Pomytkin et al. Molecular Brain (2019) 12:112
https://doi.org/10.1186/s13041-019-0533-5

Molecular Brain

MICRO REPORT Open Access

Excitotoxic glutamate causes neuronal insulin resistance by inhibiting insulin receptor/Akt/mTOR pathway

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Abstract

Aim: An impaired biological response to insulin in the brain, known as central insulin resistance, was identified during stroke and traumatic brain injury, for which glutamate excitotoxicity is a common pathogenic factor. The exact molecular link between excitotoxicity and central insulin resistance remains unclear. To explore this issue, the present study aimed to investigate the effects of glutamate-evoked increases in intracellular free Ca^{2+} concentrations $[Ca^{2+}]_i$ and mitochondrial depolarisations, two key factors associated with excitotoxicity, on the insulin-induced activation of the insulin receptor (IR) and components of the Akt/ mammalian target of rapamycin (mTOR) pathway in primary cultures of rat cortical neurons.

Methods: Changes in $[Ca^{2+}]_i$ and mitochondrial inner membrane potentials ($\Delta\Psi_m$) were monitored in rat cultured cortical neurons, using the fluorescent indicators Fura-FF and Rhodamine 123, respectively. The levels of active, phosphorylated signalling molecules associated with the IR/Akt/mTOR pathway were measured with the multiplex fluorescent immunoassay.

Results: When significant mitochondrial depolarisations occurred due to glutamate-evoked massive influxes of Ca^{2+} into the cells, insulin induced 48% less activation of the IR (assessed by IR tyrosine phosphorylation, $pY^{1150/1151}$), 72% less activation of Akt (assessed by Akt serine phosphorylation, pS^{473}), 44% less activation of mTOR (assessed by mTOR pS^{2448}), and 38% less inhibition of glycogen synthase kinase β (GSK3β) (assessed by GSK3β pS^{9}) compared with respective controls. These results suggested that excitotoxic glutamate inhibits signalling via the IR/Akt/mTOR pathway at multiple levels, including the IR, resulting in the development of acute neuronal insulin resistance within minutes, as an early pathological event associated with excitotoxicity.

Keywords: Insulin, Glutamate excitotoxicity, Central insulin resistance

Main text

An acute impairment in the biological response to insulin in the brain, known as central insulin resistance, was identified during stroke [1] and traumatic brain injury [2], for which excitotoxicity, which is caused by excessive glutamate release, is a key pathogenic factor [3].

The exact molecular link between insulin resistance and glutamate excitotoxicity remains unclear. Based on published data, an abnormal rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and decreased mitochondrial inner membrane potential ($\Delta\Psi_m$) are factors associated with the excitotoxic glutamate [4–6], which could potentially affect insulin signalling. The presence of Ca^{2+} (1 mM) has been shown to reduce the insulin-induced tyrosine phosphorylation of the insulin receptor (IR) in hippocampal synaptic preparations [7]. Glutamate has been shown to reduce the tyrosine phosphorylation of the IR when added after the prolonged insulin-mediated stimulation of hippocampal neuronal cultures [8]. Protonophore-induced decreases in $\Delta\Psi_m$ have been shown to evoke concomitant decreases in the IR tyrosine phosphorylation in response to insulin, indicating that mitochondrial depolarisation is an independent causative factor for neuronal insulin resistance [9]. However, the

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effects of glutamate-evoked changes in $[Ca^{2+}]_i$ and $\Delta\Psi_m$ on the insulin-induced activation of the IR/Akt/mammalian target of rapamycin (mTOR) and glycogen synthase kinase (GSK)3β pathways have never been studied. Here, we investigated whether excitotoxic glutamate affects the insulin-induced phosphorylation of IR/Akt/mTOR pathway components during significant mitochondrial depolarisations caused by the massive influx of Ca^{2+} into the cells.

To determine the times during which glutamate induces significant mitochondrial depolarisation, rat cortical neurons were exposed to 100 μM glutamate and changes in $[Ca^{2+}]_i$, and $\Delta\Psi_m$ were monitored for 30 min. Detailed methods for the preparation of primary cortical neuronal cultures and measuring $[Ca^{2+}]_i$ and $\Delta\Psi_m$ are described in Additional file 1. As expected, glutamate evoked a rapid increase in $[Ca^{2+}]_i$, followed by a decreased in $\Delta\Psi_m$ in the cells (Fig. 1a and b, Additional file 2: Tables S3 and S4). After 30 min of glutamate exposure, the mean $[Ca^{2+}]_i$ level increased by 2.4-fold above baseline ($F_{148,8732} = 44.8$, $P < 0.0001$), and the mean $\Delta\Psi_m$ value significantly decreased by 1.6-fold below baseline ($F_{148,8732} = 182.8$, $P < 0.0001$) (Fig. 1a–c, one-way analysis of variance [ANOVA] with repeated measures, followed

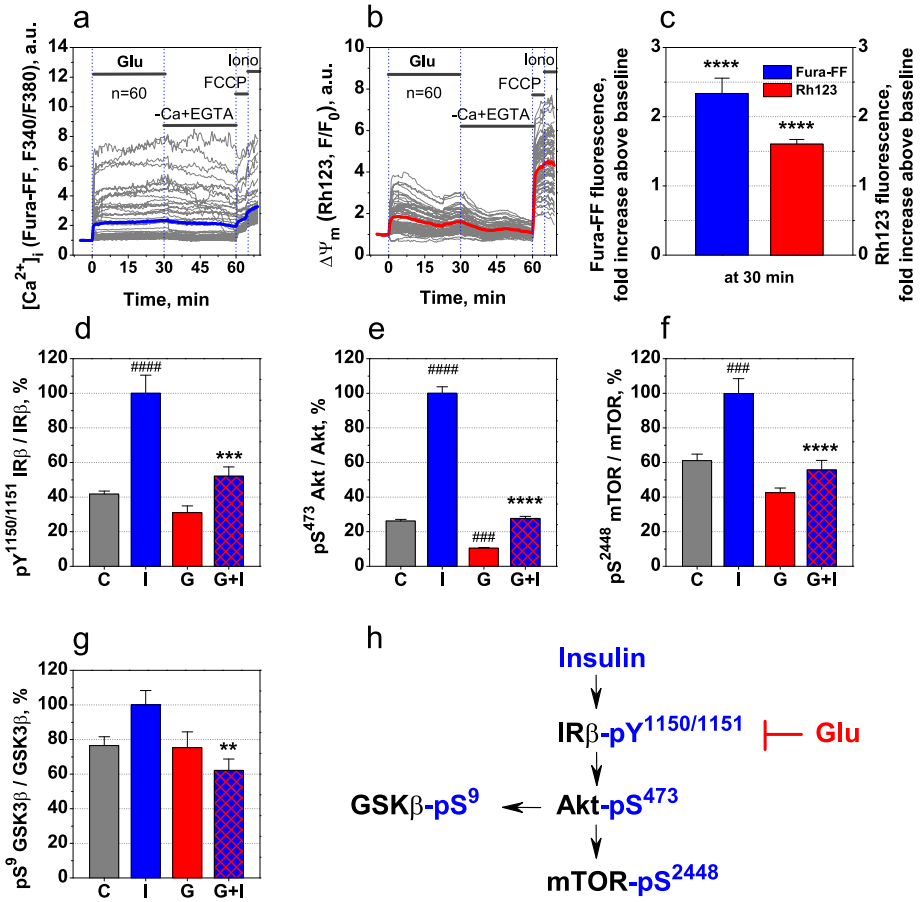

Fig. 1 Excitotoxic glutamate inhibits IR/Akt/mTOR pathway**a** Dynamics of $[Ca^{2+}]_i$ and **b** $\Delta\Psi_m$ in single rat cortical neurons, loaded simultaneously with Fura-FF and Rh123 dyes and exposed to 100 μM glutamate. Grey lines represent sixty single neurons. Blue and red lines represent the respective means of $[Ca^{2+}]_i$ and $\Delta\Psi_m$, averaged across sixty individual neurons at every time point. **c** Fura-FF and Rh123 fluorescence 30 min after the onset of glutamate exposure, expressed as the fold increase over baseline (at − 5 min). Data are the mean ± SEM from sixty neurons. $^{****}P < 0.0001$ compared to respective baselines (one-way ANOVA with repeated measures, followed by Tukey's post hoc test). **d-g** Levels of **d** IRβ $pY^{1150/1151}$, **e** Akt pS^{473}, **f** mTOR pS^{2448}, and **g** GSK3β pS^{9} in rat cortical neurons exposed to 0 nM (C) or 100 nM insulin for 15 min (I), 100 μM glutamate for 30 min **g**, or sequentially to 100 μM glutamate for 30 min and 100 nM insulin for 15 min (G + I). Bar graphs represent the levels of the phosphoproteins, normalised against respective total protein levels, in cell lysates and expressed as a percentage of levels in insulin-treated cells (group I). Each value represents the mean ± SEM from six independent cultures (cell populations obtained from twelve separate rats, two per culture). $^{###}P < 0.001$, $^{####}P < 0.0001$ compared with untreated control **c**; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ compared with insulin **i** (one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons). **h** Scheme illustrating the inhibitory effects of glutamate on the insulin-induced activation of the IR/Akt/mTOR pathway.

by Tukey's post hoc test). Therefore, the 30-min interval for glutamate exposure was selected for subsequent experiments.

Next, we investigated the effects of glutamate on the insulin-induced activation of signalling molecules in the IR/Akt/mTOR and GSK3β pathways. Rat cortical neuronal cultures were sequentially exposed to either 100 μM excitotoxic glutamate concentration or no glutamate for 30 min, then stimulated with 100 nM insulin for 15 min and lysed. The levels of the tyrosine-phosphorylated IR β-subunit (IRβ $pY^{1150/1151}$), the serine-phosphorylated Ser/Thr kinase Akt (Akt pS^{473}), the serine-phosphorylated mammalian target of rapamycin (mTOR pS^{2448}), and the serine-phosphorylated glycogen synthase kinase 3β (GSK3β pS^{9}) were measured in the cell lysates and normalised against the total levels of these proteins, as outlined in Additional file 1. A one-way ANOVA analysis revealed a significant difference between groups for IRβ $pY^{1150/1151}$ (Fig. 1d, $F_{3,20} = 23.62$, $P < 0.0001$), Akt pS^{473} (Fig. 1e, $F_{3,20} = 388.6$, $P < 0.0001$), mTOR pS^{2448} (Fig. 1f, $F_{3,20} = 19.69$, $P < 0.0001$), and GSK3β pS^{9} (Fig. 1g, $F_{3,20} = 4.48$, $P = 0.0146$). Tukey's multiple comparisons test showed that insulin stimulation resulted in a significant increase in IRβ $pY^{1150/1151}$ (Fig. 1d, $P < 0.0001$), Akt pS^{473} (Fig. 1e, $P < 0.0001$), and mTOR pS^{2448} (Fig. 1f, $P < 0.001$) levels compared with their respective controls (Additional file 1: Tables S1 and S2). In the glutamate pre-treated neurons, insulin induced 48% less IR phosphorylation at $pY^{1150/1151}$ (Fig. 1d, $P < 0.001$), 72% less Akt phosphorylation at pS^{473} (Fig. 1e, $P < 0.0001$), 44% less mTOR phosphorylation at pS^{2448} (Fig. 1f, $P < 0.0001$), and 38% less GSK3β phosphorylation at pS^{9} (Fig. 1g, $P < 0.01$) compared with non-glutamate treated controls. Neuronal IR becomes fully active following autophosphorylation at $Y^{1150/1151}$ [10], which triggers downstream signalling via the Akt/mTOR and GSK3β pathways. The serine-phosphorylation of Akt at S^{473} [11] and mTOR at S^{2448} [12] are crucial for their activation, whereas the serine-phosphorylation of GSK3β at S^{9} [13] leads to its inhibition. Therefore, our results suggested that excitotoxic glutamate inhibits insulin-induced IR activation and the downstream IR/Akt/mTOR signalling pathway (Fig. 1h).

The primary finding of the present study was that excitotoxic glutamate inhibits the IR/Akt/mTOR pathway, resulting in the development of acute neuronal insulin resistance during periods of significant mitochondrial depolarisation caused by glutamate-evoked massive influxes of Ca^{2+}. This rapid loss of neuronal insulin sensitivity appears to be one of the earliest pathological events associated with glutamate excitotoxicity. These results are in complete agreement with our previous findings that mitochondria control IR autophosphorylation in neurons and that mitochondrial depolarisation causes the loss of insulin sensitivity during the IR autophosphorylation stage [9, 14]. Recently we showed that pre-treatment with insulin prevents the glutamate-evoked increases in $[Ca^{2+}]_i$ and decreases in $\Delta\Psi_m$, protecting rat cortical neurons against excitotoxicity [15]. The glutamate effect and the protective effects of insulin were both completely abrogated by MK 801, an inhibitor of Ca^{2+} influx, via the N-methyl-D-aspartate (NMDA) receptor and the plasmalemmal Na^+/Ca^{2+} exchanger operating in reverse mode [15]. Collectively, these findings suggested that the modulation of intracellular Ca^{2+} levels plays a critical role in negative cross-talk between insulin and glutamate signalling during excitotoxicity. Glutamate induces an increase in $[Ca^{2+}]_i$ and a decrease in $\Delta\Psi_m$, which inhibit IR activation. In turn, insulin prevents the glutamate-evoked rise in $[Ca^{2+}]_i$ and mitochondrial depolarisation, protecting against excitotoxicity.

In conclusion, this study showed that glutamate excitotoxicity is causative for central insulin resistance and may induce the acute loss of insulin signalling within minutes under mitochondrial depolarisation conditions. Therefore, the use of agents designed to prevent mitochondrial depolarisation may be a reasonable approach to the treatment of acute neuronal insulin resistance during excitotoxicity.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13041-019-0533-5.

Additional file 1: Figure S1. Representative images of fluorescence staining of cell culture on (A) DAPI, (B) GFAP, (C) bIIITubulin, and (D) Merge, scalebar 100 μm. (A), **Table S1.** Raw data of phosphoproteins measurements in cell lysates, **Table S2.** Results of statistical analysis.

Additional file 2: Table S3. Raw data of [Ca2+]i (Fura-FF f340/f380 fluorescence) measurements.

Abbreviations

$[Ca^{2+}]_i$: Intracellular free Ca^{2+} concentration; Akt: The Ser/Thr kinase Akt; ANOVA: analysis of variance; GSK3β: The glycogen synthase kinase 3β; IR: Insulin receptor; IRβ: Insulin receptor β-subunit; mTOR: The mammalian target of rapamycin; $\Delta\Psi_m$: Mitochondrial inner membrane potential

Acknowledgements

Not applicable.

Authors' contributions

IK, AS, VP, and IP conceived and designed the experiments. IP wrote the article. IK and ZB performed experiments. IK, VP, and IP analyzed the data. All authors read and approved the final manuscript.

Funding

This work was supported by Russian Ministry of Health, the project N. AAAA-A19–119012590191-3.

Availability of data and materials

All data are available in the Additional file 1 and Additional file 2.

Ethics approval and consent to participate

Experiments with animals were performed in accordance with the ethical principles and regulatory documents recommended by the European Convention on the Protection of Vertebrate Animals used for experiments (Guide for the Animals and Eighth Edition. 2010), as well as in accordance with the "Good Laboratory Rules practice", approved by order of the Ministry of Health of the Russian Federation No. 199n of 04/01/2016. The experimental procedures were approved by the Ethics Committee at National Medical Research Center for Children's Health, Russian Ministry of Health.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 14 August 2019 Accepted: 11 December 2019
Published online: 19 December 2019

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