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

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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.

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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).

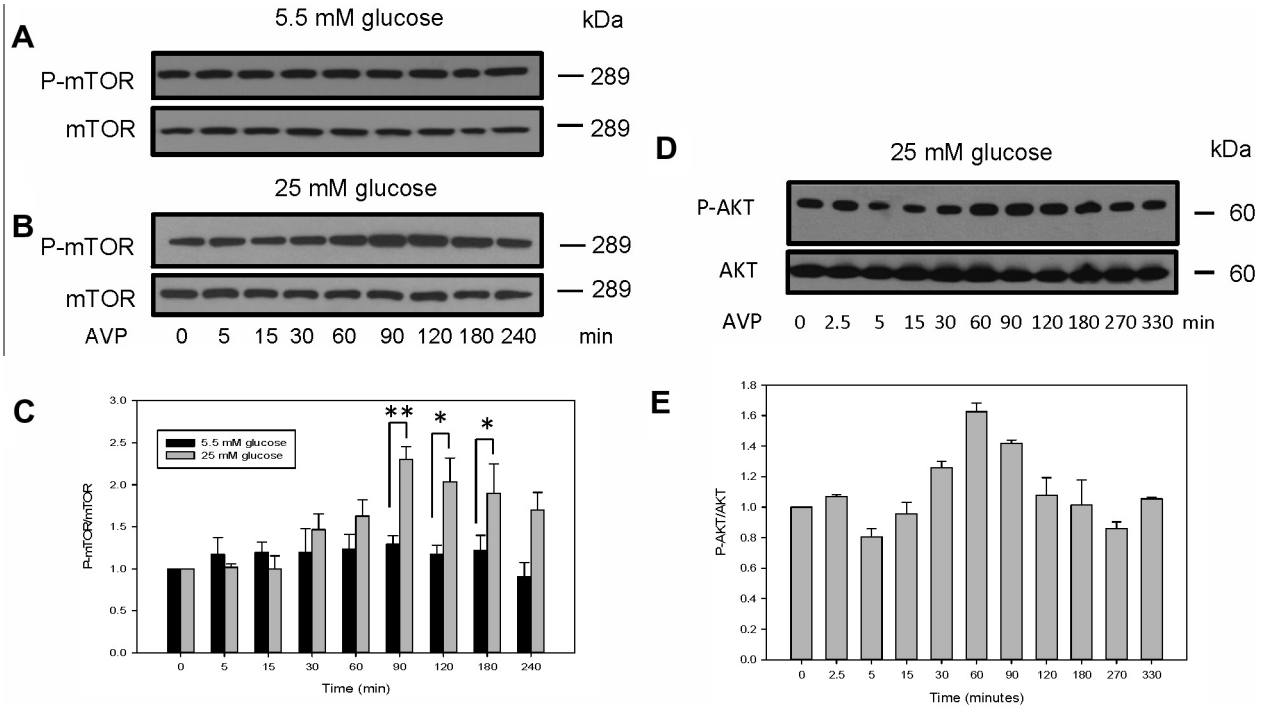

Fig. 1. Vasopressin induces phosphorylation of mTOR and Akt in A-10 cells cultured in high glucose medium. A-10 cells were cultured in DMEM containing 5.5 mM (LG) (A) or 25 mM (HG) (B) glucose followed by stimulation with AVP for different time periods. Cell extracts were subjected to Western blotting and the degree of mTOR phosphorylation was determined using a phospho-site (Ser 2448) antibody. Immunoblots were quantified using UN-SCAN-IT densitometry software. Values were normalized to that in the absence of AVP (basal) and expressed as the ratio phospho-mTOR/total mTOR (C). The statistical significance of the data was determined by one-way repeated measures analysis of variance (ANOVA) followed by Holm-Sidak method by multiple comparisons versus time zero (without AVP treatment). Anova showed that the differences in the mean values among the AVP-treated HG group (gray column) are statistically significant; whereas, those of the AVP-treated LG group (black column) are not. Values are mean ± SE of 4 independent experiments. * indicates $p < 0.05$ and ** $p < 0.01$. The degree of Akt phosphorylation was evaluated using an anti phospho Akt (Ser 473) antibody and detected using peroxidase-labeled secondary antibody at a dilution of 1:50,000 followed by chemiluminescence (D). Ratios of P-Akt and Akt signal were measured by quantitative densitometry (E). Data represent the mean ± SE of three experiments.

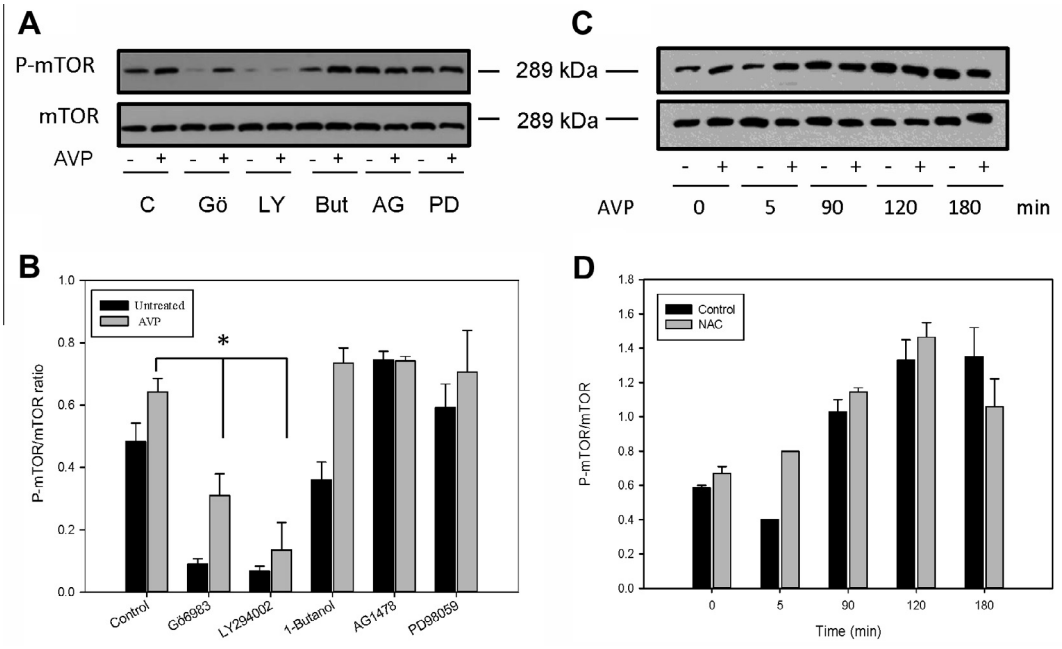

Fig. 2. PI3K is involved in the AVP-induced mTOR phosphorylation. A-10 cells cultured in DMEM containing 25 mM glucose treated without or with 50 nM AVP for 120 min (control, C). Prior to AVP stimulation cells were treated for 30 min with the PKC inhibitor Gö6983 (10 μM), the PI3K inhibitor LY294002 (10 μM), the PLD inhibitor 1-butanol (0.3%), the EGFR kinase inhibitor AG1478 (1 μM), the MEK inhibitor PD98059 (50 μM). The degree of mTOR phosphorylation was evaluated using an anti phospho mTOR (Ser 2428) antibody and detected using peroxidase-labeled secondary antibody at a dilution of 1:50,000 followed by chemiluminescence (A). P-mTOR and mTOR signals were measured by densitometry and expressed as a P-mTOR/mTOR ratio (B). Cells were treated with 1 mM N-acetyl-L-cysteine prior to the AVP stimulation for the indicated time (C) and P-mTOR and mTOR signals were determined by densitometry and expressed as a P-mTOR/mTOR ratio (D). Data represent the mean ± SE of three experiments.

3.3. The AVP-induced mTOR activation down-regulates autophagy and increases cell proliferation

Because mTORC1 controls the autophagy mechanism, such that, an increase in the phosphorylation of mTORC1 inhibits the autophagy; therefore, to assess the extent of autophagy we determined the autophagosome markers LC3 I and II (microtubule-associated protein1 light chain 3) by Western blotting after treating A-10 cells with AVP for different time. There was a marked decrease in the LC3II band after incubating cells with 50 nM AVP for 60 min, which lasted until 240 min, indicating a decrease in the autophagy activity (Figs. 3A and B). Having demonstrated that AVP is able to reduce autophagy activity, next we analyzed whether the AVP-dependent activation of mTORC1 increases cell proliferation. To evaluate this possibility, [^{3}H] Thymidine incorporation was determined in cell maintained in normal and high glucose and stimulated with AVP. After 48 h of AVP stimulation cells maintained in high extracellular glucose concentration showed a significant increase in the thymidine incorporation as compared with those maintained in normal glucose concentration (Fig. 4). The AVP-induced thymidine incorporation in cells maintained in high glucose concentration was completely abolished by rapamycin (Fig. 4), which is known to form a complex with FKBP12 (FK binding protein) cytosolic protein [20] that then binds to and inhibits mTORC1 function. This result clearly demonstrates that the AVP-induced proliferation in A-10 cells exposed to high extracellular glucose concentration is mediated by mTORC1.

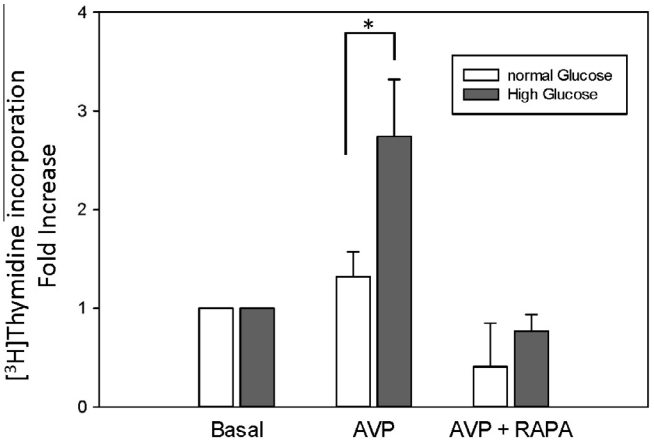

Fig. 4. AVP induces cell proliferation by mTOR activation in A-10 cells cultured in high glucose medium. A-10 cells maintained in DMEM containing 5.5 mM glucose (LG) or in 25 mM glucose (HG) and stimulated with 50 nM AVP for 48 h. DNA synthesis was measured by the incorporation of [^{3}H]Thymidine. The incorporation of thymidine increased more than three times compared to that of cells maintained in 5.5 mM glucose and treated with AVP (A). The inhibition of mTORC1 with rapamycin (0.1 μM) completely blocked the AVP-induced cell proliferation (B). Data represent the mean ± SE of triplicates.

4. Discussion

We demonstrated that a chronic exposure of smooth muscle cells to high extracellular glucose concentration enhances the AVP-induced mTOR phosphorylation. This response could be neither found in AVP-stimulated cells, which were incubated with normal extracellular glucose nor in cells with an acute exposure (48–72 h) to high extracellular glucose concentration. The inhibition of the AVP-induced mTOR phosphorylation by LY294002 indicates that PI3K was involved in the mTOR activation. Indeed, numerous studies have demonstrated that GPCR can regulate PI3Ks mainly of the class IB. Moreover, it has been shown the involvement of Gβγ in activating p110γ catalytic subunit of PI3K, both in vitro as well as *in vivo* [21,22].

Once PI3K is activated, it phosphorylates PtdIns(3,4)P_2 generating PtdIns(3,4,5)P_3 which binds to the PH (pleckstrin homology) domain of Akt, promoting its translocation to the plasma membrane [23]. In the membrane, Akt can be phosphorylated by PDK1 at Thr308 residue; whereas, the phosphorylation at Ser473 residue is carried out by mTORC2, indicating that mTOR can act either as a substrate, as well as, an effector of Akt [24]. The

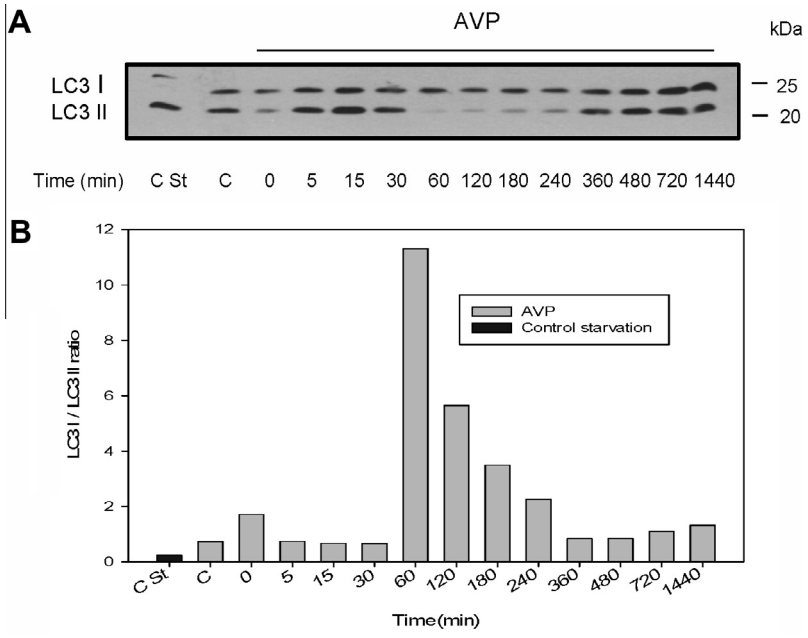

Fig. 3. AVP inhibits autophagy in A-10 cells cultured in high glucose medium. A-10 cells were cultured in DMEM containing 25 mM glucose followed by stimulation of vasopressin for the indicated time period. Western blot were carried out using anti-LC3 antibody (A). Quantification of immunoblots was carried out by densitometry and values are expressed as a ratio of LC3I/LC3II (B).

phosphorylation of both residues is required for full activation of Akt [25], which, in turn, phosphorylates to mTOR in two adjacent sites, that is, Thr2446 and Ser2448 [26]. Moreover, the phosphorylation of mTOR at residue S2448 by Akt is correlated with the activation of mTORC1 [27]. Furthermore, Akt is also able to directly phosphorylate TSC2, which as a heterodimer with TSC1 is a negative regulator of mTORC1 [25]. Accordingly, PI3K plays a vital role in controlling mTORC1 activity by regulating of Akt phosphorylation, on one hand, by directly phosphorylating mTOR, and, on the other, by inhibiting TSC1/2 activity and hence stimulating mTOR phosphorylation.

An intriguing question is why mTORC1 is solely activated by AVP when cells are maintained in high extracellular glucose concentration. One possible explanation would be that the high concentration of extracellular glucose leads to alterations of the mechanisms regulating the signaling pathways involved in the regulation of vascular tone, and in cell proliferation. In this context, it has been reported that both endothelial and smooth muscle cells maintained in high glucose concentrations have an augmented DAG concentration, which translates into an increase in PKC activity [28,29]. Alternatively, the high glucose concentration produces ROS (Reactive Oxygen Species), which could also alter signaling pathways. Indeed, Swiss 3T3 fibroblasts or HEK 293 cells subjected to oxidative stress showed PI3K-dependent activation of Akt/PKB, which is an upstream of mTOR cascade [30]. Likewise, ROS play an essential role in the Interleukin 7-mediated viability and proliferation by activation of PI3K/Akt/mTOR pathway in T-cell lymphoblastic leukemia cells [31]. However, in our cell model we did not find changes in Akt nor mTOR phosphorylation when cells are exposed to high extracellular glucose concentration. Moreover, the incubation of cells with NAC, a scavenger of ROS, did not modify the AVP-induced mTOR phosphorylation. These data ruled out the possible participation of ROS on the AVP-stimulated mTOR activation.

It is well known that mTOR activation induces cell proliferation by up-regulating cell cycle-related genes and that its inhibition by rapamycin results in cell cycle arrest [25,32,33]. Growth factors are able to induce cell proliferation and, on the other hand, AVP is able to transactivate the EGFR [16,34]. However, AG1478, an EGFR kinase inhibitor, did not alter the AVP-induced mTOR activation suggesting that the AVP-induced mTOR activation is carried out by an EGFR-independent transactivation mechanism. Most importantly, the inhibition of AVP-induced thymidine incorporation by rapamycin demonstrates that this proliferative effect of AVP is mediated by mTORC1 activation. Taken these results together clearly show that AVP is capable of reducing autophagy activity and concomitantly increasing cell proliferation via mTOR pathway activation.

Therefore, high extracellular glucose concentration itself or by the generation of ROS might lead to alterations of the mechanisms regulating the signaling pathways involved in the regulation of vascular tone, and in cell proliferation. Here, we showed that AVP, which is secreted due to the hyperglycemia-induced osmolality increase, might also be contributing to vascular complications associated with diabetes mellitus.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.169.

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