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## Ischemic neuroprotectant PKC $\epsilon$ restores mitochondrial glutamate oxaloacetate transaminase in the neuronal NADH shuttle after ischemic injury

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### Abstract

The preservation of mitochondrial function is a major protective strategy for cerebral ischemic injuries. Previously our laboratory demonstrated that protein kinase C epsilon (PKC $\epsilon$ ) promotes the synthesis of mitochondrial Nicotinamide adenine dinucleotide (NAD<sup>+</sup>). NAD<sup>+</sup> along with its reducing equivalent, NADH, is an essential co-factor needed for energy production from glycolysis and oxidative phosphorylation. Yet, NAD<sup>+</sup>/NADH are impermeable to the inner mitochondrial membrane and their import into the mitochondria requires the activity of specific shuttles. The most important neuronal NAD<sup>+</sup>/NADH shuttle is the malate-aspartate shuttle (MAS). The MAS has been implicated in synaptic function and is potentially dysregulated during cerebral ischemia. The aim of this study was to determine if metabolic changes induced by PKC $\epsilon$  preconditioning involved regulation of the MAS. Using primary neuronal cultures, we observed that the activation of PKC $\epsilon$  enhanced mitochondrial respiration and glycolysis *in vitro*. Conversely, inhibition of the MAS resulted in decreased oxidative phosphorylation and glycolytic capacity. We further demonstrated that activation of PKC $\epsilon$  increased the phosphorylation of key components of the MAS in rat brain synaptosomal fractions. Additionally, PKC $\epsilon$  increased the enzyme activity of Glutamic oxaloacetic transaminase 2 (GOT2), an effect that was dependent on

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#### Compliance with Ethics Requirements

All institutional and national guidelines for the care and use of laboratory animals were followed. Animal usage and experimentation were approved by the Institutional Animal Care and Use Committee at the University of Miami and was in accordance with the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

#### Conflict of Interest

The authors declare that they have no conflict of interest.

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the import of PKC $\epsilon$  into the mitochondria and phosphorylation of GOT2. Furthermore, PKC $\epsilon$  activation was able to rescue against decreased GOT2 activity induced by ischemia. These findings reveal novel protective targets and mechanisms against ischemic injury, which involves PKC $\epsilon$ -mediated phosphorylation and activation of GOT2 in the MAS.

### Keywords

cerebral ischemia; ischemia tolerance; ischemic preconditioning; conditioning; malate aspartate shuttle; mitochondria

### Introduction

Worldwide, stroke is one of the leading causes of death and long-term disability [1]. The current FDA-approved treatments for stroke are recombinant tissue plasminogen activator and mechanical thrombectomy [1]. Although highly effective, these treatments can only benefit a small percentage of stroke patients leaving the majority of patients without treatment [1]. Preclinical studies have shown that ischemic preconditioning (IPC), which involves the induction of a sub-lethal ischemic insult prior to a lethal one, confers protection by activating intracellular adaptive mechanisms [2–4]. Our laboratory and others have previously demonstrated that preconditioning-induced neuroprotection is mediated, in part, by protein kinase C epsilon (PKC $\epsilon$ ): a serine/threonine kinase [5, 6].

Several downstream effectors of PKC $\epsilon$  have been identified in the mitochondria. Our lab has previously shown that PKC $\epsilon$  translocates to synaptosomal mitochondria, where it increases the phosphorylation of proteins of the mitochondrial electron transport chain and enhances mitochondrial respiration [7]. Given that the high-energy demand in the brain is primarily met by mitochondrial ATP production, the preservation of mitochondrial function is a major protective strategy for cerebral ischemic injuries [8–10].

PKC $\epsilon$  also targets the nicotinamide adenine dinucleotide (NAD $^{+}$ ) pathway in the mitochondria [11]. NAD $^{+}$  is a necessary co-factor needed for energy production from glycolysis and oxidative phosphorylation [12]. During glycolysis, NAD $^{+}$  is reduced to NADH, which is then oxidized by complex I of the mitochondrial electron transport chain to generate the proton gradient necessary for ATP production. Yet, NAD $^{+}$  and NADH do not cross the inner mitochondrial membrane [13]. The reducing equivalents from the cytosol produced by glycolysis are imported into the mitochondrial matrix via the NADH shuttles [12, 14]. The malate-aspartate shuttle (MAS), which tightly controls neuronal metabolism, is considered the most important NAD $^{+}$ /NADH shuttle in neurons [14]. Although PKC $\epsilon$ -mediated activation of the MAS was reported in the heart [15], it is still unclear whether PKC $\epsilon$  regulates cellular metabolism via the MAS in neurons.

The MAS plays a prominent role in neuronal mitochondrial respiration. Knock out of the aspartate-glutamate carrier (AGC), one of several MAS components, results in a 46% drop in mitochondrial respiration in primary neurons [16]. One of the mediators of MAS activity is calcium (Ca $^{2+}$ ). Removal of Ca $^{2+}$  inhibits MAS-promoted mitochondrial respiration [16]. Given the fundamental role of calcium signaling in synaptic function, it is likely that the

MAS is involved in synaptic transmission which is triggered by presynaptic  $\text{Ca}^{2+}$  signaling. Importantly, the MAS is selectively activated by small  $\text{Ca}^{2+}$  signals under physiological conditions. In pathological conditions of cerebral ischemia, excitotoxicity-induced calcium overload activates the mitochondrial  $\text{Ca}^{2+}$  uniporter, but not the MAS [17], implicating dysfunctional regulation of the MAS in cerebral ischemic injury.

Because of the crucial role of the MAS in neuronal metabolism and synaptic function, and its potential dysregulation during ischemia, we sought to determine if PKC $\epsilon$  preconditioning/neuroprotection mediates cellular metabolic changes in the brain via MAS modulation. In this study, we identified proteins involved in the MAS that became phosphorylated after PKC $\epsilon$  activation. We defined the mechanisms by which PKC $\epsilon$ -mediated phosphorylation regulated the activity of the MAS. Furthermore, we showed that PKC $\epsilon$  activation was capable of rescuing against the ischemia-induced decrease of MAS activity. Overall, our study reveals a novel neuroprotective mechanism of PKC $\epsilon$  that involves increasing the activity of a component of MAS: Glutamic oxaloacetic transaminase 2 (GOT2).

## Materials and Methods

### Animals

Animal usage and experimentation were approved by the Institutional Animal Care and Use Committee at the University of Miami and was in accordance with the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals. Sprague-Dawley (SD) rats were obtained from Charles River (Wilmington, MA) and housed in a supervised facility of the division of veterinary resources.

### Rat Primary Neuronal Cultures and Astrocyte Cultures

Primary neuronal cultures were prepared from embryonic 18–20 day old pups. Embryos were harvested, their cortical tissue was homogenized and dissociated into single cells. Cells were plated on poly-D-lysine-coated tissue culture plates in neuronal plating medium (minimum essential media, 1% Glutamax, 5% fetal bovine serum, and 15 mM glucose). Medium was replaced 2hrs later with neuronal culture medium (neurobasal plus medium supplemented with 2% B27, 0.5 mM Glutamax, Thermo Fisher Scientific, Waltham, MA). Neurons were seeded at 2,500,000 cells/well in 6-well plates or 40,000 cells/well in Seahorse Extracellular Flux Analyzer Miniplates (Agilent, Santa Clara, CA). Cultures underwent half media changes every third day for 7 to 10 days before treatment. Primary astrocyte cultures were prepared from 1–2 day old pups. Their cortical tissue was homogenized and dissociated into single cells. Cells were plated in a T-25 flask with astrocytic plating medium (Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% Glutamax, 1% penicillin-streptomycin, Thermo Fisher Scientific). Astrocytes were trypsinized when confluent and seeded at 40,000 cells/well in Seahorse Extracellular Flux Analyzer Miniplates (Agilent) 1 day before experiments.

## Pharmacological Treatments

For *in vitro* PKC $\epsilon$  preconditioning, cultures were treated 2 days prior to experimental analysis. For PKC $\epsilon$  activation, cells were exposed for 1 hour to 500 nM of control peptide TAT (Sigma, St. Louis, MO), specific PKC $\epsilon$  activator TAT-conjugated  $\Psi$ eRACK (CPC scientific, Sunnyvale, CA), with or without 500 nM Hsp90 inhibitor, 17-AAG (Tocris, Bristol, UK). For inhibition of the MAS, cultures were exposed to 20  $\mu$ M or 80  $\mu$ M of vehicle DMSO (Sigma) or GOT enzyme inhibitor, Aminooxyacetic acid (Tocris) for 48 hours. For proteomic analysis, activation of PKC $\epsilon$  was induced by pre-incubating hippocampal synaptosomes with 1  $\mu$ M TAT or TAT-conjugated  $\Psi$ eRACK for 15 minutes at 4°C before isolation of mitochondria. For *in vivo* experiments, 12- to 14-week-old male SD rats were intraperitoneal injected with 0.2 mg/kg TAT or TAT-conjugated  $\Psi$ eRACK. TAT or  $\Psi$ eRACK was dissolved in saline. 17-AAG or Aminooxyacetic acid was dissolved in DMSO.

## Oxygen Consumption Rate and Glycolytic Rate

Oxygen consumption rate and glycolytic rate were measured using the Seahorse Extracellular Flux Analyzer (Agilent) as described previously [18, 19] with minor modifications. To measure oxygen consumption rate, neurons or astrocytes were washed 3 times with neurobasal plus media or seahorse media (XF Base Medium-minimum Dulbecco's Modified Eagle Medium supplemented with 20 mM glucose, 1 mM sodium pyruvate, and 2 mM glutamine), respectively. To measure glycolytic rate, neurobasal plus media or seahorse media (XF Base Medium-minimum Dulbecco's Modified Eagle Medium supplemented with 2 mM glutamine) was used in neurons or astrocytes, respectively. Cells were then incubated in a CO<sub>2</sub>-free incubator at 37°C for 45 minutes prior to the plate run. Oxygen consumption rate and glycolytic rate were normalized to cell counts as measured by Hoechst staining.

## Isolation of Synaptosomes from Cortices or Hippocampus

Synaptosomes from rat cortices or hippocampus were isolated according to previously published procedures [7]. Mitochondria from hippocampal synaptosomes were used for proteomic analysis and cortical synaptosomes were used for further immunoprecipitation and Western blot. In brief, rats were decapitated under isoflurane anesthesia. The cortices or hippocampi were removed immediately and immersed into cold (4°C) isolation medium, consisting of 0.32 M sucrose, 1 mg/ml BSA, 0.25 mM Dithiothreitol and 1mM EDTA. The minced tissue was homogenized in a glass Teflon homogenizer. The homogenate was centrifuged at 500 x g for 5 minutes, and the resulting supernatant was layered on a preformed Percoll gradient consisting of 23, 15, 10, and 3% (v/v) Percoll. The gradients were centrifuged at 32,500 x g for 5 minutes and the cortical synaptosomes were collected at the interface between 15–23% layers. To improve the yield, hippocampal synaptosomes were collected from 10–15 and 15–23% layers. The synaptosomes were then washed successively with isolation media and 0.32 M sucrose by centrifugation at 15,000 x g for 10 minutes. The purified synaptosomal samples were resuspended in 0.32M sucrose. All procedures were carried out at 4°C.

## Phosphoprotein Staining and Mass Spectrometry

Hippocampal synaptosomes from rats were isolated as described above. The samples were then placed inside a nitrogen cell bomb and put under a pressure of 1200 psi for 7.5 min to isolate the total mitochondria [7]. The mitochondria obtained were subsequently used for proteomic analysis. Briefly, mitochondrial proteins were separated on a 4–20% acrylamide gel (Invitrogen Corporation, Carlsbad, CA). Phosphoproteins in the gradient gel were identified using Pierce Phosphoprotein Staining Kit (Thermo Fisher Scientific) as per manufacturer's instructions and subsequently stained with Coomassie blue (Pierce Biotechnology, Rockford, IL) to confirm equal protein loading. For protein identification, gel slices were excised and digested *in situ* with sequencing-grade trypsin (Promega Biosciences, Inc., Madison, WI). The peptide mixtures extracted from the gel were further concentrated on the peptide trap (Cap Trap, Michrom BioResources, Auburn, CA). This was followed by a separation of the peptides using BioBasic C18 PicoFrit microcapillary column (New Objective, Woburn, MA) by a linear gradient of 7–93% acetonitrile in water and 0.1% formic acid over 45 minutes. To collect the full MS and tandem MS/MS spectra, peptides eluted from the column were analyzed by a DECA-XP Plus ion trap mass spectrometer equipped with a nano-LC electrospray ionization source (Thermo Fisher Scientific). For data analysis, BioWorks 3.2 software (Thermo Fisher Scientific), based on the SEQUEST algorithm was used to search the NCBI NR non-redundant protein databases (NCBI NR). Peptide/spectra matches were retained if the following criteria were met: XCor scores higher than 1.5 for peptides charged +1, higher than 2.0 for peptides charged +2, and higher than 2.5 for peptides charged +3 and peptide probability lower than 0.05.

## Immunoprecipitation and Immunoblotting

The immunoprecipitation was carried out using the Pierce Classic IP Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The synaptosomal fraction or cell lysate (500 µg) was immunoprecipitated with anti-phosphoserine, anti-phosphothreonine, or anti-phosphotyrosine antibodies. The resulting pellet was then subjected to immunoblotting with anti-GOT1, GOT2, or OGC. To determine the protein levels of GOT1 and GOT2, protein concentration was determined by the BioRad DC™ Protein Assay. Forty micrograms of protein was loaded into a 4–20% SDS polyacrylamide gel (BioRad, Hercules, CA). Proteins were then transferred onto a nitrocellulose membrane (Biorad) and blocked for 1 hour at RT. Incubation in primary antibody was achieved overnight at 4°C. Following addition of HRP-conjugated secondary antibodies (1:5,000, GE healthcare, Little Chalfont, UK) for 1 h, blots were developed with ECL reagents (ThermoFisher Scientific, Waltham, MA) and visualized with X-ray film (Denville Scientific, Holliston, MA). Blots were then digitalized and analyzed in Image J software (NIH) by densitometry. The following primary antibodies were used: Actin (Cell Signal, Danvers, MA, 8H10D10), GOT1 (Proteintech, Rosemont, IL, 14886-1-AP), GOT2 (Proteintech, 14800-1-AP), GOT2 (Millipore, MABS417), OGC (Santa Cruz, Dallas, TX, sc-515593), Phospho-serine (Sigma, St. Louis, MO, P3430), Phosphothreonine (Sigma, P3555), Phospho-tyrosine (Cell Signal, 9411).

### Subcellular Fractionation

Cultures were fractionated into cytosolic and mitochondrial compartments as described previously [20, 21]. Briefly, cells were homogenized through a 27-gauge needle in hypotonic buffer (250 mM sucrose, 50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, Phosphatase and Protease inhibitor (Thermo Fisher Scientific), pH 7.4). The homogenate was then subjected to centrifugation at 800 x g for 10 minutes to remove nuclei, and 11,000 x g for 10 minutes to sediment mitochondria [21]. The mitochondria was washed twice by resuspending the pellet in suspension buffer (250 mM sucrose, 50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, pH 7.4) and repeating the 11,000 x g sedimentation. The purified fractions were then collected to measure GOT enzyme activity as described below.

### Assay of GOT Activity

GOT enzyme activity was measured using Aspartate Aminotransferase Activity Colorimetric Assay Kit (Biovision, San Francisco, CA) according to the manufacturer's instructions. Mitochondria were disrupted by four freeze-thaw cycles before measurements. Enzyme activity was normalized to protein concentrations.

### Oxygen and Glucose Deprivation (OGD)

The OGD experiments were performed as our lab previously described [19]. Briefly, primary neurons were incubated in glucose-free OGD medium and exposed to OGD for 2 hours under a hypoxia chamber (Coy Lab Products, Grass Lake MI) flushed with 90% N<sub>2</sub>, 5% CO<sub>2</sub>, 5% H<sub>2</sub> at 37 °C. The cultures were then returned to maintaining media and the normoxic incubator. The OGD media constituted 1.26 mM CaCl<sub>2</sub>, 5.37 mM KCl, 0.44 mM KH<sub>2</sub>PO<sub>3</sub>, 0.49 mM MgCl<sub>2</sub>, 0.41 mM MgSO<sub>4</sub>, 136.9 mM NaCl, 4.17 mM NaHCO<sub>3</sub>, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM sucrose, and 10 mM HEPES adjusted to pH 7.4. Sham OGD constitutes cells exposed to a complete media change without OGD. A schematic diagram of the experimental design is indicated in Fig. 7.

### Real-Time Polymerase Chain Reaction

Please refer to the online-supplement. The information about the primers used are found in Supplementary Table 1.

### Statistical analysis

Rats were randomly assigned to groups throughout. Data acquisition and analyses were conducted in a blinded manner when possible. Data are presented as mean ± SEM. Statistical analysis was performed using Prism6 software (GraphPad, San Diego, CA). The respective statistical test for each experiment is indicated in the figure legends. Significance is denoted in each figure. In all cases, a P value of <0.05 was considered as statistically significant.

## Results

### PKC $\epsilon$ preconditioning increases the oxygen consumption and glycolytic rate in neurons

PKC $\epsilon$  is a crucial mediator of preconditioning-induced ischemic tolerance [5, 6, 22, 23]. Previously, our laboratory demonstrated that transient activation of PKC $\epsilon$  for 1 hour induced

an increase in the concentration of mitochondrial NAD<sup>+</sup> in neuronal-glia cortical cultures 48 hours later [11]. Increases in NAD<sup>+</sup> can lead to the activation of important signaling pathways, but its primary function is metabolic. To specify whether preconditioning with a specific PKC $\epsilon$  activator,  $\Psi$ eRACK, induced metabolic changes, we performed live cell bioenergetics analysis in primary neuronal cultures using the Seahorse Bioscience Technology. The measurements of oxidative phosphorylation and glycolytic rate obtained from this assay are illustrated in Fig. 1a and Fig. 1c, respectively. In primary neurons, PKC $\epsilon$  preconditioning through the specific PKC $\epsilon$  activator,  $\Psi$ eRACK (500 nM), induced a modest increase in the basal oxygen consumption rate (13%,  $P < 0.05$ ) and oxygen consumption linked to ATP production (17%,  $P < 0.05$ ) compared to the control neurons treated with TAT carrier peptide. PKC $\epsilon$  activation also increased maximal oxygen consumption rate (22%,  $P < 0.05$ ) in response to energetic stress (Fig. 1b) compared to the TAT control. Similarly, we observed increased basal (14%,  $P < 0.05$ ) and maximal glycolytic rate (21%,  $P < 0.05$ ) in the PKC $\epsilon$  preconditioned neurons compared to TAT-treated control (Fig. 1d). Together, these data show that PKC $\epsilon$  preconditioning increases oxidative phosphorylation and glycolysis in neurons.

### **PKC $\epsilon$ activation enhances phosphorylation of proteins in the malate aspartate shuttle in the brain**

To determine the potential substrates of PKC $\epsilon$  upon activation, phospho-proteins were identified in mitochondria isolated from rat hippocampal synaptosomes treated with TAT or  $\Psi$ eRACK (1  $\mu$ M) for 15 mins. A protein band of 37 kDa size that demonstrated increased phosphorylation by 23% with  $\Psi$ eRACK treatment ( $n = 3$  for TAT and  $\Psi$ eRACK each,  $p = 0.064$ , data not shown) was subjected to proteomic analysis. Protein identification was performed using MS/MS analysis, which identified the presence of GOT2, Glutamic oxaloacetic transaminase 1 (GOT1) and Oxoglutarate carrier (OGC) in the band (Table 1). These proteins are essential components of the malate aspartate shuttle (MAS), which is involved in transferring reducing equivalents (NADH) generated by glycolysis across the mitochondrial inner membrane, thus, tightly controlling neuronal metabolism (illustrated in Fig. 6b). Since the proteomic data suggested increases in GOT phosphorylation, we proceeded to determine the phosphorylation by Western blot. The levels of GOT2 and OGC phosphorylation were determined in cortical synaptosomes from rats 48 hours after treatment with TAT or  $\Psi$ eRACK (0.2 mg/kg, i.p.). After PKC $\epsilon$  activation, we observed a significant increase in the serine phosphorylation of GOT2 (214%,  $P < 0.05$ ) and OGC (174%,  $P < 0.01$ ) (Fig. 2a–c) compared to the TAT control. We did not observe significant changes in threonine and tyrosine phosphorylation. Together, the phosphoproteomic analysis and Western blot analysis reveal that PKC $\epsilon$  activation increases phosphorylation of proteins in the MAS in the brain. These data, along with our previous observations of enhanced respiration and glycolysis in neurons, suggest that PKC $\epsilon$  phosphorylates proteins in the MAS and increases activity of the MAS.

### **Effects of PKC $\epsilon$ activation on the phosphorylation levels of GOT2 *in vitro***

Given the increased levels of cellular respiration and glycolysis, together with enhanced phosphorylation of MAS proteins, we hypothesized that PKC $\epsilon$ -mediated phosphorylation of GOT increases its enzyme activity. Because the MAS is found predominantly in neurons

[14], we used an *in vitro* model of primary neuronal cultures. To test the hypothesis, we firstly determined the levels of protein phosphorylation of GOT2 in neurons 48 hours after PKC $\epsilon$  preconditioning. Similar to our data in synaptosomes (Fig. 2), we observed a significant increase in the serine phosphorylation of GOT2 in PKC $\epsilon$  treated neurons (197%,  $P<0.05$ ) (Fig. 3a) compared to the TAT treated control neurons. The threonine and tyrosine phosphorylation of GOT2 were not significantly altered by activation of PKC $\epsilon$  (Fig. 3b, c). The protein levels of tyrosine phosphorylation in GOT2 were very low requiring higher primary antibody concentration and longer exposure time. These data demonstrate that PKC $\epsilon$  activation induces an increase in the serine-phosphorylation of GOT2 in primary neurons.

### PKC $\epsilon$ activation does not alter the mRNA and protein levels of GOT2 in neurons

It has been reported that transgenic mice overexpressing PKC $\epsilon$  exhibit increased levels of GOT1 and malate dehydrogenase 1 (MDH1) in the heart [24]. To determine if PKC $\epsilon$  preconditioning, in addition to phosphorylating MAS components, also alters the expression of the MAS components in neurons, we measured the mRNA levels of MDH1, MDH2, AGC, GOT1, GOT2, and OGC using real-time qPCR analysis. Expression of the MAS components did not differ between the control-TAT and specific PKC $\epsilon$ -activator  $\Psi\epsilon$ RACK group at 24 hours (Fig. 3d) and 48 hours (Fig. 3e) following the 1-hour treatment. Additionally, we did not observe significant changes in the protein levels of GOT1 (Supplementary Fig. 1) and GOT2 (Fig. 3f) after PKC $\epsilon$  activation, which is consistent with our data of non-altered mRNA expression of GOT1 and GOT2. These data suggest that PKC $\epsilon$  preconditioning does not alter expression levels of the MAS components in neurons.

### PKC $\epsilon$ activation increases the enzyme activity of GOT2 via phosphorylation

To determine if PKC $\epsilon$ -mediated phosphorylation of GOT increases its enzyme activity, primary neurons were treated with TAT or  $\Psi\epsilon$ RACK (500 nM), and then fractionated 48 hours later to allow specific investigation of GOT1 or GOT2. The enrichment of GOT1 and GOT2 is shown by Western blot from cytosolic or mitochondrial fractions (Fig. 4a). We observed that PKC $\epsilon$  preconditioning induced a significant increase in the enzyme activity of GOT2 (GOT in the mitochondrial fraction, 15.8%,  $P<0.05$ ) (Fig. 4b). Next, we sought to determine if PKC $\epsilon$ -mediated phosphorylation of GOT2 is required for the increased enzyme activity. Previously, our group and others reported that PKC $\epsilon$  translocates to mitochondria and mediates mitochondrial protection [7, 25, 26]. Although PKC $\epsilon$  lacks a mitochondrial targeting sequence, it is imported into the mitochondria through the cytoprotective protein chaperone, heat shock protein 90 (Hsp 90) [25, 26]. Our laboratory has demonstrated that inhibition of the activity of Hsp90, using 17-AAG, blocks the import of PKC $\epsilon$  into the mitochondria and inhibits its role in regulating mitochondrial proteins [26]. Therefore, neurons were treated with vehicle (DMSO) or Hsp90 inhibitor (17-AAG, 500 nM) in the presence of  $\Psi\epsilon$ RACK (500 nM). Inhibition of Hsp90 resulted in a significant decrease in PKC $\epsilon$ -mediated serine-phosphorylation of GOT2 (73.1%,  $P<0.05$ ) (Fig. 4c). In addition, we observed decreased activity of GOT2 (14.2%,  $P<0.05$ ) (Fig. 4d). Together, these results suggest that PKC $\epsilon$  increases the enzyme activity of GOT2, an effect that is dependent on PKC $\epsilon$  import into the mitochondria and phosphorylation of the enzyme.



### **Inhibition of MAS activity alters mitochondrial respiration and glycolysis in neurons**

Since PKC $\epsilon$  activation increases the enzyme activity of GOT2 and increases cellular metabolic rates in neurons, we postulated that inhibition of GOT activity results in suppressed respiration and glycolysis. To this end, we used aminooxyacetic acid (AOA), a non-specific inhibitor of amino acid transaminase that inhibits GOT in the MAS [27, 28] and assessed live cell bioenergetics analysis. As predicted, AOA (20  $\mu$ M or 80  $\mu$ M) -mediated inhibition of GOT induced a significant reduction in basal oxygen consumption rate (20  $\mu$ M--20%, 80  $\mu$ M--29%,  $P<0.01$ ), oxygen consumption linked to ATP production (20 $\mu$ M--22%,  $P<0.05$ ; 80 $\mu$ M--33%,  $P<0.01$ ), and maximal oxygen consumption rate (20  $\mu$ M--18%, 80  $\mu$ M--28%,  $P<0.05$ ) (Fig. 5a, b) compared to the DMSO control. Interestingly, the inhibitor increased the basal glycolytic rate (20  $\mu$ M--27%, 80  $\mu$ M--46%,  $P<0.05$ ) (Fig. 5c, d), which may be an adaptive metabolic change in response to the constrained mitochondrial respiration. However, the maximal glycolytic rate that corresponds to a stressor is significantly decreased with the inhibition (20  $\mu$ M--17%,  $P<0.01$ ; 80 $\mu$ M--41%,  $P<0.05$ ) compared to the DMSO treated control neurons. Together, these data suggest that inhibition of GOT, and subsequent disruption of MAS activity, decreases mitochondrial respiration and, under energetic stress, suppresses glycolytic capacity as well.

### **PKC $\epsilon$ preconditioning restores the decreased activity of GOT2 following ischemia reperfusion injury**

Given that interruption of the MAS results in impaired cellular metabolism, we sought to elucidate the effects of ischemic injury on GOT2 activity and determine if enzymatic activity in this context is modulated by PKC $\epsilon$ . Primary neurons exposed to TAT or  $\Psi$  $\epsilon$ RACK (500 nM) were subjected to oxygen and glucose deprivation (OGD) or Sham OGD for 2 hours. Activity of GOT2 was assessed 12 hours following this treatment. The results revealed decreased activity of GOT2 (11.7%,  $P<0.05$ ) in the TAT-treated OGD group compared to the TAT-treated Sham OGD group. PKC $\epsilon$  preconditioning was able to rescue the decreased GOT2 activity. PKC $\epsilon$ -treated OGD neurons exhibited higher GOT2 activity (12.26%,  $P<0.05$ ) (Fig. 6a) compared to the TAT-treated OGD neurons and was statistically similar to sham OGD, reflecting the protection induced by PKC $\epsilon$  activation.

## **Discussion**

Our study identifies a novel protective mechanism of PKC $\epsilon$ -mediated regulation of the MAS. PKC $\epsilon$  preconditioning promotes cellular metabolism by increasing mitochondrial respiration and anaerobic glycolysis. This is likely through the activation of the MAS as reflected by increased phosphorylation and enzyme activity of some of the MAS component(s). Specifically, PKC $\epsilon$  activation increased the phosphorylation and activity of GOT2. Finally, PKC $\epsilon$  preconditioning restored the decreased activity of GOT2 caused by ischemia-reperfusion injury.

Cerebral ischemia induces profound disturbances in metabolic pathways. Sustained ischemia leads to the deprivation of intracellular glucose and oxygen, which results in the inhibition of glycolysis and mitochondrial respiration. One of the many protective roles of preconditioning is to prevent ATP depletion following ischemic injury. Indeed, resveratrol, a

polyphenolic compound that mimics ischemic preconditioning, was shown to increase the glycolytic rate and protect against ischemia-induced ATP depletion [18, 19]. In addition, our lab previously reported that PKC $\epsilon$  increases the phosphorylation of proteins of the mitochondrial electron transport chain and enhances mitochondrial respiration in synaptosomes from preconditioned animals [7], suggesting preconditioning improves the capability of oxidative phosphorylation. Consistent with these findings, we observed increased oxygen consumption and glycolysis after PKC $\epsilon$  preconditioning in primary neurons. In addition, PKC $\epsilon$  preconditioning increased mitochondrial respiration linked to ATP production. This regulation is likely crucial for protection against cerebral ischemia given the susceptibility of the brain to energy derangements.

Our phosphoproteomic analysis and Western blot results show increased serine phosphorylation, with no significant changes in the threonine and tyrosine phosphorylation of MAS components. The increased serine phosphorylation may result from direct phosphorylation by PKC $\epsilon$ , or indirect phosphorylation via PKC $\epsilon$ -induced activation of other kinases. For example, serine/threonine kinase mitogen-activated protein kinase (MAPK) is one of the downstream transducers of PKC $\epsilon$  preconditioning. It was shown that preconditioning induced protection is at least, partly, mediated by PKC $\epsilon$  dependent regulation of MAPK/ERK [29, 30], MAPK/JNK [31–33] and p38 MAPK [31–33]. Tyrosine kinase Lck, a member of the Src family, is another downstream component of PKC $\epsilon$  signaling [34–36]. In cardiac cells, PKC $\epsilon$  phosphorylates, activates, and forms a complex with Lck, which is required for the cardiac protection induced by preconditioning [35]. Similarly, in primary neurons and a mouse model of cerebral focal ischemia, it was shown that the PKC $\epsilon$ -Lck axis is a key mediator of ischemic preconditioning induced neuroprotection [36].

Interestingly, MAS is highly expressed in neurons, with relatively low expression in astrocytes [14]. The loss of the aspartate glutamate carrier (AGC, a MAS component) results in a significant decrease in oxygen consumption in neurons [16]. In contrast, AGC-deficient astrocytes display no differences in mitochondrial respiration [37], indicating the low activity of the MAS in astrocytes. In line with these findings, we did not observe metabolic alterations regarding oxygen consumption and glycolytic rate in primary astrocytes (Supplementary Fig. 2). This may be due to cell-specific effects mediated by PKC $\epsilon$  that elicit different mechanisms of protection in neurons and astrocytes [38]. We did not observe significant changes in mRNA expression of the MAS components in neurons. In the heart of transgenic mice overexpressing PKC $\epsilon$ , there is increased protein expression of MAS components (MDH1 and GOT1) [24], suggesting PKC $\epsilon$  may increase MDH1 and GOT1 at the transcriptional level in the heart. This discrepancy may reflect tissue specific regulation of PKC $\epsilon$  on the MAS or a difference between the constitutive activation of PKC $\epsilon$  by transgenic overexpression and a transient preconditioning treatment as implemented in our study. Still, these findings support a regulatory effect on the MAS by the PKC $\epsilon$  signaling pathway.

To characterize the contributions of the MAS in cellular metabolism, we used a pharmacological inhibitor, AOA. Although AOA is a non-specific inhibitor of amino acid transaminases, it has been shown to inhibit the MAS [27, 28] and has been used for

inhibition of the MAS in neurons [39, 40], as well as in different types of cells [41–44]. Our data show that inhibition of the MAS decreases oxygen consumption and glycolysis, demonstrating the MAS plays a pivotal role in cellular metabolism. Importantly, deficiency of the MAS was reported in ischemic stroke. In post-mortem brain tissue from patients who suffered from ischemia, proteins that participate in the MAS were downregulated in the infarcted samples compared to the controls [45]. Therefore, dysregulation of the MAS may account for the failure of energy metabolism following cerebral ischemia.

Consistent with previous findings of MAS deficiency following ischemic stroke, we also observed reduced activity of GOT2 following ischemic injury in primary neurons. As an essential component of the MAS, GOT2 is also implicated in cerebral ischemia. As mentioned above, GOT2 was decreased in infarcted brain tissue compared to non-infarcted controls [45]. On the contrary, overexpression or activation of GOT in mice improved the anatomical and functional outcomes after stroke [46, 47]. Here, we found that PKC $\epsilon$  preconditioning increased the activity of GOT2, an effect that was lost by the blockade of PKC $\epsilon$ -mediated phosphorylation of the enzyme. Furthermore, PKC $\epsilon$  preconditioning alleviated the decreased activity of GOT2 after ischemic injury, suggesting PKC $\epsilon$  mediates post-ischemic protective mechanisms against the dysfunctional MAS. This is likely to restore cellular ATP production and protect against the energy crisis following ischemic injury. Indeed, previous findings highlighted an essential role of the MAS during the reperfusion phase. Inhibition of the MAS during reperfusion was shown to disrupt recovery and block preconditioning-induced protection in heart [48]. Whether restoration of MAS activity after ischemic injury is related to altered cerebral blood flow (CBF) remains undefined. Previous studies suggest that PKC $\epsilon$  preconditioning may play different roles in the regulation of CBF in reperfusion. Our lab previously showed that, in rats undergoing global cerebral ischemia, PKC $\epsilon$  preconditioning resulted in a significant reduction in CBF during the reperfusion phase [49]. However, in a mouse model of focal cerebral ischemia, we observed that PKC $\epsilon$  preconditioning did not change the CBF during reperfusion [50]. Consistent with the latter finding, another study showed that there were no significant differences in CBF between PKC $\epsilon$ -null mice and control mice during the reperfusion phase [51]. These discrepancies may derive from the disparities of cerebral ischemic models used in these experiments.

Although PKC $\epsilon$  preconditioned neurons exhibit an increased trend in the phosphorylation of GOT1 (Supplementary Fig. 3), we did not observe a significant change in the enzyme activity (Supplementary Fig. 4). This may reflect differential regulation of GOT1 and GOT2 that has been reported previously. Posttraumatic injury of the hippocampus gave rise to a significant decrease in the expression of both GOT1 and GOT2 in the dentate gyrus [56]. However, in the CA1 region, only protein expression of GOT2 was significantly reduced [56]. In addition, the enzyme activity of GOT2, but not GOT1, was significantly decreased in ischemic and hypoxic rat retinas [57]. In contrast, hyperoxia induced a significant increase in the expression of GOT1 in the brain while GOT2 remained unaltered [58]. Since previous studies reported an aspartate-depleting role of GOT1 and an aspartate-producing role of GOT2 [59, 60], our results may indicate increased biosynthesis of aspartate, a metabolite that has been shown to affect mitochondrial function [61]. Whether PKC $\epsilon$  preconditioning increases aspartate production warrants future investigation. It is also

noteworthy that the MAS is composed of multiple proteins including cytosolic and mitochondrial enzymes as well as mitochondrial carriers. The shuttle shares substrates with glycolysis in the cytosol and the tricarboxylic acid (TCA) cycle in the mitochondria. Therefore, it is difficult to dissect out the regulation of the MAS comprehensively. Future techniques that allow live assessments of specific changes in cytosolic and mitochondrial metabolites may allow for better characterization of the dynamic flux of the MAS.

In the synaptosomes from rat cortices, we observed increased phosphorylation of OGC after PKC $\epsilon$  activation. Whether PKC $\epsilon$ -induced phosphorylation of OGC is functionally important and whether OGC is essential in PKC $\epsilon$ -mediated protection remain to be determined. OGC is a mitochondrial carrier protein that exchanges malate and  $\alpha$ -ketoglutarate across the inner mitochondrial membrane [52]. In addition to its important roles in the MAS, it is also part of the oxoglutarate/isocitrate shuttle, which transports the reducing equivalents of NADPH from mitochondria into the cytosol [53]. Considering that OGC is involved in the transports of these important equivalents of NAD and NADP, it is tempting to speculate that OGC plays important roles in ischemic preconditioning. Future studies from our lab will address these potential mechanisms.

A caveat in our study was that we utilized an *in vitro* model of ischemic injury (OGD). This approach enables us to control external factors and study direct effects on the MAS by PKC $\epsilon$  activation. In addition, the *in vitro* culture of primary neurons allows us to determine cell-specific roles of PKC $\epsilon$  and further dissect out specific mechanisms. However, we are cognizant that *in vitro* models do not fully recapitulate the structure and network complexity that exist *in vivo*, which may involve several different mechanisms. Therefore, *in vivo* studies in animal models are needed to facilitate a better understanding of PKC $\epsilon$ -mediated regulation of the MAS.

A final note about clinical relevance and the clinical translation of ischemic preconditioning, which continues to be a promising therapeutic strategy. A meta-analysis of randomized controlled trials showed that remote ischemic conditioning (RIC) significantly reduced the incidence of stroke and improved post-stroke patient outcomes [62]. In addition, in patients undergoing carotid artery surgeries, the volume and incidence of post-surgery brain lesions was significantly decreased in the RIC group [63]. Future studies will need to evaluate the possibility of using PKC $\epsilon$  peptide agonists (i.e.,  $\Psi\epsilon$ RACK), as the one used in our study, as a potential therapeutic strategy against stroke. Furthermore, the clinical efficacy of other direct modulators of GOT2 remains to be evaluated. Previous studies in mice showed that GOT overexpression reduced ischemic stroke lesion volumes and improved poststroke sensorimotor functions [46]. Thus, agents like  $\Psi\epsilon$ RACK or any other direct activator of GOT2 may prove to be a novel therapeutic agent against stroke. Finally, although GOT 2 is a promising target, more basic research is needed to further characterize other MAS components in order to elucidate its translational application.

In summary, our study illustrates a novel neuroprotective mechanism whereby PKC $\epsilon$  increases the activity of GOT2 in the MAS. We show that PKC $\epsilon$  phosphorylates proteins in the MAS, which is crucial for cellular metabolism. These findings lend support for protective mechanisms of the MAS, the NAD<sup>+</sup>/NADH shuttle, as a potential therapeutic

intervention to protect against energy failure from cerebral ischemia. More broadly, our study supports future investigation into the MAS in other neurological disorders such as cerebrovascular and neurodegenerative diseases, in which cellular metabolic stress is commonly involved in the pathogenesis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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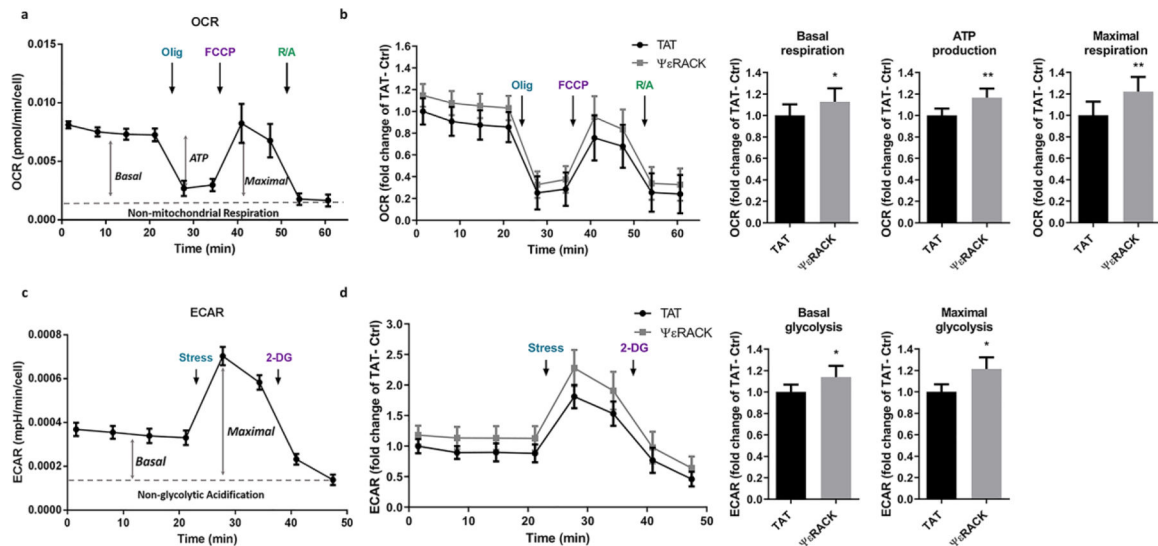
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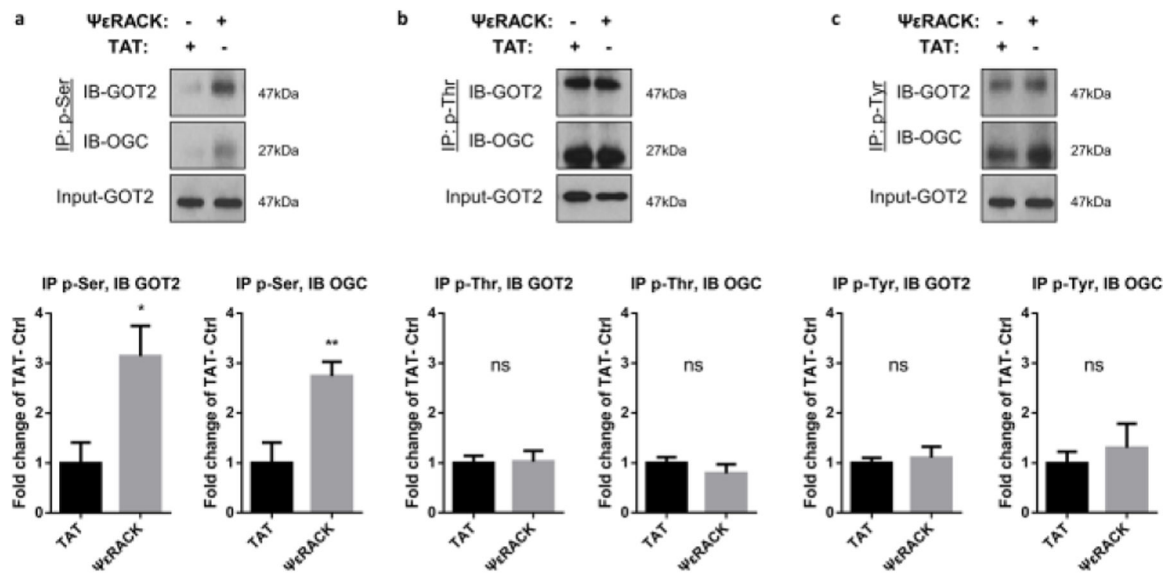
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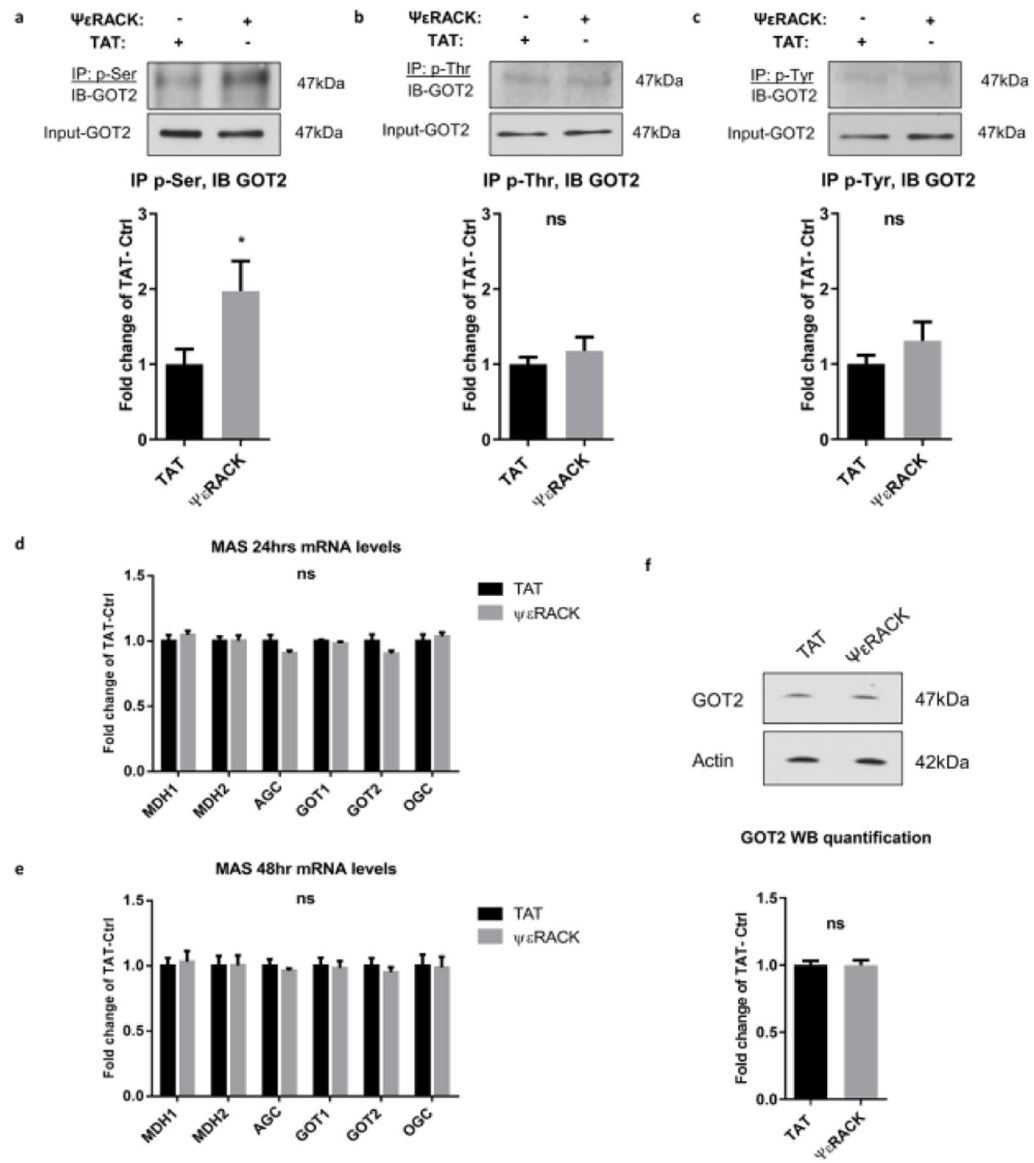


**Fig. 1. Activation of PKC $\epsilon$  increases the respiratory and glycolytic rate in primary neurons.** (a) Representative profile of the oxygen consumption rate (OCR) showing the sequential injection of metabolic inhibitors: complex V inhibition by oligomycin (Olig), mitochondrial uncoupling by FCCP, complex I and complex III inhibition by rotenone and antimycin (R/A); arrow indicates application. Basal: Basal respiration rate; ATP: oxygen consumption rate linked to ATP production; Maximal: maximal uncoupled respiration rate. (b) Primary neurons were exposed to control -TAT or specific PKC $\epsilon$  activator- $\Psi\epsilon$ RACK (500 nM) for 1 hour. OCR was measured 48 hours later using the Seahorse Analyzer. Values were normalized to cell counts and represented as fold change of TAT control (n=4, mean  $\pm$  SEM, \* $P$ <0.05, Two-tailed paired Student's t-test). (c) Representative profile of the extracellular acidification rate (ECAR) showing the sequential injection of metabolic inhibitors. Energetic stress cocktail: complex V inhibition by oligomycin and mitochondrial uncoupling by FCCP; glycolysis inhibition by 2-Deoxyglucose (2-DG); arrow indicates application. Basal: Basal glycolysis rate; Maximal: maximal glycolytic rate. (d) Primary neurons were treated with TAT or  $\Psi\epsilon$ RACK (500 nM) for 1 hour. ECAR was measured 48 hours later using the Seahorse Analyzer. Values were normalized to cell counts and represented as fold change of TAT control (n=4, mean  $\pm$  SEM, \*  $P$ <0.05, Two-tailed paired Student's t-test).



**Fig. 2. Activation of PKC $\epsilon$  increases serine phosphorylation of the MAS components GOT2 and OGC in synaptosomes.**

Synaptosomes from rat cortices were isolated 48 hours after TAT or  $\Psi$ eRACK (0.2 mg/kg i.p.) treatment. Proteins were immunoprecipitated with (a) phospho-serine, (b) phospho-threonine, (c) phospho-tyrosine, and immunoblotted for GOT2 and OGC. Input-GOT2 acts as a loading control. Quantification fold change of TAT control and representative blot as shown above (n=4–5, mean  $\pm$  SEM, \*  $P$ <0.05, \*\*  $P$ <0.01, ns = non-significant, Two-tailed unpaired Student’s t-test). GOT2: Glutamic oxaloacetic transaminase 2; OGC: Oxoglutarate carrier.



**Fig. 3. Effects of PKCε activation on the phosphorylation levels of GOT2 in primary neurons.** (a-c) Neurons were exposed to TAT or ΨεRACK (500 nM) for 1 hour. Forty-eight hours after treatment, protein from cells was immunoprecipitated with (a) phospho-serine, (b) phospho-threonine, (c) phospho-tyrosine, and immunoblotted for GOT2. Input-GOT2 represents as a loading control. Quantification fold change of TAT control and representative blot as shown above. (d-f) PKCε activation does not alter the expression of MAS components in neurons. Real-time qPCR was performed (d) 24 hours and (e) 48 hours following the 1-hour treatment of TAT or ΨεRACK (500 nM) in primary neurons. mRNA levels were normalized to GAPDH and shown as fold change of TAT control. (f) Western blots of GOT2 48 hours following the 1-hour treatment with TAT or ΨεRACK (500 nM) in neurons. Actin acts as a loading control. Quantification fold change of TAT control and representative blot as shown above (n=5-7, mean ± SEM, \* P<0.05, ns = non-significant, Two-tailed unpaired Student's t-test). MDH1: Malate dehydrogenase 1; MDH2: Malate

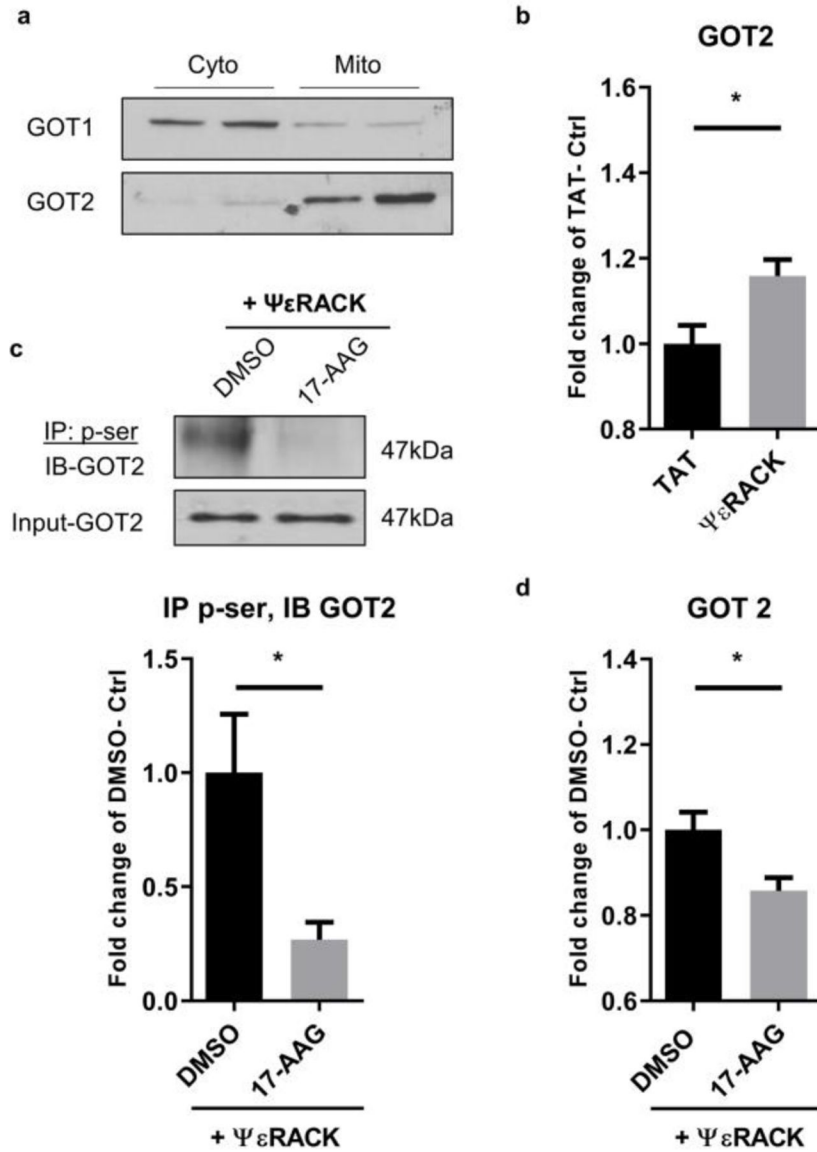
dehydrogenase 2; AGC: Aspartate glutamate carrier; GOT1: Glutamic oxaloacetic transaminase 1; GOT2: Glutamic oxaloacetic transaminase 2; OGC: Oxoglutarate carrier.

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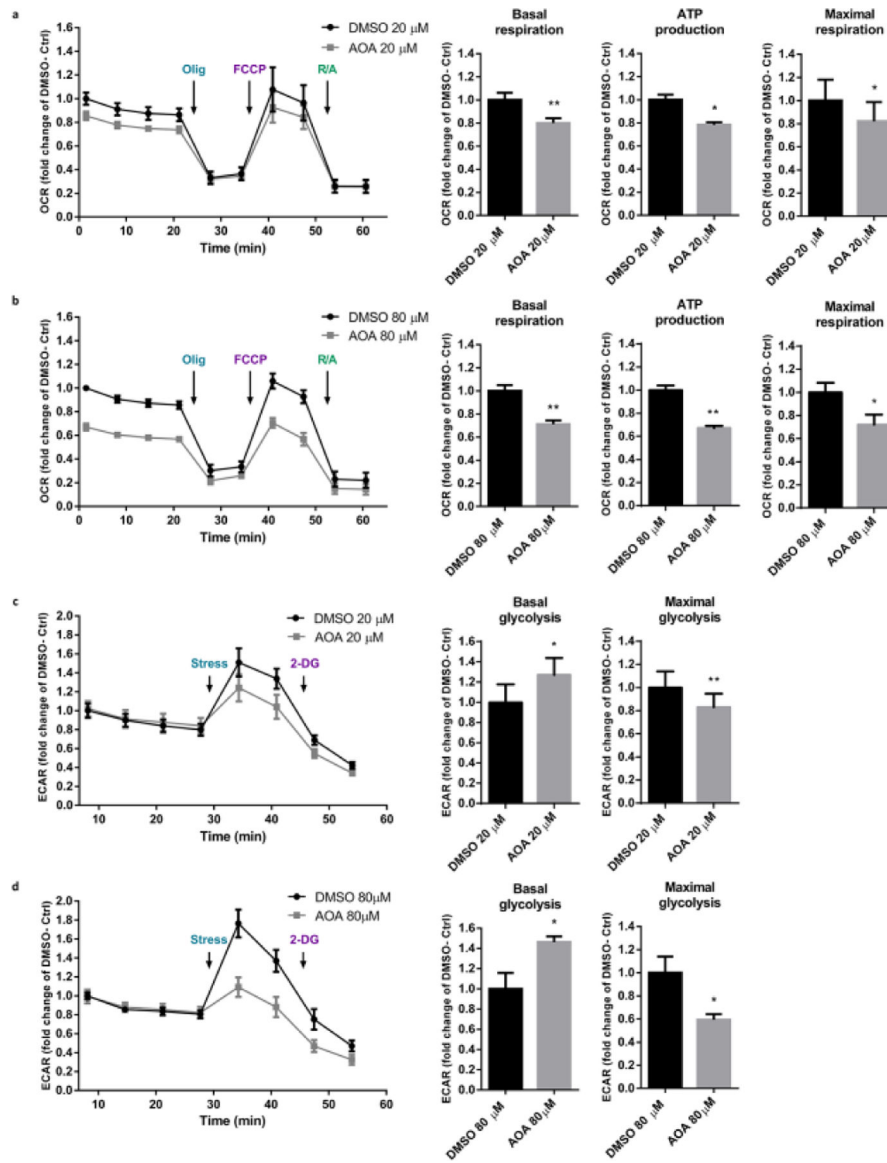
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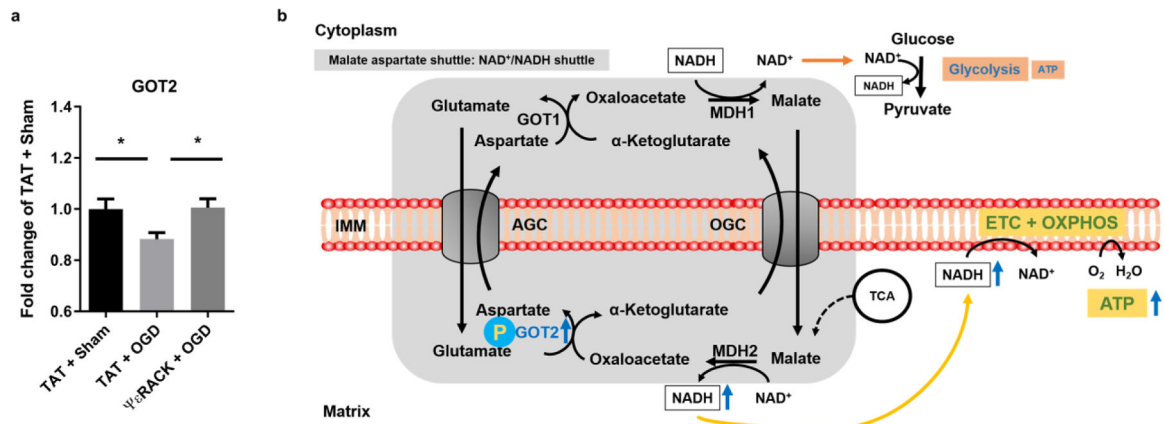
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**Fig. 4. PKCε activation increases the enzyme activity of GOT2 via phosphorylation.** (a) Western blot reveals enriched GOT1 in the cytosolic fraction and GOT2 in the mitochondrial fraction. (b) Primary neurons were exposed to TAT or ΨεRACK (500 nM) for 1 hour. Forty-eight hours later, activity of GOT2 (GOT in the mitochondrial fraction) was measured using Biovision AST colorimetric assay kit and normalized to protein concentration. Values are shown as fold-change of TAT control. (c, d) Neurons were treated with 500 nM vehicle (DMSO) or Hsp90 inhibitor (17-AAG) in the presence of ΨεRACK (500 nM). (c) Representative Western blot for serine-phosphorylation of GOT2. Input-GOT2 acts as a loading control. Quantification fold change of TAT control as shown above. (d) Activity of GOT2 is represented as fold change of control (n=5–7, mean ± SEM, \*  $P < 0.05$ , ns = non-significant, Two-tailed unpaired Student's t-test).



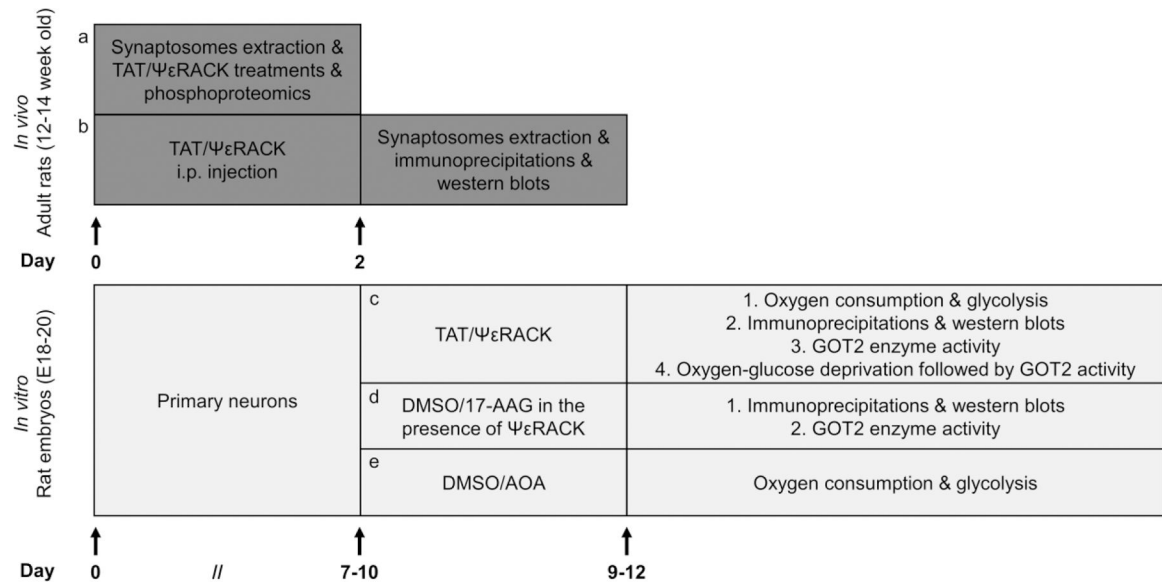
**Fig. 5. Respiratory and glycolytic rates are impaired with the inhibition of GOT in neurons.** Primary neurons were treated with vehicle (DMSO) or GOT enzyme inhibitor, Aminoxyacetic acid (AOA), for 48 hours. OCR and ECAR were assessed using the Seahorse Biosciences Technology. Values were normalized to cell counts and represented as fold change of DMSO control. **(a, b)** Measurements and quantifications of OCR with **(a)** 20 μM and **(b)** 80 μM DMSO or AOA. **(c, d)** Measurements and quantifications of ECAR with **(c)** 20 μM, and **(d)** 80 μM DMSO or AOA (n=4-5, mean ± SEM, \*  $P < 0.05$ , \*\*  $P < 0.01$ , Two-tailed paired Student's t-test).



**Fig. 6. PKCε rescues decreased GOT2 activity after ischemia reperfusion injury.**

Primary neurons exposed to TAT or ΨeRACK (500 nM) were subjected to oxygen and glucose deprivation (OGD) for 2 hours. Activity of GOT2 was assessed 12 hours following OGD using Biovision AST colorimetric assay and normalized to protein concentration. Values are represented as fold change of control (TAT + sham). **(a)** In TAT-treated neurons, GOT2 activity was decreased after 2 hours of OGD compared to Sham OGD. In neurons subjected to OGD, PKCε preconditioning rescued the decreased activity of GOT2 compared to TAT treated group (n=10, mean ± SEM, \* *P*<0.05, One-way ANOVA, post-hoc Bonferroni). **(b)** Schematic diagram of the proposed effects of PKCε on GOT2. Our results suggest that PKCε activation increases the phosphorylation and enzyme activity of GOT2. This leads to increased activity of the NAD<sup>+</sup>/NADH shuttle, and enhanced respiratory and glycolytic rates. Ischemic injury reduces the enzyme activity of GOT2 that gives rise to the impaired respiratory and glycolytic rates. GOT1: Glutamic oxaloacetic transaminase 1; GOT2: Glutamic oxaloacetic transaminase 2; AGC: Aspartate glutamate carrier; OGC: Oxoglutarate carrier; MDH1: Malate dehydrogenase 1; MDH2: Malate dehydrogenase 2; NAD<sup>+</sup>/NADH: Nicotinamide adenine dinucleotide; ATP: Adenosine triphosphate; IMM: Inner mitochondrial membrane; ETC: Electron transport chain; OXPHOS: Oxidative phosphorylation; TCA: Tricarboxylic acid cycle.





**Fig. 7. Schematic diagram of the experimental design.**

Briefly, (a, b) synaptosomes were extracted from adult rats. Phosphoproteomic analysis, immunoprecipitation and immunoblots were used to examine PKC $\epsilon$ -activated phosphorylation. (c-e) Primary neurons were prepared from embryonic 18–20 day old pups. Oxygen consumption and glycolysis were evaluated by Seahorse Bioscience Technology. PKC $\epsilon$ -mediated phosphorylation was determined by immunoprecipitation and western blots. A colorimetric assay was utilized to assess the GOT2 activity. We used oxygen-glucose deprivation as an *in vitro* model to evaluate the effects of ischemic injury on GOT2 activity. For pharmacological treatments and experimental details please refer to “Materials and Methods”.

**Table 1.**  
**Proteomic analysis suggested proteins in the malate-aspartate shuttle phosphorylated by PKC $\epsilon$ .**

Phospho-proteins were identified in mitochondria isolated from rat hippocampal synaptosomes treated with TAT or  $\Psi\epsilon$ RACK (1  $\mu$ M) for 15mins. A protein band of 37 kDa size that demonstrated increased phosphorylation by 23% with  $\Psi\epsilon$ RACK treatment (n = 3 for TAT and  $\Psi\epsilon$ RACK each, p = 0.064, data not shown) was subjected to proteomic analysis, which identified phosphoproteins as shown in the table above. GOT1: Glutamic oxaloacetic transaminase 1; GOT2: Glutamic oxaloacetic transaminase 2; OGC: Oxoglutarate carrier. An illustration of MAS components is shown in Fig. 6b.

Accession NCBI NR	Protein	MW KDa	Peptides identified	Score Xcalibur
6980972	Glutamic oxaloacetic transaminase 2 (GOT2)	47	11	120.22
6980970	Glutamic oxaloacetic transaminase 1 (GOT1)	46	3	40.22
21312994	Oxoglutarate carrier (OGC)	34	6	52.23