Characterizing Heme Signaling and Bioavailability in Neurodegeneration

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Abstract:

Alzheimer’s disease is a neurodegenerative condition that results in cognitive decline, impacting over 40 million people worldwide. Alzheimer’s is characterized by amyloid beta plaques, tau tangles and neuroinflammation. Recently, neuroinflammation has emerged as a major player in disease progression, impacting astrocytes, microglia, and neurons. Prior work conducted in the Wood Lab suggests that the blood-derived factors heme and hemoglobin (Hb) modulate neuroinflammation, yet little work has been done to understand their effects on cell signaling. This study aims to expand on previous work analyzing heme interactions by determining the kinetics and bioavailability of heme using ratiometric heme sensors and studying inhibition of signaling pathways to determine modulation of heme signaling. Through experiments with ratiometric heme sensors, it can be determined if these changes in signaling are due to uptake of heme and intracellular interference of signaling pathways or if heme is externally interfering with these cells via extracellular mechanism. Results of these experiments show that astrocytes uptake heme, revealing that heme intracellular interactions may play a role in regulating downstream signaling. Furthermore, cell signaling experiments were conducted using primary mouse astrocytes and treatment with various inhibitors of the PI3K/Akt pathway. Results revealed that heme signaling is partially regulated by the mTOR pathway, as treatment with rapamycin, an mTOR inhibitor, resulted in decreased HO-1 expression. Understanding how heme is internalized by these cells and the mechanism of action, as well as which pathways modulate these effects will be useful for developing future heme-based therapies.

Introduction:

Alzheimer's disease (AD) is a neurodegenerative disease that is characterized by decline of cognitive function and neuronal death. This disease is quickly becoming a worldwide epidemic, with a new incidence every seven seconds.[1] Alzheimer's is the most common cause of dementia, defined as decline in cognitive ability, accounting for 50-60% of cases globally.[2] Alzheimer's disease has a multifaceted pathology and the disease can present in patients with a variety of signs and symptoms, contributing to the complexity of research aiming to discover a cure.

Because Alzheimer's disease is such a complex disease, there are a variety of key pathological players impacting disease progression such as the development of tau tangles, amyloid beta plaques, and neuroinflammation.[1] Though these prominent pathologies have been identified, the mechanisms by which these players form and interact with other cellular components are still unknown.[1] Emerging research suggests a major role for neuroinflammation in disease progression. Furthermore, heme, a bloodborne component found in red blood cells and specifically hemoglobin, has been identified as a key factor in pathology because of its impact on neuroinflammatory pathways through suppression of astrocyte activity. In a healthy cell, astrocytes work to clear pathogens and debris from the brain and heme suppresses this activity, leading to impaired clearance of amyloid beta, a molecule that aggregates to form plaques in AD.[3] These plaques can induce further cognitive decline by impairing normal neuronal function.[3] One major component of AD pathology is a breakdown of vasculature in the brain, known as a leaky blood brain barrier; this allows blood factors to come in contact with glial cells.

Although previous work has suggested that heme and blood derived factors play an important role in AD pathology, there is a lack of knowledge with regard to the2 specific molecular mechanisms and impact these may have on glial cells.[3] Prior work conducted in my

lab to study the interaction of blood factors and glial cells, suggests that the blood-derived factors, heme and hemoglobin (Hb) may modulate neuroinflammation in AD. Further work is necessary to understand the effects of heme on the cell signaling pathways that modulate inflammation in the brain. Cell signaling is complex and preliminary data suggests that modulating these pathways with either inhibitors or activators may be able to restore glial function, which may lead to future therapeutic approaches to AD.[3]

To understand the role of heme in Alzheimer's disease, experiments will be designed to analyze the bioavailability of heme in relation to glial cells to determine if the mechanism in which the interaction occurs is extracellular and utilizes a receptor or if the heme is internalized by the cell and further changes occur along cell signaling pathways. This work will study inhibition and activation of the PI3K/Akt intracellular signaling pathway in astrocytes to determine causes for changes in cell signaling and inflammatory response in the presence of heme. Understanding signaling pathways and their interactions with blood derived factors such as heme will provide a target for possible Alzheimer's disease therapy in the future.

Literature Review

Alzheimer's disease (AD) has a multifaceted pathology involving various cell types and hallmarks such as amyloid beta plaques and neurofibrillary tangles. Because the pathology of the disease, like most neurodegenerative disorders, is so complex, there are many conflicting theories about the progression of AD and which cellular interactions drive pathophysiology.[1] Although theories range from amyloid-beta-based studies to tau tangle theories, recent research identifies that neuroinflammation could play a major role in increasing cognitive decline and contributing to neuronal death.[4] Thus, a systems approach has been identified as an appropriate way to address neurodegenerative diseases with complicated pathologies like AD, as they allow for examination of multiple variables, in a systematic way to isolate a causative mechanism.[5] Furthermore, when studying neuroinflammation, research from my lab has identified heme, an oxidative molecule that is upregulated in AD tissue, as modulatory to glial function.[3]

During the past few decades, the understanding of the pathophysiology of AD has been changing. In particular, neurofibrillary tangles were once widely accepted as central drivers of pathology. Yet, as more data is uncovered characterizing the pathology of AD, these tangles are now understood to be a response to neuronal damage.[1] Furthermore, amyloid beta plaques are no longer thought to be a sole trigger responsible for pathology. However, clinical trials using secretase inhibitors to block beta and gamma inhibitors in hopes of halting the formation of amyloid beta have proven ineffective in restoring cognitive function or slowing neuronal death.[1] It is now understood that various cellular mechanisms can play a part in Alzheimer's pathology including oxidative stress, amyloid beta production, neurofibrillary tangle accumulation, inflammation and many others. This wide array of factors contributes to the diverse opinions and wide array of theories concerning the mechanism of AD pathophysiology.[1] A variety of therapies

are currently in testing, targeting each of these mechanisms, yet very few have proven successful.[1] Only aducanumab, a drug developed by BioGen, has proven to show some success, although results are controversial and the drug has yet to be approved by the FDA.[6]

Due to the complexity and decade long duration of neurodegenerative diseases, it is difficult to isolate causative mechanisms and determine if neuronal death is driven by aggregation of various molecules or if these cellular changes are a response to neurodegeneration and death. Research suggests that a systems biology approach that utilizes multivariate tools and transgenic mouse models is a promising method for studying and identifying causative mechanisms in Alzheimer's disease.[5] Recent studies using a systems approach have suggested that amyloid beta is necessary, but not enough for driving pathological events like neuronal death in AD. Furthermore, this work reveals that glial changes in response to inflammatory cytokines driven by amyloid beta plaques may play a larger role in driving pathology than previously believed, highlighting the importance of studying neuroinflammation as a catalyst to cognitive decline.[5,7]

In addition, emerging evidence identifies inflammation as having a causal role in Alzheimer's pathogenesis.[4] AD is widely known to be linked to an abnormal inflammatory immune response. However, there are conflicting opinions on whether neuroinflammation is neuroprotective or harmful. Researchers describe the roles of various glial cells in an inflammatory response and how these changes can impact pathogenesis (Figure 1).[4,7] Microglia are known to bind to amyloid beta via phagocytic receptors, yet activation of microglia can be beneficial or detrimental to diseased brain tissue, depending on the stage of the disease.[4] Furthermore, microglial activation causes a cascade of cytotoxic molecules to be produced, which can induce dysfunction in astrocytes and neurons, leading to further cognitive decline.[2]

Astrocytes play a role in inflammation by acting as scavengers, releasing cytokines that can elicit an inflammatory response, and forming a physical barrier between brain tissue and vasculature. Astrocyte activation can be measured through GFAP expression.[4] More in-depth research analyzes the role of neuroinflammatory cytokines, indicating they create an extreme proinflammatory environment.[3] Studies suggest that activation and dysfunction of glial cells, such as microglia and astrocytes, ultimately amplify AD pathology and neurodegeneration. Targeting inflammation could lead to mediation of neurotoxic events, halted cognitive decline, and increased clinical benefits.[2] Understanding the role of neuroinflammation in propagating Alzheimer's and the implications of glial cell interactions is important in understanding the overall pathology of this complex disease.

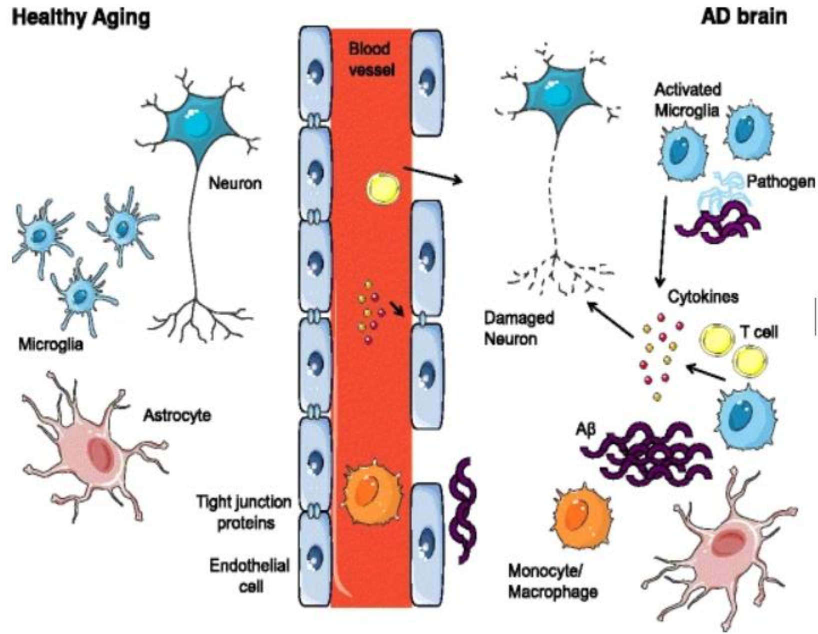

Figure 1. Comparison of Glial Activity in Healthy Brain versus an AD Brain.[6]

Multiple studies reveal that hemoglobin and heme levels are significantly elevated in Alzheimer's brain tissue and can cause changes in glial activity.[8,9] These levels are increased in humans and in APP/PS1 genetically modified Alzheimer's mouse models potentially as a consequence of a leaky blood brain barrier, which is characteristic of AD pathology.[9] Chuang et al. found that heme is the binding component of hemoglobin.[8] Their work suggests that the binding of heme to amyloid beta prevents toxic oxidative species from being generated by heme,

an oxidizing agent. Though they identify that heme binds to amyloid beta, Chuang et al. noted that mechanisms of binding are still unclear.[8] Further studies analyzing elevation of heme in AD brain tissue explain that heme binding to amyloid beta decreases the bioavailability, suggesting a potential heme deficiency in the brain, although there is little to back up this claim.[9] Other researchers believe heme may play a negative role in Alzheimer's disease, as heme-amyloid beta complexes are identified as having peroxidase activity and can suppress immune function.[10]

Though heme is identified as a player in pathology, little work has been done to outline mechanisms of heme interactions and how this molecule contributes to declining cognitive function. Heme theory in Alzheimer's pathogenesis is undervalued due to a lack of knowledge on the chemistry of heme interactions. Ghosh et al. performed work that aims to fill this gap by exploring the active binding sites of heme and the reactivity of this bound complex.[11] Their work revealed specific binding sites of heme and note these molecular interactions are associated with "pathologies like down-regulation of NO, hypometabolism, abnormal heme homeostasis, and overexpression of heme oxygenase."[11]

More recently, by establishing a method for elucidating immunomodulatory effects of proteins, Sankar et al. studied heme interactions at a physiologically relevant level of heme and identify heme as a key immunomodulator.[3] Results demonstrate that heme plays a role in suppressing amyloid beta mediated glial responses, forming a bridge between heme and neuroinflammatory theories.[3] From experiments with wild type mouse primary glial cells and treatment with heme and amyloid beta, results showed that heme suppresses activity of astrocytes by reducing expression of CD36, a proinflammatory cytokine.[3] Furthermore, this work revealed a reduction in scavenger and lysosomal activity of astrocytes through experiments using

pHrodo beads.[3] pHrodo beads are a tool used to indicate phagocytotic activity, as they fluoresce in phagosomes within the cell. Results show that levels of fluorescence are decreased in the presence of heme.[3] These results are significant as scavenger activity is important in the clearance of toxic substances like amyloid beta.[4] This reduction in scavenger activity could contribute to increased cognitive decline and neurodegeneration. Sankar et al. also revealed that the PI3K/Akt is upregulated by heme; this pathway is believed to be important in regulating immune function.[3] This research concludes that heme is important in modulating inflammatory response and neuroinflammation lies on a spectrum between neuroprotective and destructive, depending on the level of glial activation.[3]

Heme has been well defined as a player in modulating neuroinflammation. Its impacts on glial function and neuronal death have been outlined, yet studies analyzing its role in signaling are lacking. According to Sankar et al., the PI3K/Akt pathway is upregulated, and this pathway is important in modulating inflammatory response and cell survival.[3,12] This pathway is found to be modulated by heme, yet there is a gap in research that evaluates the mechanisms of heme signaling interactions with further pathways in immune function and cell survival such as the MAPK pathway. The key to moving forward in understanding heme signaling interactions lies within expanding experiments involving various pathways and inhibitors as a means of attempting to restore glial function.

Through review of the various approaches to Alzheimer's disease and examination of how heme and neuroinflammation have been linked in recent studies, we can identify potential avenues for therapeutic targets based on further exploration of heme interactions. My work aims to fill this gap, as well as study bioavailability of heme using a novel genetically encoded FRET based heme sensor, created by collaborators in the Reddi Lab.[13] Understanding the

bioavailability of heme and mechanisms of signaling pathways responsible for glial disruption and inflammatory response will allow for future targets and expansions upon potential heme-based therapies for Alzheimer's disease.

Materials and Methods

Preliminary work done in my lab, the Wood Lab, has established that heme plays a major role in regulating inflammation in Alzheimer's disease.[3] My work aims to extend upon previous findings through quantitatively analyzing the interactions of heme and inflammatory cell signaling pathways as well as qualitatively studying the bioavailability of heme in these interactions. Experiments were designed to determine which pathways regulate heme degradation and mechanistically, whether heme interacts with glial cells via internalization or external factors. Analysis of the bioavailability of heme was done by transfecting heme sensors into cells. In order to study internal interactions, cells were cultured and then treated with various combinations of inhibitors. After all studies were completed, data was analyzed. Experiments in cell signaling and bioavailability were conducted using the following methods.

Cell Culture

Primary astrocyte cells were collected from postnatal day 0-1 CD1 mice (Charles River Laboratories) by the supervising graduate student under the approved IACUC protocol. Pathology studied in mouse tissue is easily translatable to clinical implications and removes the need for experimentation on human tissue. Brains were harvested and the cortices were isolated from the meninges. Cortices were broken apart by trituration in a plating medium consisting of minimum essential medium (Thermo Fisher Scientific) with 10% horse serum (Sigma), 1% antibiotic/antimycotic solution (Sigma), and 0.3% glucose solution (Sigma). Cells remained in this medium overnight in a T-75 flask to allow for adherence. The T-75 flasks were previously treated per the manufacturer's protocol with 0.1 mg/ml poly-D-lysine (Sigma), a positively charged amino acid polymer that when adhered to a surface increases the number of positively charged binding sites for cell adherance.[14] After 24 hours, the plating medium was replaced with

a culture medium consisting of astrocyte medium (ScienCell) with 2% fetal bovine serum (ScienCell), 1% penicillin/streptomycin solution (ScienCell), and 1% astrocyte growth serum (ScienCell). The cultures were maintained at 37°C and 5% CO_2 in a humidified incubator. Astrocytes were periodically passaged using trypsin (Sigma) and fresh culture media was added to allow for growth. Cells were passaged a maximum of four times and were utilized for experimentation at 80% confluency.

Cell Conditioning

For experiments aimed at analysis of heme interaction with cell signaling pathways, astrocytes were trypsinized and plated in a 96-well plate. Cells were allowed to adhere for 24 hours in preparation for treatment with various conditions. Pathway inhibitors rapamycin (Selleck Chemicals), MK2206 (Selleck Chemicals), and SB203580 (Selleck Chemicals) were applied to columns of wells of cells at a concentration of 1:1000 (Figure 2). Each inhibitor was chosen to target pathways that are known to play a role in the inflammatory response: rapamycin is an mTOR inhibitor, MK2206 inhibits the AKT pathway, and SB203580 inhibits the p38 MAPK pathway, which is typically activated by cellular stress and

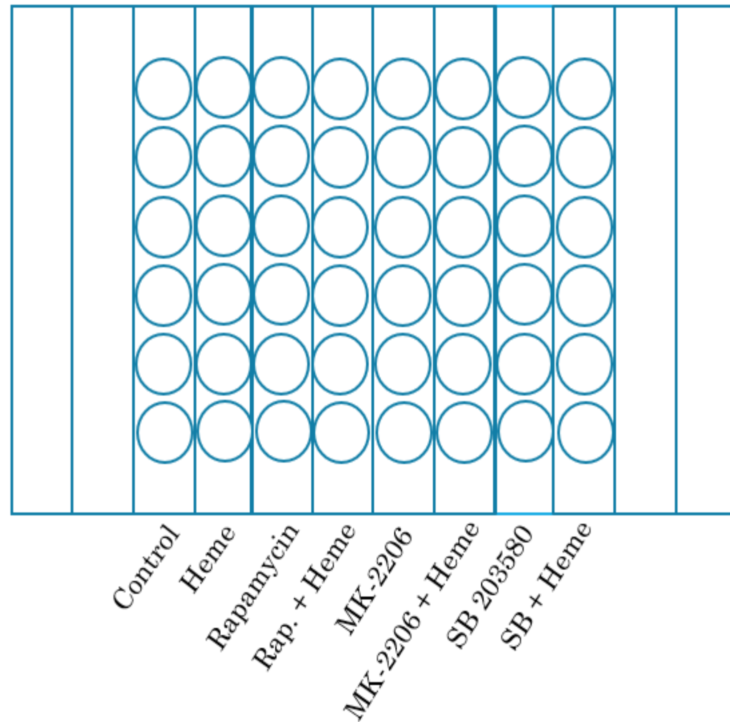

Figure 2. 96 Well Plate Layout for Inhibitor Experiment. Each circle represents one well. Each column was treated with a different treatment and conditions were done in multiples of 6.

inflammatory cytokines. After treatment with inhibitors, cells were returned to the incubator to rest at 37°C and 5% CO_2 for one hour to allow activation of the inhibitors. The inhibitor treatments were then aspirated and replaced with either fresh dilutions of the designated inhibitor or a combination of inhibitor and heme in the form of hemin chloride (25 uM, EMD Millipore) in astrocyte media. Cells were then incubated in treatments for 24 hours before analysis and imaging to determine activation of heme degrading enzymes.

Immunocytochemistry and Microscopy

After conditioning as previously described, the media was aspirated and cells were rinsed with sterile 1xPBS. Cells were then fixed using 4% paraformaldehyde and incubated for 15 minutes at room temperature. Cells were then rinsed with PBS and permeabilized by 0.1% Triton-X, followed by blocking by incubation in blocking buffer (1xPBS, bovine serum albumin, goat serum). After 1 hour, cells were incubated overnight at 4°C with an anti-heme-oxygenase-1 primary antibody (Novus) diluted in wash buffer. The wash buffer consists of PBS and bovine serum albumin. Cells were then rinsed 5 times with wash buffer and incubated in Alexa Fluor 555 (1:200) secondary antibody for 2 hours at room temperature. Cultures were kept covered to prevent light exposure and bleaching of fluorescent staining. Cells were rinsed once again with wash buffer, then counterstained by incubation in DAPI (1:1000) for 30 minutes to 1 hour at room temperature. After the counterstain, cells were washed in wash buffer and rinsed with PBS. After the final rinse, the cells were prepared for imaging by storage at 4°C in 1xPBS.

Cells were imaged via confocal microscopy using a Zeiss LSM 700 laser scanning inverted microscope. Images were acquired to quantify fluorescence and analyze changes in HO-1 expression after treatment with various pathway inhibitors. HO-1 is an enzyme that catalyzes

heme degradation and studying expression of HO-1 in the presence of inhibitors will reveal which pathways play a role in modulation.

FRET-based Sensor Transfection and Heme Uptake Quantification

For experiments to analyze heme uptake, astrocytes were seeded in T-25 flasks and allowed to adhere for one hour. The media was then aspirated from the flask and replaced with astrocyte media containing 10% heme depleted FBS and 0.5 mM succinyl acetone (Sigma), to inhibit heme synthesis. Each flask was treated with a combination of Lipofectamine LTX with PLUS Reagent (Invitrogen), opti-mem reduced serum media (Thermo-Fisher) and a plasmid containing the Förster resonance energy transfer (FRET) based heme sensor developed by collaborators in the Reddi lab(Figure 3).[13] Lipofectamine is a liposome formulation that allows plasmids to easily pass through the cell membrane and be transfected into cells.[15] Once transfected with the FRET based heme sensor, cells were treated with heme (25 μM) and analyzed to determine the bioavailability of heme. The FRET based sensor has mKATE2 and eGFP channels. mKATE2 fluoresces red and shows which cells have been successfully transfected. EGFP fluoresces green and is responsive to heme. As the cell uptakes more heme, the heme binding quenches the GFP channel, causing green fluorescence to decrease.[13] Analysis was done by fluorescent imaging and quantification of the ratio of mKATE2 to EGFP.

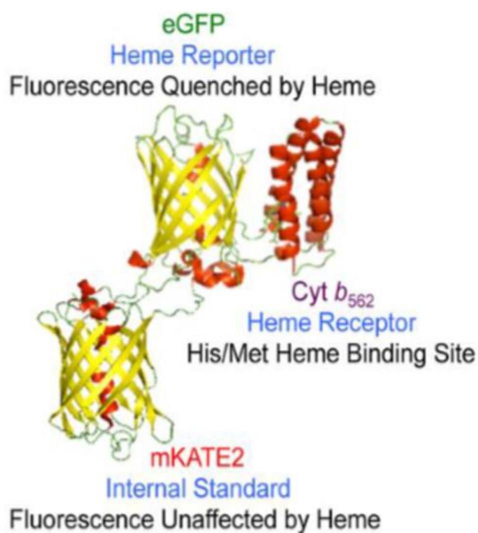

Figure 3. Diagram of Binding Sites on FRET Based Heme Sensor [12]

Results

Heme is internalized by primary astrocytes, as measured by ratiometric heme sensor proteins

Primary mouse astrocytes were transfected with FRET based heme sensor proteins, as described in the methods section, to allow for analysis of heme interaction. We used the sensors to monitor the uptake of heme in real time, which is useful for determining extracellular versus intracellular effects of heme on cell activity, allowing for a better understanding of heme bioavailability. After transfection with the heme sensor proteins, astrocytes were treated with 25 µM heme and allowed to incubate for 4 hours before observation. Succinyl acetone was used in both the control and treatment group cells, as it depletes intracellular heme to create a baseline of minimal heme binding to the sensor.

The heme sensor has a unique design and contains two fluorescent proteins, mKATE2, and EGFP, that can be compared ratiometrically to determine the uptake of heme into cells. mKATE2 fluoresces red and shows which cells have successfully transfected. EGFP fluoresces green and is responsive to heme. As the cell uptakes more heme, the heme binding quenches the GFP channel, causing green fluorescence to decrease. These values can be quantified using fluorescent imaging and the ratio can be compared to a control ratio to determine changes in heme uptake over time.

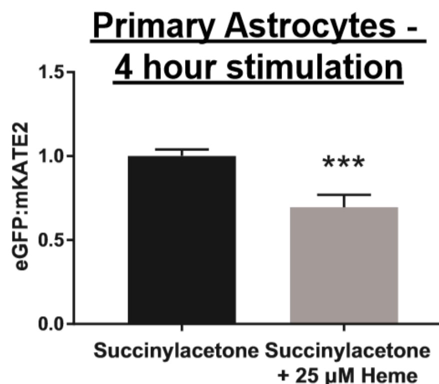

Figure 4. Treatment with heme reduces the ratio of EGFP to mKATE2, indicating internalization of heme by primary astrocytes

Results from the transfection experiment are shown in Figure 4. After incubation, the cells were imaged using Zeiss LSM 700 laser scanning inverted microscope. Images were used to quantify fluorescence of EGFP and mKATE2. Results show that cells treated with heme had a

decreased ratio of EGFP to mKATE2 when compared to the control, signifying internalization of heme by the astrocytes.

Treatment with pathway inhibitors reveals that heme interaction is mediated by the mTOR pathway

Results from the heme sensor experiment revealed that heme interacts with glial cells, specifically astrocytes, via internalization. Therefore, the pathway inhibitor study was conducted by treating cells with various inhibitors for different pathways suspected to play a role in mediating heme interaction through internal signal transduction. Cells were cultured, treated, and stained for HO-1, as described previously, and then imaged. Results from treatment with either rapamycin, MK2206, or SB203580, compared to corresponding treatment with inhibitor and heme, can be seen in Figure 5A. Red florescence signifies HO-1 expression, which is an enzyme that degrades heme into its byproducts, and blue signifies DAPI, which stains the nucleus of every live cell. Treatment with heme greatly increases HO-1 expression compared to the control; this occurs because astrocytes internalize heme and begin to break down the molecule. This result is quantified in Figure 5B. Furthermore, treatment with each individual inhibitor shows similar HO-1 expression to the control, as no heme is applied to these samples and the enzyme is not active.

When comparing all inhibitors to the control, rapamycin, an mTOR inhibitor, is the only one of the three that significantly decreased HO-1 expression. This is quantified in Figure 5C, which compares HO-1 expression in the heme treatment group compared to the rapamycin + heme treatment group. This result is presented visually in Figure 5D, which shows decreased red florescence, or HO-1 expression, in a heme and rapamycin group versus a control heme group.

Treatment with rapamycin reduces red florescence, or HO-1 expression, close to the level of control expression, indicating the mTOR pathway partially regulates internal heme interaction.

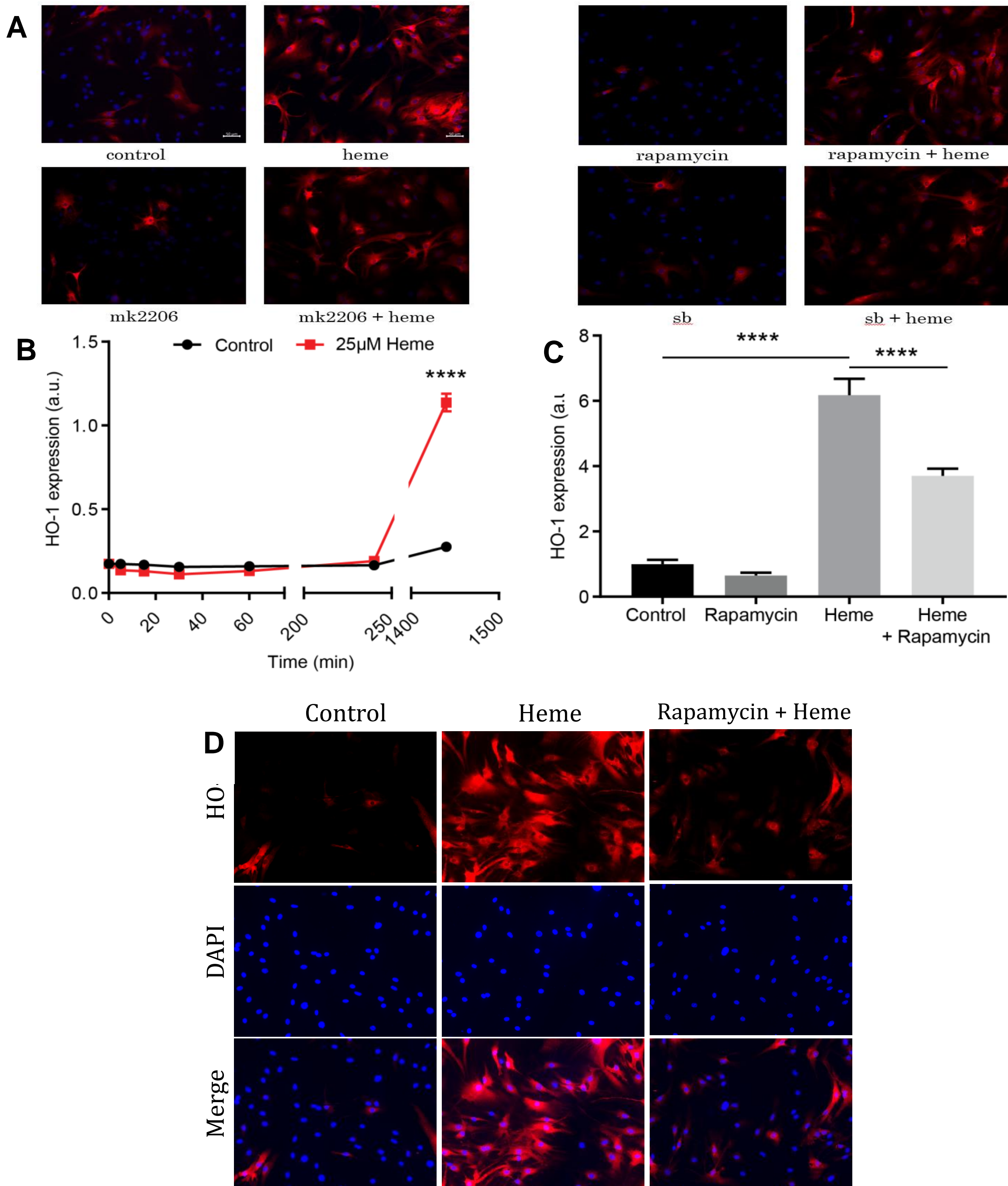

Figure 5. HO-1 expression increases with 24- hour heme treatment and is partially regulated by mTOR. A) Florescent images of astrocytes and HO-1 expression after treatment with heme and inhibitor B) Time Dependent HO-1 Expression. HO-1 expression increases with 24-hour heme treatment. C) Quantification of HO-1 expression in astrocytes treated with heme and rapamycin, the inhibitor for the mTOR pathway. D) Florescent images of astrocytes treated with heme and inhibitors. Red represents HO-1 expression and blue represents DAPI staining.

Discussion

The present study was designed with the goal to examine the mechanisms by which heme interacts with glial cells and its contributions to further cognitive decline. Experiments were performed to analyze the bioavailability of heme in relation to glial cells to determine if the mechanism by which the interaction occurs is extracellular and utilizes a receptor or if the heme is internalized by the cell and further changes occur in cell signaling pathways. FRET based heme sensors were used to measure the amount of heme that was internalized by the astrocytes and results showed that cells treated with heme had a decreased ratio of EGFP to mKATE2 when compared to the control, signifying internalization of heme by the astrocytes (Figure 4).

Results from the study are significant as they reveals that heme is internalized by cells. This signifies that astrocytes do uptake heme, which causes downstream signaling changes and potentially plays a role in regulating immune function and cell viability, although the internal pathway may not necessarily be the casual pathway. Understanding how heme is internalized by these cells will help determine a mechanism for the action of heme on cells, which will be useful for developing heme-based therapies. The results from this set of experiments helped to drive further experiments in uncovering which internal signaling pathways are responsible for mediating a response.

Experiments using pathway inhibitors were performed in order to gather information about the internal mechanism of heme interaction. Through the use of inhibitors, we can manipulate the cell's response to heme and discover which pathways may play a role in modulation. The results from this experiment, as shown in Figure 5, reveal that rapamycin was the only inhibitor that significantly reduced HO-1 expression in the presence of heme in astrocytes, as quantified and seen visually by red fluorescence. Rapamycin is an mTOR pathway inhibitor; because treatment with this inhibitor revealed a decrease in HO-1 expression, and ultimately less heme degradation.

Therefore, we can conclude that the mTOR pathway plays a role in the modulation of internal heme interaction with astrocytes.

Overall, findings from the present study are significance as they provide a starting place for future work aiming to expand on the role of heme in Alzheimer's. Further research can be directed to delve deeper into uncovering specific mechanisms by which the mTOR pathway plays a role in modulating the way heme interacts with glial cells. By determining the pathway responsible for these modulations, we can contribute to a knowledge base for future research to target this pathway in a therapeutic manner and potentially halt the impacts of heme on cognitive decline in Alzheimer's disease.

Conclusion

This study aimed to fill a gap in current research regarding Alzheimer's disease, by studying the bioavailability of heme and the pathways that may play a role in modulating heme interaction with glial cells. My research examined how heme and neuroinflammation have been linked in recent studies and identifies potential avenues for therapeutic targets based on further exploration of heme interactions. Heme bioavailability was studied using a novel genetically encoded FRET based heme sensor, created by collaborators. Prior research shows heme can lead to further neurodegeneration and cognitive decline in patients. Experiments using heme sensors revealed that heme is internalized by astrocytes. To further examine this, pathway inhibitors were used to manipulate cellular response to treatment with heme and uncovered that the mTOR pathway plays a role in regulating HO-1 expression and internal heme interaction.

Future work based off this study should focus on expanding the scope of heme sensor and inhibitor experiments to examine heme interactions in other glial cells types such as neurons and microglia. Heme may interact with these cell lines differently and understanding the effects on mechanism in these cells is important to assess the overall role of heme in increasing cognitive decline. The role in internal versus external pathway interactions should be further studied to determine which has a definitive casual role, or if both are involved. Furthermore, mTOR was determined to partially mediate internal heme interactions as seen in decreased HO-1 expression when inhibited. This pathway should be further explored through more in-depth testing, such as a Luminex assay, so that it can be fully characterized as a potential target for future therapy. Overall, understanding the bioavailability of heme and mechanisms of signaling pathways responsible for glial disruption and inflammatory response is important as it will allow for future targets and expansion of potential heme-based therapies for Alzheimer's disease.

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