AN ABSTRACT OF THE THESIS OF

Helen T. Hobbs for the degree of Honors Baccalaureate of Science in Biochemistry and Biophysics presented on August 15th, 2014. Title: Quantification of Lipoic Acid Induced Changes in Nrf2-Mediated Stress Response

Abstract approved:_____

Tory Hagen

As organisms age they lose the ability to respond to both exogenous and endogenous stresses, therefore placing them at a greater risk for the development of many diseases. The transcription factor Nrf2 is responsible for the induction of many oxidative stress response genes and therefore 3proteins. Additionally, Nrf2-mediated stress response has been shown to decline with age. In rats and mice, the compound α-lipoic acid (LA) appears to reverse this age related loss, however the effectiveness of LA in humans has not yet been demonstrated. In this study salivary levels of the Nrf2-mediated protein aldehyde dehydrogenase 3A1 (ALDH3A1) were measured during both LA and placebo supplementation in human subjects. Results indicate that ALDH3A1 levels show a delayed increase as a result of LA. In addition to ALDH3A1 levels in saliva, expression Nrf2-regulated genes heme oxygenase 1 (HO1) and γ-glutamylcysteine ligase (GCLC) in white blood cells were measured via RT-qPCR. GCLC expression in white blood cells did not change following LA supplementation, but HO1 levels decreased, which may be indicative of an overall increase in glutathione (GSH). This study was part of a small, pilot clinical trial and the data obtained from this study suggest possible method in which LA induced changes in Nrf2-mediated stress response can be studied in humans.

Key words: Nrf2, Aging, Oxidative Stress, Lipoic Acid

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Quantification of Lipoic Acid Induced Changes in Nrf2-Mediated Stress Response

by

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Helen T. Hobbs, Author

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Introduction

Human life expectancy, especially that of people living in wealthy nations, has risen dramatically in the past 200 years[1]. For instance, the life expectancy for Swedish women has steadily increased by approximately three months per year for over 160 years[1]. This increase, as well as growing populations, has led to a rise in the number of people living over the age of 65. It is predicted that the population of people aged 65 years or older living in the United States will reach almost 84 million by the year 2050, representing a significant increase from the 44 million currently living in the U.S.[2] This growing demographic presents healthcare challenges for all nations. It is well documented that aging is associated with an increased risk for the development of many medical conditions including Alzheimer's, diabetes, atherosclerosis, and age-related macular degeneration[3].

I. Oxidative Stress Theory of Aging

The oxidative stress theory of aging proposes a mechanism for aging as well as the associated risk for development of chronic diseases[4]. This theory, originally proposed in 1956, indicates that the free radicals produced during aerobic respiration cause cumulative oxidative damage that results in aging and ultimately death[5]. A few of these free radicals, or oxidants, include superoxide ($O_2^{\cdot-}$) and hydroxyl radical ($\cdot OH$)[5]. Superoxide is formed via the mitochondrial respiratory chain when ubisemiquinone donates electrons to ground-state diatomic oxygen (O_2)[6]. Superoxide can be generated enzymatically by several types of proteins including various oxidases, peroxidases, and cytochrome P450 enzymes[7]. In order to counter the deleterious effects of superoxide, cells produce a family of enzymes known as superoxide dismutases to convert superoxide

into water and oxygen (O_2) or hydrogen peroxide (H_2O_2)[6,8]. H_2O_2 is reactive itself and may damage proteins via the oxidation of cysteine and methionine residues[7]. Additionally, in the presence of iron or copper ions H_2O_2 can also form •OH and $^{-}$OH, which are highly reactive oxygen species and capable of causing significant cellular damage[8].

The damage caused by reactive oxygen species (ROS) such as O^{-}_2, H_2O_2, and •OH or reactive nitrogen species, such as nitric oxide (NO), is due to an imbalance between ROS/RNS and a biological system's ability to detoxify the reactive intermediates and repair the damage done by these intermediates, a state known as oxidative stress[9,10]. Such imbalances are found in the aging phenotype[5,11,12]. When cells are not able to repair and prevent further oxidative damage, oxidants can react with biomolecules, including lipids, nucleic acids, and proteins, and therefore cause damage to these molecules that can lead to loss of function and mutagenesis[5].

II. Glutathione: An essential detoxification molecule

One of the most important detoxification molecules in mammals is glutathione (GSH), which paradoxically decreases with age in some human, rat, and mouse tissues[11,13].

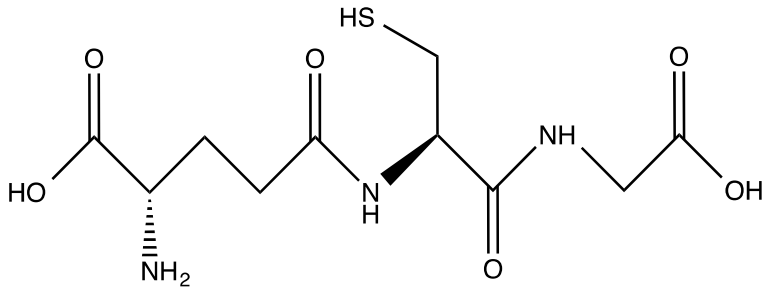

Figure 1. Structure of glutathione (GSH).

In the cell, GSH scavenges free radicals, H_2O_2, and lipid peroxides through the oxidation of a thiol group to form glutathione disulfide (GSSG). GSH is also a substrate for

glutathione peroxidases and glutathione s-transferases[13,14], which rapidly detoxify peroxides and electrophilic xenobiotics, but in the process cause GSH to become oxidized to GSSG. Glutathione reductase (GR) reduces GSSG to reform GSH using nicotinamide adenine dinucleotide phosphate as the electron donor[15].

GSH is synthesized from the ligation of glutamate, cysteine, and glycine where the linkage between glutamate and cysteine is in an isopeptide linkage instead of the usual alpha-peptide bond. GSH is synthesized in almost all cells of the body by the concerted action of two cytosolic enzymes: γ-glutamylcysteine ligase (GCL) and GSH synthetase[14]. GCL catalyzes the rate-limiting step of GSH synthesis, the formation of γ-glutamylcysteine from cysteine and glutamate[14,16]. GCL is regulated by non-allosteric feedback competitive inhibition with glutamate by GSH and the concentrations of L-cysteine available in the cell[16]. GSH synthetase then catalyzes the formation of GSH from γ-glutamylcysteine and glycine[14].

In addition to the regulation of its synthetic pathway, GSH levels in the cell are also controlled pre-translationally[14]. This is accomplished through the activation of a regulatory sequence of DNA known as the antioxidant response element 4 (ARE) by a transcription factor known as nuclear factor erythroid-derived 2-like 2 (Nrf2)[14,17,18]. AREs are enhancer sequences found in the promoter regions of many antioxidant and detoxification genes, including GCL, GSH s-transferase 2a, aldehyde dehydrogenase 3A1, and heme oxygenase I[18]. When the cell is under oxidative stress Nrf2 translocates into the nucleus where it binds to an ARE enhancer sequence and begins the transcription of proteins needed to counter the stress. As previously mentioned, GSH levels have been

shown to decline with age, and part of this decline may be related to a decline in Nrf2 mediated stress response that is observed in aging populations[11,19,20].

III. Lipoic Acid: Reversing the age-related decline in oxidative stress response

An attenuated ability to respond to stresses, both exogenous and endogenous, presents a significant and potentially fatal problem for an organism. Our study has focused on the compound α-lipoic acid (LA), which when fed to rats has been shown to reverse the age-related loss of Nrf2-mediated stress response[11]. Old rats that were fed diets supplemented with LA showed dramatically improved Nrf2-mediated stress response, similar to that observed in younger animals[11].

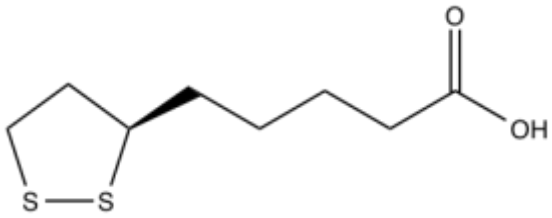

Figure 2. Structure of α-lipoic acid (LA).

These results suggest that the age related decrease in antioxidant and detoxification defenses may not be inevitable and that dietary supplements, such as LA, could be used in animals, including humans, to improve stress response. Currently, the effectiveness of LA in the Nrf2 mediated stress defense in humans has not been fully explored in the literature.

IV. Hypothesis

The goal of this project was to develop a non-invasive method to quantify changes in the levels Nrf2-mediated stress response proteins following LA supplementation in human subjects. We hypothesized that ALDH3A1 levels in human saliva and HO1 and GCLC levels in white blood cells would be affected by LA supplementation. An increase in the expression of these proteins and genes may indicate

an LA-induced reversal of the age-related loss of Nrf2-mediated stress response due to LA.

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Chapter 1:

Lipoic acid increases the level of ALDH3A1 in human saliva.

1.1 Introduction

As of 2005, over 300 proteins have been identified in human saliva[1]. These proteins begin the digestion process, facilitate taste perception, maintain tooth enamel, and form the first line of defense against potentially dangerous pathogens, including many microorganisms and small compounds, including exogenous sources of reactive oxygen species that can cause oxidative damage to cells and tissues[2,3]. These potentially damaging ROS enter the body through the mouth and are commonly found in cigarette smoke and some food products[4,5]. Some antioxidant proteins are secreted in the saliva to begin the detoxification process as soon as possible.

One such enzyme is aldehyde dehydrogenase 3A1 (ALDH3A1)[6,7]. ALDH3A1's function in the saliva is to oxidize long and medium chain aldehydes, including both aliphatic and aromatic compounds, into their corresponding acids[8].

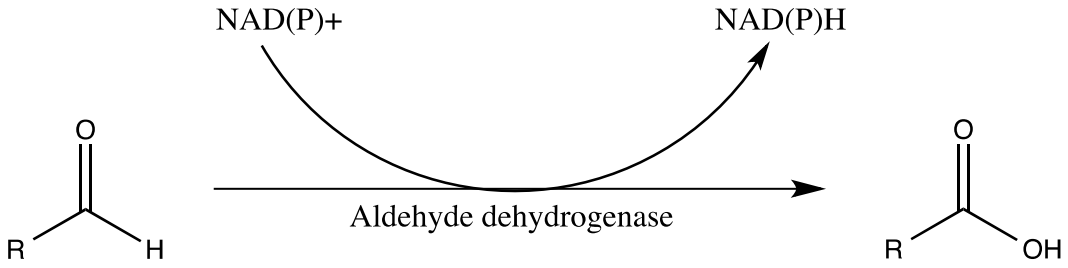

Figure 3. General mechanism for aldehyde dehydrogenase enzymes. Using NADP+ or NAD+ as the electron acceptor, aldehyde dehydrogenase oxidizes potentially damaging aldehydes to corresponding and less reactive acids.

ALDH3A1 is known to be transcriptionally regulated by a xenobiotic response element (XRE)[9]. Like AREs, XREs are upstream enhancers that upon binding a transcriptional activator, aryl hydrocarbon receptor (AhR), induce the transcription of proteins needed to combat harmful xenobiotics, such as exogenous aldehydes[10]. However evidence suggests that ALDH3A1 may also be regulated by the Nrf2-ARE pathway as it can be transiently

induced by electrophiles that are known to elicit an ARE response but do not induce the AhR-XRE pathway[9]. Crosstalk between the AhR-XRE and Nrf2-ARE pathways is becoming increasingly better understood and has also been observed in the regulation of the antioxidant protein NAD(P)H:quinone oxidoreductase (NQO1)[10,11]. This crosstalk may occur due the presence of an XRE in the promoter of the Nrf2 gene[12].

Human saliva is easily collected and therefore may provide a non-invasive method for investigating the effects of LA on levels of stress response proteins in humans. We hypothesize that LA will affect levels of ALDH3A1 in human saliva compared to the placebo.

1.2 Methods and Materials

Clinical Trial Design – Samples were collected during a double-blind, randomized, placebo controlled study (IRB Study #: 3755, Approval Date: 12/14/2010) in which ten elderly subjects (>70 years of age) were randomly separated into two groups of five people. One group was given two 500 mg tablets of LA, a gift from USANA Health Sciences (Salt Lake City, Utah), and the other group took two placebo tablets. Both groups took the tablets daily for three weeks. This three week period was followed by a two week wash-out period in which individuals took no tablets. After this wash-out period, the two groups were given the type of tablet they did not receive in the first arm of the trial for another three weeks.

Sample Collection – Individuals collected daily saliva samples using Salivol sample swabs and vials. For each collection, individuals held the swab under their tongue for 1-2

minutes and then placed the swab into the collection tube. Collection tubes were stored in the freezer until delivered to OSU at the time of blood collection. Individuals observed the following protocol for saliva collection: During the first week of each arm of the trial saliva was collected four times each day, the first as soon as possible after waking in the morning, the second about 15 minutes after the first sample but before breakfast and supplement, the third sample in early afternoon (about 2 pm), the fourth sample about 30 minutes after the evening meal. Frozen swabs were thawed and then centrifuged to extract the saliva.

Total Protein Quantification - The microplate protocol for determination of unknown concentrations and the Thermo Scientific Pierce BCA Protein Assay Kit were used to quantify the total protein present in each saliva sample. A standard curve was made using bovine serum albumin (BSA). Protein concentrations of the saliva samples were interpolated from this curve.

Western Blots - 14 µg protein samples were suspended in a 1X sample buffer containing bromophenol blue and beta-mercaptoethanol and then heated for five minutes at 95 ^{o}C. Samples were loaded and electrophoresed on 4–20% Criterion™ TGX™ precast gels in Tris running buffer. Proteins were transferred to a nitrocellulose membrane, which was then placed in a 5% bovine serum albumin (BSA) blocking solution. Membranes were probed with a primary antibody against human ALDH3A1 (Abcam). A fluorescence conjugated goat anti-mouse antibody was then applied to each membrane and detected

using a LICOR scanner. Band density was quantified using the program ImageJ and normalized to the placebo Day 1, time-point 4 sample for each subject.

Data Analysis - The daily levels of salivary ALDH3A1 were determined by calculating the area under the curve formed by time points A-D (Figure 4) measured each day.

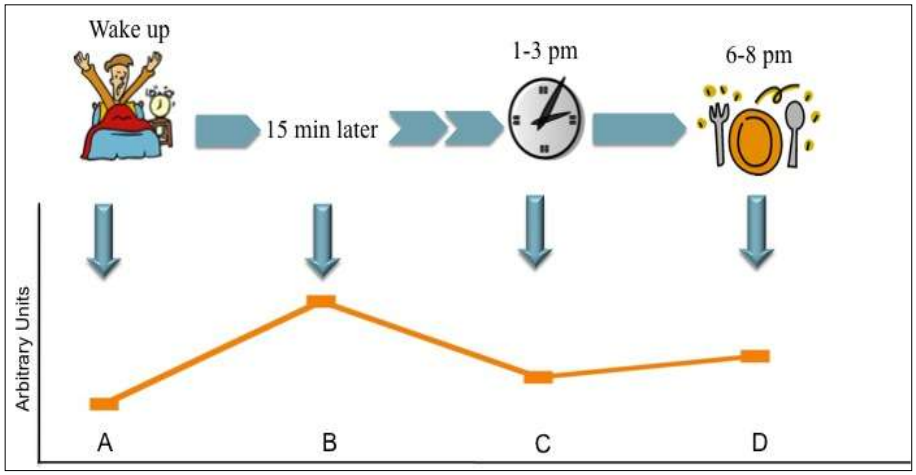

Figure 4. ALDH3A1 protein bands on western blots were converted to arbitrary units using ImageJ to determine relative expression. For each day the relative expressions at each time point were plotted on a single line. In order to obtain a daily measurement of ALDH3A expression area under the curve was calculated for each line and therefore each day.

Percent difference was calculated by dividing the daily area under the curve value for each day the subject took placebo by the daily area under the curve value for lipoic acid days. Due to their similar ranges, the data for subjects 1 and 3 could be combined.

1.3 Results

The daily levels of ALDH3A1 during the first week of each supplementation (LA or placebo) are depicted in Figure 5. There was significant variability within the four

subjects (80-2000 a.u.), and this, in addition to the small sample size, made statistical analysis impossible.

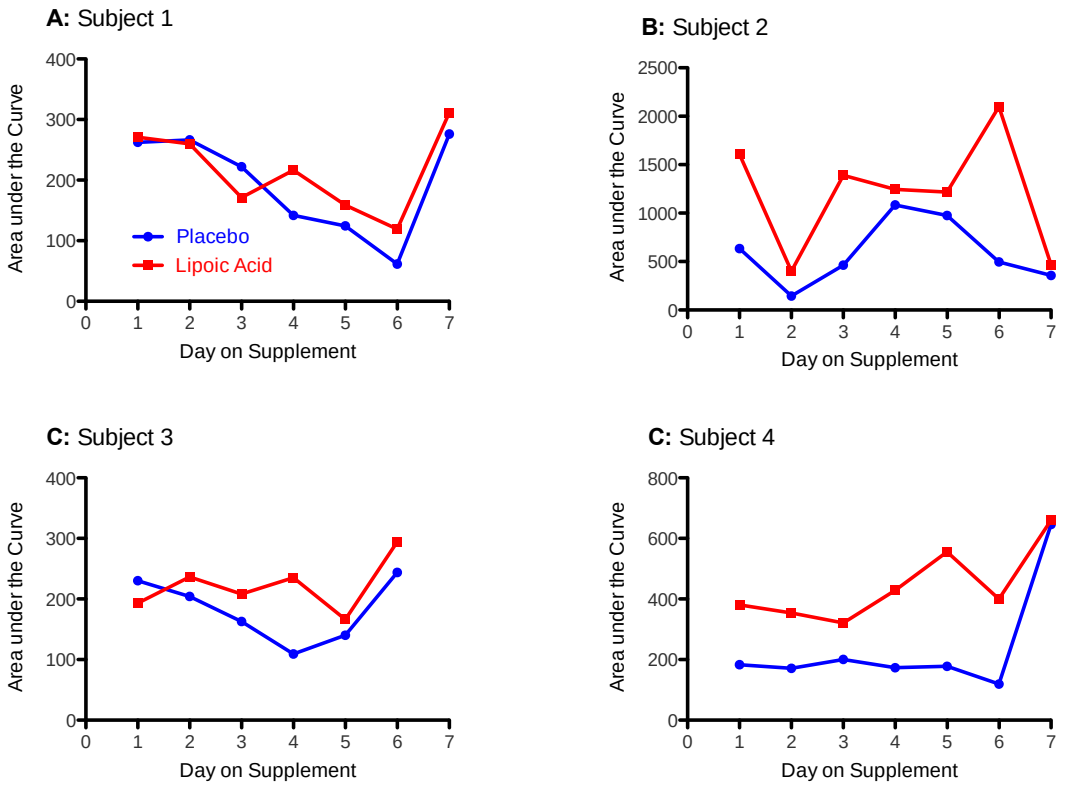

Figure 5. Area under the curve (AUC) measurements of salivary ALDH3A1 levels in subjects 1-4 on placebo (blue) and on LA (red). Subjects display a similar overall pattern in which there is an increase in ALDH3A protein levels approximately 4 days after beginning LA. All subjects show a dramatic change in levels on the weekend, days 6 and 7.

However, when examining ALDH3A1 levels over time, a weekly trend could be observed. Subjects on the LA supplement experienced an increase in ALDH3A1 levels relative to placebo on approximately day four of supplementation. Prior to this increase, LA supplementation followed a similar pattern as those subjects on placebo: a downward trend in salivary ALDH3A1. LA treatment increased ALDH3A1 to a level similar to that observed on the first day of the supplementation period. In this sense LA appears to have a leveling effect on salivary ALDH3A1. The levels of ALDH3A1 on the weekends

varied greatly in all four subjects. This result was unexpected and could not be accounted from lifestyle issues as all subjects were retired and had similar daily routines throughout the week. This weekend phenomenon has also been observed by other members of the Hagen lab while studying other compounds in human saliva.

Data obtained from subjects 1 and 3 fell within a similar range and could therefore be averaged in order to highlight a common pattern of salivary ALDH3A1 levels. Due to the extreme differences noticed on the weekend these days are not included in Figure 6, nor was data from subjects 2 or 4 as they did not fall within a similar range.

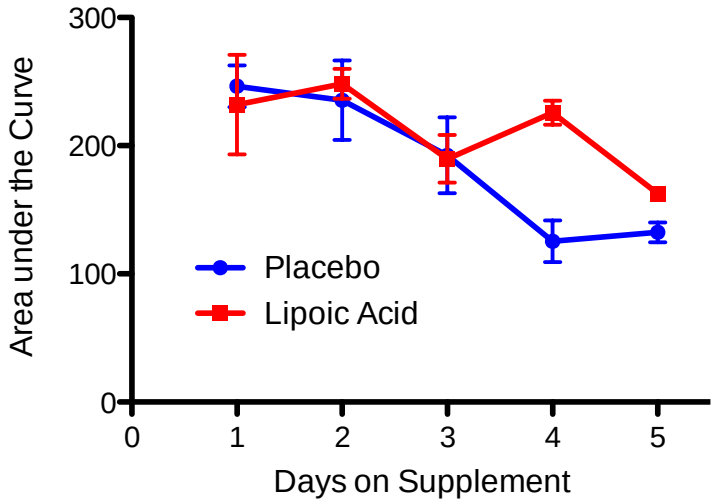

Figure 6. Combined area under the curve calculations on LA (red) and placebo (blue) of ALDH3A expression in the subjects 1 and 3. These subjects show a similar pattern of an increase on day 4 after beginning LA supplementation compared to the decrease in expression observed during placebo.

The difference between LA and placebo observed on day 4 of supplementation is illustrated as a percent difference from placebo in Figure 7. On day four of LA supplementation the increase in salivary ALDH3A1 is approximately 80% compared to

placebo levels. Following this increase, levels began to decline but did not return to baseline levels.

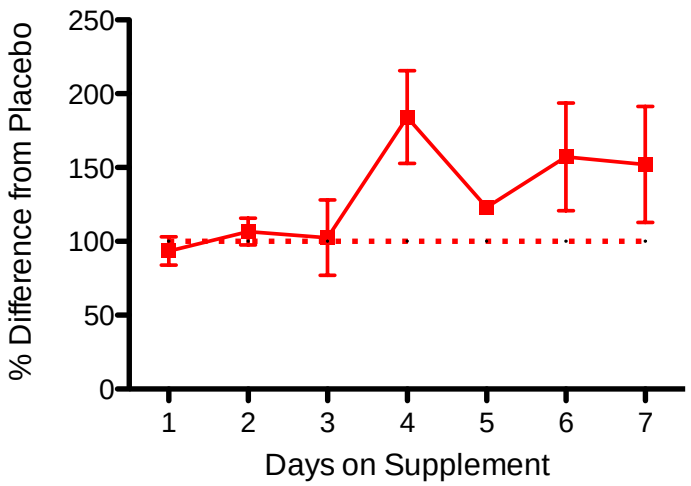

Figure 7. The percent difference of salivary ALDH3A1 expression on LA compared to placebo in subjects 1 and 3. During the first several days of treatment there is no significant change but there is an increase of 180% on day four indicating a higher expression of ALDH3A on LA for both individuals. ALDH3A levels decrease after day four but not to the baseline levels.

1.4 Discussion

In two of the four subjects there was a substantial increase of approximately 180% in salivary ALDH3A1 levels three days after beginning LA supplementation. This pattern of a delayed increase in ALDH3A1 is also present in subject 4 (Figure 5 D), however the rise is more gradual, continuing through day 5 of LA supplementation before decreasing. Although the small sample size from this pilot study prohibits the data from reaching statistical significance, this trend implies that LA is affecting salivary ALDH3A1 levels. This increase in ALDH3A1 could potentially signify an increase in the cellular ability to respond to oxidative stress via Nrf2 mediated stress response.

The pattern observed in ALDH3A1 is similar to that which is seen in salivary cortisol levels (Fig. 8).

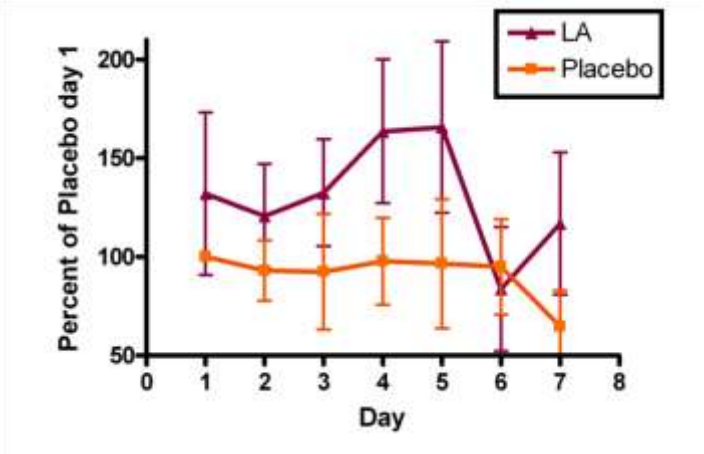

Figure 8. Salivary cortisol levels measured via ELISA and presented as a percent of cortisol level on the first day on placebo. LA causes an increase of salivary cortisol starting approximately three days after beginning the supplement

Cortisol, a member of a class of hormones known as glucocorticoids, is released in response to both exogenous and endogenous stresses[13,14]. The similarity we observed between salivary ALDH3A1 and cortisol levels in >70 years old men may suggest that the levels of these molecules are related. The observed rise in cortisol levels may imply that LA acts as a mild stressor that the body responds to by increasing the levels of cortisol and other stress response proteins, including Nrf2 mediated proteins such as ALDH3A1.

In addition to providing a possible insight into the mechanism of LA, our data indicates that ALDH3A1 may be a potential candidate for observing the effects of LA on Nrf2 mediated stress response in aging humans. However, this was a small pilot study, and in order to confirm that the expression pattern we see in subjects 1 and 3 (Fig. 5 A&C) is significant we would like to conduct a larger clinical trial. Additionally, we would like to measure ALDH3A1 mRNA expression in cheek cells, which can be obtained easily and non-invasively. Finally, we would like to investigate the response of other salivary proteins and molecules to LA supplementation. Two potential candidates for further study include glutathione[17] and glutathione-s-transferase α (Gsta)[18] . It has been shown that levels of these molecules are regulated by Nrf2 and have been observed

to decrease in aging humans. It would be of interest, therefore, to examine whether these molecules are similarly affected by LA as ALDH3A1.

1.5 Conclusion

From this study we conclude that ALDH3A1 may be a good biomarker for quantifying the effects of LA on Nrf2-mediated stress response. In order to determine whether ALDH3A1 is in fact a good biomarker we will need to compare it to other Nrf2-mediated proteins and genes throughout the body and ensure that the response to LA observed in the saliva is due to LA and not other causes. In addition to providing a non-invasive method for measuring Nrf2-mediated stress response, ALDH3A1 may also provide insight into LA's mechanism in cells and the organism as whole, specifically in relation to cortisol. In the future we would like to continue to study ALDH3A1 in a larger clinical trial that includes studies aimed at understanding the relation between cortisol and ALDH3A1.

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Chapter 2:

The effects of LA on GCLC and HO1 expression in human white blood cells

2.1 Introduction

Previous studies have indicated that ALDH3A1 is regulated by the transcription factor, Nrf2. Furthermore, our lab has shown that LA induces Nrf2-mediated gene expression. We wanted to determine whether the effects of LA on ALDH3A1 observed in the saliva were consistent with other known Nrf2 regulated genes affected by age, including γ-glutamylcysteine ligase catalytic subunit (GCLC) and heme oxygenase I (HO1).

GCLC is the catalytic subunit of heterodimeric GCL, which is one of the two enzymes required for the synthesis of GSH, a powerful antioxidant[1]. The other protein in GCL is γ-glutamylcysteine synthetase modulatory subunit (GCLM) and is responsible for improving catalytic efficiency[1]. GCLC expression is inducible via the Nrf2-ARE pathway[2–4]. Therefore as Nrf2 mediated stress response declines with aging[5–7], so do the inducible levels of GCLC therefore inhibiting the ability of the organism to respond to stresses with glutathione (GSH).

Similar to GCLC, HO1 expression can be induced by oxidative stress via the Nrf2-ARE pathway[8–10]. HO1 catalyzes the rate-limiting step of the catabolism of heme into Fe^{2+}, carbon monoxide (CO), and biliverdin[10–12]. Respectively, these breakdown products are thought to contribute to the effectiveness of HO1 as a stress response protein. CO acts as a potent anti-inflammatory agent[13], and biliverdin is converted to bilirubin, which scavenges free radicals often in the form of lipid peroxides[14]. The release of Fe^{2+} from heme by HO1 leads to the expression of ferritin, an iron-sequestering protein, and an ATPase pump that removes free iron from the cell[12]. It has been shown

that removal of free iron is essential to protecting the cell from oxidative damages as Fe^{2+} can react with hydrogen peroxide to form a hydroxyl radical[15].

Both GCLC and HO1 are important stress response proteins induced by Nrf2, and therefore both genes may be affected by LA supplementation. For instance, in rat hepatocytes GCLC expression was increased by LA supplementation[5], and HO1 has been shown to be induced by LA in mouse retinal neurons [16]. The expression of these two genes in white blood cells suggests that these cells could provide an easily accessible sample in which Nrf2-mediated stress response induced by LA could be studied.

2.2 Methods and Materials

Clinical Trial Design – Samples were collected during a double-blind, randomized, placebo controlled study (IRB Study #: 3755, Approval Date: 12/14/2010) in which ten elderly subjects (>70 years of age) were randomly separated into two groups of five people. One group was given two 500 mg tablets of LA, a gift from USANA Health Sciences (Salt Lake City, Utah), and the other group took two placebo tablets. Both groups took the tablets daily for three weeks. This three week period was followed by a two week wash-out period in which individuals took no tablets. After this wash-out period, the two groups were given the type of tablet they did not receive in the first arm of the trial for another three weeks.

Sample Collection - All blood collection were performed between 7 and 9 am, before the LA or placebo tablets were taken that day. Before donating blood, individuals fasted over night. A total of two 7.5mL samples were drawn at each visit. For the first week of each arm of the trial individuals donated blood on the first, third, and fifth day. White

blood cells were separated from one of the 7.5 ml vials of blood donated and were isolated using ficoll and differential centrifugation (Sigma)

Quantitative real-time PCR - Total mRNA was extracted from white blood cells using the Qiagen RNeasy mini kit. White blood cells were lysed using a 20-gauge needle and 1 mL syringe. mRNA was eluted in 30µl of RNase free water and concentrations were measured using the Nanodrop. Using ThermoScript RT-PCR Systems (Invitrogen, Carlsbad, CA) 3µg of each mRNA sample was used to make corresponding cDNA. Real-time PCR was performed using 1µl of cDNA in triplicates using Taqman probes and buffers. Transcript levels of GCLC were determined relative to those of the housekeeping gene, eukaryotic translation initiation factor 2a (eiF2a). Transcript levels of HO1 were compared to those of housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Calculation of fold-change in gene expression - A Ct (cycle threshold) value refers to the PCR cycle number at which fluorescence crossed the fluorescence threshold, a signal just above background fluorescence. The average Ct of the triplicate Ct's was calculated for both the target gene and the housekeeping gene from each sample. The difference (ΔCt_{mean}) between these two Ct_{mean}'s was calculated for each sample. To calculate relative expression of GCLC, $\Delta\Delta Ct$ was calculated. $\Delta\Delta Ct$ represents the difference between the sample and the sample collected on day 3 on supplement of the placebo arm.

2.3 Results

The expression of GCLC in the white blood cells from subjects 1, 3, and 4 did not change on days 1,3, and 5 of each arm of the trial as measured by RT-qPCR (Figure 9).

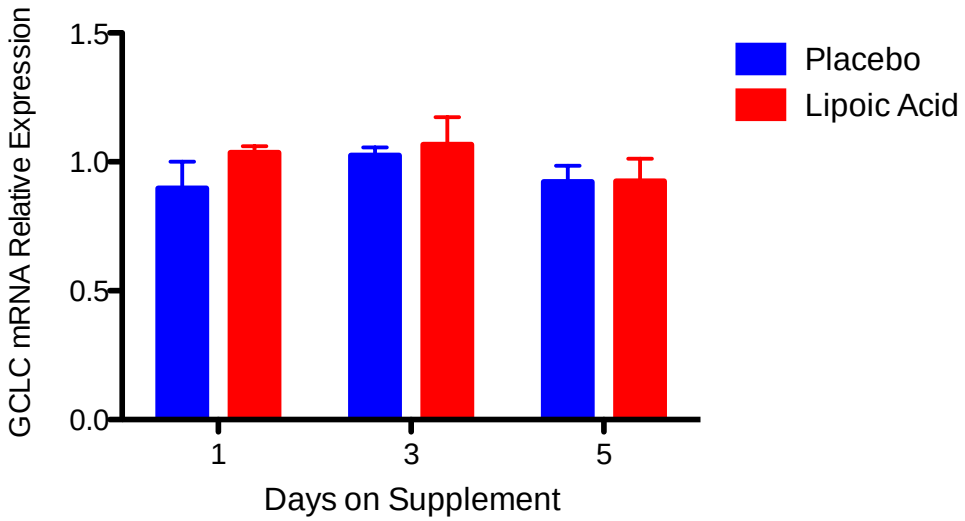

Figure 9. Relative expression, with respect to housekeeping gene eiF2α, of GCLC while taking LA supplements (red) compared to placebos (blue) as measured by Taqman qPCR (n=3). No supplement induced change in expression is observed.

The expression of HO1 in white blood cells varied notably in all four of the subjects. This variability can be seen in Figure 10 as the three subjects have distinct trends in the expression of HOI compared to the housekeeping gene GAPDH, ΔCt. An increase in ΔCt corresponds to a decrease in the expression of the gene of interest, HO1. In addition to the small n-value interpretation of this data is difficult due to the missing days of subject 1 and 3.

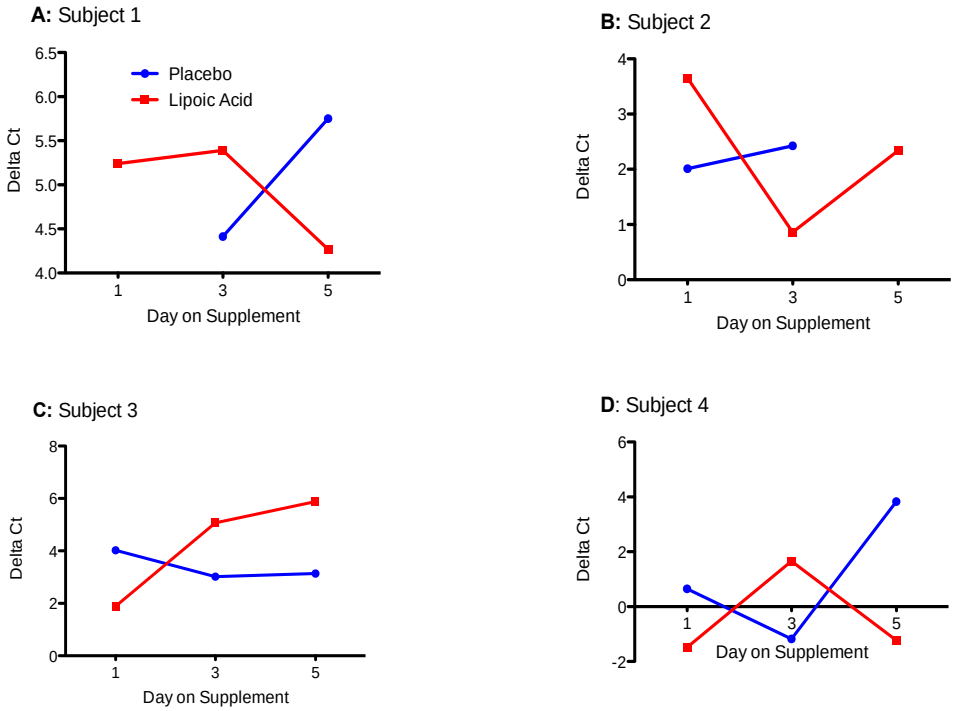

Figure 10. Relative expression, with respect to housekeeping gene GAPDH, of HOI while taking LA supplements (red) compared to placebos (blue) as measured by Taqman qPCR.

Subjects 1, 3, and 4 show similar trends in the expression of HO1 compared to placebo. This trend shows a decrease in HO1expression during the first three days the subject is taking LA supplements. This trend is opposite to an early increase in HO1 expression that is during the placebo arm of the trial.

2.4 Discussion

Although the sample size of this study is too small to draw statistically significant conclusions, there are some interesting patterns in expression of these genes that are worthy of further investigation. One of these patterns is the decrease in HO1 expression observed on day three of LA supplementation in three of the four subjects (Fig.10). This effect may be due to the overall decrease in oxidative stress due to the increased levels of

GSH. An increase in GSH would result in a decrease in the quantity of ROS present in the cell, therefore eliminating the organism's need to catabolize heme in order to remove free iron from the cell.

Additionally, LA supplementation had no observable effect on the expression of GCLC in the white blood cells of old (>75 years) human males (Fig. 9) compared to expression in subjects taking a placebo. This is different than the previously measured increase of hepatic GCLC protein levels in rats[5]. A potential explanation for this marked difference is that different tissues respond distinctly to stresses. LA is taken up and concentrated in the liver, which may prevent it from attaining effective levels in other tissues, such as white blood cells[5]. It is also possible that LA exerts its effects on GCLC in a different time frame than that which we measured.

The ease with which white blood cells are obtained suggested that Nrf2-regulated genes in white blood cells may be useful as markers of the effects of LA on Nrf2-mediated stress response. However, the two genes selected for this study do not appear to be suitable candidates. In future studies we would like to extend this trial to include more >75 years old individuals as well as individuals under the age of 30 in order to compare the effects of LA in old and young individuals. All of the subjects in the presented data were male, but in the future we would like to include female subjects as well because gender has been shown to influence oxidative stress response[19].

2.5 Conclusion

We report that although the small sample size precludes us from drawing definitive conclusions HO1 and GCLC expression in white blood cells may not be

suitable as candidates for a non-invasive method for studying Nrf2-mediated stress response in humans. There are other Nrf2-mediated stress response proteins that we would like to further investigate, including glutathione-s-transferase. This pilot study has provided us with invaluable insight into how to design future studies.

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Conclusion

The data presented in this thesis suggests that LA does affect the Nrf2 mediated stress response in humans. It also indicates salivary ALDH3A1 may be a good marker for studying the effects of LA in humans, as ALDH3A1 levels were shown to increase compared to placebo levels in three of four >75 years old males approximately three days after beginning LA supplementation. However, in order to determine whether ALDH3A1 is a good biomarker its expression and protein levels will need to be further assessed in conjunction with other known stress response molecules, such as GSH.

In addition to salivary ALDH3A1 protein, HO1 mRNA appears to be affected by LA. However, as opposed to an increase we observe a decrease in three subjects on approximately day three of supplementation. This decrease may be the result of an increase in GSH caused by LA, and this is an area in need of further investigation. In the future we would like to measure intercellular glutathione levels in white blood cells as well as other Nrf2-mediated proteins and genes.

The data collected in this clinical trial has provided insight into how to better design future clinical trials as well as indicated salivary ALDH3A1 as a suitable candidate for measuring the effects of LA on Nrf2-mediated stress response in humans. Due to the complexity of human stress response pathways as well as the inherent diversity between individuals it is likely that a combination of genes and gene products will be required to understand the effects of LA on stress response. The study of natural, dietary supplements, such as LA, has the potential to not only improve the health of aging individuals but also greatly contribute our understanding of the aging process and its many effects on human health.

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