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Effects of VGF Derived Bioactive Peptide TLQP-21 on ERK/AKT Signalling in SH-SY5Y Cells

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Abstract

Growing functional information regarding bioactive TLQP-21 has led to the ubiquitous demand for identification of receptor dependent or independent pathways associated to the peptide TLQP-21. To date, little, in some cases, nothing is known about the signalling mechanisms in which the peptide TLQP-21 and its receptor(s) take(s) part. For this reason the study was carried out to in order to know the read out that either or both of the two proteins, ERK/AKT were modulated in SH-SY5Y cell model. SDS-PAGE, Western blot analysis and immunodetection were performed with the treated SH-SY5Y cells by TLQP-21 and control samples (only SH-SY5Y cells, without TLQP-21 treatment) which obtained after solubilization with lysis buffer. The result found here confirmed that in SH-SY5Y cells, TLQP-21 did not bring any alternation in the expression of ERK and AKT proteins. Of note, past studies confirmed the modulatory effect of TLQP-21 on different proteins in different cell lines while some other studies nullified, but confirming the effect on some other proteins. The significance of the study lies in fact that this is the first study in human cell line, regarding cell signalling by TLQP-21. In the end, the signalling pathway of the human TLQP-21 will help to pharmacological modulation of those diseases of human related to TLQP-21.

Keywords: VGF, TLQP-21, ERK, AKT, SH-SY5Y.

AMS Classification: 92C55.

1. Introduction

VGF (a non-acronymic name) gene was originally identified as a nerve growth factor (NGF) responsive gene and should not be confused with VEGF (vascular endothelial growth factor). Andrea Levi first determined the existence of VGF mRNA in 1985 at the National Institute of Health in the laboratory of Bruce Macdonald Paterson and this observation came out at the time of exploring on neural sympathetic and sensory differentiation induced by nerve growth factor (NGF), here rat pheochromocytoma PC12 cells were used as a model (Levi et al., 1985). NGF33.1, a nervous system-specific mRNA was cloned by treatment of PC12 cells with NGF. After elucidating the nucleic acid as well as amino acid sequences of the NGF33.1 cDNA clone, Levi et al. (1985) designated this clone corresponding to the NGF-inducible mRNA as VGF. The term 'VGF' derived from the selection of this clone from plate **V** of the nerve **G**rowth **F**actor induced PC12 cell cDNA library (Levi et al., 1985; Possenti et al., 1989).

Out of several bioactive peptides derived from VGF, TLQP-21 is the most studied and of huge importance because of its multi-physiological roles: Energy expenditure (Possenti et al., 2012; Jethwa et al., 2007; Bartolomucci et al., 2006), metabolism (Bartolomucci et al., 2008), obesity (Possenti et al., 2012), glucose-stimulated insulin secretion (GSIS) (Stephens et al., 2012), pain modulation (Fairbanks et al., 2014; Chen et al., 2013; Rizzi et al., 2008), hypertension (Fargali et al., 2014), gastric contractility (Severini et al., 2009; Bartolomucci et al., 2008), gastric acid secretion (Sibilia et al.,2012; Sibilia et al., 2010a; 2010b) reproduction (Aguilar et al., 2013; Pinilla et al.,2011), stress (Razzoli et al., 2012, Bartolomucciet al., 2011), neuroprotective agent (Severini et al., 2008), anorexia (Bartolomucci et al., 2006; Jethwa et al., 2007).

To our knowledge no proteomic or phosphoproteomic studies have yet focused on the effect of VGF derived bioactive peptide TLQP-21 on ERK/AKT signalling in SH-SY5Y cells. Recently a proteomic analysis was carried outusing protein extracts of control and SH-SY5Y cells treated with the peptide, to know which proteins are modulated (Akhter& Requena, 2018; Akhter, 2015).

In addition to the phosphoproteomic study of SH-SY5Y cells treated with TLPQ-21, another analysis of total protein expression was carried on using the same cell extracts. Comparison of simple 1D SDS-PAGE gels stained with SYPRO® Ruby protein gel stain and Pro Q® Diamond staining was done. Several bands with

altered intensity in the SYPRO® Ruby experiment were of the same positions in the gels as those in the Pro Q® Diamond, suggesting that both techniques were detecting, to some extent, similar changes. This also suggested that the proteins with altered phosphorylation status were probably abundant proteins, hence their changes were "picked up" by SYPRO®Ruby staining. Furthermore, mass spectrometry analysis of the bands with changed intensity confirmed a list of proteins with altered expression levels (Akhter & Requena, 2018a; Akhter, 2015).

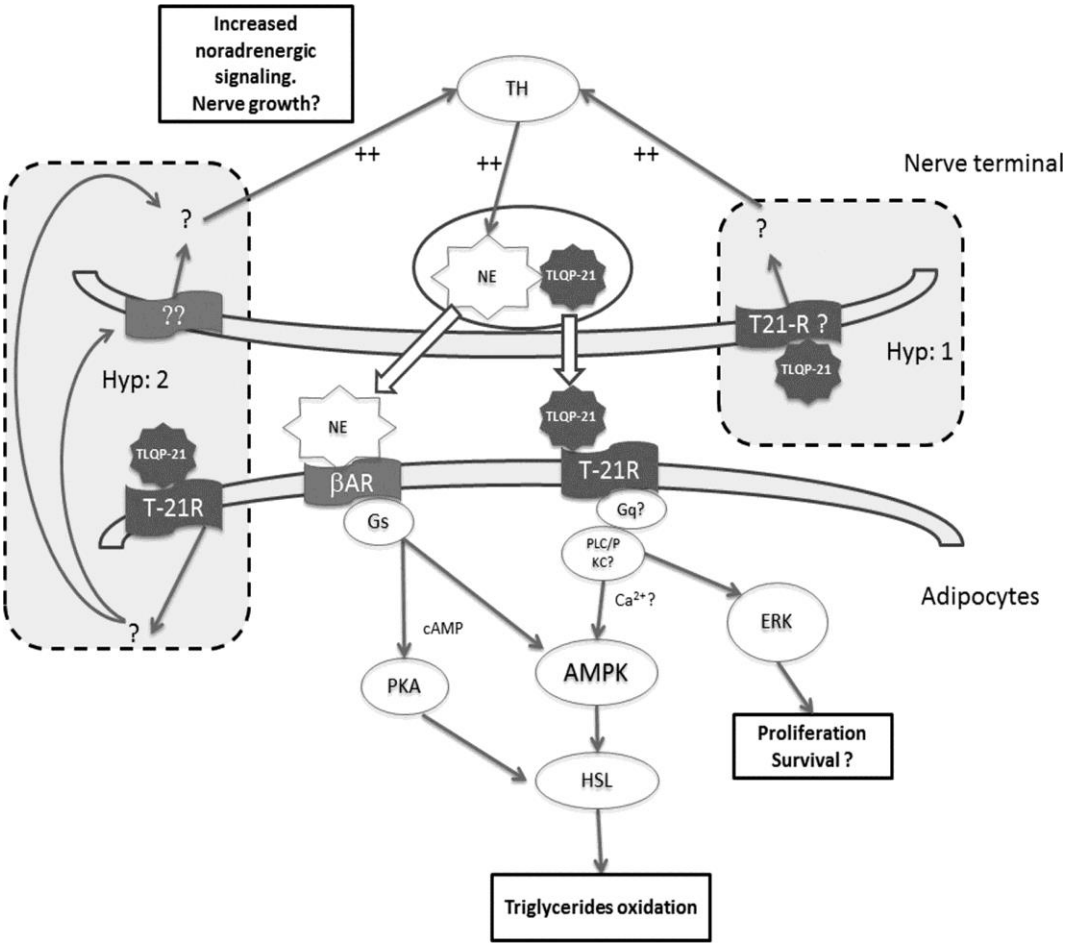

Figure 1: Proposed mechanism of action of TLQP-21: Intracellular signalling downstream in adipocyte membrane after activation of a putative TLQP-21 receptor activation (From Possenti, et al., 2012).

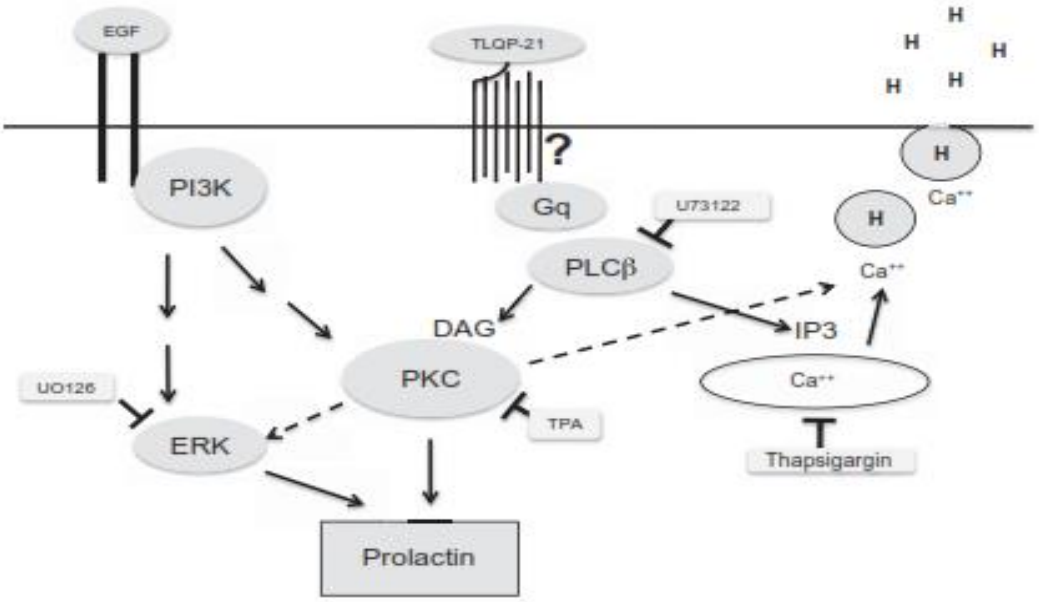

Figure 2: Schematic demonstration of TLQP-21 intracellular signalling (From Passeri et al., 2013).

It was a big challenge to detect the receptor(s) of VGF-derived peptides and their cellular signalling mechanisms, but recently some receptors have been identified by using powerful proteomic methods, namely C3AR1, gC1qR and HSPA8. Their functions are not similar at all. Among these receptors, C3AR1 and gC1qR are receptor of rodent model. And HSPA8 is the first putative receptor for human model(Akhter, 2020; Akhter & Requena, 2018a; Akhter & Requena, 2018b; Akhter & Requena, 2018c; Akhter et al., 2017; Akhter, 2015).

In several biological functions, TLQP-21 follows diverse signal transduction pathway. TLQP-21 is unable to activate the cAMP/PKA pathway as PKA substrates were not substantially phosphorylated by TLQP-21 (Carmen et al., 2006). But likewise other peptides, binding with a seven transmembrane domain receptor, TLQP-21 will activate a Gq protein, stimulate a phospholipase B, increase in ERK, DAG and PI3K (phosphoinositide 3-kinase) and actívate PKC and intracellular calcium mobilization (Passeri et al., 2013; Possenti, et al., 2012; Figure 1 and 2).

Taken together all the data into consideration, as presented in the Figure 1 and 2; ERK/AKT signalling pathway was chosen to perform a study to have a read out whether the peptide potentiates ERK/AKT signalling in the SH-SY5Y cell model or not.

2. Materials and Methodology

2.1 SH-SY5Y Cell Culture

From patent depository for Europe; catalogue number-94030304 from European Collection of Authenticated Cell Cultures (ECACC) of England, an International Depository Authority, SH-SY5Y cell line was bought. It was taken from a four years-old female, a metastatic bone tumor patient, with neuroblastoma as a third time cloned, proceeded in order like this: SK-N-SH → SH-SY → SH-SY5 → SH-SY5Y, subline of the neuroblastoma cell line SK-N-SH31-32 (Biedler et al., 1973; 1978). Of note, the cell line was established in 1970. Cells were grown in a incubator which CO2-humidified at 5% rate (Life Sciences), on 100×20 mm Falcon Petri dishes at 37 ˚C with the culture medium composed of 1:1 Earle's Balanced Salt Solution (EBSS) (Sigma Aldrich) and F12HAM (Sigma Aldrich) supplemented with 15% fetal bovine serum (FBS) (Gibco), 1%Glutamine (Gln) (Sigma Aldrich), 1% Non-Essential Amino Acids (NEAA) (SigmaAldrich), and 1% Penicilin-Streptomicin (P/S) (Invitrogen) in 100×20 mm Falcon Petridishes (Life Sciences). The cells were always used at less than 20 passages.

2.2 Protein Extraction

Petri dishes with confluent SH-SY5Y cells were incubated for 6 hours with the peptide TLQP-21 at a concentration of 1µg/ml. SH-SY5Y cells in Petri dishes were washed twice with cold PBS, followed by solubilization in lysis buffer, composed of 20 mM HEPES, 2mM EGTA, 1mM DTT, 1mM sodium orthovanadate, 1% Triton X-100, 10% Glycerol, 2µM leupeptin, 400 µM PMSF, 50 µM ß-glycerophosphate, 100 µg/ml trasylol. Following solubilization in lysis buffer, the cells were tussled keeping them on ice, for 10 minutes. A periodic vortex was done with each 5 minutes interval to incubate them on ice for half an hour. Solution of the cells were centrifuged for 0.3 hour at rpm of 14000g at 4 ˚C. And it was followed by supernatant collection. Using instantly the supernatant and/or the rest of the supernatant was preserved for use afterwards further after a

while. The BCA assay kit (Pierce Chemical) for protein was used for quantifying the protein concentration.

2.3 SDS-PAGE, Western blot analysis and immunodetection

Cell homogenate samples (20 µl) were boiled in 2X Laemmli buffer (Bio-Rad) for 10minutes at 100 °C, spun, and loaded to SDS-PAGE gel. After electrophoresis (200V, 01h), proteins were transferred onto Polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) in the condition of 0.8 mA/cm^2 for 90 minutes using semi-dry method (Trans-blot SD semi-dry transfer cell, Biorad). The PVDF membrane was then blocked by 5% BSA (Bovine Serum Albumin) in TBS-T solution (Tris-buffered saline with 0.1% tween 20) for 01 hour at room temperature, followed by three washes by TBS-T each for 10 minutes and then, the membranes were probed by primary antibodies in TBS-T, as specified in the Table 1.

Table 1: Specifications of the antibodies used.

Antibody	Commercial source	Dilution	Other specifications
pERK(Thr202/Tyr204)	Cell signalling	1:2000	Overnight incubation
ERK	Cell signalling	1:1000	Overnight incubation
p^{AKT}(Ser473)	Cell signalling	1:1000	Overnight incubation
AKT	Cell signalling	1:1000	Overnight incubation
GAPDH	Sigma-Aldrich	1: 5000	Overnight incubation

Then the PVDF membrane was washed 3 times with TBS-T for 10 minutes each wash, followed by incubation with the secondary Anti-rabbit antibody (Dako) at 1 : 2000 dilution in TBS-T. The control was performed using a GAPDH antibody, as specified in the Table 1. The membrane was washed 3 times with TBS-T for 10 minutes each wash. Subsequently, the membrane was incubated with chemiluminescence solution Luminata Forte Western HRP substrate (Merck Millipore) for 5 minutes covering the membrane containing container to protect it

from light and was developed using Hypercassette and Amersham hyperfilm (GE Healthcare).

3. Result and Discussions

With protein extracts obtained from TLQP-21 treated cells and not treated (control) cells, the following Western blot analyses were performed. The result obtained in this case showed no difference in expression of the proteins in SH-SY5Y cells. Results shown are from a single experiment and are representative of three separate experiments.

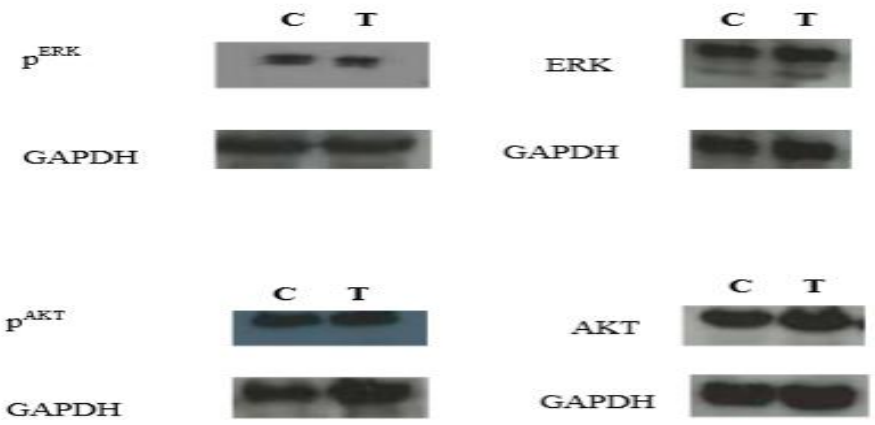

Figure 3: Representative Western blot showing that treatment of TLQP-21 did not alter in the expression of pERK, ERK, pAKT (Ser473) and AKT.
(C- Not treated with peptide, Control; T- Treated with TLQP-21. GAPDH was checked as load control).

The present study aimed to perceivethe effect of VGF derived bioactive peptide TLQP-21 on ERK/AKT signalling in SH-SY5Y cells. For this purpose, in the past, phosphoproteomic and total proteomic studies of SH-SY5Y cells treated with human TLPQ-21were performed, suggesting that the peptide brought changes in signal transduction pathway (Akhter & Requena, 2018a; Akhter, 2015). Since the characterization of signallingpathwaysis a difficult task (Laugesenetet al., 2004; Vyetrogon et al., 2007), here immunochemical validation and detection methods were used.

To our knowledge, no cell signalling studies have yet focused on human cell line treated with human TLQP-21. Of note, in TLQP-21 treated rat pituitary tumor cell lines (GH3), no difference was found in pERK and pAMPK though there was difference in expression of pAKT and p-p38 (Passeri et al., 2013). In another study, TLQP-21 was found to significantly activate ERK ½ in rat cerebellar granule cells (CGCs). Akt phosphorylation was found to be increased after 15 minute of treatment with TLQP-21, while phosphorylation of Akt increased further after that time using insulin-like growth factor-1 (IGF-1). After 24 and 48 h of incubation, IGF-1 treatment increased Akt phosphorylation while TLQP-21 did not modify the amount of phosphorylated Akt, although total Akt and α-tubulin were found to be increased in both cases in rat CGCs (Severini et al., 2008).

In another study of TLQP-21 induced signal transduction pathway in mice 3T3-L1 adipocytes, TLQP-21 did not bring any change in expression of Akt (Ser473), PKC (protein kinase C; pan Ser660), p38 (Thr180/Tyr182)and JNK (c-Jun N-terminal kinase; Thr183/Tyr185, PKA and HSL, whereasTLQP-21increased phosphorylation of AMPK and ERK (Possenti et al., 2012). All these previous study suggest that the signalling cascade induced by the peptide TLQP-21 is still ambiguous. More studies are needed to clarify this issue.

4. Conclusion

In SH-SY5Y cells, TLQP-21 did not bring any alternation in the expression of ERK and AKT proteins. To the best of our knowledge, this made the first move regarding the study to see the effect of the peptide TLQP-21 in human cell line whether it modulate ERK and AKT. The significance of the study is that it provides a starting point for further investigation into cell signalling by TLQP-21. Deeper proteomic analysis involving DIGE, cICAT, and iTRAQ, followed by 2D Gel- or LC-MALDI TOF/TOF will help us for better understanding in this regard.

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Contribution of the authors

Conceptualization: Shamim Akhter, Jesus R. Requena; Data curation: Shamim Akhter, Jesus R. Requena; Formal analysis: Shamim Akhter; Funding acquisition: Jesus R. Requena. Investigation: Shamim Akhter; Methodology: Shamim Akhter; Project administration: Shamim Akhter, Jesus R. Requena; Supervision: Jesus R. Requena; Validation: Shamim Akhter, Jesus R. Requena; Writing – original draft: Shamim Akhter; Writing – review & editing: Shamim Akhter, Jesus R. Requena.

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