glibenclamide, iberiotoxin, and sulpiride were obtained from Sigma-Aldrich (St. Louis, MO). Salvinorin A (SA, purity > 98%) was obtained from Apple Pharms (Asheville NC, USA). Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin (IgG), Alexa Fluor 488-conjugated goat anti-rabbit IgG with 10 nm colloidal gold conjugate, Antifade Mounting Medium with DAPI, Minimum Essential

Medium (MEM) powder, glucose-free Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). Anti-RFP antibody and Dyngo-4a were obtained from Abcam (Cambridge, MA). Geneticin was obtained from Cellgro Mediatech (Herndon, VA). Lab-Tek II Slide Chamber was obtained from Thermo Scientific (Waltham, MA). Hypoxia system with gas mixture (95% N₂, 5% CO₂) was obtained from Airgas USA, LLC (Cherry Hill, NJ). All other chemicals used were of reagent grade and obtained from Sigma-Aldrich.

Cell lines

N2A-mKOR-tdT and N2A-mKOR-FLAG cells were prepared as described previously.^{25–27} Briefly, the red fluorescent protein tdTomato (tdT) was fused to the C-terminus and the FLAG epitope was tagged to the N-terminus of the mKOR to make mKOR-tdT and mKOR-FLAG fusion proteins. Next, mKOR-tdT and mKOR-FLAG were separately transfected and expressed in Neuro 2A (N2A) cells. Cells were cultured in MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. 0.2% Geneticin and 0.005% Blasticidin were used for the selection of N2A-mKOR-tdT and N2A-mKOR-FLAG respectively. Cells were kept in a humidified atmosphere consisting of 95% air and 5% CO₂ at 37°C.

Oxygen-glucose deprivation

To simulate OGD, cells were washed twice with the glucose-free DMEM, and incubated in a plexiglass chamber (Billups-Rothenberg Inc. Del Mar, CA, USA) and flushed with hypoxic gas mixture for 5 min at the flow rate of 20 L/min. The glass chamber was then sealed and disconnected from the gas source.^{28,29} Partial pressures of oxygen and carbon dioxide of OGD medium were measured by a blood gas analyzer (RapidLab@248, Siemens Diagnostics, Tarrytown, NY).

Cell viability assay

MTT (3-(4, 5-dimethylthia-2-yl)-2, 5-diphenyl-tetrazolium bromide) colorimetric assay was used to assess cell viability. N2A-mKOR-tdT and N2A-mKOR-FLAG cells were seeded (4×10^4 cells/well) in a 96-well plate and cultured overnight. Cells were then incubated in a normal growth conditions (control group) or OGD conditions (OGD group) for 0.5, 1, 2, 4, and 8 h. After each treatment, cells were returned to a normal growth conditions and incubated with MTT in a final concentration of 0.5 mg/mL for 4 h at 37°C. Then, medium was removed and cells were incubated with 10% sodium dodecyl sulfate (SDS) in 1mM HCl for 4 h at 37°C. Optical density (OD) of each well was measured at 570 nm using a microplate reader (Multiscan MK3, Thermo Labsystems, USA). Cell viability (CV(%)) was calculated as the ratio of the OD of individual treatment to the OD of control group.

OGD induced KOR trafficking by fluorescence and confocal microscopy

N2A-mKOR-tdT or N2A-mKOR-FLAG cells were seeded to poly-D-lysine coated 8-well-chambered coverglasses (maker), and cultured to 80–90% confluence. Both cells were divided into three groups: control group, OGD group and re-oxygenation group (OGDR

group). For N2A-mKOR-tdT cells in control group, cells were washed twice with serum-free MEM, and incubated in normal growth medium containing glucose in humidified atmosphere at 37°C, 95% air and 5% CO₂ for 1 h. Concurrently, in the OGD group, cells were washed twice with the glucose-free DMEM, and then incubated under OGD conditions for 0.5 or 1 h. Cells in the OGDR group were preincubated under OGD conditions for 1 h before transferring to the normal growth medium containing glucose, and then incubated in humidified atmosphere at 37°C, 95% air and 5% CO₂ for 1 h for re-oxygenation. After each procedure, cells were washed three times with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Fixed cells were then washed with PBS and mounted using Antifade Mounting Medium with DAPI. Images of mKOR-tdT were acquired using inverted fluorescence microscope with a 40× objective (Olympus IX70; Olympus, Melville, NY) through a Texas Red channel (excitation 560 nm, emission 620 nm). Nuclear images were captured through a DAPI channel (excitation 400 nm, emission 460 nm).

N2A-mKOR-FLAG cells were used as well-established reference for the detection of KOR trafficking and were prepared for immunofluorescence staining as described previously.^{17,26} Briefly, M1 mouse anti-FLAG monoclonal antibody was diluted in the MEM (4μg/mL) containing 0.05% Triton-X100, 4% normal goat serum and 1mM CaCl₂ marked as feeding medium. N2A-mKOR-FLAG cells were washed twice with the FBS-free MEM and then incubated in the feeding medium at 37°C for 1 h. Subsequently, N2A-mKOR-FLAG cells were cultured in normal growth medium, OGD conditions for 0.5 to 1 h or OGD conditions for 1 h followed by 1 h re-oxygenation in normal growth medium. Then N2A-mKOR-FLAG cells were washed with ice-cold Hanks Balanced Salt Solution (HBSS), fixed with 4% paraformaldehyde and washed 5 min with Tris-HCl buffer solution (TBS) at room temperature. Next, cells were incubated with 2 μg/mL Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) in 0.05% triton-X100/4% normal goat serum/PBS for 30min. After washing three times with PBS, cells were mounted and images of mKOR-FLAG were acquired through FITC channel with excitation at 490 nm and emission at 520 nm.

N2A-mKOR-tdT cells pretreated by nor-BNI (a selective KOR antagonist), Dyngo-4a, glibenclamide (an adenosine triphosphate sensitive K⁺ channels inhibitor), iberiotoxin (a selective high-conductance calcium-activated K⁺ channel inhibitor), or sulpiride (a dopamine D2-receptor antagonist) for 0.5 h were used to investigate whether they could block the OGD related KOR trafficking.

To discriminate whether the redistributed KOR aggregated in the cell-surface layer or moved into the cytoplasm, N2A-mKOR-tdT and N2A-mKOR-FLAG cells were fixed or stained with immunofluorescence as described above and observed by a confocal microscopy using a 63× oil immersion objective (Leica TCS SP6, Germany). RsRed channel or FITC channel was selected respectively for the observation of N2A-mKOR-tdT cells or N2A-mKOR-FLAG cells. Z-stack was performed with the z-step size of 10 μm. For each experiment, cells were observed under the same conditions at least 3 times in each group.

Agonist induced KOR trafficking by fluorescence microscopy

To visualize the agonist induced KOR trafficking, cells treated with 10 μM SA for 30min were used to demonstrate the agonist induced KOR internalization. Cells pretreated with

nor-BNI and Dynngo-4a were used to demonstrate the inhibition of the agonist induced KOR internalization. Fluorescence microscopy was performed as described above. For each experiment, cells were observed under the same conditions at least 3 times in each group.

OGD induced KOR trafficking by single cell imaging flow cytometry

N2A-mKOR-tdT cells were prepared as a single cell suspension at 10^7 cells/mL concentration. After 1 h treatment with or without OGD, circularity feature of mKOR-tdT was captured by ImageStreamX imaging flow cytometer (Amnis, Seattle, WA) and analyzed by the IDEAS software (EMD Millipore, MA). The circularity feature, which is given by circularity index, measures the degree of the mKOR-tdT's deviation from a circle. Its measurement is based on the average distance of the object boundary from its center divided by the variation of this distance. Thus, the closer the object to a circle, higher is the feature value. The circularity and contrast were used to determine the trafficking of mKOR-tdT.

OGD induced KOR trafficking located by immunoelectron microscopy

For electron microscopy, N2A-mKOR-tdT cells were cultured in T25 flasks and divided to control group without OGD, and OGD group receiving the 1 h OGD treatment as described above. After 20 min fixation with 4% paraformaldehyde/NaAc (pH=7.3) at room temperature, the cells were gently scraped, centrifuged with the speed of 1200r/min for 5 min, and subjected to -80°C by high-pressure freezing machine with working pressure of 2300bar (HPM 010, Boeckeler Instruments, Arizona, USA). Cells were then embedded in hydrophilic resin medium HM20 and polymerized with UV light. 80 nm ultrathin sections were cut from the resin-embedded blocks at room temperature by diamond knife and collected onto the 300 mesh nickel grids. Grids were rehydrated with PBS for 10 min, blocked with 1%BSA/2% normal goat serum/PBS for 30 min, and incubated with the anti-RFG primary antibody (1:500) at room temperature. Sections incubated in 1%BSA/PBS were used as negative control. The sections were then washed with PBS, incubated with 1%BSA/PBS for 20 min and immunostained with Alexa Fluor 488-conjugated goat anti-rabbit IgG with 10 nm colloidal gold conjugate for 40 min in a humidified container. Grids were washed with PBS four times 5 min each in porcelain plate wells and fixed with 2% glutaraldehyde/PBS. Next, grids were washed with aqua dest three times, 5 min each, and briefly stained with uranyl acetate and lead citrate. Finally, grids were dried, randomly assigned and examined in a double-blind fashion using a Jeol-JEM100CX electron microscope (JEOL Ltd., Tokyo, Japan) at 60 kV. KOR internalization in various groups studied was quantified by calculating the ratio of gold particles in cytoplasm to the total gold particles for each untrasection with a remarkable nucleus.

Statistical analysis

All data are shown as mean $\pm$ S.D. unless otherwise indicated. Sample sizes were estimated based on our previous laboratory experience. Statistical significance among the groups was determined by Student's two-tailed *t* test or one-way ANOVA followed by Tukey's test. $P < 0.05$ was considered as statistically significant. Cell cultures were randomly assigned to the experimental groups using random numbers and processed identically throughout the whole experiments. Statistical analyses were performed with Graph-Pad Prism (Version 6.01, GraphPad Software, Inc. CA). Images were analyzed using ImageJ (1.48v, National

Institutes of Health, MD). All valid data were included to the analysis, without excluding outliers unless otherwise stated.

Results

OGD and cell viability

The partial pressure of oxygen in OGD group of N2A-mKOR-tdT cells was significantly lower than that in the control group. There was no statistically significant difference among OGD groups (Fig. 1-A). No significant difference was observed in partial pressure of carbon dioxide among groups of N2A-mKOR-tdT cells (Fig. 1-B). There were no significant cell viability changes within 2 h of OGD applied to N2A-mKOR-tdT (Fig. 1C) or N2A-mKOR-FLAG (Fig. 1D), indicating that OGD within 2 h was suitable for the investigation of hypoxia induced KOR trafficking. Significant cell death was observed only after 4 hours of OGD ($P < 0.001$, Fig. 1-C, D).

OGD induced KORs internalization

Under normoxia conditions, most KORs were located on the cell-surface. However, after 30 min of OGD treatment, KOR trafficking was observed in both N2A-mKOR-tdT and N2A-mKOR-FLAG cells. Detected fluorescence signals indicated the KORs moved into the cytoplasm. This phenomenon became prominent with the prolonged hypoxia (Fig. 2-A, B, C). After 1 h of re-oxygenation, most of the KORs recycled back to the cell-surface in both cell lines (Fig. 2-D). To reconcile internalization of KORs with changes in the receptor trafficking after the OGD treatment, confocal microscopy was performed. A z-stack of optical section, 10 μm in thickness was captured, and similar movement of KORs was observed (Fig. 3).

SA, a selective KOR agonist induced KOR internalization, which was similar to that of OGD induced KOR trafficking (Fig. 4). Importantly, both Dyno-4a and nor-BIN, inhibited OGD induced KORs movement as well as that induced by SA (Fig. 5A), indicating that KOR movement under OGD conditions was an internalization event. Pretreatment with both Dyno-4a and nor-BIN displayed no toxicity on the N2A-mKOR-tdT cells (Fig. 5B).

The results from fluorescence imaging flow cytometry show that mKOR-tdT of cells in normoxia had a higher circularity index than that of in hypoxia (Fig. 6-A). Single cell imaging illustrated that OGD induced intense fluorescence gathered in the intracellular space, while nor-BNI inhibited such morphological changes (Fig. 6-B).

After 4 hours of OGD, KOR trafficking could no longer be blocked or reversed by re-oxygenation (data not shown), due to irreversible cellular damage induced by prolonged hypoxia. This was consistent with the results of MTT assay. Treatment of cells with glibenclamide, iberiotoxin, or sulpiride failed to block the OGD-induced KOR internalization, indicating that adenosine triphosphate sensitive and high-conductance calcium-activated potassium channels, as wells as dopamine D₂-receptors are not involved in OGD-induced KOR internalization (data not shown).

OGD-induced subcellular KOR trafficking observed by electron microscopy

Immunogold labeling of ultrathin sections of the cells revealed subcellular localization of KORs. Under normal oxygen, the cell was in good shape and the nucleus was large-spindle (Fig. 7A); however, under hypoxia, many swollen mitochondria were present, the nucleus was shrunken, and the nucleolus was observed all over the nucleus (Fig. 7-B). Under normal oxygen, most gold particles associated with KORs were distributed on the cell-surface (Fig. 7C). After 1 h of OGD treatment, gold particles moved into the cytoplasm and distributed in the periphery of the nucleus. Fewer particles were found on the cell-surface (Fig. 7D). Perinuclear distribution coincided with the results of the fluorescence microscopy. To quantify the movement of KORs, 10 sections with distinct nuclei were examined, and the gold particles were counted (five sections on one grid, two grids under each condition). There was no significant difference in quantity of total particles between the control group and the OGD group. During normoxia, 62.7% and 37.3% particles were associated with the cell-surface and the cytoplasm respectively. OGD significantly increased the portion of gold particles to 85% in the cytoplasm ($p < 0.01$) (Fig. 7E, F).

Discussion

In this study, the effect of OGD on the translocation of KOR in the mouse Neuro-2A neuroblastoma cells was investigated. In normoxia, the majority of KORs were located on the cell membrane. OGD induced significant internalization of KORs. Such internalization can be blocked by specific KOR antagonists, or reversed by re-oxygenation. OGD-induced KOR internalization might share the similar mechanism with the agonist-activated KOR internalization, since specific KOR antagonists can block it. These findings suggest that KOR might play a critical role in modulating hypoxia.

GPCR-mediated signal transduction depends on the receptors density and activity for ligand binding on the cell-surface.^{30,31} Agonist activation triggers changes of receptors density and activity by three distinct processes. Receptor desensitization (from seconds to hours) is the alternation of the receptor function but not receptor amount, while internalization (from minutes to hours) is a rapid agonist-induced receptor movement from cell-surface to cytoplasm. Reversible movement is critical for receptor resensitization. Down-regulation (from hours to days) refers to a reduction of total receptor due to less gene expression.^{30–32} Previous study demonstrated that 30 min U50,488 exposure promoted 30–40% of total KORs internalization, which began with phosphorylation of KORs by G protein-coupled receptor kinases (GRKs) following β -arrestin recruitment and uncoupling KOR from G protein. Uncoupled KORs then clustered into the clathrin-coated pits to form endocytic vesicles. Removal of U50,488 resulted in gradual recycling of KORs to the cell-surface over an hour.^{11,32} It was also reported that 15-min acute salt loading could elicit increased KORs in the presynaptic plasma membrane, which was due to the cell membrane depolarization.³² All the above reports indicate that multiple external factors such as agonist treatment or physical stimulation might change KORs distribution.

Explicit KOR distribution is important for KOR-related signal transduction and therapeutic efficacy. Prolonged hypoxia (9%, 7 days) selectively decreased DOR ligand binding, but not MOR or KOR in mice brains, and such changes might be due to the hypoxia-stimulated

endogenous δ agonist release and DOR down-regulation.³³ KOR ligand binding decreased between 6 and 24 hours after permanent occlusion of the distal middle cerebral artery (MCAO) in the infarct frontoparietal cortices of mice, which was slower than DOR ligand binding (1 to 3 hours) and MOR ligand binding (6 to 12 hours). Similarly, KOR ligand binding decreased later (12 to 48 hours after MCAO) than DOR ligand binding (6 to 12 hours) in the infarct border zone.³⁴ The ligand binding affinity reflects both the available receptor expression and the receptor activity. However, the change of receptor distribution by this method is determined indirectly and the subcellular distribution of opioid receptor cannot be revealed under hypoxia.

In this study, the change of KORs trafficking was observed directly. The redistribution time window and phenomenon are similar to agonist induced KOR internalization, which can be blocked by the KOR antagonist. Endocytosis inhibitors can block such event. All these findings support the notion that OGD induces KOR-activated internalization in Neuro-2A cells. External stress was reported to activate KOR via increased endogenous dynorphin release *in vivo*.³⁵ However, the mechanisms involved in OGD-induced KOR activation and internalization were different since there should have no endorphin secretion in an *in vitro* model. Exposure to severe hypoxia for 30 min lead to significant 5-HT (serotonin) receptor internalization by up to 40% in the adult ovine common carotid artery, which might be due to GRK-related receptor phosphorylation.³⁶ Similarly, 1 hour OGD was found to decrease GABA_A receptors in cultured hippocampal neurons via accelerated internalization. Decreased adenosine triphosphate levels, elevated levels of protein kinase C activity and calcium-activated phosphatases might be involved in this process.³⁷ Acute hypoxia first activates oxygen-regulated ion channels in the cell membrane, which triggers inhibition of potassium channel, activation of sodium channel, membrane depolarization and potentiation of calcium influx.^{38–40} Mitogen-Activated Protein Kinases (MAPKs), a family of serine-threonine protein kinases including, extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 proteins, are activated by diverse stimuli including cellular stress and hypoxia. Hypoxia induced phosphorylation of p38 and p42/44 MAPK in a neural-like PC12 cell line.^{41,42} In human ovarian carcinoma cells, activated p38/MAPKs were significantly increased between 30 min and 120 min after acute hypoxia and declined thereafter.⁴³ Although it is not yet known which signal transduction cascades lead to hypoxic activated MAPKs, the increased calcium and mitochondrial reactive oxygen species (ROS) might initiates phosphorylation of MAPK.^{42,44,45} Similarly, stimulation of KOR was reported to activate MAPK, which was independent of KOR internalization.¹⁴ It seems that both hypoxia and activation of KOR might have the same downstream cellular signaling pathway. Recently it was reported that GPCR-mediated ERK activation had a reciprocal effect on GPCR trafficking, which reduced the cell-surface expression of GPCRs and increased the intracellular expression.⁴⁶ Thus, it is unlikely that the acute or short term hypoxia induced KOR activation and internalization observed in this study share the similar mechanism noted above. Despite the effort to investigate the potential roles of some of the ion channels and dopamine receptors, we failed to elucidate the explicit molecular mechanism of hypoxia -induced KOR activation. Further studies are needed. Consistently, with long-time agonist-induced KOR down-regulation, exposure to OGD for more than 4 h

impaired the KOR recycling. Neither re-oxygenation nor antagonists could reverse such KOR movement with decreased KOR numbers in the cell membrane.

While we cannot apply the findings in this study to all the neuronal cells, we believe that the discovery related KOR internalization using neuroblastoma cell is applicable to other neuronal cells since SA, a highly selective KOR agonist, induced KOR internalization that can be blocked by KOR antagonist nor-BNI. However, considering signal disruption activity of nor-BNI,⁴⁷ additional mechanistic studies are required to elucidate whether hypoxia may induce or disrupt KOR-dependent signaling pathways. The exact clinical implication and significance of the KOR internalization induced by hypoxia is not known based on the current *in vitro* data. It is possible that such KOR internalization is a protective physiological mechanism to avoid harm from hypoxia for neurons. The indirect evidence for such statement is that SA, the highly selectively KOR agonist, can induce KOR internalization similar to that by hypoxia and SA could protect the brain from hypoxia and ischemia, resulting improved neurological outcome as we have demonstrated in a rodent model of brain ischemia.⁴⁸ Further experiments are needed to investigate whether naïve KORs in primary neuronal cultures undergo similar trafficking as in neuroblastoma cells with KOR over-expression.

Conclusion

Hypoxia induces reversible KOR internalization, which was inhibited by selective KOR antagonists or dynamin inhibitor, and can be reversed by re-oxygenation in neuroblastoma cells, indicating the modulating effects between KOR and hypoxia via KOR activation and dynamin dependent mechanism.

Acknowledgments

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Fig. 1.

OGD and cell viability. (A) All OGD groups of N2A-mKOR-tdT cells maintained with 95% N₂ and 5% CO₂ from 0.5h to 12 h had significantly decreased partial pressure of O₂ (mmHg) in culture medium compared with that in the control group without OGD (n=6 per each group). (B) There was no significant difference among partial pressure of CO₂ (mmHg) in culture medium with OGD of N2A-mKOR-tdT cells from 0.5 h to 12 h (n=6 per each group). OGD induced time-dependent decrease of cell viability in both N2A-mKOR-tdT (C) and N2A-mKOR-FLAG (D) cells after 4 h of treatment (n=12 per each group). OGD, oxygen-glucose deprivation; KOR, kappa opioid receptor; N2A-mKOR-tdT, Neuro2A cells stably transfected with mouse KOR-tdTomato fusion protein; N2A-mKOR-FLAG, Neuro2A cells stably transfected with Flag-tagged mouse KOR. Data are shown as mean $\pm$ SD; ***, $P < 0.001$.

Fig. 2.

OGD-induced KOR trafficking of N2A-mKOR-tdT and N2A-mKOR-FLAG cells with a similar pattern. N2A-mKOR-tdT cells expressing mKOR-tdTomato fusion protein (left, red) were either untreated (A) or treated with OGD for 0.5 h (B) or 1 h (C) before the fixation. N2A-mKOR-FLAG cells labeled with anti-FLAG monoclonal antibody were fixed followed by immunostaining with anti-FLAG Alexa Fluor 488-conjugated antibody (right, green). Both mKOR-tdT and mKOR-FLAG were distributed on the cell-surface of untreated group. However, both mKOR-tdT and mKOR-FLAG were internalized as punctuated under OGD. Moreover, OGD-induced change of KOR trafficking could be reversed by re-oxygenation (D). The figures represent one of the three independent experiments with similar results. Examples of mKOR-tdT and mKOR-FLAG are indicated by yellow arrow. Scale bar: A–D, 20 μ m. KOR, kappa opioid receptor; OGD, oxygen-glucose deprivation; N2A-mKOR-tdT, Neuro2A cells stably transfected with mouse KOR–tdTomato fusion protein; N2A-mKOR-FLAG, Neuro2A cells stably transfected with Flag-tagged mouse KOR.

Fig. 3.

OGD-induced KOR trafficking was further confirmed by confocal microscopy. N2A-mKOR-tdT (left, red) and N2A-mKOR-FLAG cells (right, green) were either untreated (A) or treated with OGD for 1h (B) following the fixation and immunostaining as described in Fig. 2. A single 10 μ m layer out of z-stack is shown. Examples of mKOR-tdT and mKOR-FLAG are indicated by yellow arrow. Scale bar: A–B, 20 μ m. OGD, oxygen-glucose deprivation; KOR, kappa opioid receptor; N2A-mKOR-tdT, Neuro2A cells stably transfected with mouse KOR–tdTomato fusion protein; N2A-mKOR-FLAG, Neuro2A cells stably transfected with Flag-tagged mouse KOR.

Fig. 4.

OGD-induced KOR trafficking shared similar pattern of KOR agonist –induced KOR internalization. N2A-mKOR-tdT cells were untreated in control (A), or incubated with 1 μ M Salvinorin A (SA), a selective KOR agonist, for 1 h (B), or treated with OGD for 1 h (C). Cells were fixed, mounted with DAPI and observed by an inverted fluorescence microscope. SA induced KOR internalization, with decrease of mKOR-tdT in the cell-surface and increase of crescent-shape mKOR-tdT in the cytoplasm, which was similar to OGD induced mKOR-tdT trafficking in the left panel. Merged images in the right panel show that both the internalized KORs by SA and OGD were clustered in the periphery of the nucleus. Examples of mKOR-tdT are indicated by yellow arrow. Scale bar: 20 μ m. KOR, kappa opioid receptor; OGD, oxygen-glucose deprivation; SA, Salvinorin A; N2A-mKOR-tdT, Neuro2A cells stably transfected with mouse KOR–tdTomato fusion protein.

Fig. 5.

KOR antagonist or internalization inhibitor blocked the OGD-induced KOR internalization. N2A-mKOR-tdT cells pretreated with 10 μ M KOR selective antagonist nor-BIN or 30 μ M dynamin 1 inhibitor Dyngo-4a for half an hour inhibited both salvinorin A (SA) and OGD induced KOR internalization. Examples of mKOR-tdT are indicated by yellow arrow. (a) SA/OGD, (b) nor-BIN+SA/OGD, (c) Dyngo-4a+ SA/OGD. Scale bar: 20 μ m. (B) Cell viability by MTT assay after 1 μ M SA or OGD treatment of the N2A-mKOR-tdT cells for 1 h either with or without 10 μ M nor-BNI or 30 μ M Dyngo-4a pretreatment for 30 min. Data are shown as mean $\pm$ SD (n=6). KOR, kappa opioid receptor; OGD, oxygen-glucose deprivation; MTT, (3-(4, 5- dimethylthia- 2ol -2-yl)-2, 5- diphenyl-tetrazolium bromide); N2A-mKOR-tdT, Neuro2A cells stably transfected with mouse KOR-tdTomato fusion protein; SA, salvinorin A; nor-BNI, norbinaltorphimine.

Fig. 6. OGD decreased circularity feature and increased contrast of mKOR-tdT with imaging flow cytometry observation. Cells in normoxia had a higher circularity index than that in hypoxia (A). OGD induced a decrease in circularity and an increase in contrast of mKOR-tdT. KOR antagonist nor-BNI inhibited this change (B). OGD, oxygen-glucose deprivation; KOR, kappa opioid receptor; mKOR-tdT, mouse KOR-tdTomato fusion protein; nor-BNI, norbinaltorphimine.

Fig. 7.

KOR trafficking under OGD was observed with immunoelectron microscopy. (A) Healthy cells in normoxia had large-spindle nucleus; (B) Nucleus shrinkage and cytoplasmic vacuolization were observed in OGD group; (C) KOR-conjugated gold particles located around the cell membrane (Green arrows: cell membrane; Blue arrows: gold particles); (D) KOR-conjugated gold particles distributed in the periphery of nucleus area. (E) There was no difference in the total gold particles between normoxia and OGD groups, however more cytoplasmic particles were founded in OGD group ($n=10$ per each group). (F) Distribution of gold particles within the cytoplasm after 1 h of normoxia (Control) or OGD conditions ($n=10$ per each group). KOR, kappa opioid receptor; OGD, oxygen-glucose deprivation; Total, the total number of particles in one cell; CM, cell membrane particles number; CP, cytoplasmic particles number. Data are shown as mean $\pm$ SD; ***, $P < 0.001$.