Research Article

Journal of Pharmaceutics and Therapeutics

The Post-Ischemic Increase in GluA2 Ser880 Phosphorylation Involves NADPH Oxidase

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Received date: September 03, 2018; Accepted date: September 11, 2018; Published date: September 16, 2018

Abstract

Most 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptors (AMPA receptors) expressed on adult hippocampal pyramidal neurons contain the edited form of GluA2 (Q607R) and are thus impermeable to Ca^{2+} / Zn^{2+} entry. Following ischemic injury, these receptors undergo a subunit composition change, switching from a GluA2-containing Ca^{2+} / Zn^{2+} -impermeable AMPAR to a GluA2-lacking Ca^{2+} / Zn^{2+} -permeable AMPAR. Recent studies indicate that an oxidative stress signaling pathway is responsible for the I/R-induced changes in AMPAR subunit composition. Studies suggest that nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), a superoxide generator, is the source that initiates the oxidative stress-signaling cascade during post-ischemic reperfusion. The objective of the present study was to determine if suppression of NADPH oxidase activity prevents the increase in phosphorylation and subsequent internalization of the GluA2 AMPAR subunit during reperfusion of post-ischemic hippocampal slices. In this study, we demonstrated that exposure of adult rat hippocampal slices to oxygen glucose deprivation/reperfusion (OGD/R) results in an increase in Ser880 phosphorylation of the GluA2 subunit. The increase in Ser880 phosphorylation resulted in the dissociation of GluA2 from the scaffolding proteins Glutamate receptor-interacting protein 1 (GRIP1) and AMPAR binding protein (ABP), thus enabling the association of GluA2 with protein interacting with C kinase 1 (PICK1). OGD/R also resulted in an increase in the association of activated protein kinase C α (PKC α) with PICK1. We have found that pharmacological inhibition of NADPH oxidase with apocynin diminishes the OGD/R-induced increase in activated PKC α association with PICK1 and subsequent Ser880 phosphorylation of GluA2. Suppression of NADPH oxidase activity also blunted OGD/R-induced decreased association of GluA2 with the scaffolding proteins GRIP1 and ABP. Protein phosphatase 2A (PP2A), which regulates PKC α activity by dephosphorylating the kinase, was inactivated by OGD/R-induced increase in tyrosine phosphorylation of the phosphatase (Y307). Inhibition of NADPH oxidase activity ameliorated OGD/R-induced PP2A phosphorylation and inactivation. Our findings are consistent with a model of OGD/R-induced Ser880 phosphorylation of GluA2 that implicates NADPH oxidase mediated inactivation of PP2A and sustained PKC α phosphorylation of GluA2.

Keywords: Ischemia, NADPH oxidase, AMPA receptor, GluA2, PICK1, PKC α , GRIP1, ABP

Abbreviations: AMPA: 2-Amino-3-(3-Hydroxy-5-Methyl-Isloxazol-4-yl) Propanoic Acid; NADPH oxidase: Nicotinamide Adenine Dinucleotide Phosphate-Oxidase; GRIP1: Glutamate Receptor-Interacting Protein 1; ABP: AMPA receptor Binding Protein; PICK1: Protein Interacting with C Kinase 1; PKC α : Protein Kinase C Alpha; PP2A: Protein Phosphatase 2A; NMDAR: N-Methyl D-Aspartate Receptor; OGD/R: Oxygen-Glucose Deprivation/Reperfusion; LTP/

LTD: Long-Term Potentiation/Depression; CNS: Central Nervous System; PSD-95: Post-Synaptic Density Protein; DlgA: Drosophila Disc Large Tumor Suppressor; PDZ domain: Post-synaptic density protein (PSD-95), Drosophila disc large tumor suppressor (DlgA), Zonula occludens-1 protein (Zo-1) domain

Introduction

AMPA receptors, which arise from 4 genes (GLUA1-GLUA4) to form a functional receptor tetramer, mediate the majority of fast excitatory synaptic transmission in the CNS. While all AMPAR subunits share a large degree of homology, the presence or absence of a GluA2 subunit contained within the functional AMPAR tetramer is important in determining channel properties and ionic permeability. Most AMPARs expressed on adult hippocampal pyramidal neurons contain the edited form of GluA2 (Q607R), thereby rendering GluA2-containing AMPARs impermeable to divalent cation (Ca^{2+} and Zn^{2+}) conductance [1],[2]. Following ischemia/reperfusion however, AMPARs undergo a subunit composition switch from GluA2 containing $\text{Ca}^{2+}/\text{Zn}^{2+}$ -impermeable AMPA receptors to GluA2-lacking $\text{Ca}^{2+}/\text{Zn}^{2+}$ -permeable AMPARs, thus allowing the AMPAR to conduct $\text{Ca}^{2+}/\text{Zn}^{2+}$ and contributes to delayed neuronal death [3]. This change in AMPAR expression pattern is influenced by both the removal of the GluA2-containing receptors and the insertion of the GluA2-lacking receptors to the membrane surface.

Under physiological conditions, GluA2 endocytosis is mediated by PKC α dependent phosphorylation of serine residue 880 (Ser880) [4]. PKC α , when activated by calcium influx, is trafficked to the plasma membrane by PICK1 [5], where it binds the GluA2 scaffolding protein ABP and subsequently phosphorylates GluA2 [6]. Phosphorylation of GluA2 promotes the dissociation of GluA2 with the scaffolding protein GRIP1, an interaction that is important in anchoring AMPARs to the plasma membrane [5]. Additionally, Ser880 phosphorylation of GluA2 enables the increased binding of the subunit to PICK1 [4], an interaction that has been shown to be important in decreasing surface GluA2 levels [7],[8]. As a result, the population of GluA2-containing AMPARs is reduced, ultimately allowing for an increase in calcium permeable GluA2-lacking AMPARs, a shift important for synaptic strengthening under physiological conditions [9], but ultimately deleterious if uncontrolled under pathological conditions such as stroke [10].

In this study, we show that the treatment of acute adult rat hippocampal slices to oxygen-glucose deprivation/reperfusion (OGD/R) results in a sustained phosphorylation of GluA2, leading to the dissociation of GluA2 from GRIP

and ABP, both of which favor the association of GluA2 with PICK1. Additionally, the inhibition of NADPH oxidase prevented the OGD/R-induced increase in Ser880 phosphorylation of GluA2, consequently attenuating the loss of GRIP1/ABP-GluA2 interaction and preventing GluA2/PICK1 complex formation. Further, inhibition of NADPH oxidase dampened the increase in association of the activated form of PCK α with PICK1, a necessary step prior to phosphorylation of GluA2, as well as diminished the OGD/R induced inactivation of protein phosphatase 2A (PP2A). While the exact mechanism through which NADPH oxidase influences GluA2 Ser880 phosphorylation has yet to be elucidated, these findings identify this ROS generator as a key participant in the OGD/R induced Ser880 phosphorylation of hippocampal GluA2 subunits and diminished membrane surface expression of GluA2-containing AMPARs.

Experimental Procedures

Preparation of acute rat hippocampal slices

Adult male (6-8 week) Sprague-Dawley rats (Charles River Labs, Wilmington, MA, USA) were anesthetized with isoflurane and rapidly decapitated. The entire brain was then removed and placed in an ice-cold cutting solution (75mM Sucrose, 80mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4 , 24 mM NaHCO_3 , 25 mM Glucose, 4 mM MgCl_2 , 1mM L-Ascorbic Acid, 3 mM Na Pyruvate, 0.5 mM CaCl_2 , pH 7.4). Both hippocampi were then quickly dissected away from the brain and coronal 400 micron thick slices were made using a vibratome tissue chopper (McIlwain, Schwalbach, Germany). Slices were subsequently equilibrated in oxygenated (95% O_2 , 5% CO_2) artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 2.5 mM KCl, 1.25 mM KH_2PO_4 , 26 mM NaHCO_3 , 10 mM Glucose, 1.5 mM MgCl_2 , 2.5 mM CaCl_2 , pH 7.4) for 90 minutes prior to treatment with OGD/R. Fresh aCSF was switched out every 30 minutes during equilibration. Pre-treatment with 30 μM apocynin (Sigma, St. Louis, MO, USA) or 1:1000 vehicle (DMSO) occurred during the final 30 minutes of the equilibration process and was present throughout the duration of the experiment, either during time matched normoxia treated controls or OGD and subsequent reperfusion.

Oxygen-glucose deprivation/reperfusion of hippocampal slices

Following equilibration in oxygenated aCSF, slices to be treated with OGD/R were rinsed with aCSF without glucose (aCSF with 10 mM Mannitol substituted for 10 mM Glucose, pH 7.4) to remove glucose from the slices, and incubated for 40 minutes in a hypoxic glove box (Coy Laboratories, Grass Lake, MI, USA) containing a gas mixture of 0% O₂, 95% N₂, 5% CO₂ with aCSF minus glucose. The aCSF minus glucose media used for OGD was placed in 0% O₂ overnight to ensure complete anoxia. Following OGD, slices were removed from the hypoxic glove box and transferred back to oxygenated aCSF containing glucose for the time points indicated. Normoxic controls were left in aCSF containing glucose throughout the entire experiment and were time matched to the last of the OGD/R time points.

Lysate preparation

Slices were removed from aCSF at the indicated time point and rinsed with ice cold PBS (pH 7.4). For total protein level detection, slices were placed in tubes containing lysis buffer (250 mM Sucrose, 20 mM HEPES, 2 mM EDTA, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM AEBSF, 1% protease and phosphatase inhibitor cocktail (Thermo, Rockland, IL, USA), 1% Triton X-100, 0.01% saponin, pH 7.4) and lysed immediately via sonication for a 3 separate 5 second bursts at 25% power output with a VirTis Ultrasonic Cell Disrupter 100 (Gardiner, NY, USA). Samples were then spun at 1,000 x g to remove nuclei and cellular debris and a bicinchoninic acid assay (BCA, Thermo) assay was performed to determine protein content. Samples were then denatured in Laemmli buffer and heat (100°C, 10 minutes) and resolved via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were then transferred to a nitrocellulose membrane (Bio-Rad, Berkeley, CA, USA) for subsequent detection via immunoblotting.

Immunoblotting

Blots were blocked for 1 hour at room temp with either 5% BSA (for phospho-antibody detection) or 5% non-fat dry milk (for all others) in Tris Buffered Saline, 0.1% Tween 20, pH 7.5 (TBS-T). After blocking, blots were incubated with primary antibody overnight at 4°C at the concentration indicated. The affinity purified rabbit-monoclonal GluA2 antibody (1:2000), Protein Kinase C α (1:2000), and phospho-Protein Kinase C α (Thr497) (1:2000) were purchased from Epitomics (Burlingame, CA, USA). The affinity purified goat polyclonal PICK1

antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The affinity purified rabbit polyclonal GRIP1 (1:1000) and ABP (1:1000) antibodies were purchased from Chemicon (Billerica, MA, USA). The rabbit monoclonal protein phosphatase type 2A (PP2A) (1:1000) and phospho-PP2A (Tyr307) (1:1000) antibodies were purchased from Epitomics (Burlingame, CA, USA). Goat anti-Mouse-HRP and Goat anti-Rabbit-HRP secondary antibodies (1:2000) were purchased from Jackson Immuno Research (West Grove, PA, USA). The Donkey anti-Goat-HRP secondary antibody was purchased from Santa Cruz. Immunoreactive bands were visualized and captured with a Fuji imaging system using enhanced chemiluminescence after adding HRP conjugated secondary antibodies. Bands were analyzed using Fuji Image-Gauge software. Blots were stripped and re-probed up to 4 times using Restore plus Western Blotting Stripping Buffer (Thermo).

Immunoprecipitation

To visualize the PICK1-PKC α and PICK1-GluA2 protein complex, rat hippocampal slices were lysed in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40, and 1% protease and phosphatase inhibitor cocktail, pH = 7.5. To immunoprecipitate GluA2-GRIP1, GluA2-ABP, and GluA2-PICK1, rat hippocampal slices were lysed in "GluA2 complex lysis Buffer" containing 20 mM HEPES, 2 mM EDTA, 2 mM EGTA, 0.1 mM DTT, 100 mM KCl, 1% Triton X-100 and 1% protease and phosphatase inhibitor cocktail, pH = 7.2. Protein concentration was then determined using a BCA assay, and lysates (500 μ g/sample in 500 μ l) were then pre-cleared using Protein-A/G 50/50 mix of agarose beads for 1 hour at 4°C followed by incubation with either a PICK1 (1:50) or GluA2 (1:200) antibody overnight at 4°C. The immunocomplex was then incubated for 4 hours with 50 μ L Protein-A/G beads at 4°C with rotation before being washed 3 times with lysis buffer. Samples were then eluted from the agarose beads by treatment with Laemmli buffer and heat (100°C) and subjected to 7.5% SDS-PAGE. After transfer to nitrocellulose membranes, blots were blocked as and incubated overnight at 4°C as previously described. Immunoreactive bands were analyzed using Fuji Image-Gauge software.

Statistical analysis

Indicated statistical tests were performed using GraphPad Prism Software (La Jolla, CA, USA).

Results

Oxygen-glucose deprivation/reperfusion results in an increase in GluA2 Ser880 phosphorylation and interaction between activated PKC α , GluA2 with PICK1

Aberrant endocytic trafficking of GluA2 AMPA receptor subunit is preceded by and dependent on PKC α -PICK1-induced phosphorylation of the GluA2 subunit following cerebral trauma [11],[12]. We sought to determine whether OGD/R exposure of adult rat hippocampal slices lead to an increase in GluA2 Ser880 phosphorylation. Secondly, we examined whether OGD/R exposure increased association between GluA2, and activated PKC α (phosphThr497) with PICK1. We observed in slices subjected to 40-minutes of OGD incubation, an increase in GluA2 Ser880 phosphorylation that was maximally significant at 30-minutes of reperfusion (Figure 1A and 1B). OGD alone at 40-minutes or at 60-minutes did not lead to an increase in GluA2 Ser880 phosphorylation. Increase phosphorylation at serine residue 880 on GluA2 enables interaction of GluA2 with PICK1, an event demonstrated to reduce surface expression of GluA2 [7],[8]. We next sought to determine whether the OGD/R-induced increase in Ser880 phosphorylation of GluA2 observed in rat hippocampal slices (Figure 1A and 1B) functionally lead to an increase in GluA2-PICK1 association. Temporally consistent with the OGD/R-induced increase in GluA2 Ser880 phosphorylation status, our results indicate a significant increase in GluA2-PICK1 association during reperfusion of OGD-treated slices (Figure 2A and 2B). These results serve as a correlate with the increase in GluA2 Ser880 observed during the reperfusion of OGD-treated slices, and suggest a prevented recycling of GluA2 via PICK1-mediated membrane exclusion. PICK1 functions as a transport and targeting protein that directs the PKC α -dependent GluA2 Ser880 phosphorylation [5],[6]. However, it has not been directly demonstrated whether OGD/R exposure increases interaction between activated PKC α and PICK1. Therefore, we next sought to determine whether OGD/R exposure leads to an increase association between activated PKC α . There was a significant increase in the interaction between activated PKC α and PICK1 following OGD/reperfusion (Figure 3A). Interestingly, OGD alone did not result in a significant PKC α -PICK1 association, but the reperfusion of OGD-treated slices did, ultimately resulting in a rapid and sustained phospho-PKC α -PICK1 interaction upon reperfusion. Collectively, these initial results are consistent with OGD/R promoting the activation of PKC α , which associates with PICK1 to promote the Ser880 phosphorylation of the AMPAR

GluA2 subunit.

Figure 1: OGD/R exposure of adult rat hippocampal slices promotes the Ser880 phosphorylation of GluA2. Representative Western blot (A) from 3 independent experiments of hippocampal slices illustrates the time course of GluA2 Ser880 phosphorylation. Quantification (B) of GluA2 Ser880 phosphorylation levels expressed as a ratio of phospho-GluA2(Ser880) to total GluA2 protein levels expressed as fold change over control $\pm$ S.D from three separate experiments. (asterisk * in (B) indicates a p < 0.05 from normoxic controls; ANOVA with post hoc Bonferroni test).

Inhibition of NADPH oxidase with apocynin decreases the OGD/R-induced increase in PKC α activation and subsequent Ser880 phosphorylation of GluA2

Apocynin, an inhibitor of NADPH oxidase activity, has been demonstrated by our lab and others to decrease ROS [13], decrease damage to post-synaptic proteins [14], and provide neuroprotection [15] during ischemia/reperfusion. Oxidative stress has also been implicated in affecting ionic homeostasis during ischemia/reperfusion, through the enhancement of NMDAR currents [16],[17]. Phospholipase C mediated mobilization of intracellular

calcium stores [18], mitochondrial calcium release [13],[19] and the opening of cation channels [20] such as the transient receptor potential (melastatin) 2 (TRPM2). Therefore, we sought to determine if the reduction in oxidative stress observed with the inhibition of NADPH oxidase during OGD/R resulted in an attenuation of activated PKC α associated with PICK1. Our results illustrate that hippocampal slices pre-treated with apocynin (30 μ M) prior to OGD/R showed a significant reduction in the activated form of PKC α associated with PICK1 (Figure 3A and 3B), indicating that NADPH oxidase-mediated oxidative stress was involved in the OGD/R-induced increase in PKC α activity. Additionally, inhibition of NADPH oxidase activity also dampened the OGD/R-induced increase in GluA2 Ser880 phosphorylation (Figure 3C and 3D). These experiments show that the increase in the oxidant environment following OGD/R resulting from NADPH oxidase activity contributes to the sustained activation of PKC α associated with PICK1 and the subsequent rise in GluA2 Ser880 phosphorylation.

Inhibition of NADPH oxidase prevents the OGD/R-induced increase in GluA2-PICK1 association and OGD/R-induced loss of GluA2-GRIP1 association

On the C-terminal tail of GluA2, the sequence (IESVKI) serves as the PKC α mediated phosphorylation site at Ser880 and also as a PDZ binding domain. When GluA2 is in the dephospho-Ser880 state, it preferentially binds the PDZ binding scaffolding proteins GRIP1 and ABP, which have been demonstrated to be important in retaining GluA2 at the plasma membrane [21],[22]. Upon Ser880 phosphorylation, the affinity for GRIP1 and ABP is reduced and the binding of another PDZ binding protein, PICK1 is promoted [4], potentially facilitating the surface removal of GluA2. Therefore, experiments were performed to determine if the decrease in Ser880 GluA2 phosphorylation observed with NADPH oxidase inhibition during OGD/R resulted in maintenance of GluA2-GRIP1/ABP anchoring, as well as a decrease in GluA2-PICK1 association. Consistent with the findings from the previous figure (Figure 3) of an OGD/R-induced increase in GluA2 Ser880 phosphorylation, inhibition of NADPH oxidase with apocynin prevented the disruption of GluA2-GRIP1/ABP anchoring (Figure 4A,4B,4C and 4D). Additionally, the interaction between GluA2-PICK1 was diminished (Figure 4E and 4F), further confirming the role of NADPH oxidase in promoting the OGD/R-induced increase in GluA2 Ser880 phosphorylation and the interaction of GluA2 with its PDZ binding partners GRIP1, ABP, and PICK1.

Figure 2: OGD/R promotes the association of PICK1 with GluA2. Lysates were prepared from hippocampal slices subjected to time matched normoxia, OGD alone, or OGD/R and immunoprecipitated with a PICK1 antibody (A). Also shown in (A) is the GluA2 immunoreactive band resulting from the supernatant following immunoprecipitation of PICK1, in addition to total levels of both PICK1 and GluA2. Quantification (B) of the PICK1-GluA2 immunocomplex band expressed as fold change over control $\pm$ S.D from three separate experiments. (asterisk * in (B) indicates a $p < 0.01$ from normoxic controls; ANOVA with post hoc Bonferroni test).

Inhibition of NADPH oxidase prevents the OGD/R-induced inactivation of PP2A

PP2A has been shown to play a role in regulating PKC activity [23]. Studies have demonstrated that oxidative stress induced by H₂O₂ inactivates PP2A, antioxidant treatment prevents this inactivation [24]. We demonstrated an OGD/R-induced increase in PKC α activity; this sustained phosphorylation may be mediated by the inactivation of PP2A. Therefore, experiments were performed to determine if PP2A undergoes OGD/R-induced inactivation, and if so, does reduction of oxidative stress by inhibition of NADPH oxidase prevent

PP2A inactivation. We show an OGD/R-induced increase in PP2A Tyr307 phosphorylation (Figure 5A and 5B), which was diminished in the presence of apocynin (Figure 6A and 6B). Our results indicate that NADPH oxidase-mediated oxidative stress is involved in the OGD/R-induced inactivation of PP2A.

Discussion

Multiple lines of evidence have shown that the activation of $\text{Ca}^{2+}/\text{Zn}^{2+}$ -permeable AMPARs following ischemic injury significantly contributes to neuronal death [12][25],[26],[27],[28],[29]. While it has been demonstrated that ischemia results in a decrease in GluA2 protein levels and an increase in AMPAR-mediated Ca^{2+} currents [30], the exact mechanistic signals resulting in GluA2-mediated surface removal have yet to be fully elucidated.

In this study, the data presented demonstrates that OGD/R-induced reduction of surface GluA2 AMPAR subunits involves NADPH oxidase dependent phosphorylation of the subunit. We found that inhibition of NADPH oxidase activity with apocynin during OGD/R not only dampened localized activation of PKC α with PICK1, but also attenuated the OGD/R-induced increase in Ser880 phosphorylation of GluA2. Furthermore, the decrease in Ser880 phosphorylation observed with NADPH oxidase inhibition during OGD/R also resulted in a decreased GluA2 interaction with PICK1, in addition to maintaining association of GluA2 with the membrane anchoring proteins GRIP1/ABP, ultimately rescuing the OGD/R-induced loss of GluA2 protein levels. We found that inhibition of NADPH oxidase activity with apocynin during OGD/R also diminished the OGD/R-induced inactivation of PP2A.

The PKC α -mediated Ser880 phosphorylation of GluA2 [31] has been shown to be important in the surface removal of GluA2 [4]. While some studies have indirectly implicated that increased Ser880 phosphorylation of GluA2 occurs during ischemia/reperfusion [12], no studies to date have directly shown that an increase in Ser880 occurs with such treatment. We directly found that Ser880 phosphorylation of GluA2 increased significantly during reperfusion of OGD-exposed slices, but not during OGD without reperfusion. Our data also indicated that the association of activated PKC α with PICK1, a step necessary prior to Ser880 phosphorylation of GluA2 [6], occurred maximally during reperfusion of OGD treated slices and not during OGD alone, data that is consistent with the time course established for the

increase in Ser880 phosphorylation of GluA2.

Phosphorylation of GluA2 at Ser880 has been shown to disrupt the GluA2 association with GRIP1/ABP, proteins that are important in the anchoring of AMPARs to the cell surface [32]. Dissociation from GRIP1/ABP exposes the PDZ binding domain on GluA2 where the Ser880 residue resides, and when phosphorylated, consequently allows increased association with PICK1 [22], a protein important in restricting GluA2 from the cell surface [7],[8]. Ischemia has been shown previously to enhance the interaction of PICK1 with GluA2 and decrease the association of GRIP1/ABP with GluA2 [12]. Consistent with these findings, the data presented in this study illustrates a significant increase in PICK1-GluA2 association as the GRIP1/ABP interaction with GluA2 decreases. Interestingly, inhibition of NADPH oxidase with apocynin maintains GluA2 anchoring to GRIP1/ABP and decreases PICK1-GluA2 association following OGD/R, findings in line with the decrease in GluA2 Ser880 phosphorylation observed with NADPH oxidase inhibition during OGD/R.

Additional studies need be performed to determine the precise manner in which NADPH oxidase contributes to the OGD/R-induced increase in PKC α activation and GluA2 Ser880 phosphorylation. The first possibility involves the direct redox-modulation of PKC α via NADPH oxidase derived ROS. Published reports have demonstrated that PKC activity is increased with oxidative stress [33], which can be attenuated with anti-oxidant treatment using α -tocopherol [34] or apocynin treatment [35]. However, studies have demonstrated that α -tocopherol reduces PKC α activity through non-antioxidant effects [36], potentially confounding the published results of redox-sensitive PKC activity. However, a reduction in glutathione levels has been reported to result in drastic increases in PKC activity [33]. Further research must be completed to fully understand the relationship between ROS and direct PKC activation.

A second possibility for OGD/R-induced NADPH oxidase-mediated increases in PKC α activity includes phosphatase inactivation. Protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) have been demonstrated to be important in regulating PKC activity [23]. Studies have shown that H_2O_2 -induced oxidative stress inactivates both PP1 and PP2A, which is prevented with antioxidant treatment [24]. Additionally, PP1 has been identified as the phosphatase responsible for the Ser880 dephosphorylation of GluA2 [37]. OGD/R-induced ROS-mediated inactivation of this phosphatase may therefore

serve as an important mediator in not only regulating PKC activity, but also GluA2 Ser880 phosphorylation. Furthermore, studies have demonstrated that the filamentous actin binding protein neurabin is important in targeting PP1 to the post-synapse for GluA2 Ser880 dephosphorylation [37]. Additionally, oxidative stress has been shown to result in the de-polymerization of actin [38], thereby potentially decreasing the synaptic localization of PP1 necessary for GluA2 Ser880 dephosphorylation. Decreased synaptic localization in addition to increased phosphatase inactivation could be important steps involved in the sustained activation of PKC α and phosphorylation of GluA2, events that are

attenuated with apocynin by decreasing NADPH oxidase-mediated oxidative stress during OGD/R.

A third possibility for the mechanism in which OGD/R-induced NADPH oxidase activity mediates PKC α activation involves enhancing intracellular Ca²⁺ concentrations. PKC α activity is positively regulated by increases in Ca²⁺ [39], therefore any enhancement in Ca²⁺ concentrations will increase PKC α activity. Oxidative stress and NADPH oxidase activity has been linked to effecting Ca²⁺ levels through NMDARs [14], phospholipase C-dependent release of intracellular Ca²⁺ stores [18], mitochondrial Ca²⁺ release [13],[19] as well as the opening of cation

Figure 3: The OGD/R-induced increase in activated PKC α associated with PICK1 and rise in Ser880 phosphorylation of GluA2 is blunted with inhibition of NADPH oxidase. Representative Western blot (A) from 3 independent experiments of hippocampal slices illustrates the pattern of PKC α (phospho-Thr497)-PICK1 association of slices exposed to OGD/R in the presence of vehicle (1:1000 DMSO) or apocynin (30 μ M) pre-treatment. (C) Representative Western blot of the OGD/R-induced increase in GluA2 Ser880 phosphorylation. Quantification (B) of the density of the phospho-PKC- α (Thr497)-PICK1 immunoreactive band expressed as fold change over control. Quantification (D) of GluA2 Ser880 phosphorylation levels expressed as a ratio of phospho-GluA2 (Ser880) to total GluA2 protein levels. Data represent fold change over control $\pm$ S.D from three separate experiments. (asterisk * in (B) and (D) indicates a p <0.05 from normoxic controls; ANOVA with post hoc Bonferroni test).

Figure 4: Inhibition of NADPH oxidase restores the OGD/R-induced loss of GluA2-GRIP1/ABP anchoring and prevents the association of GluA2 with PICK1. Lysates were prepared from hippocampal slices subjected to time matched normoxia, OGD alone, or OGD/R in the presence of vehicle (1:1000) or apocynin (30 μ M), followed by immunoprecipitation of GluA2 and subsequent immunoblotting for GRIP1 (A), ABP (C) and PICK1 (E) complex formation. Quantification (B, D, F) of the density of the GluA2-GRIP1, GluA2-ABP, and GluA2-PICK1 immunocomplex band are expressed as fold change over control $\pm$ S.D from three separate experiments. (Asterisk * indicates a $p < 0.05$; ANOVA with post hoc Bonferroni test).

Figure 5: PP2A undergoes Y307 phosphorylation inactivation following OGD/R exposure. Representative Western blot (A) from 3 independent experiments of hippocampal slices illustrates the time course of PP2A Y307 phosphorylation. Quantification (B) of PP2A Y307 phosphorylation levels. Data represent fold change over control $\pm$ S.D from three separate experiments. (asterisk * in (B) indicates a $p < 0.05$ from normoxic controls; ANOVA with post hoc Bonferroni test).

channels [20]. Therefore, inhibition of NADPH oxidase activity during OGD/R could potentially accomplish the dampening of PKC α activity not only through the maintenance of PP1 and PP2A phosphatase activity, but also through the attenuation of oxidative stress-enhanced rises in Ca^{2+} entry and intracellular Ca^{2+} release necessary for PKC α activation.

Taken together, this study demonstrates the effect of OGD/R-induced NADPH oxidase-mediated oxidative stress on GluA2 Ser880 phosphorylation and subsequent effect on membrane scaffolding. Though these data J Pharm Sci Therap, 4(1):170-181 (2018)

Figure 6: Inhibition of NADPH oxidase attenuates the OGD/R-induced increase in PP2A Y307 phosphorylation. Representative Western blots (A) from 3 independent experiments of hippocampal slices illustrates the time course of PP2A Y307 phosphorylation in the presence of vehicle (1:1000) or apocynin (30 μM). Quantification (B) of PP2A phosphorylation levels. Data represent fold change over control $\pm$ S.D from three separate experiments. (asterisk * in (B) indicates a $p < 0.05$ from normoxic controls; ANOVA with post hoc Bonferroni test).

provide a novel mechanism for the OGD/R-induced loss of GluA2 surface proteins, further studies will be necessary to fully characterize the upstream signaling pathways involved in mediating PKC α activation and the resultant increase in GluA2 Ser880 phosphorylation.

Funding

This work was supported by NIH grants S21 MD00236, P20RR015583, and R15NS074418.

The authors would also like to acknowledge Riley M. St.

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