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INHIBITION OF mTOR KINASE AFFECTS ASTROCYTES GFAP mRNA EXPRESSION IN *IN VITRO* CONDITIONS

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ABSTRACT

In this *in vitro* study the effects of rapamycin – mTOR kinase inhibitor, on the activity of kinase complexes componenents Rictor and Raptor, and on astrocytes activity were investigated. Astrocytes were incubated for 24 hours in various glucose conditions: normoglycemia (CONTROL : 4.5 g glucose.L^{-1}), high glucose (H1: 9 g glucose.L^{-1}), very high glucose (H2:13.5g glucose.L^{-1}). Two concentrations of rapamycin (1nM and 10nM) were added to the culture medium. The activity of kinase complexes was evaluated by quantitative PCR measurements of mRNA expressions for Raptor, Rictor and markers of astrocytes activity – GFAP. Significant differences have been detected between control and experimental groups. Observed data suggested that rapamycin reduced mTOR kinase activity in control group and increased activity of astrocytes in elevated glucose conditions *in vitro*.

Keywords: mTOR, astrocytes, GFAP mRNA expression, rapamycin

INTRODUCTION

mTOR kinase (mammalian target of rapamycin) is a member of a large family of serine/threonine kinases. The main function of this kinase is to maintain cell homeostasis by controlling the transcription, translation, autophagy, proliferation and coordinating the catabolic and anabolic processes, by monitoring the level of nutrients, the level of energy and growth factors **(Kapahi *et al.*, 2010; Sengupta *et al.*, 2010)**. The broad spectrum of mTOR kinase is possible due to the fact that it forms two distinct complexes with different functions: mTORC1 and mTORC2 **(Martin and Hall, 2005; Kapahi *et al.*, 2010)**.
The general elements of construction of the two complexes are mTOR, mLST8/GβL (mammalian lethal with Sec13 protein 8) and Deptor (DEP domain-containing mTOR-interacting protein) **(Maiese *et al.*, 2012).** Other structural elements are specific to the individual complexes. TORC1 complex additionally includes Raptor (regulatory associated protein of mTOR) and PRAS40 (praline-rich Akt substrate 40kDa). The mTORC2 complex has additional elements like Rictor (rapamycin-insensitive companion of mTOR), mSIN1 (mammalian stress-activated protein kinase interacting protein 1), and Protor-1 (praline-rich repeat protein-5, PRR5) **(Sarbassov *et al.*, 2005)**.
Both complexes, mTORC1 and mTORC2 oversee multiple cellular functions **(Maiese *et al.*, 2013; Chong *et al.*, 2012).** mTORC1 is primarily responsible for the controls protein translation by regulating the activity of the eukaryotic initiation factor 4E-binding protein-1 (4EBP1) and the serine/threonine kinase ribosomal protein p70S6K **(Gingras *et al.*, 1998). The** mTORC2 controls cytoskeleton reorganization **(Sarbassov *et al.*, 2004)** and contact between cells **(Gulhati *et al.*, 2011)**. The role of mTOR kinase is based on the control many aspects of cellular metabolism including amino acid biosynthesis, glucose homeostasis **(Thomas *et al.*, 2004**) and also fat metabolism **(Kim and Chen, 2004)**.
The best known inhibitor of the mTOR pathway is rapamycin which inhibits mainly mTORC1 functions **(Polak and Hall, 2009),** however chronic administration of high doses of rapamycin contributes also to the inhibition of the TORC2 **(Foster and Fingar, 2010).**
The mTOR kinase has become an important target of the research in the last decade**.** Its impaired activity is more often mentioned as one of the pathological factor in the development of neurodegenerative diseases such as Parkinson's or Alzheimer's **(Webb *et al.*, 2003; Bandhyopadhyay *et al.*, 2007; Perycz *et al.*, 2007),** associated with metabolic disorders **(de la Monte and Wands, 2008; Carvalho, 2013).**
Although numerous studies have been done on the influence of the mTOR on neuron functions **(Costa-Mattioli *et al.*, 2009; Cao and Obrietan, 2010)**, the effect of this pathway on the astrocytes has not been so far fully understood.
Astrocytes activation called astrogliosis has been implicated in the pathogenesis of several neurological conditions, such as neurodegenerative diseases **(Verkratsky, 2013)**, infection, trauma or ischemia (**Lisi *et al.*, 2012).** Astrocytes can be a good model to study diabetes in CNS **(Latacz *et al.*, 2013a)**. The latest results confirm a deleterious role of activated astrocytes in impaired glucose and insulin states **(Liao *et al.*, 2011)**. The state of reactive astrocytes is often correlated with a reduced level of expression of the main cellular marker of astrocytes - GFAP (glial fibrillary acid protein). Studies using a streptozotocin-induced type 1 diabetic rat revealed decreased astrocytes GFAP expression in main CNS structures **(Coleman *et al.*, 2004)** and different data also confirmed that decrease in GFAP expression is associated with detrimental conditions in the CNS **(Pekny and Pekna, 2004).** It has been also confirmed that mTOR inhibitior – rapamycin, influences on astrocytes activity *in vitro* **(Latacz *et al.*, 2013b).**
Thus, the aim of the study was measure expression of GFAP mRNA and mTOR kinase components: Raptor and Rictor mRNA after cells exposition to the rapamycin in the culture medium supplemented with elevated glucose concentrations.

MATERIAL AND METHODS

The study was performed on primary cortical actrocytes from fetal Sprague-Dawley rats (Life Technologies, Invitrogen, Poland). The first step of the experiment was the multiplicative growth of astrocytes. Cells were seeded at 2 x 10^4cell/cm^2. Cultures were successfully grown in 25cm^2 flasks to 100% confluence in 85% Dulbecco's Modified Eagle Medium containing 4.5g/L glucose and 15% fetal bovine serum (Life Technologies, Invitrogen, Poland). Standard physical growth conditions for rat primary cortical astrocytes (37ºC in a humidified atmosphere of 5% CO_2 in air) were used. Medium was changed every 4 days. The next stage of the experiment was 24 hours incubation of the cells in three different mediums: normoglycemic (CONTROL: 4.5g glucose.L^{-1}) as a control group, high glucose (H1: 9g glucose.L^{-1}) and very high glucose (H2: 13.5g glucose.L^{-1}) with supplementation of 1nM (R1) and 10nM (R2) of rapamycin. Glucose and rapamycin used in cell culture were provided by Sigma-Aldrich (Poland).

RNA extraction and cDNA synthesis

Cells were removed from the plates using StemPro Accutase Cell Dissociation Reagent (Life Technologies, Poland) and total RNA was extracted from cells using Total RNA Mini Kit (A&A Biotechnology, Poland). Concentration and purity of the RNA samples were determined by UV spectroscopy at 260/280 nm. The first strand cDNA was transcribed from 1µg RNA with MultiScribe Reverse Transcriptase (50U/µl, Applied Biosystem, USA) using random primers at 25°C for 10 minutes followed by 37°C for 120 minutes and 85°C for 5 seconds. The cDNA was reconstituted in 50µl of sterilized water and 100 ng of the cDNA solution was used as a template.

Quantitative PCR

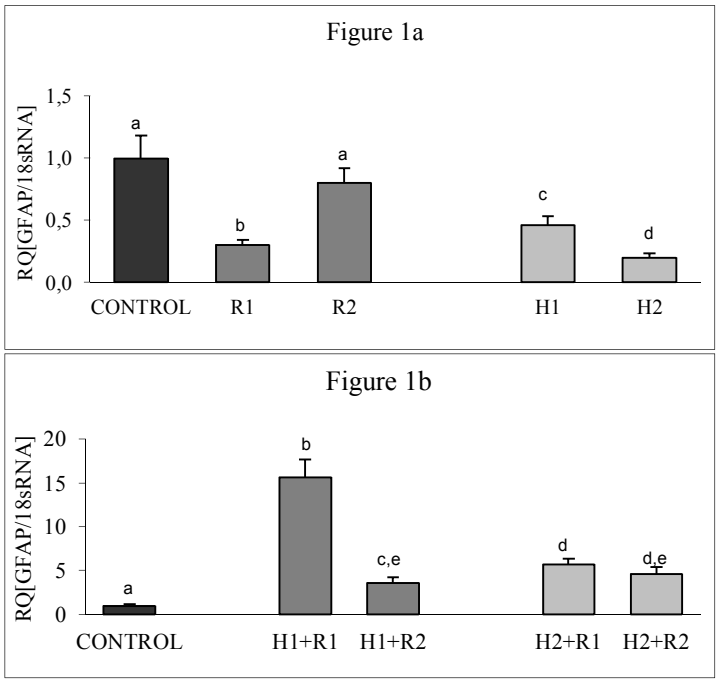
Quantitative PCR analysis was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, USA) with the Universal Master Mix and TaqMan chemistry (Applied Biosystems, USA). The reactions were as follows: an initial step of 50°C for 2 minutes, and then 95°C for 10 minutes, to activate the AmpliTaq Gold DNA polymerase, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. As a reference gene the 18sRNA mRNA was used (Applied Biosystems, USA). The GFAP, Raptor and Rictor expression were assessed using a designed gene expression assay (Applied Biosystems, USA).
The method of $2^{-\Delta\Delta Ct}$ with 18sRNA as an internal control and the sample from control group as an internal calibrator was used to present a change in gene expression.

Statistical analysis

The results were expressed as means ± standard deviations (SD). Comparisons of the group means were made using ANOVA followed by Tukey's post-hoc test (PQStat 1.4.6 Program, Poland). Statistical significance was set at $P<0.01$

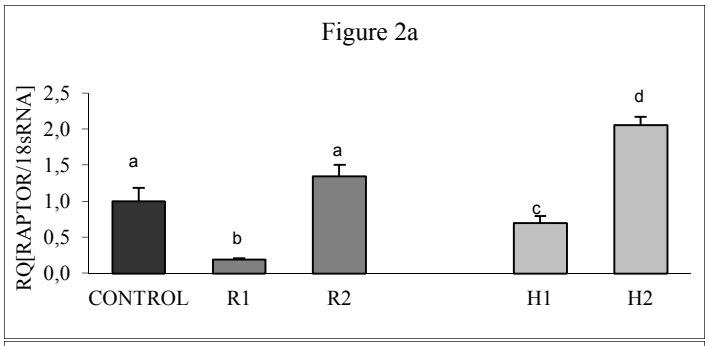
RESULTS AND DISCUSSION

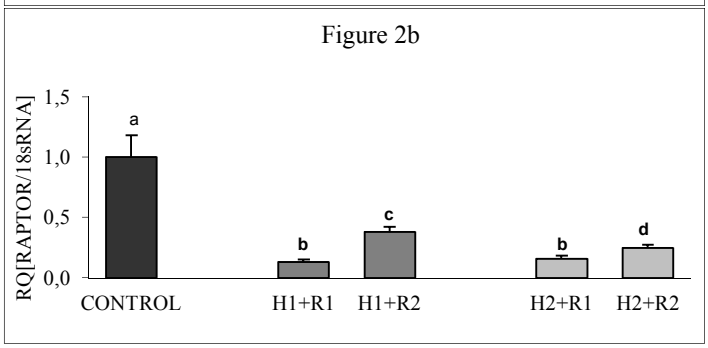
The study revealed that mTOR kinase is a potent factor which can modulate the activity of astrocytes in normoglycemia and glycemic abnormalities like elevated glucose concentration.
The obtained results **(Fig.1a)** indicated that in normoglycemia only concentration at 1nM of rapamycin decreased significantly the expression of GFAP mRNA (to 0.31±0.04 GFAP/18sRNA, $P<0.01$). Lower and higher concentrations of glucose reduced the level of expression (H1: 0.46±0.07, H2:0.19±0.03 GFAP/18sRNA, $P<0.01$). It was also noticed **(Fig.1b)** that both doses of rapamycin significantly increased the expression of astrocytes GFAP mRNA in high glucose (H1R1: 3.60±0.65 GFAP/18sRNA, $P<0.01$) and very high glucose conditions (4.60±0.82 GFAP/18sRNA, $P<0.01$) compare to control.



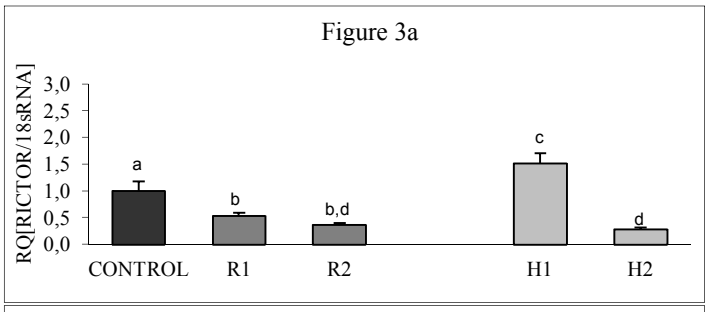
Figures 1a: Expression of GFAP mRNA in astrocytes after 24 hours incubation in normoglycemic (CONTROL), high glucose (H1) , very high glucose (H2) conditions ,and with 1nM (R1) and 10nM (R2) of rapamycin. **1b**. Expression of GFAP mRNA in astrocytes after 24 hours incubation in normoglycemic (CONTROL), high glucose (H1) and very high glucose (H2) conditions in combination with 1nM (R1) and 10nM (R2) of rapamycin. (X±SD, values with different superscript letters differ significantly, $P<0.01$).

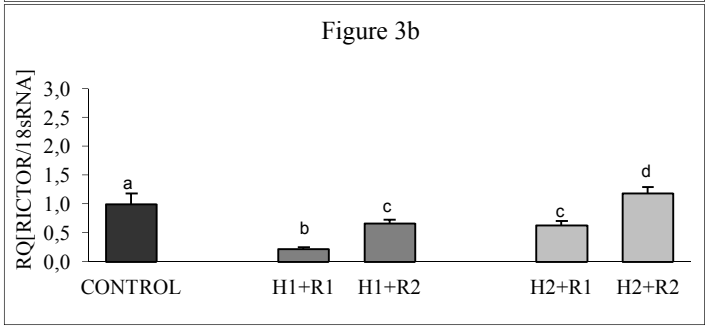
It was showed that in group with high glucose (H1) expression of Raptor mRNA was not changed significantly (**Fig. 2a.**) but it was observed an increase in mRNA expression for Rictor protein (**Fig.3a.** 1.51±0.2 GFAP/18sRNA, $P<0.01$). In H2 group (very high glucose) Raptor expression was increased twofold (**Fig. 2a.** 2.06±0.11 GFAP/18sRNA, $P<0.01$) while Rictor expression was significantly lowered (**Fig.3a.** 0.28±0.04 GFAP/18sRNA, $P<0.01$).
The obtained results also indicate that lower dose of rapamycin (1nM) in all elevated glucose conditions (H1 and H2) has a stronger inhibitory effect on both complexes mTORC1 and mTORC2 by downregulation of its components: Raptor (**Fig.2b.** H1+R1, H+2R1) and Rictor (**Fig.3b.** H1+R1, H2+R2).





Figures 2a: Expression of Raptor mRNA in astrocytes after 24 hours incubation in normoglycemic (CONTROL), high glucose (H1) , very high glucose (H2) conditions ,and with 1nM (R1) and 10nM (R2) of rapamycin. **2b**. Expression of Raptor mRNA in astrocytes after 24 hours incubation in normoglycemic (CONTROL), high glucose (H1) and very high glucose (H2) conditions in combination with 1nM (R1) and 10nM (R2) of rapamycin. (X±SD, values with different superscript letters differ significantly, $P<0.01$).





Figures: 3a. Expression of Rictor mRNA in astrocytes after 24 hours incubation in normoglycemic (CONTROL), high glucose (H1) , very high glucose (H2) conditions ,and with 1nM (R1) and 10nM (R2) of rapamycin. **3b**. Expression of Rictor mRNA in astrocytes after 24 hours incubation in normoglycemic (CONTROL), high glucose (H1) and very high glucose (H2) conditions in combination with 1nM (R1) and 10nM (R2) of rapamycin. (X±SD, values with different superscript letters differ significantly, $P<0.01$).

The obtained results clearly showed that depending on glucose concentration astrocytes reveal different activity features what can be manifested as a change of GFAP mRNA expression. mTOR kinase inhibition by rapamycin can increase GFAP mRNA expression (Fig. 1a and 1b) which may have great importance for the maintaining proper functioning of the CNS. Previous studies have confirmed that decreases in GFAP expression were associated with unfavorable conditions in the CNS **(Pekny and Pekna, 2004)**. Research on GFAP knock-out mice and decrease in astrocyte glial fibrillary acidic protein expression in type 1 diabetic rats **(Barber *et al.*, 2000; Coleman *et al.*, 2004; Coleman *et al.*, 2010)** emphasized the role of this protein in the proper white matter vascularization, maitenance the blood–brain barrier **(Bouchard *et al.*, 2002, Horani and Mooradian, 2003)** and in long-term potentiation (LTP) which is a long-lasting enhancement in signal transmission between two neurons **(Kamal *et al.*, 2000; McCall *et al.*, 1996)**. Decreased activity of both mTORC1 (Fig.2.) and mTORC2 (Fig.3.) in elevated glucose conditions appears to have a beneficial effect on the physiology of astrocytes by raising the GFAP mRNA expression (Fig.1.).

The data showed a significant modulating effect of the mTOR inhibitor on the activity of astrocytes (Fig.1a and 1b) and the involvement of particular complexes - mTORC1 and mTORC2 of this pathway. This glial cells which take part in maintenance of the neuronal homeostasis in the central nervous system may be a good therapeutic target e.g. for inhibition of neurodegenerative diseases development related to glycemic abnormalities like hyperglycemia. Using an appropriate concentration of mTOR inhibitor it is possible to stimulate activity of astroglial cells and support neuron functions.

CONCLUSION

Based on the obtained data of the expression of mTOR components mRNA and GFAP mRNA we can conclude that lower dose of rapamycin at a concentration of 1 nM in the culture medium is much more efficient than higher dose (10nM) and effectively reduces the expression of mRNA for both mTOR kinase protein subunits: Raptor and Rictor, under conditions of elevated glucose levels. At the same time inhibition of mTOR pathway results in an increased activity of astrocytes, what is documented by elevated GFAP mRNA expression. These observations indicate that rapamycin effects on astrocytes function can be significant in prevention of the central nervous system pathology related to impaired glucose conditions. Future perspectives should be focused on verification astrocytes and mTOR kinase role in glycemic abnormalities in CNS.

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