Research Article
Pamukkale Medical Journal
doi:https://dx.doi.org/10.31362/patd.1338766

HT22 cell differentiation reduces insulin receptor levels

HT22 hücre farklılaşması insülin reseptör seviyelerini azaltır

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Posted date:11.08.2023 Acceptance date:27.11.2023

Abstract

Purpose: The brain is an insulin-sensitive organ and has widespread insulin receptor (IR) expression. IR signaling in the brain is essential for neuronal development, feeding behavior, body weight, and cognitive processes such as attention, learning, and memory. HT22 cells, which are derived from parent HT4 cells that are immortalized from primary mouse hippocampal neuronal cells are used in research related to insulin signaling. However, the role of these cells in insulin signaling is not known. In this study, we aimed to examine IR levels in cells differentiated using neurobasal medium.

Material and methods: For the study, briefly, the cells were seeded in 6-well plates at 2x105 cells/well for 24 h. After the cells reached 80% confluence, the normal growth medium was replaced with a differentiation medium and the cells were incubated for 72 hours at 37^0C in 5% CO_2. Western blot procedure was used to determine the expression of the IR.

Result: Our results show that differentiation of HT22 cells stimulates neurite outgrowth. Furthermore, IR protein levels were significantly downregulated in differentiated HT22 cells.

Conclusion: This finding may require careful consideration of the use of neurobasal medium in conditions where IR signaling is important.

Keywords: HT22 cell, differentiation, insulin receptor.

Tunc Ata M, Altintas F. HT22 cell differentiation reduces insulin receptor levels. Pam Med J 2024;17:79-85.

Öz

Amaç: Beyin, yaygın insülin reseptörü (IR) ekspresyonu olan insüline duyarlı bir organ olarak kabul edilmektedir. Beyindeki IR sinyali; nöronal gelişim, beslenme davranışı, vücut ağırlığı, dikkat, öğrenme ve hafıza gibi bilişsel süreçler için gereklidir. Primer fare hipokampal nöronal hücrelerinden ölümsüzleştirilen ana HT4 hücrelerinden türetilen HT22 hücreleri, insülin sinyali ile ilgili araştırmalarda kullanılmaktadır. Bununla birlikte, bu hücrelerin insülin sinyallemesindeki rolü bilinmemektedir. Bu çalışmada, nörobazal besiyeri kullanılarak farklılaştırılmış hücrelerde IR düzeylerini incelemeyi amaçladık.

Gereç ve yöntem: Çalışma için öncelikle, hücreler 24 saat boyunca 2x105 hücre/kuyu olacak şekilde 6 oyuklu plakalara ekildi. Hücreler %80 konfluansa ulaştıktan sonra normal büyüme ortamı farklılaşma ortamı ile değiştirildi ve hücreler 72 saat 37^0C'de %5 CO_2'de inkübe edildi. İnsülin reseptörünün (IR) ekspresyonunu belirlemek için western blot prosedürü kullanıldı.

Bulgular: Sonuçlarımız, HT22 hücrelerinin farklılaşmasının nörit büyümesini desteklediğini göstermektedir. Ayrıca, IR protein seviyeleri farklılaşmış HT22 hücrelerinde önemli ölçüde azalmıştır.

Sonuç: Bu bulgu, IR sinyalinin önemli olduğu durumlarda nörobazal ortamın kullanımının dikkatli bir şekilde değerlendirilmesini gerektirebilir.

Anahtar kelimeler: HT22 hücre, farklılaşma, insülin reseptörü.

Tunç Ata M, Altıntaş F. HT22 hücre farklılaşması insülin reseptör seviyelerini azaltır. Pam Tıp Derg 2024;17:79-85.

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Introduction

Insulin is a significant growth factor that binds to the insulin receptor (IR) in the brain and then activates intracellular signaling pathways. The IR density is the highest in the olfactory bulb, hypothalamus, hippocampus, cerebral cortex, striatum, and cerebellum in the brain [1, 2] The widespread distribution of IR suggests that insulin signaling has important and diverse roles in the brain. Neuronal insulin signaling is known to regulate synaptic plasticity [3]. Evidence that insulin signaling is impaired in dementia indicates that this signaling is particularly important in maintaining or reversing symptoms in neurodegenerative diseases [4]. In addition, brain insulin resistance is the inability of brain cells to respond to insulin [5]. The reason for this could be due to the downregulation of IR, an inability of IR to bind insulin, or faulty activation of the insulin signaling cascade. This dysfunction may occur as the impairment of neuroplasticity, receptor regulation in neurons, or the impairment of processes more directly implicated in insulin metabolism, such as neuronal glucose uptake in neurons expressing GLUT4, or inflammatory responses to insulin [6]. In that respect, knowing the exact mechanism of brain insulin signaling and, its receptor is clinically important. Neuronal cell models are frequently used in studies on insulin signaling and IR.

HT22 cells are known as the mouse hippocampal neuroblastoma cell line and have similar properties to nerve cells and are used to understand neural development, synaptic functions, and neurodegeneration. In recent years, a medium called neurobasal medium has become a tool for HT22 cells to differentiate and acquire neural phenotypes. A study on the differentiation of HT22 cells showed that mature hippocampal neurons express very low cholinergic markers and glutamate receptors [7, 8]. However, the differentiation of HT22 cells caused increased levels of N-methyl-D-aspartate receptor (NMDAR) mRNA, making them more susceptible to glutamate-induced excitotoxicity [8]. In addition, another study showed that the differentiation of HT22 cells enhanced their functional serotonergic properties [9].

In line with these effects revealed by the differentiation of neuronal cells, we aimed to investigate the effect of HT22 cell differentiation with the neurobasal medium on IR expression level. Examining IR levels on differentiated HT22 cells may contribute to the understanding of insulin signaling and interactions in nerve cells.

Material and method

Antibodies and reagents

Neurobasal Medium (21103049), Dulbecco's modified Eagle's medium (DMEM) (2375262), and penicillin/streptomycin (2087433) were obtained from Gibco (Grand Island, NY, United States). N-2 supplement (100X) (CP18-2070) and fetal bovine serum (FBS) were purchased from Capricorn Scientific (Grand Island, NY, United States). L-glutamine (15323117) and Tripsin-EDTA (0.25%) (03-052-1B) were obtained from Biological Industries (Israel). Antibodies used in this study were as follows: IR (rabbit polyclonal, E-AB-60069, USA), β-actin (BTLab, BT-AP00213).

Cell culture

This study was carried out using HT22 mouse hippocampal cells. HT22 were cultured in DMEM supplemented with 10% FBS and antibiotics-penicillin 100 IU/mL and streptomycin 100 mg/mL, at 37^{0}C in 5% CO_2. Briefly, HT22 cells were seeded into 60 mm petri dishes, allowed to reach 80% confluence, and passaged. After obtaining a sufficient number of cells, they were then seeded in 6-well plates at a density of 2x10^5 cells/well. It was incubated at 37°C for 24 hours. HT22 differentiation was performed by previous literature [9], with a slight modification (Neurobasal medium containing 2 mmol/L-glutamine and 100xN2 supplement) for 72 h. HT22 cell proliferation, passages, and follow-up of cells were monitored with an inverted microscope. The number of undifferentiated samples used in the study was five and the number of differentiated samples was four. Differentiation medium: neurobasal medium, Pen-strep (1% v/v), L-glutamine (1% v/v), N2 supplement (1% v/v). Undifferentiation medium: DMEM, FBS (10% v/v), Pen-strep (1% v/v), L-glutamine (1% v/v)

Western blot procedure

HT22 cells were seeded at 2x10^5 cells/well in 6-well plates and incubated for 24h at 37^{0}C. Following the incubation, the cells were washed twice with phosphate-buffered saline

(PBS). The cells were scraped from the wells by using 500 µl of radioimmunoprecipitation assay buffer (RIPA) in the presence of a protein inhibitor cocktail (Santa Cruz Biotechnology, sc- 24948). Cell lysates were centrifuged for 12 min at 10000×g, 4˚C, and the supernatant was collected. Total protein extraction kit was used to determine the protein concentration. (DC protein assay-Bio-Rad). After denaturation with 2X Laemmli sample dilution buffer (1:1) for 5 minutes at 95^0C, 50 µg/µl of proteins were separated by 4-10% SDS-PAGE (Novex, Invitrogen) and transferred onto membranes (Invitrogen) using a wet transfer method (Bio-Rad Wet/Tank Blotting Systems). Membranes were blocked for 1 hour at room temperature by using 5% skim milk powder in PBS-Tween (PBST). Membranes were incubated at 4^0C overnight with the appropriate primary antibodies (1:1000) in PBST containing 5% skim milk powder. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000) (E-AB-1003) for 1 h at room temperature. After washing with PBST, SignalFire ECL Reagent (Cell Signaling) was placed on the membrane and specific binding was detected using a UVP Biospectrum chemiluminescence detection system (HR-410, USA). Specific bands were quantified using Image J studio software. Signals were normalized to the selected reference protein. Antibodies used in this study were as follows: IR (rabbit polyclonal, E-AB-60069, USA), β-actin (BTLab, BT-AP00213).

Statistical analysis

Data analysis was done with the GraphPad package program. Continuous variables were expressed as the mean±standard deviation (SD). The Shapiro-Wilk test was used to determine the suitability of the data for normal distribution. The Independent Samples T-test was used to compare the groups. In analyses, $p<0.05$ was considered statistically significant.

Results

Figure 1 shows 3-day images of HT22 cells in a normal growth medium and differentiation medium. In the differentiation medium, HT22 cells showed an increase in the number and length of cellular neurites, similar to that of mature neurons. Moreover, differentiated HT22 cells expressed significantly less IR levels than cells grown in a normal growth medium ($p=0.0028$, Figure 2).

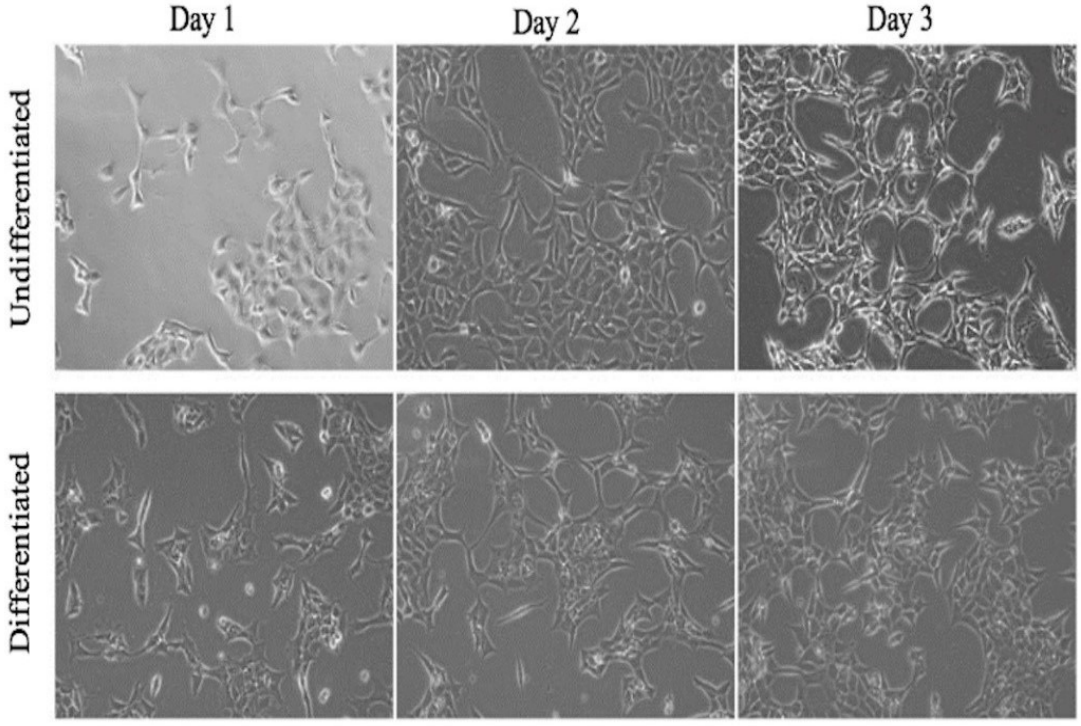

Figure 1. First three-day image of differentiated and undifferentiated mouse hippocampal HT22 cells

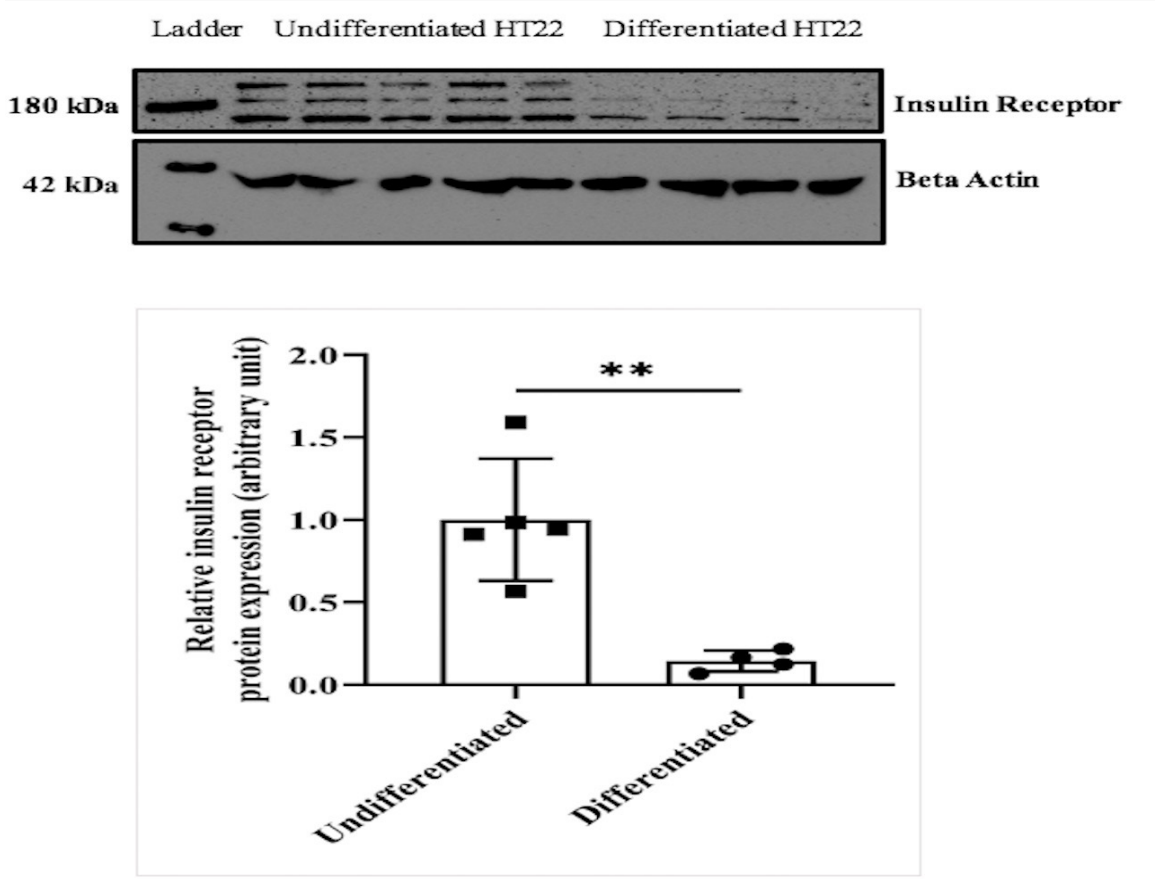

Figure 2. IR expression level in differentiated and undifferentiated HT22 cells after three days IR: insulin receptor

Discussion

In the brain, insulin facilitates differentiation, proliferation, and neuritis growth [10-12], while it also has a neuro-preservative role in preventing β-amyloid toxicity, oxidative stress, and damage caused by apoptosis. [13-16]. Conversely, these protective effects of insulin are diminished when brain insulin-receptor signaling and level are impaired [17], resulting in brain insulin resistance associated with cognitive dysfunction [18, 19].

When the literature is examined, numerous studies using different cell models and applications show that insulin and its receptors in the brain play key roles in biological functions [20-22]. One of the cell models used in these studies, HT22, is an immortalized mouse hippocampal cell line. It is an important research topic that the results of this cell line may change with their differentiation. In line with this information, we aimed to examine the IR level in differentiated HT22 cells using the neurobasal medium, which is a differentiation medium. Our results show that the differentiation of HT22 cells supports neurite growth and reduces IR levels. As far as we know, this is the first study to examine the level of IR in HT22 cells differentiated by the neurobasal medium.

Differentiation of neuronal cells in vivo has been reported to stimulate neurite outgrowth and promote up-regulation of NMDAR and choline acetyltransferase, thus displaying phenotypic features similar to those of mature hippocampal neurons [9]. Neural differentiation is achieved by complex cellular processes, including morphological changes. Maekawa et al. [23] in their study aiming to examine the pathogenesis of retinal ganglion cell diseases, observed enhanced neurite outgrowth after differentiation of mouse embryonic stem cells in neurobasal-A medium supplemented with B27 for 14 days. Yang et al. [24] aimed to

investigate the potential neurotrophic effects of a neurobasal medium containing glucagon like peptide-1 (GLP-1), retinoic acid, and B27 on neuronal differentiation. In this study, it was shown that retinoic acid and GLP-1 can trigger the morphological differentiation of SH-SY5Y cells. Consistent with the above results, we obtained an enhanced neurite morphology in HT22 cells differentiated using the neurobasal medium. N-2 supplement is provided as a 100X concentrate and is intended for use with neurobasal medium supplemented with growth factors such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). The differentiation of HT22 cells may be due to the growth factors present in the N-2 supplement added into the neurobasal medium. This result showed that our differentiation procedure was effectively accomplished.

Numerous studies examining the positive effect of insulin on neuronal cells emphasize the critical function of insulin stimulation and IR. When these studies are examined; Bassit et al. [25] showed that administration of insulin (10 nM, 24 hours) with human adipose tissue-derived stem cell exosomes after H_2O_2 treatment in undifferentiated HT22 cells increased proliferation in HT22 cells via protein kinase C delta (PKCδII). They also showed that it significantly enhances the relationship between metastatic-related pulmonary adenocarcinoma transcript 1 (MALAT1) and serine and arginine rich splicing factor 2 (SRSF2), which significantly supports cell vitality and proliferation of insulin in these cells. In another study, the positive effects of insulin (10 nM) on apoptosis induced by H_2O_2 (200 μM) in undifferentiated HT22 cells were investigated. In this study, it was shown that insulin administration inhibits apoptosis and upregulates pAKT, pGSK3β, and β-catenin protein expression levels. However, it was stated that these changes were abolished when IR in HT22 cells were silenced [26]. Another study examined the efficacy of the GLP-1 receptor agonist exendin-4 in activating insulin signaling and reducing tau phosphorylation in undifferentiated HT22 neuronal cells. In this study, insulin (100 nM) has been shown to play a fundamental role in exendin-4 eliciting these effects [20].

On the other hand, there are studies in the literature showing that real responses of the cell can be obtained by differentiation of these cells [27, 28]. Studies of differentiated neuronal cells have shown that these cells represent a better hippocampal neuron model than undifferentiated cells. For example, Lim et al. [9] showed that differentiation of HT22 cells (Neurobasal medium containing 2 mmol/ L-glutamine and. 5x N-2 supplement, 3 days), promotes neurite outgrowth and up-regulation of the NMDAR and choline acetyltransferase, as in primary cultured hippocampal neurons. In this study, proteins required for serotonergic neurotransmission were also upregulated in differentiated HT22 cells. With these findings, the authors showed that the differentiation of HT22 cells enhanced their functional serotonergic properties. Zhao et al. [29] investigated the responses of differentiated and undifferentiated HT22 cells to homocysteine toxicity. In this study, both cell models were exposed to homocysteine toxicity at different concentrations, and cell death was evaluated. It has been observed that undifferentiated and differentiated cells have different expression levels of the NMDA glutamate receptor. Our data showed a decrease in IR levels in differentiated HT22 cells.

In conclusion, although the data we obtained from this study indicate that the differentiation of HT22 cells negatively affects the level of the IR these cells will not be a suitable in vitro model for assessing the signaling mechanism of insulin. The lack of B27 supplementation in the neurobasal medium used in our study may be a limitation of this study.

This study has limitation. The results of the study are based on western blot results. These results need to be supported by other experiments (rPCR analysis).

Conflict of interest: The authors declare that they have no conflict of interest.

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Ethics committee approval: The study is a cell culture study that does not require ethics committee approval.

Author contributions

Conceptualization, M.T.A. and F.A. Data curation M.T.A and F.A. Formal analysis F.A. Investigation M.T.A. and F.A. Methodology M.T.A. Supervision M.T.A. Validation, F.A. Visualization, F.A. Writing – original draft, M.T.A. Reviewing, M.T.A. and F.A. All authors have read and agree to the published version of the manuscript.