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Vasopressin activates Akt/mTOR pathway in smooth muscle cells cultured in high glucose concentration

Daniela K. Montes [a], Marianne Brenet [a], Vanessa C. Muñoz [a], Patricia V. Burgos [a], Carolina I. Villanueva [a], Carlos D. Figueroa [b], Carlos B. González [a,c,*]

[a] Department of Physiology, Universidad Austral de Chile, Valdivia 509-9200, Chile
[b] Department of Anatomy, Histology and Pathology, Universidad Austral de Chile, Valdivia 509-9200, Chile
[c] Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX 77555, United States

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ABSTRACT

Mammalian target of rapamycin (mTOR) complex is a key regulator of autophagy, cell growth and proliferation. Here, we studied the effects of arginine vasopressin (AVP) on mTOR activation in vascular smooth muscle cells cultured in high glucose concentration.

AVP induced the mTOR phosphorylation in A-10 cells grown in high glucose, in contrast to cells cultured in normal glucose; wherein, only basal phosphorylation was observed. The AVP-induced mTOR phosphorylation was inhibited by a PI3K inhibitor. Moreover, the AVP-induced mTOR activation inhibited autophagy and increased thymidine incorporation in cells grown in high glucose. This increase was abolished by rapamycin which inhibits the mTORC1 complex formation.

Our results suggest that AVP stimulates mTOR phosphorylation by activating the PI3K/Akt signaling pathway and, subsequently, inhibits autophagy and raises cell proliferation in A-10 cells maintained in high glucose concentration.

1. Introduction

Hyperglycemia is one of the complications of diabetes leading to arteriosclerosis and hypertension. Changes in nutrients, as glucose, result in changes of the AMP/ATP ratio, such that, low ATP level is detected by the AMPK which, in turn, phosphorylates TSC2. The activated TSC2 reduces mTOR complex 1 activity by inhibiting the small GTPase protein Rheb [1]. On the other hand, nutrients can also regulate mTORC1 by activating PLD and, therefore, production of phosphatidic acid, which binds and stabilizes mTORC1 complex [2,3]. In addition, mTORC1 is also positively regulated by growth factors and mitogens through two key signaling pathways; the PI3K/Akt and Ras/RAF/ERK pathways that activate mTORC1 [4–6] by inhibiting the GTPase-activating protein activity of the TSC1/TSC2 complex and thus, promoting mTORC1 function, which eventually can, on one hand, stimulates protein translation and hence cell growth and proliferation and on the other, inhibits autophagy. Therefore, mTOR pathway integrates signals from nutrients, energy balance and hormones to regulate a variety of processes, including autophagy, metabolism, cell growth and proliferation.

High extracellular glucose concentration, such as that found in poorly controlled diabetic syndrome, produce an osmotically-induced AVP secretion from the neurohypophysis [7]. Indeed, patients undergoing a hyperglycemic condition have high levels of plasma AVP; almost the double compared with normal subjects and simultaneously, a decrease in the AVP content in the neural lobe due to AVP hypersecretion [8,9]. Recently, it has been found, in population studies, an association of an increase in the AVP system activity and diabetes mellitus [10–12].

It is well known that AVP increases cell proliferation by EGFR transactivation and hence activation of the MEK/ERK pathway [13–16]. Since two factors affecting the mTOR activity, that is, nutrients and mitogens, converges in the diabetic mellitus, we decided to analyze the effect of AVP on mTOR activity, when smooth muscle cells are cultured in a high glucose concentration, to shed light on the mechanisms of hyperglycemia as a risk factor for cardiovascular diseases.

2. Methods

2.1. Cell culture

A-10 cells (ATCC CRL 1476), derived from smooth muscle cells of rat aorta, were cultured to subconfluency on 60 mm dishes in

Abbreviations: AVP, arginine vasopressin; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; PI3K, phosphatidyl inositol-3 kinase; AMPK, AMP-activated protein kinase; PKC, protein kinase C, Akt or protein kinase B; TSC1/TSC2, tubero sclerosis protein 1 and 2; Rheb, ras homolog enriched in brain; PLD, phospholipase D; ERK, extracellular regulated-kinase.

* Corresponding author at: Edif Ciencias Biomédicas, Of. 406, Universidad Austral de Chile, Campus Isla Teja, Valdivia, Chile. Fax: +56 63 222 1513.
E-mail address: cbgonzal@uach.cl (C.B. González).

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DMEM containing 10% fetal bovine serum and 5.5 mM glucose (LG, low glucose) or 25 mM glucose (HG, high glucose) for ten days. After serum starvation for 16 h the cells were treated with AVP in the absence or presence of inhibitors. The reaction was stopped by the addition of 100 μl of ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris/HCl pH 7.4, 0.5% sodium deoxycholic acid 1% Nonidet P40, 0.1% SDS, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 0.1 mM phenylmethane-sulphonyl fluoride, 1 μg/mL leupeptin, 5 μg/mL soy bean trypsin inhibitor). Proteins were determined and a volume containing approximately 15 μg of protein was mixed with electrophoresis sample buffer. In some experiments, cells were incubated for 30 min with each of the following inhibitors: 10 μM Gö6983, 50 μM PD98059, 1 μM AG 1478, 10 μM LY294002, 0.1 μM Rapamycin, 0.3% 1-butanol or 1 mM N-acetyl-L-cysteine (NAC) prior to stimulation with AVP.

2.2. Western blotting

Cell extracts were fractionated using 8% polyacrylamide gel electrophoresis, to detect mTOR or 12% polyacrylamide gels to detect Akt. Proteins were electrotransferred onto nitrocellulose filters using 0.05% SDS in the transfer buffer (20 mM Tris–glycine pH 8.3 and 20% methanol). Blots were incubated with anti-phospho mTOR (Ser2448) or anti-phospho Akt (S473) antibodies at a dilution of 1:1000 (Cell Signaling Technology, Inc., MA, USA). Blots were then incubated with peroxidase-labeled secondary antibody at a dilution of 1:50,000 followed by chemiluminescence (SuperSignal, Thermo Scientific, IL, USA).

2.3. Cell proliferation assay

Vascular smooth muscle A-10 cells were seeded in 96-well plates at a cell density of 5×10^3 cells per well. Cells were synchronized for 24 h and then stimulated with AVP (50 nM) for 48 h. DNA synthesis was measured by the incorporation of [^{3}H]Thymidine. All the values are reported as the mean ± SE of triplicate experiments. To standardize the results, absolute counts were converted to a percentage of the control.

2.4. Data analysis

Densitometric analyses of gel digital images were carried out using the UN-SCAN-IT gel software (Silk Scientific, Inc., Orem, UT, USA) and the relative phosphorylated protein density level was normalized by comparison to the total protein signal. Values are expressed as means ± SE. Statistical analyses were performed by one-way repeated measures analysis of variance followed by Holm-Sidak method by multiple comparisons versus time zero (without AVP treatment) using SigmaPlot version 12 software. When it was necessary, the data were analyzed by *t*-test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. AVP stimulates mTOR phosphorylation in A-10 cells cultures in a high extracellular glucose concentration

A-10 cells were maintained for 16 h without fetal bovine serum in DMEM containing 5.5 mM glucose, and then the concentration of glucose was raised to 25 mM and cells maintained for different times from 15 min to 72 h. The analysis of the phosphorylation of mTOR by Western blotting using a specific antibody against the phosphorylated serine residue 2448 showed no significant variations in the phosphorylation state at any time after changing the extracellular glucose concentration (Figs. 1S A and B). The stimulation of cells, maintained in normal extracellular glucose concentration (5.5 mM), with 50 nM AVP showed no significant increase in the phosphorylation of mTOR up to 180 min after stimulation (Figs. 1A and C). In contrast, cells, which were maintained in high extracellular glucose concentration (25 mM) for 10 days and then stimulated with 50 nM AVP for the same time, displayed a statistically significant increase in the phosphorylation of mTOR between 90 to 180 min (Figs. 1B and C). Similarly, there was an increase in Ser 473 phosphorylation of Akt, which is a serine/threonine kinase upstream regulator of mTOR, in cells maintained in high extracellular glucose concentration and stimulated with AVP (Figs. 1D and E) as compared to that cultured in normal glucose concentration (not shown).

3.2. The AVP-induced mTOR phosphorylation is mediated by the activation of the PI3K/Akt pathway

In order to define the pathway by which AVP is activating mTOR, several enzyme inhibitors were used. To determine the involvement of the PI3K/Akt in the AVP-induced mTOR activation, cells were incubated with the PI3K inhibitor LY294002. Cells incubated with this inhibitor showed a significant inhibition of mTOR phosphorylation under basal condition compared with the control. However, AVP was able, after 2 h stimulation of cells treated with LY294002, to slightly increase the mTOR phosphorylation over the control without the inhibitor (Figs. 2A and B). Similarly, the treatment with the PKC inhibitor Gö6983 also showed an inhibition of the mTOR activation after stimulation with AVP; however, there was an increase in the mTOR phosphorylation but without reaching the level to that of the control without the inhibitor (Figs. 2A and B). Since PI3K is activated by tyrosine kinase receptors and, on the other hand, the AVP V1 receptor transactivates the EGFR we used AG1478, an inhibitor of the tyrosine kinase activity of the EGFR, to determine whether the V1 receptor was stimulating PI3K by EGFR transactivation. However, this inhibitor had no effect on the AVP-induced mTOR phosphorylation after 2 h of stimulation of cells maintained under high extracellular glucose concentration. This result rules out the possibility that AVP-induced mTOR phosphorylation is carried out by EGFR transactivation (Figs. 2A and B). Furthermore, to determine whether the MEK/MAPK pathway was involved in mTOR AVP-induced phosphorylation, cells were incubated with the MEK inhibitor PD98059 and then stimulated for 2 h with AVP. In this circumstance, the inhibition of MEK did not block the AVP-induced mTOR phosphorylation disregarding the participation of this pathway.

To evaluate the potential involvement of PLD and the production of phosphatidic acid (PA) in mechanism of the AVP-induced mTOR phosphorylation, cells were treated for 15 min with 1-butanol, which significantly attenuated the basal phosphorylation of mTOR; however, did not inhibit the AVP-induced mTOR phosphorylation (Figs. 2A and B). This result might be explained as the structural stabilizing role that PA plays on mTOR and the regulatory-associated proteins [3,17]. Since PA plays a crucial role in stabilizing the mTORC1 complex and thus, for its activity, the overriding effect of AVP on the mTOR activation in 1-butanol-treated cells might be explained by the production of PA by alternative pathways. In fact, PA can be generated from diacylglycerol (DAG) by diacylglycerol kinase [18,19].

To determine whether oxidative environment generated by the high glucose concentration accounted for the AKT/mTOR activation, A-10 cells were incubated with NAC, a scavenger of reactive oxygen species (ROS), previous to AVP stimulation. However, NAC did not modify the AVP-induced mTOR phosphorylation after 90 to 180 min of AVP incubation (Figs. 2C and D).