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Analysis of the potency of various low molecular weight chemical chaperones to prevent protein aggregation

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ABSTRACT

Newly translated proteins must undergo proper folding to ensure their function. To enter a low energy state, misfolded proteins form aggregates, which are associated with many degenerative diseases, such as Huntington's disease and chronic kidney disease (CKD). Recent studies have shown the use of low molecular weight chemical chaperones to be an effective method of reducing protein aggregation in various cell types. This study demonstrates a novel non-biased assay to assess the molecular efficacy of these compounds at preventing protein misfolding and/or aggregation. This assay utilizes a thioflavin T fluorescent stain to provide a qualitative and quantitative measure of protein misfolding within cells. The functionality of this method was first assessed in renal proximal tubule epithelial cells treated with various endoplasmic reticulum (ER) stress inducers. Once established in the renal model system, we analyzed the ability of some known chemical chaperones to reduce ER stress. A total of five different compounds were selected: 4-phenylbutyrate (4-PBA), docosahexaenoic acid (DHA), tauroursodeoxycholic acid, trehalose, and glycerol. The dose-dependent effects of these compounds at reducing thapsigargin-induced ER stress was then analyzed, and used to determine their EC_{50} values. Of the chaperones, 4-PBA and DHA provided the greatest reduction of ER stress and did so at relatively low concentrations. Upon analyzing the efficiency of these compounds and their corresponding structures, it was determined that chaperones with a localized hydrophilic, polar end followed by a long hydrophobic chain, such as 4-PBA and DHA, were most effective at reducing ER stress. This study provides some insight into the use of low molecular weight chemical chaperones and may serve as the first step towards developing new chaperones of greater potency thereby providing potential treatments for diseases caused by protein aggregation.

1. Introduction

Proteins are responsible for the molecular processes within the body and are vital for its survival. The function of any protein is dependent on its structure, which is divided into four major domains: primary, secondary, tertiary, and quaternary. The primary structure is the amino acid makeup of the protein and is outlined in nucleic acids. This is what most significantly determines the ultimate structure of all proteins and therefore their function. The structure is further developed through unique and precise foldings that allow for the proper and optimal functionality of the proteins. The amino acid sequence is translated from messenger RNA in the ribosome, with the endoplasmic reticulum (ER) being the center for synthesis of secretory, transmembrane and ER luminal proteins. Following synthesis of a relatively small amino acid sequence, strong interactions within the sequence and between its surroundings cause it to quickly fold to reach a low thermodynamic state, and therefore a stable conformation [15]. In long sequences, however, different regions are not close enough to experience such strong forces, and instead fold in certain ways to reach a reduced thermodynamic state. This conformation is not usually the most stable structure, which is required for proper function. In unstable structures the sequences reside in local thermodynamic minimum, which make it difficult for the sequences to later unfold and then refold into the proper structure. Molecules known as chaperones overcome this problem by providing long amino acid sequences

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Abbreviations

LWCC	Low molecular weight chemical chaperone
ThT	Thioflavin T
ER	Endoplasmic reticulum
4-PBA	4-phenylbutyrate
DHA	Docosahexaenoic acid
TUDCA	Tauroursodeoxycholic acid
Tg	Thapsigargin
Tm	Tunicamycin
UPR	Unfolded protein response
Tr	Trehalose
Gly	Glycerol
CKD	Chronic kidney disease
DMSO	Dimethylsulfoxide
PBS	Phosphate-buffered saline

with a suitable environment and enough energy in the form of ATP to fold into the proper structure [7]. Chaperones were first observed in yeast, in which their ability to aid in protein folding and reduce protein aggregation was understood [13]. Misfolded proteins are detrimental to the cell as they may not function correctly and form aggregates causing stress within organelles [2]. When amino acid sequences are in an incorrect conformation, certain hydrophobic regions, which are usually enclosed in the interior of the protein after proper folding, are exposed to the aqueous environment. In an attempt to further reach a lower thermodynamic state, these misfolded proteins bind to other exposed hydrophobic regions on other misfolded proteins. This allows the hydrophobic regions of these sequences to reduce their interaction with the aqueous environment; however, in the process they form protein aggregates. This misfolding and subsequent aggregation frequently occurs and is natural for a cell, and the amount of aggregation is maintained to a relatively low amount by chaperones. Under certain physiological and pathological conditions however, this aggregation can become too great and can cause severe stress within certain regions of the cell, specifically the ER. This ER stress leads to the activation of the unfolded protein response (UPR) pathway through which the cells undergo apoptosis when ER stress is too high [4]. Over time, this cell death can have physiologically notable effects due to the loss of suitable function of the organ the cells make up. This process is implicated in the pathogenesis of many degenerative diseases such as Huntington's disease, inflammatory bowel disease and chronic kidney disease (CKD) [14] [11], [4]. In chronic kidney disease, treatments to limit disease progression are of great importance [10] and drugs that limit renal protein misfolding may aid in this objective.

There are three major classes of low molecular weight chemical chaperones (LWCC): carbohydrates (glycerol, sorbitol), amino acids and derivatives (glycine, proline), and methylamines (betaine, trimethylamine *N*-oxide) [6]. As their name suggests, LWCC are small compounds that aid in the proper folding and reduce aggregation of proteins [12]. The specific method through which this is done however is unclear and may be unique for every molecule, however, it is believed that they stabilize improperly folded protein and prevent non-productive interactions with other resident proteins. These molecules have proven effective at alleviating ER stress in many different *in vivo* and *in vitro* models including neural, respiratory, and renal [14] [8], [3]. Additionally, most LWCC can pass through the blood-brain barrier, which allows them to be an effective treatment for neurodegenerative disease such as Parkinson's disease and Huntington's disease, where ER stress is considered one of the mechanisms of disease pathology [6]. These compounds however need to be administered at high concentrations to allow for noticeable reduction of ER stress; therefore they may lead to non-specific effects and become toxic. Hence, it is important to understand the association between the structure of these LWCC and the effects they cause, so that a more selective and effective molecule at reducing ER stress can be developed. In this study, we analyze the effectiveness of five known LWCC at reducing ER stress in human renal proximal tubule epithelial cells. ER stress is induced by thapsigargin and is quantitatively and qualitatively measured through the use of the thioflavin T (ThT) stain. ThT is a molecule that exhibits enhanced fluorescence when bound to protein aggregates, which can be visualized under a DAPI filter (410–530 nm) [1]. Therefore this stain allows for the visualization and quantification of relative ER stress within cells. The molecules that are investigated in this study include: 4-phenylbutyrate (4-PBA), docosahexaenoic acid (DHA), tauroursodeoxycholic acid (TUDCA), trehalose, and glycerol. Once the potency and efficacy of these compounds is determined, the structure of the molecules is then analyzed to postulate key functional groups and structural features that allow for ER stress reduction. Through this, we can begin to work towards developing a novel LWCC that would allow for a localized and potent effect of ER stress reduction.

2. Materials and methods

2.1. Cell culture

Immortalized human proximal tubule epithelial (HK-2) cells were grown in a 50% Dublecco's Modified Eagles Medium (Gibco, 11965) and 50% F12 medium, supplemented with 1% penicillin-streptomycin (Invitrogen), and 10% fetal bovine serum (Invitrogen). The media for the cells was changed every second or third day and the cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The cells were grown in Falcon T75 culture flasks and when the cells reached 90% confluence they were passaged using a 1X trypsin/EDTA solution. Cells were then placed into Falcon 6- or 12-well plates with autoclaved glass coverslips in each well. Cells were grown until they reached at least 80% confluence before any treatments were performed.

2.2. Thioflavin T staining

The treatment media was aspirated following the treatment period and the cells were incubated at 37 °C in new media, containing 5 μM ThT for 15 min. The ThT media was then aspirated and the cells were fixed for 30 min at 4 °C using 4% paraformaldehyde. The fixed cells were then washed with phosphate-buffered saline (PBS) before mounting them on microscope slides using Perma-Fluor. Following staining, cells had limited light exposure to reduce the loss of fluorescence. The slides were then imaged through 10X and 40X objectives using the DAPI filter in an Olympus IX 81 fluorescence microscope.

2.3. ER stress visualization and quantification

Images were analyzed using the MetaMorph Microscopy Automation and Image Analysis Software. ThT staining was quantitatively determined by using the 'trace region' tool to carefully select a region that surrounds one cell. The average fluorescence intensity within the selected region was then measured using the Measure Region Statistics tool. This was done for 10 different cells for each image. The fluorescence intensity was normalized to the background by selecting a region between cells and measuring the ThT staining intensity within it. Three images were taken for each

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