HSPA8, not C3AR1 trapped from SH-SY5Y cells: demonstrated by TLQP-21 avidin agarose affinity chromatography

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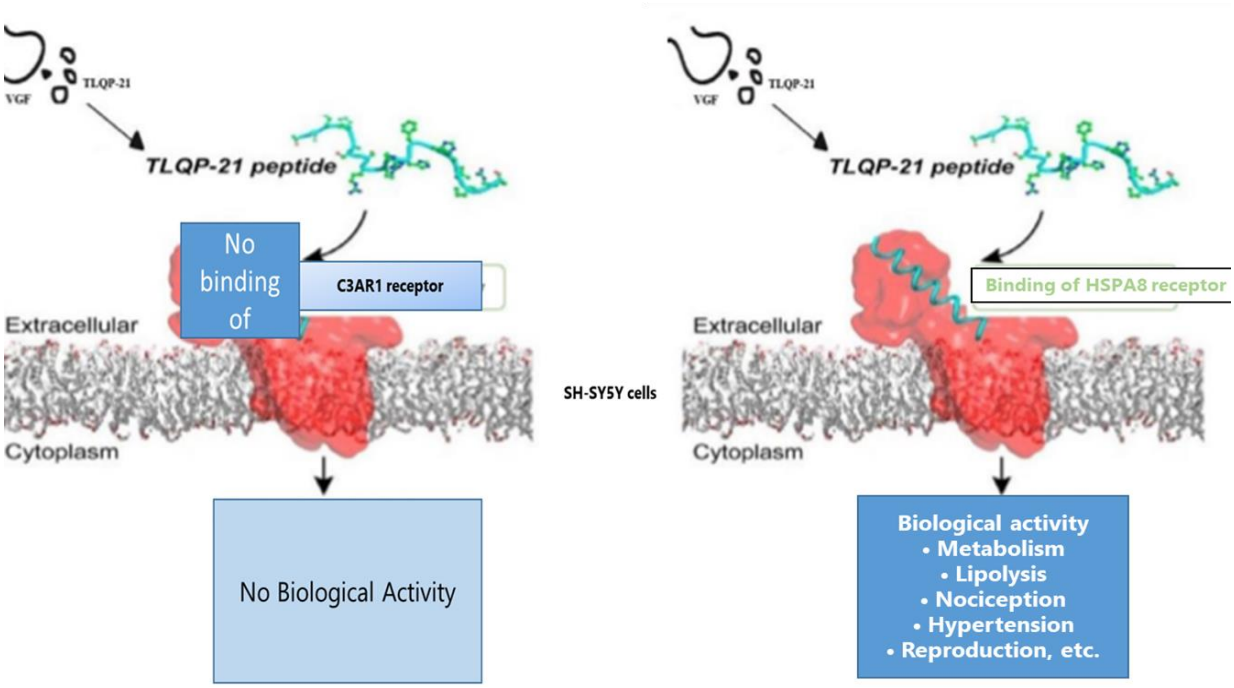
Abstract

To 'fish out' HSPA8 as a receptor of human TLQP-21 in human neuroblastoma SH-SY5Y cell; cross-linking, affinity chromatography and mass spectrometry-based protein identification techniques were used (by our group). Before that, in rodent cells, C3AR1 was found as a receptor of rodent TLQP-21 in CHO-K1 cells (by another group). So, it was of very much interest to find out whether C3AR1 can be 'fished out' as a receptor of human TLQP-21 in a human SH-SY5Y cell line or not. Here PVDF membrane (which was used for immunohistochemical validation of HSPA8 in SH-SY5Y cell line) was stripped, followed by immunohistochemical validation as before under the same condition, but C3AR1 was not found to bind with human TLQP-21. The findings concluded here that in SH-SY5Y cells, HSPA8 but not C3AR1 was documented as a receptor of human TLQP-21 using avidin agarose affinity chromatography. The significance of the study is that it provides a starting point to signpost: human TLQP-21 exerts its biological activity via HSPA8 (not C3AR1) in human SH-SY5Y cells. The further readout of TLQP-21-HSPA8 signaling can be exploited to explore new horizon in diagnosis and therapies for VGF related human diseases, especially in which TLQP-21 has been shown to affect and related with thereof.

Keywords: HSPA8, C3AR1, TLQP-21, Avidin agarose affinity chromatography, SH-SY5Y cells.



Diagrammatic representation of the summary: Binding of TLQP-21 to its receptor HSPA8 and the biological effects thereby in human SHSY-5Y cell line (right); on the other hand, in the same cell line C3AR1 was not found to bind with ligand TLQP-21, hence no biological effects thereof (Modified from Cero et al., 2014) [20].

Introduction

For the last 20 years, if not more, the biological roles (bioactivity) of TLQP-21 have been recognized and characterized in aspects of (adipo)lipolysis [1], metabolism [1-4], diabetes [5], pain modulation [6-8], hypertension/arterial pressure regulation [9], gastric motility/contractility modulation [4, 10], regulation of gastric acid secretion [11-13] , reproduction [14-15], stress [16-17], neuroprotective agent [18], anorexia [2, 3].

But till now, not enough has been elucidated about its molecular mechanism in physiological system. Being one of the bioactive peptides of VGF, the TLQP-21 was found to bind to adipocyte membranes, expressing stimulatory prolypolytic effect [1] and unique binding sites of TLQP-21 were revealed on living Chinese hamster ovary (CHO) cell lines with the aid of atomic force microscopy (AFM) [19] but there was little information about the mechanisms of action of the peptide as well as about putative receptor that mediate the TLQP-21 effects, as mentioned earlier. Complement component-3a receptor 1 (C3AR1) [20-21], and globular head of the complement component C1q receptor (gC1qR) [7] was identified as receptors of TLQP-21 in murine cell line.

The heat shock cognate 71 kDa protein A8 (HSPA8) was identified as a receptor of human TLQP-21 in SH-SY5Y cell line. Not only the cross linking and the Fluorescence-activated cell sorting (FACS) studies showed that TLQP-21 can bind to membrane associated HSPA8 in live SH-SY5Y cells but also the molecular modeling *in silico* studies determined that TLQP-21 can be fit (docked) into the peptide binding pocket of HSPA8 [22-23].

Before these findings, in 2013, and later on, in 2014, C3AR1 was explained as a receptor for TLQP-21 in rodent cell line and many of the effects of TLQP-21 by C3AR1 activation in rodent cell line but it was translatable in human cell line [20-21].

So, it was of interest here to have a study to observe whether both or either of HSPA8 and C3AR1 can be 'fished out' as receptor of human TLQP-21 in SH-SY5Y cell line.

Materials and Methods

SH-SY5Y cell culture

SH-SY5Y cells (European Collection of Cell Cultures, ECACC; catalog number-94030304), a subline from SK-N-SH cell line was established in 1970 from a metastatic bone tumor of a four year-old female with neuroblastoma SH-SY5Y which was cloned three times as of SK-N-SH → SH-SY → SH-SY5 → SH-SY5Y. [24-25].

Avidin agarose affinity chromatography

As per modified standard protocol [21-23, 26], the Pierce Monomeric Avidin Kit (Thermo) was used to conduct Avidin agarose affinity chromatography. At room temperature, to attach biotin -TLQP-21 to the column, 2 ml of solution (0.1 mM). Thus the column was ready to add SH-SY5Y cell homogenate.

SH-SY5Y cell homogenates were applied to the column already supposed to be attached with the biotin-TLQP-21. Fractions of 500 µl were collected and stored on ice until further use for analysis by SYPRO® Ruby Protein Gel Stain (Lonza), and Western blot, performed in accordance with the standard protocol [21-23, 26]. Cell lysate solution mixed to a column without biotin-TLQP-21 attached, maintaining the aforementioned environmental standards, was used here as control.

Box 1: A short description of Avidin Affinity Chromatography

> Typical biotinylated proteins were attached to avidin-coupled gel. The columns were characterized by improved stability properties exhibiting remarkably low or no leakage. In this context, biotin of high specificity and moderate affinity was bound to the immobilized monomeric avidin protein. In more details: avidin with strong binding features was immobilized in monomeric avidin immobilization. These high affinity biotin-binding binding sites were first blocked with biotin-containing buffer (biotin blocking and elution buffer). Biotin molecules were eluted from monomers with the glycine solution (regeneration buffer, pH 2.8) revealing the reversible binding sites. Thus, the monomeric avidin agarose resin resulted in the high-binding capability with least nonspecific binding and last of all, excellent recovery of biotinylated molecules was performed.

To regenerate column after use, it was washed two times with 4 ml of regeneration buffer to strip the column of residual bound biotin without losing the ability to bind another biotinylated sample. For storage, column was washed with 5 ml of PBS containing the preservative 0.01% sodium azide. For successful maintenance and preservation, the column was topped with a supplied white tip and PBS with additional preservatives were added onto the top disc before removing the top cap. Finally, it was stored at 4°C for use next time.

Protein identification

Following Pierce monomeric avidin agarose affinity chromatography, SDS-PAGE gel run and SYPRO® Ruby Protein Gel Staining, the protein bands found in the treated sample (not found in the control sample) was identified according to the method of Shevchenko et al., (2006) [27]. As per standard protocol for Western blot analysis, samples obtained from monomeric avidin experiment were subjected to Western blot analysis.

Additionally, the same PVDF membrane was stripped by incubation in Restore Plus Western Blot Stripping Buffer (Thermo Scientific) as per instruction of the supplier and finally was probed with Anti-C3AR1 antibody (Sigma-Aldrich) at a 1:200 dilution, followed by incubation with anti-mouse secondary antibody (GE Healthcare, UK) at a 1:5000 dilution. But it could not recognize any protein in the membrane, concluding that it is not C3AR1 protein.

Results and Discussion

Validation of HSPA8

Following affinity chromatography, SDS-PAGE of eluted fractions and SYPRO® Ruby protein staining of the band (Fig. 1) and final analysis by Western blot against anti-HSPA8 antibody (Abcam) (Fig. 2) identified heat shock cognate 71 kDa protein (HSPA8) [22, 23]. Literature review revealed the known presence of HSPA8 in the cell membrane [28-32], which further strengthened the HSPA8 as candidate to be a receptor of VGF-derived peptide TLQP-21.

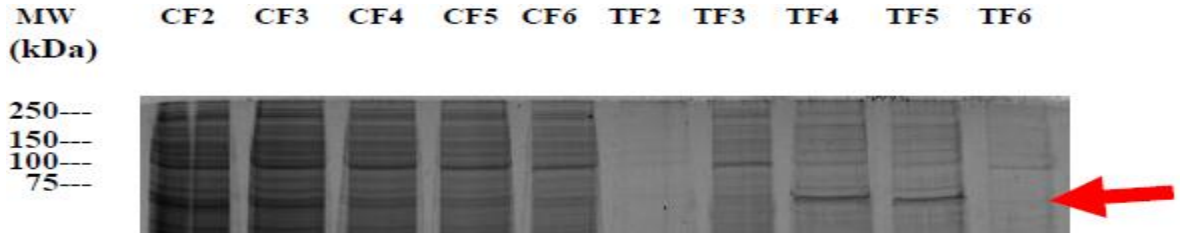


Figure 1: Biotin-TLQP-21 entrapped with a ≤ 75 kDa protein in SH-SY5Y cell homogenate. A protein band of ≤ 75 kDa was evident in fractions from the TLQP-21 column, TF 4-5 but not in the analogous elution fractions without TLQP-21 attached (control fraction, CF4-5) [22, 23].

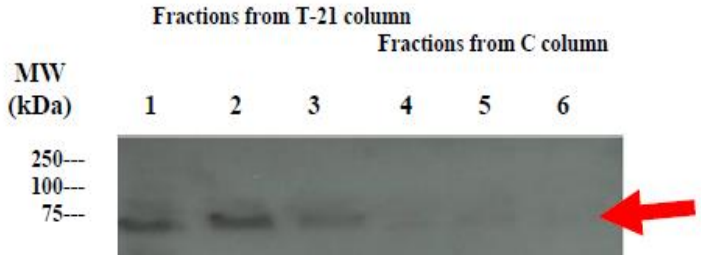


Figure 2: Immunochemical validation of HSA8 as a TLQP-21 binding protein. **T**he fractions eluted from the column filled with biotin-**T**LQP-21 ("TF", T-21 column) after Western blot analysis validated the HSPA8 at ~71 kDa, but not in the corresponding fractions eluted from a control column not loaded with biotin-TLQP-21(C column) [22, 23].

C3AR1 was not trapped by TLQP-21 affinity chromatography: Immunochemical invalidation of C3AR1: In 2013, Hannedouche et al., [21] confirmed and later in 2014, Cero et al., [20] confirmed C3AR1 as a putative receptor of the murine TLQP-21 peptide. Hence, the same PVDF membrane used for probing with Anti-HSPA8 antibody, was stripped and probed with anti-C3AR1 antibody but it was nullified here that it was not the C3AR1 protein (Fig. 3).

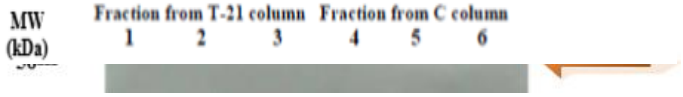


Figure 3: C3AR1, a ~53 kDa protein, does not bind biotin-TLQP-21 following Monomeric Avidin-agarose based affinity chromatography. After the immunochemical validation of HSPA8 (Fig. 2), the PVDF membrane was taken apart, stripped and incubated with C3AR1 Ab but was not detected, proving the absence of C3AR1 in the eluate.

Though the official name suggests that C3AR1 binds with C3a only, this G-protein actually binds both to C3a and C4a (complement component - 3a and 4a, respectively) [33]. Its function was thought to be involved in innate immune system roles, such as - the complement system but after the findings of its role as a receptor of rodent TLQP-21, a new dimension of study was opened. So its roles are not limited only to complement system, its roles also found in neurogenesis [33], cancer [34] and hormone release from pituitary gland [35]. Moreover, C3AR1 knockout mice were found to be resistant to diet-induced obesity and high fat diet induced insulin suggesting its role in metabolism [36].

To validate C3AR1 as a receptor of TLQP-21 in rodents' cell line, siRNA screening was performed. Earlier confirmation through transcriptomic analysis of 21 genes, 63 siRNAs were tested against them. Among them, only siRNA against C3AR1 could reduce the TLQP-21 mediated response. Hence, by virtue of defined receptor antagonist and siRNA techniques, it could be concluded that C3AR1 is a potential receptor for TLQP-21 in rodents [21].

But this finding could not translate with the human receptor. To show the TLQP-21 signaling in HEK293 cells expressing the human C3AR1 was failed but was successful in case of hamster and rat C3AR1. Since human and rodent TLQP-21 version is significantly different, the results were different, as well [21] (For the difference between human and rodent version of the peptide, see underneath). Thus several findings till now suggest that there might be another receptor for TLQP-21 in humans other than C3AR1.

As hypothesized, human versions of the peptide differ by 4 amino acids when compared to rodents (as pointed out by Bartolomucci et al., 2011 [17, 37-38] but apparently human version of TLQP-21: TLQPP**SAL**RRRH**Y**HHALPP**S**R shows extensive homology but also substantial differences with mouse TLQP-21: TLQPP**ASS**RRRH**F**HHALPP**A**R by 5 amino acids), resulting in the lower potency of the human TLQP-21 towards both of the human and rodent C3AR1 receptor [20].

Concluding remarks and future prospective

Until now, HSPA8 is the first putative receptor in humans for TLQP-21. This new discovery opens the door to new diagnostic and therapeutic approach for a broad spectrum of human diseases associated to VGF, more specifically, in cases where TLQP-21 has demonstrated effects. It goes without saying that further insight is essential into the HSPA8-TLQP-21 interaction focusing on the signaling pathways downstream.

Conflict of interest: There is no conflict of interest anywhere.

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Author Contributions

Conceptualization: MSA, JRR. Data curation: MSA, JRR. Formal analysis: MSA. Investigation: MSA. Methodology: MSA. Supervision: JRR. Validation: MSA, JRR. Writing – original draft: MSA. Writing – review & editing: MSA, JRR.

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