

Effects of Hypothermic Temperatures on Tau Hyperphosphorylation as a Model of Alzheimer's Disease in SH-SY5Y Cells

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Abstract

Alzheimer's disease, among other neurodegenerative diseases, is associated with tau hyperphosphorylation. This study evaluated SH-SY5Y cells grown in hypothermic temperatures (30 °C) and compared them against cells in normothermic conditions (37 °C) to assess tau hyperphosphorylation at varying temperatures in a time-course analysis. Enzyme-linked immunosorbent assay (ELISA) was used to measure the expression of the pT181 isoform, a biomarker of tau hyperphosphorylation. This study found that hypothermic incubation does increase tau hyperphosphorylation best at 18 hours of incubation. These results suggest that hypothermic conditions can serve as an in-vitro model for Alzheimer's research.

Introduction

Tau proteins are abundant in neurons of the central nervous system. These proteins are found in the cytoskeleton and help maintain cell structure by regulating the stabilization of microtubules, but have also been linked to progressive neurodegeneration under pathological conditions (Kong, Y., et al., 2016). Diseases associated with this degeneration of tau, known as tauopathies, include Huntington's, Parkinson's, and Alzheimer's disease (AD) (Cheon, M., Chang, I., & Hall, C., 2012). Under normal circumstances, tau is a soluble protein that is released through the cerebrospinal fluid (CSF) during sleep. However, in certain pathological conditions, some tau proteins may become hyperphosphorylated (Mukda, S., Panmanee, J., Boontem, P., & Govitrapong, P., 2016). When hyperphosphorylated, excess phosphate groups attach themselves to tau proteins and become attractive to other phosphorylated tau molecules, forming abnormally paired helical filaments (PHFs). As the formation of PHFs continues, large aggregates known as neurofibrillary tangles (NFT) are created (Mpousis, S., Thysiadis, S., Avramidis, N., Katsamakas, S., Efthimiopoulos, S., & Sarli, V., 2015). Insoluble and unable to be released through the CSF, NFTs remain in the brain, cutting off inter-neuronal communication by impairing the tau protein's ability to stabilize microtubules, resulting in cell death and associated neurodegeneration, ultimately leading to death (Chami, B., Steel, A. J., De La Monte, S. M., & Sutherland, G. T., 2016). Such tangles begin in the hippocampus and spread throughout the brain as the diseases progress (Simic, G., et al., 2016). NFT associated conditions are growing more common throughout the world. In the United States in the early 2000s, 4.5 million people had AD, the most common tauopathy. By 2010, there were 5.3 million cases in the United States alone, with a total worldwide count of 35 million cases (Rosales-Corral, S. et al., 2012). By 2050, it is estimated that roughly 106.8 million people will be subject to AD worldwide (Medhi, B., & Chakrabarty, M., 2013). With such a drastic predicted increase in the near future, AD has become a research priority in the neuroscience field.

Symptoms of AD include cognitive, behavioral, psychological, and physical degeneration, manifested through neurodeficiencies such as memory decline, difficulty with personal care, personality changes, and hallucinations (Mayo Clinic, 2017). Despite the roughly \$214 billion annually dedicated to AD research in the United States alone, progress in finding viable treatment options has been slow and expensive (Whitwell, J. L. et al., 2015). Although research has not resulted in a cure, it has revealed numerous probable causes of AD.

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In addition to the aforementioned problems of the tau protein, aggregation of the amyloid beta ($A\beta$) protein and impaired glucose metabolism have also been theorized to cause neurodegeneration (Hebda, M., et al., 2016). In the $A\beta$ hypothesis, the soluble amyloid precursor protein is spliced to create $A\beta$. This resulting protein binds to other $A\beta$ molecules, forming insoluble aggregates which, like tau NFTs, can no longer be removed through the CSF during sleep and therefore cause neuronal death (Hane, F. T., Lee, B. Y., Patoyan, A., & Leonenko, Z., 2013). In regards to the glucose metabolism hypothesis, decreased glucose metabolism rates cause increased tau hyperphosphorylation, citing aging is a main factor, as nearly 50% of people over the age of 85 have some stage of AD (Sung, H. Y., Choi, B., Jeong, J., Kong, K. A., Hwang, HJ., & Ahn, J., 2015).

There are some limited treatments based on these hypotheses which have focused on the reduction of enzymes which lead to retarded metabolic rate and have proven useful thus far (Medhi, B., & Chakrabarty, M., 2013). Studies have found that decreased glucose metabolism rates cause increased tau hyperphosphorylation. More recent data have suggested that increases in hyperphosphorylation are caused by kinase inhibitors, which lead to decreased glucose metabolism and eventually cause hypothermia (Bretteville, B. et al., 2012). Under hypothermic conditions, nerve cells produce higher amounts of tau, which become hyperphosphorylated at an increased rate. Hypothermia can be caused by incubation at low, hypothermic temperatures of 30°C rather than the 37°C which is the typical growth temperature. These hypothermic cells have AD-like conditions and can therefore serve as test subjects for AD reduction experiments both in-vivo and in-vitro. Studies in recent years have shown that hypothermic conditions can cause limited tau hyperphosphorylation at several different epitopes, or antibody destinations, of the mouse brain (Bretteville, B. et al., 2012). However, not all epitopes, including the pT181 epitope, have been tested, qualifying additional research.

Rzechorzek et al. (2015) showed that hypothermic conditions proved to be neuroprotective, meaning there was no evidence of hyperphosphorylation at any tested epitope. This contradiction revealed an existing need for further research on the subject of cellular insults resulting in tau hyperphosphorylation. To research these possibilities, an ideal system was necessary. Tau proteins are abundant throughout the body, but their presence is confined to nerve cells. Since most nerve cells do not divide, they are difficult to study and monitor over generations. However, the human neuroblastoma cell line SH-SY5Y, which has undergone three generations of mutations and cloning, does multiply and divide every 48 hours, making it a prime test subject. It is also highly sensitive to oxidative stress, exemplifying the whole of the human brain (Olivieri et al., 2000). Using small cultures of such cells, an in-vitro experiment was performed using a manipulated tau-producing system to measure the hyperphosphorylation of tau. This study was undertaken to investigate if hypothermic conditions will result an in-vitro model of the tau hyperphosphorylation seen in Alzheimer's disease when measured at the pT181 epitope.

Hypothesis

The research question to be addressed:

Will hypothermic incubation at 30°C cause AD-like tau hyperphosphorylation?

Null Hypothesis:

Incubation at 30 °C will have no change on tau hyperphosphorylation

Alternative hypothesis:

Incubation at 30 °C will cause a change in hyperphosphorylation of tau

Dependent Variable:

Expression of the pT181 isoform, a direct product of tau hyperphosphorylation

Independent Variables:

Temperature cells were exposed to that mimic hypothermic conditions
Amount of time cells were exposed to low temperatures

Statistical Analysis:

T-Test comparing reliability of difference between hypothermic and normothermic cells.

Sample Size:

Samples were run in triplicate. There were nine total in normothermic conditions, three at each time point. There were n=18 at hypothermic conditions without melatonin, n=6 at each time interval, Samples further split at analyze n=3 with previous incubation and n=3 without at each time interval

Materials

- Nature Made brand Melatonin herbal supplement
- SHSY5Y cell line human-1 vial (ATCC)
- 48 well culture plates (6)
- 0.1 M Phosphate-Buffered Saline
- 0.5 M Trypsin
- 1:1 Eagle's Minimum Essential Medium and F12 Medium (ATCC)
- 5% CO₂ humidified incubator at 30 °C
- 5% CO₂ humidified incubator at 37 °C
- 3 Human Tau [pT181] phosphoELISA™ ELISA Kits (ThermoFisher Scientific)
- P-20 micropipette and tips
- P-200 micropipette and tips
- P-1000 micropipette and tips
- Invitrogen Cell Extraction Buffer (ThermoFisher Scientific)
- 0.1 M PMSF for buffer
- Protease Inhibitor Cocktail for buffer
- Micromax RF Thermo IEC Centrifuge
- Laboratory Refrigerator (4 °C)
- Laboratory Freezer (-80 °C)
- Mortar and pestle
- 0.1 M DMSO
- 24 well plates (6)
- Ice bucket
- MicroMax Vortex

Methods

Culturing of SH-SY5Y Cells

The SH-SY5Y cells were cultured using the following conditions and methods in a CSUCI laboratory located in Aliso Hall.

- Medium: 1:1 Eagle's Minimum Essential Medium and F12 Medium
- Temperature: 37°C
- Atmosphere: 5% CO₂

Cells were thawed through gentle agitation in a 37°C water bath. Cells grew in 2 mL media as a mixture of attached and floating cells with the majority as floating. Cell density was read after 3 days of growth under above conditions and shown to be 7.4×10^5 cells per mL of media. Subculturing was performed directly into 96 well plates where experimentation occurred. Only one passage was completed.

Addition of Media and Cells to Culture Plates

Six culture trays were used to organize combinations, three labeled experimental and three labeled control. One of each type was used for each time interval of 12, 18, and 24 hours. 1 mL of media was added to the rows of each tray (specified below) and was immediately followed by the addition of 10 μ L of SH-SY5Y cells to the same wells. Trays were then incubated for their various time allotments. Experimental trays were incubated at 30°C while Controls were incubated at 37°C.

- a. Row A-E, Columns 1-6 of 12, 18, and 24 hours experimental trays.

- b. Row A-B, Columns 1-3 of 12, 18, and 24 hours control trays

Cell Extraction and Freezing

At the end of each time point the following protocol was performed. Cells remained in the freezer until all time interval had been completed, allowing all samples to be run in the ELISA simultaneously.

- a. Removed medium (broth) with floating cells and transferred to pre-labeled centrifuge tube
- b. Harvested adherent cells by washing wells with 5 μ L Trypsin then 2x 50 μ L PBS and added to pre-labeled centrifuge tube
- c. Centrifuged cell extract for 15 minutes at 14,000 rpm and 4°C. Discarded supernatant and kept pellet in pre-labeled centrifuge tube
- d. Frozen at -80°C until ready for ELISA

Cell Lysation and Preparation for ELISA

Once all cells had been frozen to prevent protein change, the tau protein was extracted through the following lysing process and used in ELISA analysis.

- a. Removed all cells from -80°C incubator
- b. Thawed Lysis Buffer [Purchased from Invitrogen – CAT# FNN0011]
- c. Added 1 μ M PMSF (.3 M stock in DMSO) and 500 μ L protease inhibitor cocktail per 5 mL Lysis buffer immediately before use
- d. Added 1 mL lysis buffer to each centrifuge tube
- e. Lysed for 30 minutes on ice with buffer:
- f. Vortexed pellet for 10 seconds at 10 minute intervals throughout the 30 minutes on ice with lysis buffer
- g. Transferred lysate into centrifuge tubes and centrifuged at 13,000 rpm for 10 minutes at 4°C
- h. Kept supernatant for ELISA analysis

ELISA analysis

An enzyme-linked immunosorbent assay (ELISA) kit was used to indirectly detect the hyperphosphorylation of tau. The ELISA kit measured the expression of the pT181 isoform, which is produced at the pT181 epitope as a direct result of hyperphosphorylation. Therefore, more pT181 expression meant more hyperphosphorylation. Absorbance was read at a wavelength 450 nm in an epifluorescent plate reader.

Results

Hypothermic Incubation as an In-Vitro Model in 12 Hour Samples

The amount of hyperphosphorylation at 12, 18, and 24 hours was measured through fluorescence of an ELISA assay that recorded the expression of the pT181 isoform, a direct result of tau hyperphosphorylation. Higher concentration of the isoform would have limited the transmittance of light through the solution, which would be shown through a high level of absorbance. At 12 hours of incubation there was little difference in the averages of absorbance between hypothermic and normothermic samples, showing an insignificant change that could not serve as an in-vitro model.

Hypothermic Incubation as an In-Vitro Model in 18 Hour Samples

At 18 hours of incubation a more drastic difference between hypothermic and normothermic conditions was observed. However, quantitative epifluorescent data of normothermic conditions showed a consistently higher value than expected, suggesting it may be an outlier. When ignoring the outlier in calculations, the averages between the two conditions have a difference of 0.8 absorbance, showing that hypothermic incubation is capable of increasing the hyperphosphorylation by nearly 40%, which is significant enough to qualify for further testing.

Hypothermic Incubation as an In-Vitro Model in 24 Hour Samples

The value of hyperphosphorylation at 24 hours was maintained from the results at 18 hours in the hypothermic samples, but the difference between absorbance at each condition was decreased due to the rapid increase in normothermic wells. The hyperphosphorylation of tau in these normothermic wells increased to a level that was greater than hypothermic conditions, though only by difference of 0.2 AU.

Evidently, hypothermic incubation did increase hyperphosphorylation, but there was an unknown circumstance in the normothermic conditions, possibly a decrease of nutrients in the cell media due cell growth in the time allotment, that also caused hyperphosphorylation that could warrant further exploration.

Statistical Analysis

Significance for hypothermic incubation was measured through T-Tests, one for each time point and with or without incubation, totaling six different tests. Alpha for all tests was measured at $\alpha=0.1$ due to the number of samples. Any p-value under 0.1 was considered enough to encourage further research, and therefore significant. The p-values for 18 hours with and without hypothermic incubation were significant for this study, 0.03 and 0.07 respectively, while all others ranged from 0.24 to 0.80, showing statistical insignificance. Although there are not enough data to confirm statistical significance out of the scope of this study, the pilot study shows a possible significance in further research experiments.

Discussion

Tau protein hyperphosphorylation is a biomarker for Alzheimer's disease. The NFTs formed are neurodegenerative and believed to be toxic to the brain. Another biomarker for AD has been proven to be A β aggregation. To test the effects of melatonin on the closely related tau protein, an in-vitro model for hyperphosphorylation had to be developed. Hypothermia has been shown to affect tau kinase inhibitors both in-vivo and in-vitro by causing glucose deprivation and metabolic deregulation (Bretteville, A., et al., 2012). Hyperphosphorylation is caused by an imbalance between kinase and phosphatase activities, promoting hypothermic incubation as a plausible in-vitro cause of tau hyperphosphorylation (Rzechorzek, N., et al., 2015).

This study found that hypothermic conditions can induce tau hyperphosphorylation slightly at 12 hours and more drastically at 18 hours, by up to 40%. However, by 24 hours of incubation the levels of tau hyperphosphorylation in the cells incubated at 30°C matched the levels of cells in the 37°C incubator. This can be attributed to the rapid cell growth and the glucose deprivation caused by a media lacking nutrients necessary for normal growth. A length of 12 hours of incubation may not have been a suitable amount of time to induce the full hyperphosphorylation of tau. At the 18 hour time interval, the largest difference of hyperphosphorylation was likely due to the nutrients available in the media and the amount of time in the hypothermic conditions. Therefore, the alternative hypothesis can be accepted at 18 hours of incubation. Initial research performed by Bretteville et al. (2012) found that hypothermia results in an increase of phospho-tau epitopes in-vivo in mice and in-vitro in metabolically active mouse brain slices at a variety of epitopes, including AT270, Tau-1, Ser199, CP13, and AT8. The findings of this study supported these findings and expanded knowledge to include hyperphosphorylation at the pT181 epitope as well.

Sources of Error

Sources of error may have occurred in the lysis process. The cell density was lower than expected due to the late arrival of the cells, so each well had a small number of cells. When centrifuged, the protein pellets were not visible, but supernatant was discarded. It is possible that a substantial portion of cells were decanted with it because of the difficulties in visually confirming that the lysis process was successful.

Further Work

Further experimentation would need to be done to establish tau hyperphosphorylation with cell degeneration. The addition of Fluoro-Jade B staining will indicate cell degeneration and apoptosis due to hyperphosphorylation. This was not able to be performed due to time and monetary restraints. Additionally, this experimental study would benefit from running a larger number of samples at both 12 and 18 hours of incubation, as these were the incubation times with the most notable differences. More trials per combination would decrease standard deviation and confirm absorbance values. The methods could be repeated using higher cell density. There were several outliers in the ELISA analysis that affected statistical analysis and modified data. Low cell count may have caused variation in cells per well as well as a highly sensitive reading by ELISA, accounting for the large deviation. All this could be accounted for through repeating the experiment with a larger sample size.

An additional study could build off of these findings by testing the effects of possible treatments in-vitro. The production of the hormone melatonin, for example, which regulates the circadian systems in the body, has been shown to decrease with age, particularly in Alzheimer's patients. In-vivo studies with lab mice have suggested that melatonin protects against A β aggregation (Mukda, S., Panmanee, J., Boontem, P., & Govitrapong, P., 2016).

This discovery could be tested against hypothermia using this model to test its preventative and reversal properties in regards to the tau hypothesis.

Conclusion

Tau hyperphosphorylation can be induced most effectively after 18 hours of incubation in hypothermic conditions. This amount of hyperphosphorylation is large enough to mimic the hyperphosphorylation that contributes to Alzheimer's disease, therefore an in-vitro model for AD and many other neurological diseases can be derived from this length and temperature of incubation.

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Conflicts of Interest Statement

Manuscript title: Effects of Hypothermic Temperatures on Tau Hyperphosphorylation as a Model of Alzheimer's Disease in SH-SY5Y Cells The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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