THE AMELIORATIVE PROPERTIES OF 3-HYDROXYL-3METHYLGLUTARYL-COENZYME A REDUCTASE INHIBITORS ON THE NITRIC OXIDE PRODUCTION OF MICROGLIAL CELLS: AN ALZHEIMER'S MODEL

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ABSTRACT

In Alzheimer's disease, β-amyloid plaques activate glial cells and induce the release of high levels of glutamate and nitric oxide (NO). Subsequently, glutamate disrupts neuronal ionic homeostasis and NO causes the formation of lethal doses of oxygen free radicals, resulting in a cascade of neuronal apoptosis. This study tested for the ameliorative effects of statins on BV-2 microglial cells, tested for any effect of statins on iNOS production, and observed mitochondrial behavior in Alzheimer's neurons. We simulated Alzheimer's conditions in BV-2 cells using lipopolysaccharide (LPS), and in neuron cells using glutamate and β-Amyloid as activators. A fluorescent JC-1 mitochondrial stain was used in neuron cells to determine the effect of malignant activators on mitochondrial distribution. An immunocytochemistry experiment using a combination of a murineαinducible nitric oxide synthase antibody and a rabbitαmurine IgG bound to FITC as markers was performed to detect iNOS levels after statin application. A nitrite assay was conducted on the prepared microglial cells after treatments with simvastatin, lovastatin, mevastatin, and pravastatin—each statin was added separately. Nitrite assays found that all statins decrease NO levels in glial cells, but particularly simvastatin to a significant extent. Potential reasons might involve simvastatin's structure or behavior. Immunocytochemistry showed that intracellular iNOS levels increased following exposure to statins; therefore, statins did not effectively lower iNOS levels and reduce NO levels elsewhere. Analysis of fluorescent mitochondrial staining showed a higher concentration of mitochondria in the soma as opposed to the neurites under stress (high levels of glutamate/ β-Amyloid), contrary to our hypothesis. We propose that neurons retract their neurites for ionic and cellular protection under stress.

Keywords: Alzheimer's disease, β -amyloid plaques, BV-2, glutamate, nitric oxide (NO), statins, iNOS, immunocytochemistry

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by neuronal cell death, which adversely affects all areas of the brain. Currently, 4.5 million Americans suffer from AD, a number that has more than doubled since 1980; by the year 2050, scientists project this exponential growth to reach approximately 16 million[1]. The risk of developing AD increases as a person gets older; 10% of AD patients are older than 65 and 50% are over 85 (see Fig. 1) [1].

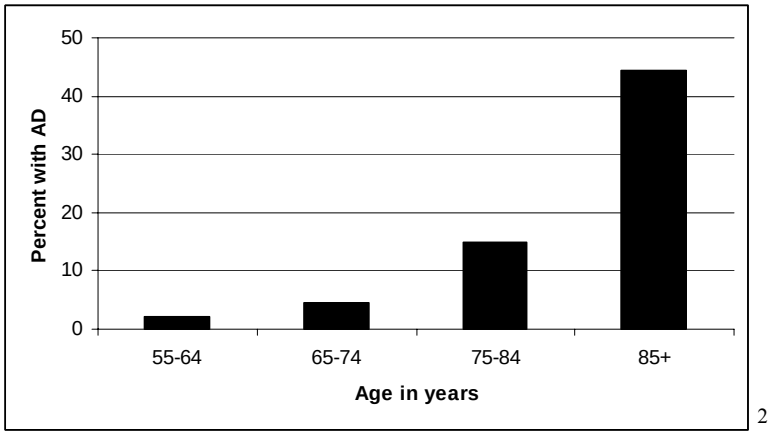

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Fig 1: AD as a function of age.

People who develop AD survive 8-20 years, a remaining life span half as long as those who have not been diagnosed. Not only are the epidemic proportions astounding, but also the estimated annual cost for AD is staggering— $174,000 per individual's lifetime and $100 billion annually for the US [1,2]. Therefore, extensive research is necessary to decrease the human and monetary cost of AD.

Outward symptoms of the irreversible and progressive brain disorder AD include a gradual loss of memory, clarity of thought, and mental skills. Patients begin to lose the ability to recognize faces, recent memories, and experience sharp mood swings and personality changes. Due to the unequal vulnerability of the disease, neuron loss begins in the hippocampus, the brain area responsible for memory and emotion, leading to an anatomical spread of neuronal death. However, the death of an AD patient is generally not due to the disease itself; instead, a secondary condition may be responsible[2].

B-Amyloid and Excitotoxicity

Clinical hallmarks of AD include amyloid plaques, intracellular neurofibrillary tangles, and a cascade of neuronal apoptosis[3]. In AD patients, mutations in the APP gene will cause an excess production of β-Amyloid (Aβ) protein, which makes up the plaques[2]. These plaques negatively affect neuronal and glial cells. Invivo, Aβ has been shown to activate microglia [3]. This activation results in the production of proinflammatory agents and glutamate, which both lead to the production of NO [4].

As the immune system of the brain, the microglial cells are responsible for maintaining homeostasis. They buffer pH, regulate the concentration of neurotransmitters, and eliminate foreign bodies from the brain. First, when activated by Aβ, glial cells release high amounts of cytokines, including Tumor Necrosis Factor- α (TNF- α) and interferon-gamma (IFN) that activate transcription factors, such as IRF-1, in the nucleus[3,9,10]. Increased levels of IRF-1 trigger high rates of transcription of the inducible NOS (iNOS) gene. iNOS is an inducible isoform of neuronal NOS and is a soluble enzyme which does not require elevated intracellular levels of Ca^{2+} for activation, unlike other forms. Therefore, increased iNOS production leads to excess NO levels through the following equation:

$$\text{L-arginine} \xrightarrow{\text{iNOS}} \text{L- citrulline} + \text{NO}$$

An excessive amount of NO, a free radical, causes a wide variety of physiological and pathological processes. When the exogenous NO enters the neuron, it reacts with the endogenous superoxide (O_2^-) to form neurotoxic peroxynitrite ($ONOO^-$). $ONOO^-$ is an unstable free radical that damages DNA and destroys the lipid bilayer of the cellular membrane. These detrimental effects ultimately lead to apoptosis and necrosis. The neuronal cell lyses and releases its contents, thereby damaging its neighboring neurons and offsetting a chain reaction of neurodegeneration[8].

In addition to the production of proinflammatory agents, activated microglial cells also increase the level of extracellular glutamate. Specifically, Aβ activated microglial cells lose their ability to absorb excess extracellular glutamate and produce high levels of glutamate. Glutamate, a neurotransmitter, activates the *N*-methyl-D-aspartate (NMDA) receptors of the neuron. Glutamate is necessary to convey sensory information and complex motor commands, and is associated with long-term potentiation, or the formation of learning and memory, in the hippocampus. However, excessive activation of the NMDA receptors with glutamate can lead to excitotoxicity. A high level of glutamate is characteristic of the disruption of energy metabolism that results from neurodegenerative disorders. Specifically, without energy the neurons cannot maintain homeostasis and undergo depolarization; consequently, the Mg^{2+} that usually blocks the NMDA receptor and inhibits calcium influx is repelled and released from the channel pore. The NMDA glutamate receptor is then overstimulated and Ca^{2+} influx reaches a critical high[5]. It is thought that many of these mechanisms which disrupt homeostasis happen on some level as a person grows older. It is only when factors such as plaques and tangles occur that the problem is exacerbated and results in a much faster rate of cell death, as in AD.

In order to prevent toxicity with the influx of Ca^{2+}, calcium ion pumps drive out the excess Ca^{2+}. However, when the mitochondria cannot produce sufficient ATP as a result of membrane depolarization and bioenergetic failure from the uptake of Ca^{2+}, the ion pumps can no longer pump out excessive Ca^{2+} and the cell begins feeling stressed and undergoes apoptosis, or programmed cell death[6]. One particular molecule that exacerbates apoptosis in neurons as a result of Ca^{2+} influx is NO, which is produced in two ways: the direct activation of NOS and mitochondrial induced activation of NOS.

First, when Ca^{2+}enters the cell, NOS, which is attached to the NMDA receptor, is activated. This results in increased levels of NO, which have been detected in patients with stroke and neurodegenerative diseases[5].

Secondly, the Ca^{2+}overload of mitochondria results in the formation of oxygen free radicals and the activation of caspases. This activation causes Ca^{2+}–dependent activation of neuronal NOS, leading to increased NO production and the formation of toxic ONOO-. Therefore, both these pathways result in the production of harmful oxygen free radicals which damage cells and lead to apoptosis.

When one neuron lyses due to apoptosis, all of the intracellular fluid, including the excessive $Ca^{2+}$and glutamate, is released into the extracellular matrix, thus affecting the surrounding neurons by causing a region of hypertoxicity. This reaction is particularly crucial in setting off a chain of apoptosis among neurons.

Alzheimer's Disease Treatments

3-hydroxyl-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are typically used to reduce cholesterol production (see Fig. 2) [12]. Statins block the rate limiting step, the action of HMG-CoA reductase in the mevalonate pathway, and, thus, reduce levels of low-density lipoprotein (LDL) cholesterol in the body. It is important to note that LDL cholesterol is the type strongly associated with coronary artery disease, and various statins have been marketed in the U.S. under brand names such as Lipitor®, Lescol®, Mevacor®, Pravachol®, and Zocor® [13]. However, statins have pleitropic actions and may be helpful in the treatment of AD. In endothelial cells, statins have shown to have a regulatory effect on nitric oxide production [12]. In one study, atorvastatin had shown the ability to attenuate iNOS production in rat spinal cord cells under conditions similar to those of spinal cord injury [14].

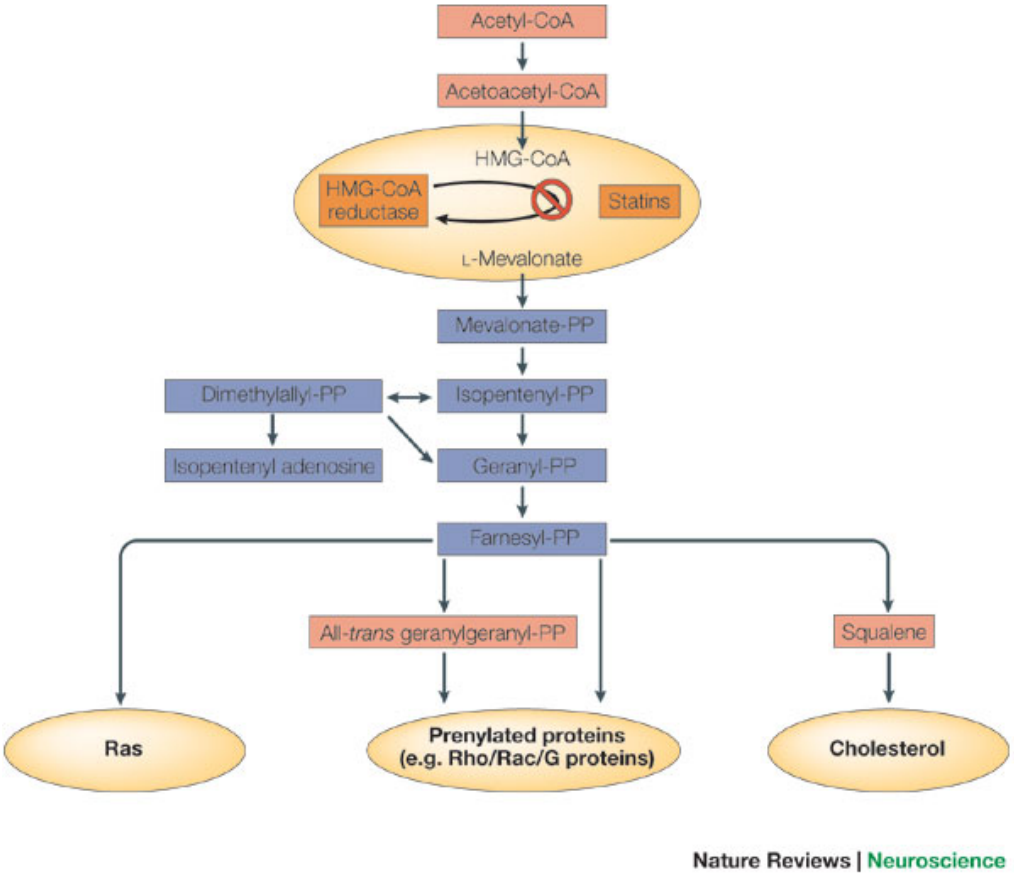

Fig 2: Pathway for decreasing cholesterol levels by statins.

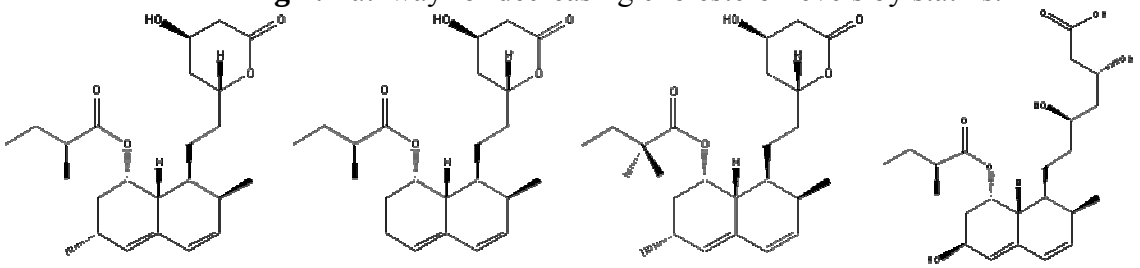

Fig 3: Comparison of different statin chemical structures.

Figure 3 shows the structural diversity of various statins used for pharmacological purposes. Lovastatin is marketed as Mevacor, simvastatin is known by its trade name Zocor, and pravastatin is known as Provochol.

Goals

The present study investigates different treatments for areas of AD pathways. Statins are reported to lower NO levels in microglial cells, but there is little known about the mechanism through which they act. These experiments are designed to give insight into which step along the NO synthetic pathway is affected by statins. We will tested separately for a statin-induced decrease in NO level and iNOS production. We hypothesized there would be a significant decrease in NO level, and an observed decline in iNOS production.

Our second purpose was to observe neuronal mitchondrial distribution under simulated AD stress. Since AD factors such as excessive glutamate and Aβ are known to initiate ionic dyshomeostasis. We hypothesized there would be a mitochondrial migration towards the neurites and the periphery of the soma, as ATP requirements for plasma membrane extrusion mechanisms would increase.

METHODS AND MATERIALS

BV-2 microglial cells were grown in T-25 flasks in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). This medium was supplemented with gentamycin (50 μg/mL), penicillin (50 units/mL), and streptomycin (50 μg/mL). The cell cultures were then placed into a 37°C incubator at 5% CO_2/95% humidified air [15]. For all conducted studies, the cells were seeded at a density of 1 X 10^5 cells/mL. After 24 hours, cells were treated with serum-free experimental medium.

LPS Treatment

BV-2 microglial cells were treated with a 500ng/mL solution of lipopolysaccharide (LPS) and then treated with 20 μM concentrations of the HMG-CoA reductase inhibitors: mevastatin, lovastatin, pravastatin, and simvastatin as prepared by Sigma Chemical Company.

After a 24-hour incubation in the DMEM/10% FBS, the cell cultures were treated experimentally in serum-free medium. The BV-2 microglial cells were then activated using LPS with or without various statins at 20 μM concentrations: mevastatin, lovastatin, pravastatin, and simvastatin. The control setups were prepared as shown in figure 1. The cell cultures were then placed into a 37° C incubator containing 5% CO_2/95% humidified for 48 hours.

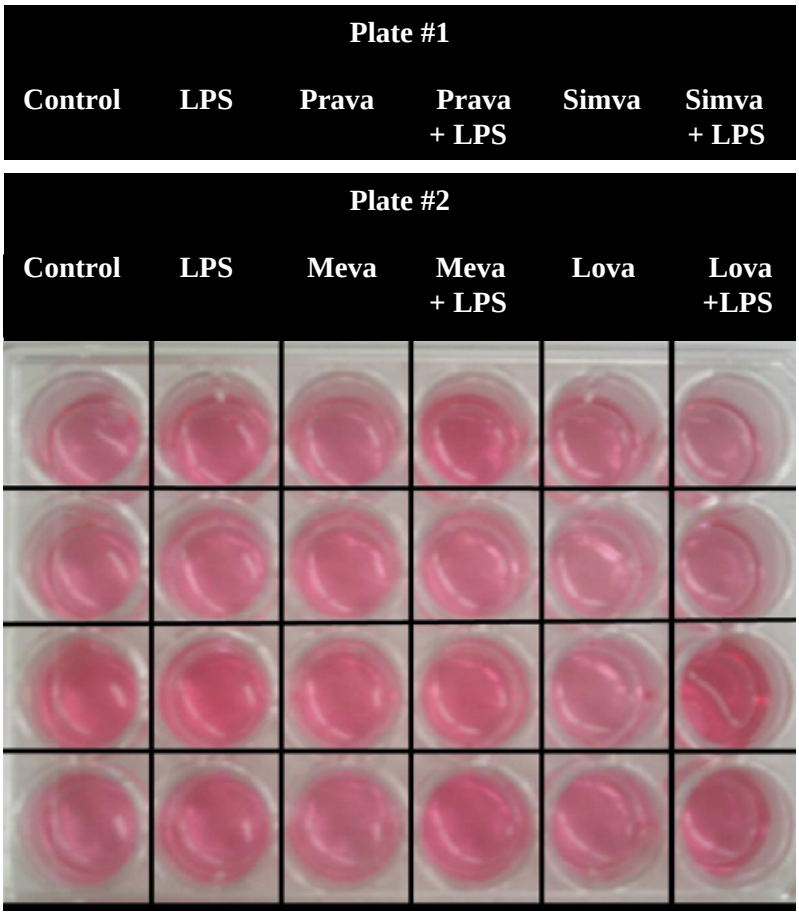

Fig 4: Two 24-well culture plates were prepared using the following treatment groups: control, LPS alone, statin alone and statin + LPS.

Nitrite Assay

Materials used in the nitrite assay included BV-2 cells in a control group, an LPS activation, and experimental treatments of LPS with mevastatin, lovastatin, pravastatin, and simvastatin. Standards were prepared by a serial dilution of $NaNO_3$ in the following concentrations: 3.125 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM, and 100 µM. A Greiss reagent was also prepared to measure nitrite production.

50 µL standard solutions of $NaNO_3$ and DMEM were made with the following concentrations: 3.125 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM, and 100 µM. 100 µL of the cell cultures were centrifuged and transferred to 96-well microtitre plate. Constant standards were compared with the experimental data. 100 µL of the Griess reagent was then added to each of the wells to measure the NO_2^- level. The microtitre plate was read at a wavelength of 540 nm with a mix time of 3 seconds.

Microplate analysis was conducted by an automated assay using the BIO-RAD Model 680 Microplate Reader. Computer software Microplate Manager, version 5.2.1 was employed to measure optical density of the experimental densities in comparison to those of the known standard solutions. Since NO is unstable, we had to measure the stable metabolite, NO_2^- instead.

Mitochondrial Stain

Materials used in mitochondrial staining included the differentiated HCN-1A cells incubated in DMEM media as described above, solutions of amyloid-β and glutamate, and the JC-1 red fluorescent stain. A Nikon Eclipse TE300 fluorescent microscope and the computer software Image Pro Plus, Version 4.1 were employed to photograph cells with both phase-contrast and a red fluorescent filter.

The JC-1 mitochondrial stain was actively taken up by viable mitochondria in the cells. A 1:3000 dilution of stock JC-1 solution was prepared in PBS and heated up to 37°C before it was added to the cells. The DMEM media was aspirated out of the culture plates and replaced promptly with media containing JC-1 solution. The plate was incubated for 15 minutes at 37^{o}C, 5% CO_2. The JC-1 media was washed out with warmed experimental media and returned to the incubator for 5 minutes.

The cells were then visualized using the Nikon microscope and Image Pro Plus. The cells were photographed using phase-contrast exposure for 250 milliseconds and with a rhodamine (red) fluorescent filter for 4 seconds. The numbers of mitochondria in the soma and neurites were counted and the relative percentages calculated.

Immunocytochemistry

Materials used in the assessment of iNOS production included the BV-2 cells activated with LPS and treated with mevastatin, lovastatin, pravastatin, and simvastatin; a murineαinducible nitric oxide synthase antibody; and a rabbitαmurine IgG bound to the fluorescent stain fluorescein isothiocyanate (FITC) (Sigma Chemical Company). The fluorescent imaging method described above was used to measure the levels of FITC with a FITC/green filter.

A mouse antibody, murineαinducible NOS antibody, was used to label the enzyme inducible nitric oxide synthase (iNOS) in microglial cells following treatment with statins and LPS. The mouse antibody was then tagged with a rabbitαmurine IgG, another antibody bound to the fluorescent stain fluorescein isothiocyanate (FITC).

BV-2 microglial cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature. They were washed in Phosphate Buffer Saline (PBS) three times for three minutes each and then incubated with 0.5% Triton x-100 for 20 minutes at room temperature. After brief wash of the cells with PBS, 100 µL of primary antibody was applied to the BV-2 cells. After 45 minutes room temperature incubation, the cells were washed in PBS three times and then a secondary antibody was applied to cells. The cells were incubated for 45 minutes and then washed in PBS three times.

Computer software Image Pro Plus, Version 4.1 was employed to measure the fluorescence intensity of iNOS by measuring FITC in the cells as well as the background. Relative fluorescence was calculated as the difference between the mean values of cell fluorescence intensity and background intensity.

Data Acquisition

Using Microsoft Excel, two-sample t-tests were conducted assuming unequal variances comparing the concentration of NO after the application of different statins to the LPS control. Therefore, there were four t-tests: Lovastatin/LPS, Mevastatin/LPS, Pravastatin/LPS, and simvastatin/LPS.

RESULTS

Statins Summary and Analysis

Using Microsoft Excel, two-sample t-tests were conducted assuming unequal variances comparing the concentration of NO_2^- after the application of different statins to the control. In addition, a further comparison is made between the LPS alone and each of the statin groups.

Analysis of data from Griess assays (n=4) indicated that statins reduced the levels of NO in cells, as measured by [NO2$^-$], stimulated with LPS. Figure 5 shows that Simvastatin and Pravastatin significantly reduce NO ($p<.004$), ($p<.02$), respectively, while other s do so, but not to a significant extent. Interestingly, NO levels were shown to be not significantly different from the control in BV2 cells treated with Lovastatin, Simvastatin, and Pravastatin. Mevastatin exhibited a slight difference significantly with ($p<.0594$). These data indicate that Simvastatin and Pravastatin have the greatest ability to both decrease NO levels and maintain these levels close to the control.

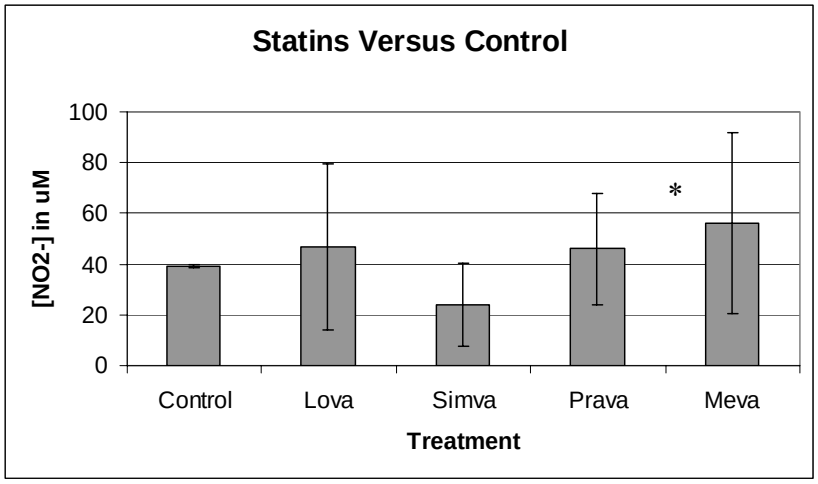

Fig 5: This graph represents the average [NO_2^-] (in uM) after the various statin and LPS treatments. The * indicates a statistically significant result.

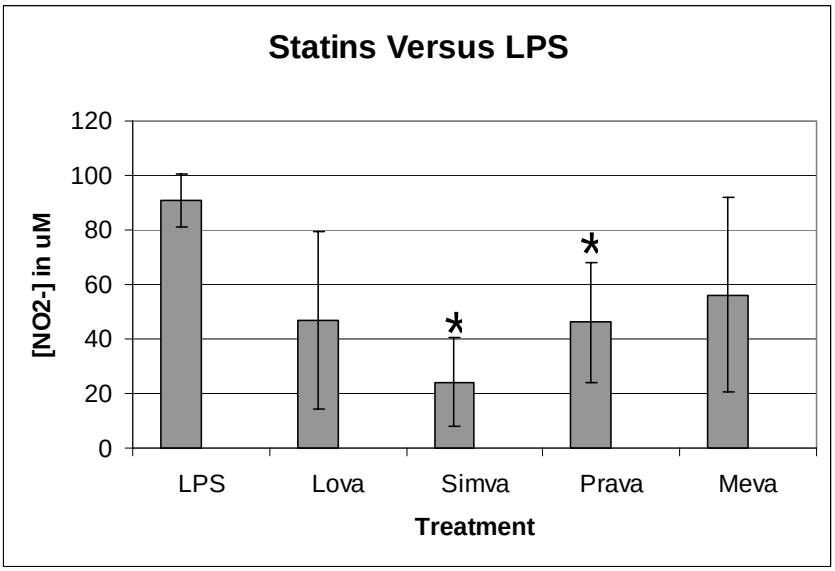

Fig 6: This graph compares the [NO_2^-] between statin+LPS-treated and LPS samples. Each statin treatment contained LPS. The * indicates a statistically significant result.

Treatment	P-Value	Treatment	P-Value
Meva + Control	0.0594	Lovastatin + LPS	0.076843
Lova + Control	0.0703	Simvastatin + LPS	0.004474
Prava + Control	0.0366	Pravastatin + LPS	0.024118
Simva + Control	0.0719	Mevastatin + LPS	0.122499

Tbl 1: Listed are the p-values from the T-tests, done with an alpha-value of 0.05. The first two columns represent the statin treatments vs. the control. The last two columns represent the statin treatments vs. the LPS.

Immunocytochemistry

Microglial cells treated with both the murine iNOS-binding antibody and the FITC associated rabbit anti-murine antibody did exhibit a low level of green fluorescence under the microscope. The cells appeared as faint green bodies, seeming to indicate that the antibodies did in fact bind to iNOS either outside of or within the cell.

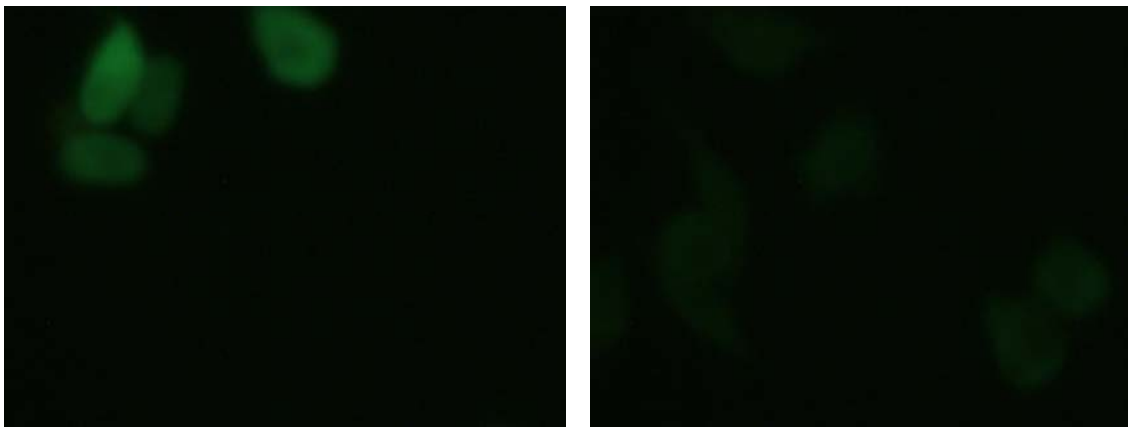

Fig 7: Fluorescence of antibody-treated microglial cells

Upon comparing the relative fluorescence (the fluorescence of a microglial body as compared to the black background), it became apparent that the fluorescent complexes were bleached out if overexposed. To keep data consistent—especially considering the importance of relative fluorescence among experimental treatments—cells were exposed for a minimal amount of time.

Figure 7, which shows weak, barely visible fluorescence, seems to indicate weak antibody binding and possible failure of the antibodies to cross the plasma membrane. In general, none of the cells seemed to have absorbed the secondary antibody into their cytoplasmic space; whether the primary antibody entered the cell is impossible to ascertain, as it cannot be visualized with the microscope. Much of the fluorescence exhibited by the microglial cells was evident on the plasma membrane only.

Despite a lack of staining clarity in most of these preparations, some fields could be used to measure the presence of iNOS staining. A histogram assessment of the relative fluorescence of each group (n=1) revealed a trend differing from that proposed in our hypothesis that the levels of iNOS would be higher in cells treated with only LPS than those treated with LPS and statins. For one statin, simvastatin, the microglial cells seemed to have more antibody-bound iNOS when they were exposed to the statin and LPS, as opposed to LPS alone. This would suggest that statins do not decisively act to inhibit iNOS synthesis, but perhaps statins may actually increase cellular production of iNOS.

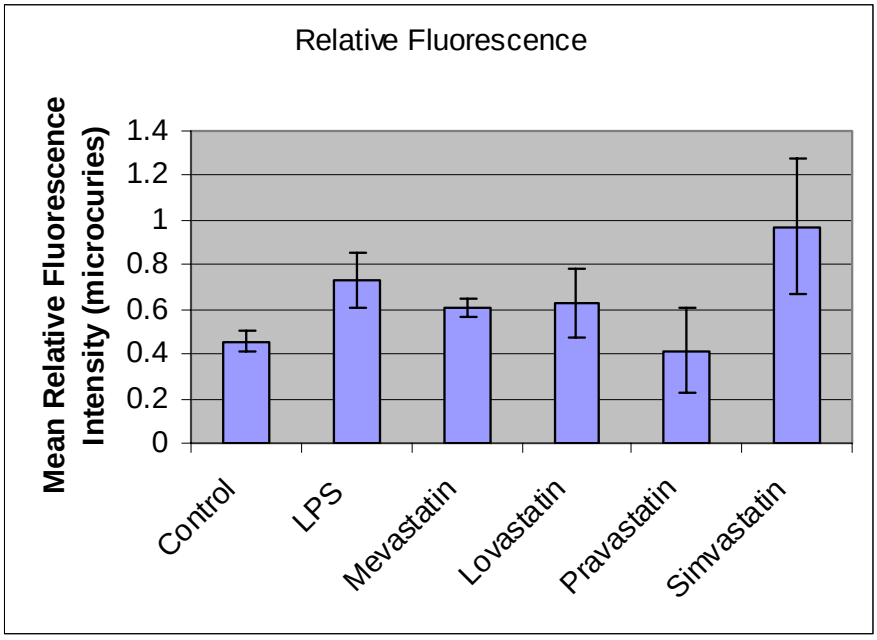

Fig 8: The relative fluorescence of antibody and LPS treated cells. The relative fluorescence is directly related to the amount of iNOS present in the cell. The p-values for each statin (Meva-.2712; Lova-.2086; Prava-.0962; Simva-.2452), found after conducting a one-tailed t-test analysis assuming unequal variances.

The relative fluorescence of the microglial cells indicates that LPS did in fact activate microglial cells and stimulate them to produce iNOS, although not to a significant extent. Mevastatin and lovastatin may have had slight ameliorative effects by reducing iNOS production, but statistically the variation in fluorescence between the LPS treated group and the mevastatin and lovastatin treated groups is insignificant. These fluctuations can be attributed to chance. Pravastatin, however, more than likely did act in some way to inhibit the production of iNOS. Simvastatin may have stimulated iNOS production, or it may have had no effect.

Neural Cell Data And Analysis

These experiments used a neuronal cell line to investigate the affect of Aβ and glutamate on mitochondrial migration. These cells were first differentiated to simulate adult brain cells. Differentiated HCN-1A cells differ from the undifferentiated HCN-1A cells in several respects, most notably in their morphological arrangement. The addition of the differentiating cocktail causes the undifferentiated HCN-1A cells to develop long, branching neurites with morphology similar to neurons present in living organisms. Surprisingly, some HCN-1A cells also seemed to differentiate a glial cell morphology, providing a substrate on which neural HCN-1A cells could anchor themselves.

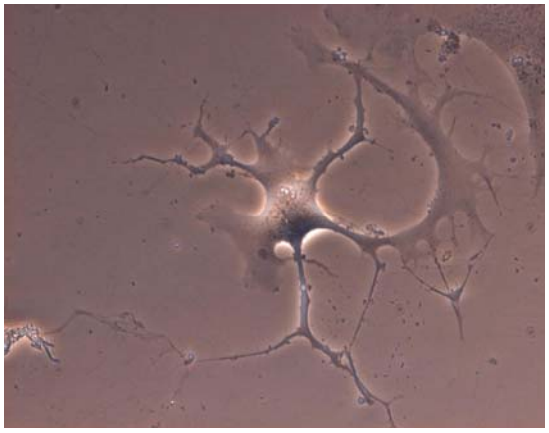

Fig 9: Differentiated HCN-1A cell (neuronal phenotype**)**

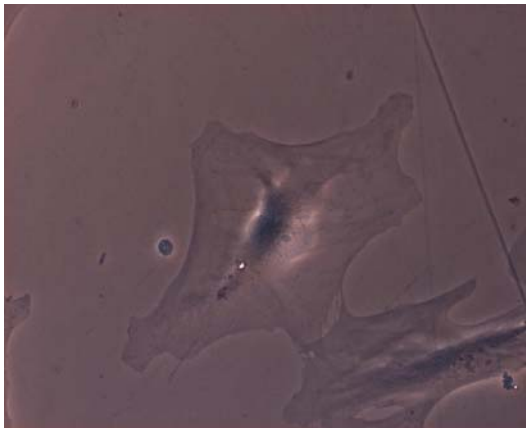

Fig 10: Glial phenotype of HCN-1A cells

HCN-1A cells treated with glutamate, beta-amyloid, or a combination of the two exhibited no noticeable morphological changes. The neural cells did not retract their neurites, nor did they appear lysed.

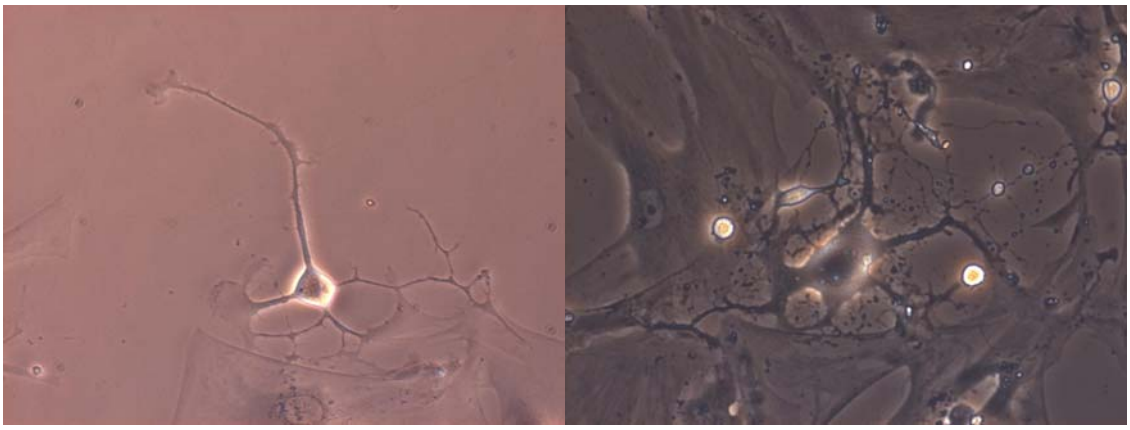

Fig 11: Left--control group HCN-1A cell. Right--HCN-1A cell treated with a-beta and glutamate

Fluorescent analysis revealed stained mitochondria present throughout both neural differentiated and glial differentiated HCN-1A cells. Mitochondria were counted manually by counting the number of red-fluorescing bodies in both the soma and the neurites of neural-differentiated HCN-1A. A count of mitochondria indicated that the number of mitochondria in the neurites was always about twice that of the mitochondria in the soma

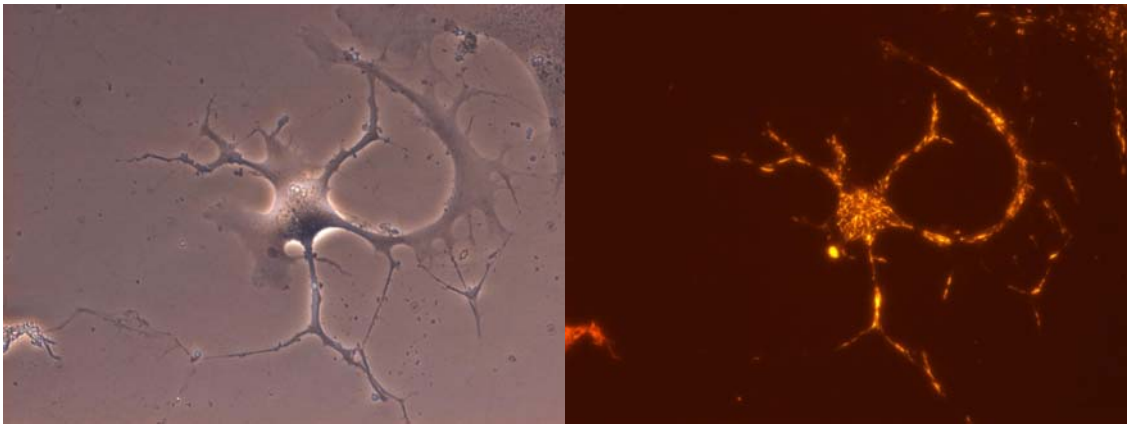

Fig 12: Fluorescence of mitochondria-specific stain JC-1 in control group cell

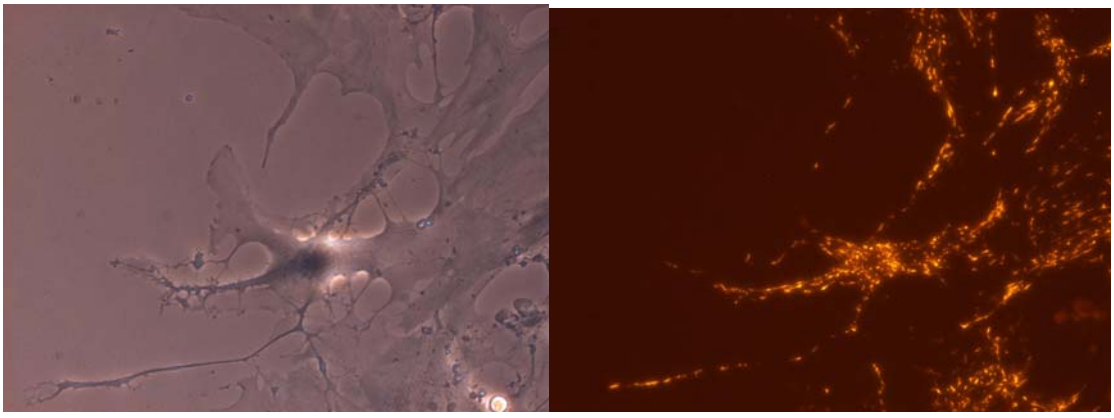

Fig 13: Fluorescence of mitochondria-specific strain JC-1 in glutamate treated cells

In observing the percent of mitochondria located in the neurites versus the soma, it was revealed that the distribution of mitochondria actually shifted towards the soma as stress factors were introduced, rather than towards the periphery as our hypothesis proposed.

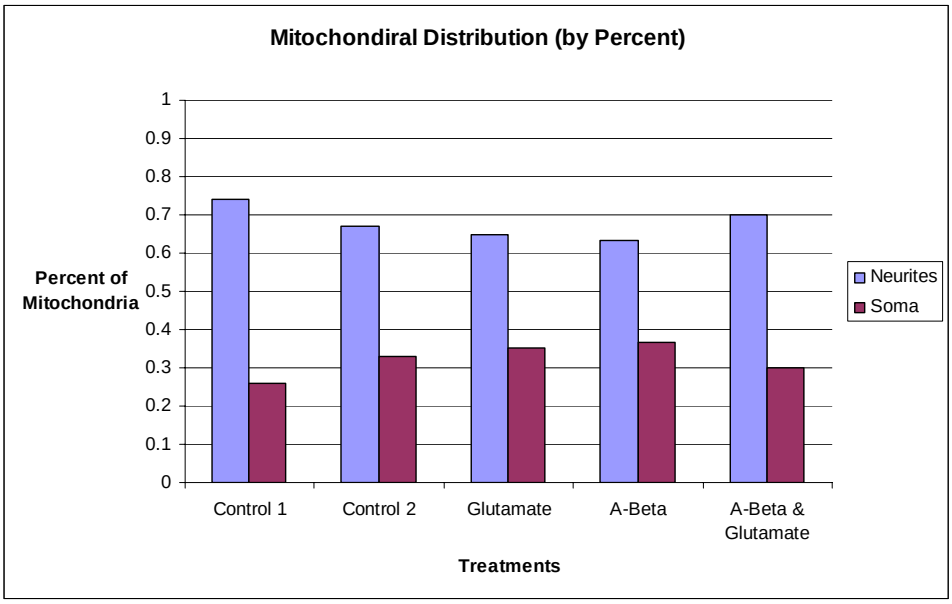

Fig 14: Percent Distribution of Mitochondria in Treated HCN-1A cells. Mitochondria actually seem to shift toward the soma with stress as opposed to towards the periphery.

Relative neurite mitochondrial density was also measured by dividing the number of mitochondria located in the neurites by the total neurite length of the given neuron. When this data was averaged, it became clear that neurite mitochondrial density was much lower in stressed cells than in cells of the control group. Glutamate stressed cells, for example, had, on average, less than half the neurite mitochondrial density of control groups cells. Mitochondria did not seem to migrate to the periphery as had been hypothesized.

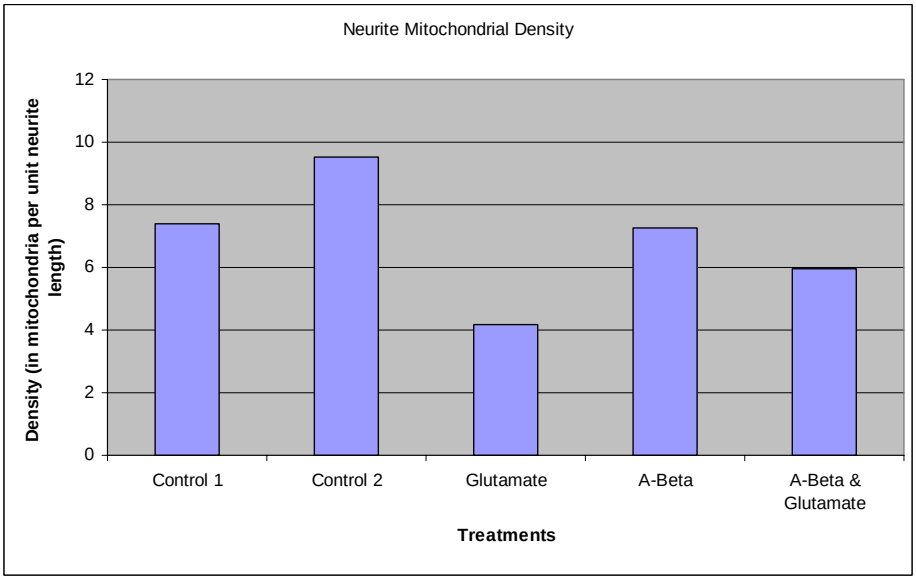

Fig 15: Density of Mitochondria in neurites. As stress increases, mitochondria in the neurites decreases.

DISCUSSION

Statins and Nitrite Assays

Out of all the statins, simvastatin and pravastatin were the only ones that showed a statistically significant effect in reducing NO. Although the other statins also decreased NO levels, the change was not enough to be significant. The expected decrease in NO levels produced by the statins in the experiment is supported by a similar experiment conducted by Pahan et. al [15].

Interestingly, the FDA has approved statins for treating hypercholesterolemia with few side effects. Lovastatin is advertised as Mevacor, Pravastatin as Provochol, and Simvastatin as Zocor [1]. However, in our experiment statins demonstrated pleiotropic properties by lowering toxic NO implicated in AD. Thus we were able to observe a different application of statins through our study of their effects on microglial cells.

We had expected more significant results, but there were some errors that may have accounted for this. During our nitrite assay, up to 6 people were working on different plates at the same time. Also, cells were seeded by various team members which could have led to an inconsistency in cell density. In addition, after analysis, several of our standard curves were slightly inaccurate, which altered the reliability of the NO readings in statin-applied wells. In addition, the data from one experiment was discarded due to improper preparations. This reduced the sample size for some of our tests and may have contributed to the insignificant results.

Although we had an n = 4 for the nitrate assays, more trials are necessary to obtain more accurate data and conclusions. Had there not been time constraints on the project, the effect of the statins on microglia would have been studied more in depth. Thus these experiments have the potential to provide information about the mechanisms of NO production in microglial cells and possibly produce future treatments for AD.

It is noteworthy to mention a potential source of data misinterpretation. Since the ethanol in which the statins were dissolved contributed to some cell death, the lowered [NO_2-] detected by the nitrate assay may have been due to both the statins and the ethanol. A possible experiment to perform in the future to explore these findings is to observe [NO_2-] with statins dissolved in a substance other than ethanol, such as one in which lipids are soluble. Another potential experiment to conduct is an MTT assay to measure cell death relative to the amount of NO_2- found with a nitrate assay.

Immunocytochemistry

The antibody staining of microglial cells revealed that iNOS was present on or near the plasma membrane. Our results, however, ran contrary to our original hypothesis that statins acted to reduce iNOS expression. In fact, recent research suggests that statins might increase iNOS production by up regulating iNOS transcription factors [15]. This means that statins must act in some other way to reduce NO levels in our models. Such information is important in the

modern world; statins are already on the market and are in clinical trials for treatment of neurodegeneration. Understanding the mechanism by which they work is of great importance, and our research indicates that they must work in some way other than preventing iNOS transcription.

When observed with fluorescence under the microscope, the cells displayed low levels of the stain on the outer surface of the cell membrane and virtually none in the cytoplasm that we could detect. This suggests that perhaps the permeabilization step in our protocol was not sufficient for the passage of the antibodies. Further experiments with permeabilizing compounds would be necessary to determine a more reliable result about iNOS presence.

Incubation periods are also an issue. The microglial cells were permeated with an incubation of 0.5% Triton x-100 for 10 minutes. They were subsequently tested with a longer incubation of 20 minutes. This may have been too short a period of time to make the membrane permeable to the antibodies. In future trials, the incubation period may be extended to several hours. It must be taken into account that these results are based on a single trial (n=1); therefore, while they provide no statistically relevant data, they offer insight into future studies on iNOS production could be conducted.

Mitochondrial Staining

As a secondary experiment, the HCN-1A neuronal cell line was used to study the effects of β-amyloid and excessive glutamate levels on the migration of mitochondria throughout the cell. We hypothesized that the mitochondria would move to the periphery of the neurites in order to provide ATP that would power ion channels to maintain the normal homeostatic ion levels in the cell. Instead, it was observed that the mitochondria were more concentrated in the soma rather than in the neurites when treated with stress factors. It is possible that rather than migrating to the neurites, the mitochondria remain centralized in the cell body as to protect the nucleus from excitotoxicity.

The HCN-1a cells retracted their mitochondria in our experiments; we hypothesize that this is compensation for excessive stress at the expense of the now-powerless neurites. While central nervous system neurons normally move their mitochondria to the periphery to maintain their ion homeostasis when stressed, it is possible that these cloned cells may instead retract their mitochondria, as they are more concerned with survival than preserving their functionality. While this does not tell us much about ion homeostasis in AD neurons, it does offer some potential insight into the workings of brain cancers. It would make interesting further research to see if HCN-1A cells responded differently to calcium influx than other neural cell lines. Such knowledge might someday be of use in brain cancer treatments.

When we tried to continue our experiments with HCN-1a cells, it was found that the HCN-1A cells were more difficult to work with than expected. Not only were they too fragile and difficult to manipulate in accordance with the experiment, but they also replicated at an extremely slow rate. This made it impractical to use the cells for this experiment.

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