Role Of Small Molecules In Rescuing Protein Folding Under Oxidative Stress

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ROLE OF SMALL MOLECULES IN RESCUING PROTEIN FOLDING

UNDER OXIDATIVE STRESS

MAHMOUD FAWZI MEGAHED HELAL KHALIL

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THIS THESIS IS DEDICATED TO MY PARENTS.

FOR THEIR ENDLESS LOVE, SUPPORT AND ENCOURAGEMENT
ROLE OF SMALL MOLECULES IN RESCUING PROTEIN FOLDING UNDER OXIDATIVE STRESS

By

MAHMOUD FAWZI MEGAHED HELAL KHALIL, BS

THESIS

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Acknowledgements

First and foremost, I have to thank my parents for their love and support throughout my life. Thank you both for giving me strength to reach for the stars and chase my dreams. My sisters and my brothers deserve my wholehearted thanks as well.

I would like to sincerely thank my supervisor, Dr. Mahesh Narayan, for allowing me to be a part of his laboratory, for his guidance and support throughout the last two years, and especially for his confidence in me. I would also like to thank Dr. Kyung-An Han and Dr. Rachid Skouta for serving as members on my thesis committee. Their comments and questions were very beneficial in my completion of the study. I learned from their insight a lot. To all my past and present lab mates, thank you for all your help. I honestly could not have done it without you. Thank you to everyone who contributed to my education. I value each and every one of you greatly.
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Introduction

Central Nervous System and Neuro-Degenerative Diseases

The nervous system is the body’s biological information highway. It sends and receives information through electrical signals to control all the biological processes and movements in the body. It is anatomically divided into two systems: central nervous system (CNS) and peripheral nervous system (PNS). The CNS is composed of the brain and the spinal cord and is responsible for receiving and interpreting signals from the PNS, while the PNS is made of all other nerves that travel to and from the CNS to tissues and organs. The consequences of an organ receiving a signal through the nervous system depend on the physiological function of the organ itself. For example, a signal sent to a muscle will result in either contraction or relaxation depending on the type of muscle and a signal received by a gland can result in an increase or decrease in secretion.

Neurodegenerative diseases are a group of diseases characterized by continuous neuronal degeneration or cell death within a specific area of the nervous system, eventually leading to damage and consequently loss of function of that part of the nervous system and the structures innervated by the affected area. A common feature of this group of diseases is an increased level of oxidative stress, which is perhaps responsible for the death of a specific neural cell population. The most common neurodegenerative diseases are Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and Amyotrophic Lateral Sclerosis (ALS), their location and most prominent macro- and micro-symptoms are described in Figure (1).
Figure 1: Most common neurodegenerative diseases, their location within the CNS, and the most prominent accompanying macro- and micro-symptom for each (Bertram and Tanzi. JCL, 2005).

**Parkinson’s Disease**

Parkinson’s disease was first described by the British physician James Parkinson. He described it as “shaking palsy” in his monograph *An Essay on the Shaking Palsy* (1), which included his observations of six patients. Further understanding of the signs and symptoms of Parkinson’s disease was undertaken later by professor Jean-Martin Charcot. In 1912, around 100 years after the first description of Parkinson’s disease by Dr. James Parkinson, the German neurologist Friedrich Lewy described the main pathological biomarker of the disease, Lewy Bodies (2). Although we are approaching the 200-year anniversary of the first scientific diagnosis of Parkinson’s and the 100-year anniversary of the first diagnosis of Lewy Bodies
lesions in a Parkinson’s patient, still many questions are yet to be answered to improve the diagnosis and management of the disease.

The prevalence of Parkinson’s disease in the general population at age 65 is approximately 1%, and it increases to approximately 5% by age 85 (3,4). Parkinson’s disease is considered the second most common neurodegenerative disease after Alzheimer’s disease (3,4). Although the mean age for the diagnosis of Parkinson’s disease is during the 6th decade of life, symptoms usually start years before clinical diagnosis and go unnoticed until more severe signs present (5).

Only around 3% of Parkinson’s cases are diagnosed before the age of 50 (4). The mean time between diagnosis of Parkinson’s disease and death is 15 years, during which the disease progress slowly (6,7). The main symptoms of Parkinson’s disease include bradykinesia (slow movement), muscular rigidity (increased tone), and gait impairment (7). Together, these clinical symptoms are called Parkinsonism Syndrome. Although several neurodegenerative diseases could cause Parkinsonism Syndrome, the main cause is Parkinson’s disease. The main pathophysiological picture behind Parkinson’s disease is the neurodegeneration of dopaminergic neural cells in the Substantia Nigra pars compacta, which leads to dysfunction of the basal ganglia, which function in the initiation and execution of voluntary movement. The symptoms of Parkinsonism Syndrome as a consequence of degeneration of the basal ganglia start developing gradually as the neurodegeneration progress (8). In the last 10 years, therapeutic agents have been available on the market to manage the symptoms of Parkinson’s disease, the most effective is dopamine replacement therapy that aims at restoring dopamine levels in the affected part of the brain (7). However, none of the available therapeutic solutions, including dopamine replacement therapy, have been proven to revert or stop the pathophysiological dysfunction and neural degeneration behind the disease. Instead, delaying symptoms development and prolonging life
expectancy are the maximal possible benefits shown to be gained from the currently available therapeutic agents.

**Protein Biosynthesis and Oxidative Folding of Proteins**

Protein biosynthesis is the process of synthesizing new proteins within biological cells. New proteins are continuously being synthesized to compensate the cells that have been lysed or exported. The process of protein biosynthesis consists of multiple steps including transcription and translation of mRNA, post-translational modifications, and protein folding.

Transcription is the process of synthesizing new mRNA molecules from the double-stranded DNA molecule (Figure 2). The process is complex and it involves temporary unwinding of the region carrying the gene within the double stranded DNA. The unwinding (sometimes called unzipping) occurs through a group of enzymes called Helicases. This process aids in creating sufficient space for proteins and molecules involved in the transcription process to carry on and consequently for the transcription process to occur. Many proteins are involved in the regulation of the transcription process, such as transcription factors, which are sometimes called sequence-specific DNA binding factors, and co-activators, which increase gene expression by binding to transcription factors. RNase is the enzyme responsible for reading the DNA template strand and synthesizing the complementary mRNA sequence. The enzyme reads the template DNA sequence from 3-prime to 5-prime end, and the complementary mRNA is synthesized in the opposite direction, from 5-prime end to 3-prime end.
Figure 2: Transcription of mRNA from DNA template. The newly synthesized mRNA strand is complementary to the template DNA strand, after substitution of Thymine (T) bases with Uracil (U) (Pearson Education Inc., 2012).

During translation, tRNA molecules are loaded with single amino acids, an amino acid per tRNA molecule. Then, loaded tRNA molecules are matched up through their anti-codon regions with the complementary three-bases codon region in the mRNA molecule that encodes for the protein to be synthesized. Both tRNA molecules and mRNA are brought together by ribosomes, which are large and complex intracellular molecules made up of rRNA strands combined with more than 50 different proteins (Figure 3). There are two different ribosomal subunits: large and small. The large subunit is responsible for binding the newly added amino acid to the growing polypeptide chain, while the small subunit is responsible for reading the mRNA codon encoding for that amino acid.
Figure 3: Translation of mRNA strand into a polypeptide chain, namely the primary structure of proteins. Since ribosomes are key in the assembly of amino acids together, the whole process occurs in the rough endoplasmic reticulum which is rich in ribosomes (Purves et al., 1994).

Posttranslational modifications are a group of chemical and physical modifications of the newly synthesized polypeptide chain that occur not only after the synthesis of the polypeptide chain is complete, but also during translation. Posttranslational modifications were found to be a part of protein biosynthesis process in eukaryotes, but not prokaryotes. These modifications are essential for the proper folding of the newly-synthesized polypeptide chain to form the proper secondary and tertiary protein native needed for proper function. Examples of these modifications are the addition of new functional groups such as acetyl and alkyl groups, changing the chemical nature of some amino acids such as converting Asparagine to Aspartic acid or Glutamine to Glutamic acid, the breakage of an existing bond between two amino acids, and the formation of a new bond between two amino acids.
One of the most common examples of posttranslational modifications is the formation of a disulfide bond between two Cysteines (Figure 4). Examples of proteins that have disulfide bonds in their native structure include immunoglobulins, digestive enzymes, albumin, and polypeptide hormones. In fact, most of proteins destined for plasma membrane and extracellular proteins contain disulfide bonds in their native structure. Oxidative protein folding is the process by which proper disulfide bonds, which are essential for the formation of the native tertiary structure, are formed (29-32). Oxidative protein folding is a complex process, because it is a combination of physical conformational folding and the chemical formation of native disulfide bonds (29-31). Moreover, formation of non-native disulfide bonds during the oxidative folding process requires further chemical thiol-disulfide exchange reactions to exchange non-native disulfide bonds for the native bonds which eventually leads to a competition between the physical conformational changes of the oxidative protein folding process and the chemical thiol-disulfide bond exchange (29-32). This competition is unique to proteins that have disulfide bonds in their native structure (33). Disulfide bond formation in these types of proteins and protein folding occur in the endoplasmic reticulum (ER) where the oxidized:reduced glutathione ratio is favorable for the oxidative folding to proceed. Within the ER, the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) ranges between 1:1 and 3:1. However, in the cytosol, the ratio increases to 30:1, which makes a highly reductive environment that is not favorable for the oxidative folding and maturation of the nascent polypeptide chain into the tertiary structure. Protein traffic in the endoplasmic reticulum at any moment includes mainly three types of proteins: properly-folded native-structured proteins that are biologically functional and to be secreted to the cell membrane or outside the cell, terminally misfolded proteins to be secreted to the cytosol for proteosomal degradation, and newly synthesized polypeptides still to be folded (9-11). Properly folded proteins are secreted outside the endoplasmic reticulum via the secretory
pathway, while terminally misfolded proteins are excreted via the translocatory pathway. A major determinant of whether proteins will be secreted through the secretory pathway or the translocatory pathway is the ability of the newly synthesized protein to rapidly acquire its native disulfide bonds during the oxidative folding process (12-14). Beside the favorable oxidative environment within the ER, many other cofactors and chaperones play important roles assisting the oxidative folding process. Among these is Protein Disulfide Isomerase (PDI) (Figure 5). PDI is the chief endoplasmic reticulum resident oxidoreductase chaperone. It catalyzes proper folding of disulfide bond containing proteins within the endoplasmic reticulum during the oxidative folding process (9,15). Therefore, optimally functioning PDI is critical for keeping the homeostasis between newly synthesized polypeptides and folded secretory proteins, and consequently for preventing the overburden of the translocatory pathway (10,11). Overburdening of the translocatory pathway results in aggregates of protein such as the Lewy bodies seen in Parkinson’s disease and β-amyloid in Alzheimer’s.

![Disulfide bond between two Cysteine residues. The two Cysteines could be either within the same polypeptide chain or two different chains.](image)

Figure 4: Disulfide bond between two Cysteine residues. The two Cysteines could be either within the same polypeptide chain or two different chains.
Figure 5: Schematic of protein disulfide isomerase (PDI) indicating the redox-active a and a0 domains and the hydrophobic b and b’ domains (Gonzalez et al., 2010).

PDI works mainly through two different mechanisms: oxidation and isomerization. In both mechanisms, molecular oxygen is the terminal electron acceptor (15). The chaperone consists of five domains, two of which are responsible for its activity. These two domains contain surface exposed Cysteine residues that are responsible for both oxidoreductase and thiol-disulfide isomerize activities of PDI (16-19). Therefore modification or blockage of Cysteine affects PDI’s ability to function properly. Involvement of these Cysteines in PDI’s activities does not affect its structure (17-18). It was shown that intracellular high levels of nitrosative stress leads to nitrosylation of redox-active Cysteines in PDI molecules, leading to their blockage (20-26). This leads to the formation of S-nitroso-PDI, in which the two active domains are unable to oxidize thiol groups or isomerize thiols-disulfide bonds in nascent polypeptides during the oxidative folding process (27,28). S-nitroso-PDI were found to be abundant in isolated neural cells of Parkinson’s Diseases and Alzheimer Disease patients (27,28). When further research was done to investigate the correlation between these neurodegenerative diseases and nitrosylation of PDI, it was found that the formation of N-nitroso-PDI leads to the accumulation of alpha-synuclein and synphilin-1, the two major components of Lewy Bodies in the cytosol (36-39). However, since these two proteins do not contain disulfide bonds, and consequently are not PDI
substrates, it was concluded that there is a cross-talk between folding events within the ER and aggregation of terminally misfolded protein within the cytosol (40). The cross-talk was hypothesized to occur as follows: reduced catalytic activity of PDI compromised its capacity to process newly synthesized protein and forward them to the secretory pathway. This has led to overwhelming in the translocatory pathway caused by PDI substrate proteins (32, 34-35). The traffic caused by these proteins overburdens the capacity of the cytosolic housekeeping machinery (Parkin:Ubiquitin system), exceeding its capacity to tag terminally misfolded proteins. Meanwhile, high-tend-misfold proteins such as alpha-synuclein, synphilin-1 and A-beta, that need to be ubiquitin-tagged in a timely manner, remain untagged. This result in failure of proteosomal degradation of these aggregates and eventually neural cells undergo apoptosis and result in neuropathies such as Parkinson’s disease and Alzheimer’s (32, 34-35, 40). These findings made PDI of particular interest for managing these two diseases, probably along with other neurodegenerative diseases that might have the same pathophysiology involved (27,28).

Many of the disulfide bond containing proteins have been used for research purposes both in-vitro and in-vivo to study the oxidative folding process of these protein in the ER over the last three decades (40-72). Some of these include hirudin, tick anticoagulant, BPTI and bovine ribonucleaseA (RNase). Using BPTI as a model for protein folding, researchers were able to show the presence of intermediates between the fully reduced structure and the native structure of BPTI using dithiothreitol-ox/dithiothreitol-red for oxidative folding process (40). Others used bovine pancreatic ribonuclease A (RNase A) as a model for oxidative folding of proteins, and using both GSSG/GSH and dithiothreitol-ox/dithiothreitol-red agents they were able to demonstrate different pathways of folding of these proteins (40-46). In our research, we chose
to use RNase (Figure 6) (82) as a model for disulfide bond containing proteins folding, not only because of the wide variety of studies that have been done on the enzyme making it a very well defined protein both chemically and physically, but also because of its unique properties in exhibiting many different pathways through which it folds to the native structure. This makes RNase A a reliable representative of the different features that exist in the oxidative folding landscape of the disulfide bond containing protein pool. The typical oxidative folding pathway of RNase A is shown in Figure 7 (39). For redox reactions, we are using dithiothreitol-ox/dithiothreitol-red reagent. The main problem with using of GSSG/GSH as a redox reagent is the formation of mixed disulfide intermediates between the redox reagent and thiols in the protein. These adducts complicate the HPLC analysis of protein fractions by adding up to the total number of intermediates being formed during the oxidative folding process (48). Dithiothreitol-ox/dithiothreitol-red redox agent does not form mixed disulfide intermediates with protein thiols, and hence does not increase the number of intermediates formed (48). Also, since dithiothreitol-ox is a weaker oxidizing agent than GSSG, using it has the advantage of giving a greater control over the rate of the oxidative folding process (47,49).
Figure 6: Structure of bovine pancreatic ribonuclease A (RNase A) (79) a small but stable protein of 124 residues with four disulfide bonds: 26-84, 40-95, 58-110, and 65-72. The disulfide bonds 58-110 and 26-84 are fully buried in the major and minor hydrophobic cores, respectively. By contrast, the disulfide bonds 65-72 and 40-95 occur near the surface of the protein, although the 65-72 disulfide bond is part of a small hydrophobic core involving residues Val63, Ile107, Ala122, and Val124. The relatively large number of Cysteines (eight) makes the oxidative folding of RNase A one of the most complex ever studied; there are 7193 possible disulfide species, if mixed disulfide species are considered. The well-studied conformational folding of disulfide-intact RNase A (80,81) aids in structural interpretations of oxidative folding experiments (Narayan et al., 2000).
Figure 7: Oxidative folding pathway of RNase A at pH 8 (25°C) [48-53]. Unstructured isomer ensembles of disulfide-bond-containing intermediates populate the pathway and achieve a quasi-equilibrium status prior to the formation of structured intermediates. The blue lines within the image represent protein’s native disulfide bond connectivities; black lines are non-native linkages; red lines are thiolates. The values within brackets are the theoretically predicated numbers of isomers within each ensemble when dithiothreitol (DTT) is used as a redox couple. Two major pathways and two minor pathways of regeneration are depicted from the 3S and 2S ensemble, respectively (discussed in the text) (Narayan, 2012).
Materials and Method

Chemicals and reagents

RNase A and ellagic acid were purchased from the Sigma Chemical Co. They were tested for purity by reversed phase HPLC analysis, and were found to be >99% pure. They were used without further purification. Ferrous sulfate and hydrogen peroxide (30%) were purchased from the Sigma Chemical Co. Reduced dithiothreitol (DTTred) was purchased from the Fisher Scientific Co. Oxidized dithiothreitol and tetranitromethane were purchased from the Sigma Chemical Co. and used without further purification. All other chemicals were of the highest commercially available grade.

Preparation of fully reduced RNase A

Fully reduced RNase A was prepared by incubating the native protein (5mg/ml) in 9 M Urea and 20 mM DTTred (PH 8, 200 mM Tris-HCL, 1 nM EDTA) for a period of 2 hours (83). The pH was then dropped to 3 by adding 10 µL/ml acetic acid to the mixture. The mixture was then dialyzed three times against 50 mM acetic acid. Each dialysis was done for 4 hours at 4°C, before the dialysis solution is exchanged with a fresh one. The mixture was then desalted on a G-25 column using 0.1% Glacial acetic acid as a mobile phase. The desalted fully reduced protein was then kept in -80°C until further use.
Oxidative folding of fully reduced RNase A.

Oxidative folding of previously fully-reduced RNase A was done by incubating fully reduced RNase A (50 nM) with 100 mM DTTox (PH 8, 200 mM Tris-HCL, 1 mM EDTA, 25°C). Time was recorded, and aliquots were periodically withdrawn every 3 hours from the regeneration mixture. Aliquots were subjected to reduction pulse (2 mM DTTred for 1 min) (84), then the pH was decreased to 3 by addition of 40 µL/ml glacial acetic acid. Aliquots were then desalted using a G-25 column and then dialyzed with reversed phase HPLC using a C-18 column (Supleco Discovery® BIO Wide Pore C18, 5 µm, 25 cm * 4.6 mm). Acetonitrile gradient (1%/min) was used as a mobile phase (85).

Oxidative folding of fully reduced RNase A under nitrosative and oxidative stress.

In other experiments, oxidative folding of fully reduced RNase A was done as described above but after incubating the regeneration mixture with tetranitromethane (10 µM final concentration by dilution of a stock of 2 mM acetonitrile solution). In other experiments, oxidative stress environment was created by adding 5 µl/ml of freshly prepared mixture of 1:2 ferrous sulfate to hydrogen peroxide (1 mM : 2 mM) solution in acetonitrile. Aliquots withdrawal and analysis was done as above.

In other experiments, ellagic acid (100 µM final concentration) was introduced to the regeneration mixture before the addition of tetranitromethane. The same was repeated and ellagic acid was added to the regeneration mixture before the addition of ferrous sulfate-hydrogen peroxide mixture. Aliquots withdrawal and analysis was done as above.

The regeneration rate of the native structure of RNase A (N) from the fully reduced structure (R) was determined by integrating the areas under the peaks corresponding to the native
structure, the fully reduced structure, and structured intermediates at each time point. The fractional increase of the native structure was plotted against time. The plotted data were fitted to a single-exponential function and the rate constant was calculated for the regeneration process (37, 86).

**NOx scavenge assays and Mass Spectroscopy.**

Ellagic acid (final concentration of 1 mM obtained by dissolving the neat compound) was dissolved in ethyl acetate before adding tetranitromethane (final concentration of 1 mM, obtained by diluting the neat compound). Incubation was done for 24 hours, and then ethyl acetate was evaporated using rotor evaporator (80°C). The solid remaining layer was then dissolved in methanol and subjected to ESI-FTMS (LTQXL, Thermo Fisher Scientific, San Jose, CA).
DPPH (1,1-diphenyl-1-picrylhydrazyl) assay

Introduction.

DPPH antioxidant assay was first demonstrated in 1958 by Blois. He was able to demonstrate the ability of DPPH molecule to accept hydrogen atom from a Cysteine molecule (89). However, it was not until the late 80s that DPPH started drawing attention for characterizing the antioxidant properties of different compounds (90). 1,1-diphenyl-1-picrylhydrazyl is a kind of stable radical. It has an unpaired valence electron at one atom of nitrogen bridge (91). DPPH antioxidant assay is used worldwide for the quantification of radical-scavenging capacity. It is considered one of the most standard and easiest colorimetric assays used for evaluation of the antioxidant properties of pure compounds. The higher the capacity of the compound to scavenge the DPPH radical, the stronger it’s antioxidant ability. DPPH radical is stable in solution and it has a purple color that absorbs at 517 nm in methanol. The mechanism behind DPPH antioxidant assay is the scavenging of DPPH radical by the antioxidant to be tested (91). When DPPH accepts a hydrogen atom from the antioxidant molecule, this results in the reduction of DPPH into DPPH₂, which has a yellow color (Figure 8) (88). The change from DPPH to DPPH₂, and consequently from purple to yellow, is accompanied by a reduction in absorbance at 517 nm (Figure 9) (87). Using spectrophotometric measurements, antioxidant properties of compounds being tested can be quantified. The original DPPH assay procedure has been modified throughout the last three decades by many different labs for convenience. Table 1 shows different modifications on DPPH assay protocol that have been adopted by different labs (92). These modifications included variation in DPPH concentration (22.5-250 µM), incubation time (5-60 min), pH of the reaction mixture (3.0-5.5), and the solvent used (ethanol or methanol).
Figure 8. The reaction of DPPH free radical with antioxidant. AH is donor Molecule, and A* is free radical produced (Musa et al., 2013).

Figure 9: Absorption spectra of DPPH and oxidation of DPPH radicals (Tam, 2012).
Table 1: Summary of some representative publications of antioxidant assay using DPPH (Sharma et al., 2009)

<table>
<thead>
<tr>
<th>DPPH (μM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.5</td>
<td>Mimica-Dukic et al. (2004)</td>
</tr>
<tr>
<td>50.0</td>
<td>Karioti et al. (2004)</td>
</tr>
<tr>
<td>80.0</td>
<td>Eklund et al. (2005)</td>
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<tr>
<td>100.0</td>
<td>Chen et al. (2005, 2007), Govindarajan et al. (2003), Kano et al. (2005),</td>
</tr>
<tr>
<td></td>
<td>Saito et al. (2004), and Tepe et al. (2005)</td>
</tr>
<tr>
<td>250.0</td>
<td>Alma et al. (2003) and Kim et al. (2004)</td>
</tr>
</tbody>
</table>

Reaction medium
- Ethanol
- Methanol
- Methanol buffered (pH 5.5)
- Ethanol buffered (pH 3.0)

Incubation time (min)
- 5
- 20
- 30
- 60

IC<sub>50</sub> of ascorbic acid
- 9.84 (50 μM)
- 110.77 (628 μM)

IC<sub>50</sub> of BHT
- 5.4 (25 μM)
- 19.8 (50 μM)
- 86.8 (338 μM)

Materials and method:

DPPH assay procedure and data analysis were done in collaboration with Dr. Rachid Skouta, University of Texas – El Paso. DPPH, ascorbic acid and ferrostatin-1 were purchased from Sigma Chemicals Co. and used without further purification. All other chemicals were of the highest commercially available grade. A TECAN M200 microplate reader was used for UV readings. A 20X solution of DPPH was prepared by dissolving 3.9 mg of DPPH in 10 ml of methanol (final concentration of 1 mM). Vortex and ultra-sonic waves were used for full solubilization. The 20X DPPH solution was diluted to 1X solution in methanol. Fresh dilutions were made for each experiment. Ellagic acid was dissolved in DMSO for a final concentration of 4 mM. The same concentration of 4 mM was prepared for solutions of ferrostatin-1 and ascorbic acid separately in DMSO. Then, 10 μL of each of the three solutions was added to one of the 96-Well Plate containing 190 μL of methanol, for a final volume of 200 μL and a final concentration
of 200 µM. From there, 2 fold dilutions were made to obtain final concentrations of 100, 50, 25, 12.5 and 6.25 µM. Incubation was done for 30 min in 25°C, during which UV absorbance at 517 nm was measured using TECAN microplate reader at 5 minute intervals. Percentage of inhibition in UV absorbance was calculated for each concentration at each time point. Pure methanol was used for background control of UV measurements. All experiments were repeated 3 times and data was averaged.
Results and discussion

Regeneration studies of RNase A

Figure 10 shows the regeneration profile of fully reduced RNase A. The addition of tetranitromethane (10µM final concentration) negatively affected the regeneration kinetics, shown as decreases in native-structure fractions formed over time, and consequent decreases in the oxidative folding rate constant. The data shows that the addition of ellagic acid to the regeneration mixture can rescue the oxidative folding process of RNase A under nitrosative stress conditions. This is shown by restoration of the regeneration kinetics to higher levels. Table 2 summarizes the values of rate constants for the formation of native RNase A structure under the applied conditions. The oxidative folding rate constant for RNase A is diminished after the addition of 10 µM of tetranitromethane (0.041) compared to the regeneration mixture without tetranitromethane (0.2027). The oxidative folding rate constants were restored to higher levels upon the addition of 100 µM of ellagic acid (0.2004) to a regeneration mixture containing 10 µM of tetranitromethane.

Similarly, Figure 10 shows that the regeneration profile of fully reduced RNase A is affected by oxidative stress conditions. The addition of OHx radicals into the regeneration environment (5 µL/ml) lead to decreases in the regeneration kinetics of RNase A. Data was analyzed as described above. The data shows that the addition of ellagic acid was able to restore the folding kinetics under oxidative stress conditions to higher levels and rescue the folding of RNase A to its native structure. The data in Table 2 shows that the oxidative folding rate constant for RNase
A is diminished after the addition of 5 µl/ml of the ferrous sulfate / hydrogen peroxide mixture (0.0278) compared to the regeneration mixture without OHx radicals (0.2027). The oxidative folding rate constants were restored to higher levels upon the addition of 100 µM of ellagic acid (0.2016) to a regeneration mixture containing 5 µl/ml of the ferrous sulfate / hydrogen peroxide mixture.

Figure 10. Regeneration of native RNase A formed from the fully-reduced protein (R). The regeneration conditions were 40 µM fully-reduced RNase A, 100 mM DTTox, pH 8, 200 mM Tris–HCl, 25° C.
Table 2.
Rate constants of RNase A regeneration under control conditions, under nitrosative stress conditions, and under oxidative stress conditions (100 mM DTTox, pH 8, 200 mM Tris–HCl, 25°C).

<table>
<thead>
<tr>
<th>Folding condition</th>
<th>Rate constant R→N</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>0.2027 ± 0.0049</td>
</tr>
<tr>
<td>R + 10µM TNM</td>
<td>0.0410 ± 0.0034</td>
</tr>
<tr>
<td>R + 10µM TNM + 100µM ellagic acid</td>
<td>0.2004 ± 0.0032</td>
</tr>
<tr>
<td>R + 5µl/ml OHx</td>
<td>0.0278 ± 0.0012</td>
</tr>
<tr>
<td>R + 5µl/ml OHx + 100µM ellagic acid</td>
<td>0.2016 ± 0.0025</td>
</tr>
</tbody>
</table>

NOx scavenging assays and Mass Spectroscopy.

Figure 11 is the mass spectrum of ellagic acid after incubation in nitrosative stress conditions (1 mM tetranitromethane). The addition of a 90 Da mass to ellagic acid indicates bi-nitration. Control experiments were done in the absence of tetranitromethane. Results showed the absence of di-nitro adducts of ellagic acid (Figure 11).
Figure 11. ESI-FTMS of ellagic acid (1 mM) reaction products after incubation in nitrosative stress conditions (1 mM tetranitromethane).
1,1-diphenyl-1-picrylhydrazyl assay

UV absorbance at 517 nm is related to the concentration of the antioxidant. As anticipated, absorbance decreased as the concentration of the antioxidant increased. Figure 12 shows a comparison of the antioxidant activity of ellagic acid, ferrostatin-1 and ascorbic acid. Observable color change from purple to yellow was associated with all samples tested. At 5 minutes, the radical scavenging activity of ellagic acid at 6.25 µM concentration was 41.4%, compared to 35.4% for ferrostatin-1 and 95.6% for ascorbic acid at the same concentration. At concentration of 200µM, ellagic acid showed radical scavenging activity of 90.8%, compared to 76.8% for ferrostatin-1 and 95.6% for ascorbic acid at the same concentration. The minimum difference between radical scavenging activity of ellagic acid and Fer-1 at the 12.5 µM concentration was observed after 30 minutes, with a difference of 37.4%. The maximum difference in radical scavenging activity of the same two antioxidants was observed at the same concentration (12.5 µM) at the 10 time point, with a difference of 41.0%.

Previous studies (27-28, 37) revealed that nitrosative stress conditions lead to disruption in the oxidative folding process of PDI substrate proteins. However, those results were attributed to the loss of catalytic activity of PDI chaperone through the modification of Cysteine residues of the PDI chaperone, which are responsible for both its oxidation and isomerization functions.

However, here we demonstrated that increased levels of nitrosative stress had an additional direct effect on maturation of disulfide-bond-containing proteins, without PDI catalytic activity being involved in the oxidative folding process. This is demonstrated by the decreased rate of regeneration of fully reduced RNase A in presence of NOx and OHx radicals, measured by the folding rate constant. However, the observed rate of regeneration of RNase A in the presence of
NOx and ellagic acid equaled levels observed in absence of NOx. Similar data shows that the regeneration kinetics in presence of OHx and ellagic acid are restored to those observed in absence of OHx. Although the mechanism of action and location of NOx and OHx radicals on the polyphenols are not investigated here, our study demonstrates that PDI-substrate proteins’ oxidative folding can be maintained unaffected under nitrosative and oxidative stress conditions as long as the radical-scavenging ellagic acid is present.
Figure 12. The radical scavenging activity of ellagic acid, ascorbic acid and ferrostatin-1 shown by % inhibition of UV absorbance at 517nm using DPPH assay. Six different concentrations of each antioxidant were used. Bars’ color gradient, from darker to lighter, represents concentration gradient, from the highest to the lowest. Measurements were done at six different time points, with 5 minute intervals between each.
Conclusion

Increased levels of nitrosative stress intracellularly within the endoplasmic reticulum is a key factor involved in the pathogenesis of both Parkinson's (PD) and Alzheimer's (AD) diseases. Previous in-vitro studies in our lab showed that increased levels of nitrosative stress lead to aggregation of misfolded proteins and formation of Lewy Bodies, the main biomarker of PD and AD diseases. Although this was mainly through nitrosylation of Protein Disulfide Isomerase (PDI), the chief endoplasmic reticulum (ER) resident oxidoreductase chaperone responsible for maturation of disulfide-bond-containing proteins, we demonstrate in this paper that increased levels of nitrosative stress has an additional direct effect on maturation of disulfide-bond containing proteins. Importantly, we demonstrate that the maturation of disulfide-containing proteins under conditions mimicking nitrosative stress can be rescued by the naturally occurring ellagic acid. Our data reveals that ellagic acid can serve as a lead prophylactic agent in searching for small molecules that can prevent against oxidative/nitrosative stress-related neurodegenerative diseases.

Our future scope for the study is investigating the neuroprotective effect of ellagic acid in green-fluorescent protein-tagged synphilil-1 in dopaminergic neural cell line. Synphilin-1 is a constituent of lewy bodies aggregations noticed in the cytosol of human neural cells. It is considered a main biomarker of Parkinson’s Disease. Expected positive results will be represented by decreased Synphilin-1 aggregation in the cytosol of neural cell lines exposed to oxidative stress conditions after incubation with ellagic acid. This will open the way for taking the antioxidant molecule, ellagic acid, for the next step by investigating it’s neuroprotective effect in animal models, and from there to clinical studies in humans. This work opens avenues for the design of
new prophylactic agents that are less toxic and more effective against neurodegenerative diseases, including Alzheimer’s disease and Parkinson’s Disease.


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Curriculum Vitae

Mahmoud was born the middle son among five siblings to May F. Abdel-hafez and her husband Dr. Fawzi M. Khalil. He graduated from high school in May 2004, and started his B.Sc degree in Pharmaceutical Sciences at Misr International University (Cairo, Egypt) in August of the same year. It is a 5 year program leading to a professional licensure for pharmacy practice. After graduation in June 2009, he started a 1 year post-graduate diploma in clinical pharmaceutical sciences at Ain Shams University (Cairo, Egypt) in November 2009, from which he graduated in January 2011. After more than two years of pharmacy practice in Egypt, in both hospital and retail pharmacies, Mahmoud moved to the U.S. to start his M.Sc. program in chemistry at the University of Texas at El Paso (UTEP) in the Fall of 2012. During his study at UTEP, Mahmoud was able to gain a wide range of hands-on experiences in different areas, including analytical chemistry techniques: HPLC, UV-Visible spectroscopy, mass Spectroscopy and NMR (300/600 MHz); biochemistry techniques: Molecular cloning, restriction-free cloning, site-directed mutagenesis, PCR, agarose-gel electrophoresis, DEAE DNA purification, protein expression/purification, SDS-PAGE, ELISA, LAL and western blot; and experiences in biological research during collaborations with biology department at UTEP: Drosophila handling and feeding, drosophila brain dissection, Drosophila negative-geotaxis test (studying loco-motor behavior in drosophila flies), and confocal microscopy. Mahmoud graduated with the M.Sc. in the Fall of 2015. In December 2014, he obtained the FPGEC (Foreign Pharmacy Graduate Equivalency Examination Certificate) from the American National Association Boards of Pharmacy (NABP). Later in the same month, he obtained a professional license to start practicing pharmacy as an intern (a preceding step for a full pharmacist licensure) in the U.S. Currently, Mahmoud resides at 411 New York avenue, apartment 7 in El Paso, Texas, 79902. He
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