THE EFFECT OF NOVEL ANTI-INFLAMMATORY DRUGS ON ALZHEIMER'S DISEASE

by

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ABSTRACT

Alzheimer's Disease is a neurodegenerative disease characterized by cognitive, functional, and neuronal loss. Its core pathology includes beta-amyloid protein plaque formation, neurofibrillary tangles of tau protein, and loss of microglial cell function, all of which may be facilitated or exacerbated by a prolonged neuroinflammatory response. The inflammatory signaling pathway culminates in the activation of transcription factor NF-κB, which then goes on to activate the expression of cytokines and other signaling molecules such as TNFα. One of the points of regulation for this pathway is the binding of the IκBα protein to NF-κB that prevents NF-κB from entering the nucleus. However, when the appropriate stimulus triggers the pathway, a downstream effect is the phosphorylation of IκBα by the IκB kinase, and its subsequent degradation which then releases NF-κB for translocation into the nucleus.

This project aims to elucidate the mechanism of action of novel anti-inflammatory drugs (provided by P2D Biosciences company). Previous *in vivo* studies with the compound have shown a reduction in inflammation and improved cognition, but the drug's exact point of interference in the pathway remains unclear. We hypothesize that the drugs reduce inflammation by reducing IκBα degradation, thus preventing NF-κB from turning on cytokine expression.

BV-2 mouse microglial cells were exposed to the drugs, then to LPS for various time intervals, then harvested and lysed. A Western blot procedure was performed on the lysates to visualize the amount of IκBα present, then those bands were quantified to compare against control cells that were not incubated with the drug. It follows then, that if the drugs' mechanism of action is inhibition of NF-κB release into the nucleus, then there will be increased amounts of IκBα in the treatment cells compared to the control cells as IκBα degradation is prevented.

INTRODUCTION

Alzheimer's Pathology

Alzheimer's Disease is a neurodegenerative disease characterized by cognitive, function, and neuronal loss. As the most common form of dementia, it often results in memory loss and is associated with impaired judgement and vision/spatial issues. Core pathology typically includes formation of beta-amyloid (Aβ) plaques between neurons, tau protein tangles, and a loss of microglial cell function (resident macrophages that clear away Aβ protein buildup). This disease currently affects over 5 million Americans, though this number is expected to rise as the population ages, and it presents a huge financial burden. Thus, understanding the mechanisms responsible for pathogenesis and thus identifying novel therapeutic targets is crucial.

Aβ protein is formed from the amyloid precursor protein (APP); the precise function of APP is uncertain, although it has been shown to have a role in cell health and growth. APP is expressed in neurons and has multiple proteolytic pathways that allow for the production of various soluble peptides. However, one particular cleavage pathway involving BACE1 protease and γ-secretase creates insoluble Aβ monomers, which are dumped into the extracellular space where they can eventually aggregate into Aβ oligomers, which can go on to form plaques. In their aggregated form, these Aβ assemblies can induce neurotoxicity and thus disrupt synaptic functions of the nervous system. It also may promote cell death (neurodegeneration) by facilitating the formation of peroxynitrite radicals (Murphy, O'Brien, 2011).

Tau proteins are found in mature neurons, and their job is to promote the assembly of tubulin into microtubules and provide subsequent stabilization of those microtubule structures (Iqbal, 2010). When phosphorylated, the affinity of tau for the microtubule is reduced and its binding to microtubules is decreased, allowing for intracellular trafficking to occur, then to

return tau to the microtubule, the protein is dephosphorylated (Guo, 2017; Kinney, 2018). However, when the protein is hyperphosphorylated, it tends to aggregate and form neurofibrillary tangles (NFTs), which cannot interact with tubulin nor work to promote microtubule assembly. Additionally, much of the hyperphosphorylated tau can be found in the cytosol (as opposed to in an NFT), where it inhibits microtubule assembly and actively disrupts microtubules. With these effects in consideration, intracellular trafficking, cell morphology, and other cellular processes become compromised due to the loss in microtubules and support for existing microtubules.

Microglia are the resident immune cells in the central nervous system that are constantly surveying the environment for any threats like invasion, injury or disease. Once such a threat is detected, the microglial cell is activated and undergoes morphological changes including enlargement and migration. It is hypothesized that in Alzheimer's, Aβ buildup activates the microglia and triggers localization to and phagocytosis of the plaque, as well as the release of pro-inflammatory cytokines to recruit additional microglia. However, after prolonged inflammation, microglia have demonstrated a loss in their ability to process the plaques. As a result, the initial Aβ-processing activity observed in early Alzheimer's eventually gives way to Aβ accumulations as the microglia lose their efficiency for binding and phagocytosing the proteins (Kinney, 2018).

The Role of Chronic Inflammation

Inflammation is a key defense against pathogens, injuries, and other threats to the body that has been extensively studied. Physiologically, it is associated with edema, erythema, warmth, pain, and loss of function as the damaged cells release histamine, bradykinins, and prostaglandins. In response to these signaling molecules, vasodilation occurs and capillary

permeability increases to allow for increased blood flow to the site of tissue injury. Immune cells such as neutrophils and macrophages are brought to the site to clear away pathogens or other threats (Chen, 2017). The inflammatory response is protective to the body during an acute-phase response, but it is a danger when the response becomes prolonged.

In Alzheimer's Disease, chronic inflammation is thought to actually be a result from the pathology rather than a chief cause— the accumulation of Aβ continues to activate microglia cells to release their proinflammatory cytokines, which results in the buildup of cytokines, chemokines, and reactive oxygen and nitrogen species. Data indicates that throughout this continual activation, the microglia lose their ability to effectively break down the Aβ plaques and recruit peripheral macrophages to help take over the job, but in the process, they likely exacerbate the consequences of prolonged inflammation (Jay, 2015). Additionally, microglial activation is thought to contribute to tau phosphorylation and the subsequent NFT formation. Therefore, it is believed that the prolonged neuroinflammation that is observed in Alzheimer's is a downstream effect from the core pathologies that in turn either facilitate or exacerbate those Aβ and tau protein aggregations (Kinney, 2018).

The NF-κB Pathway and Inflammation

One of the important pathways leading to inflammation involves the transcription factor NF-κB, which acts as the "switch" that turns on the transcription of various pro-inflammatory cytokines, chemokines, and other mediators. NF-κB is regulated via constitutive binding to an inhibitory protein called IκBα that sequesters it in the cytoplasm, away from its target genes. Site-specific phosphorylation of IκBα by IκB Kinase (IKK) induces ubiquitination and degradation of IκBα by the proteosome, thus freeing NF-κB to travel into the nucleus to induce transcription of its target genes. (Liu, 2017). IKK activity is regulated via phosphorylation from

the signaling cascade that is triggered upon receptor activation by appropriate stimuli, which includes microbial products/substances such as lipopolysaccharide (LPS), and intercellular cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Chen, 2017). Additionally, overexpression of these cytokines and the subsequent NF-κB upregulation have been implicated in other inflammatory diseases such as rheumatoid arthritis (Barnabei, 2021).

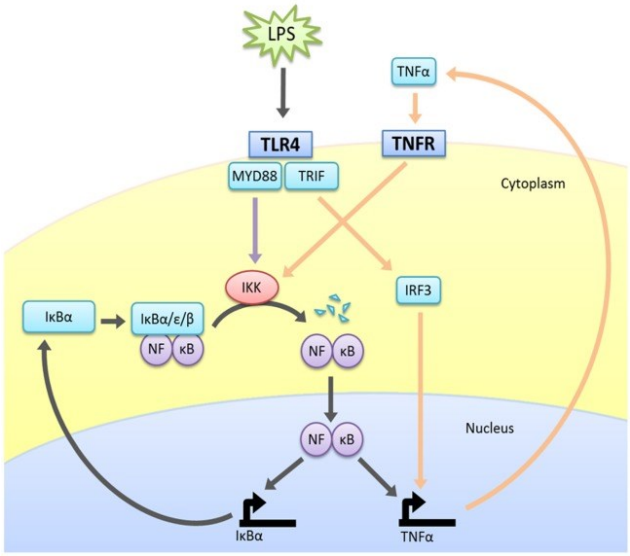


Fig. 1: The NF-KB pathway upon LPS binding with the TLR4 receptor. IKK is activated, then phosphorylates IκBα which results in its degradation. NF-κB is free to enter the nucleus and promote transcription of cytokines and IκBα.

Of the genes influenced by NF-κB activation, two are important to note. TNF-α, as mentioned before, is also capable of stimulating the NF-κB pathway, so the upregulation of this protein creates a positive feedback loop as the resulting TNF-α goes on to trigger the pathway again. The upregulation of IκBα is relevant because it is the protein responsible for inhibiting NF-κB— therefore, NF-κB effectively causes its own demise by promoting the creation of its own inhibitor.

LPS is a pathogen-associated molecular pattern present in the outer membrane of Gram-negative bacteria. Because it is commonly found in bacteria and not produced in mammalian cells, it is a strong inducer of the innate and adaptive immune response, and recognition and binding of this molecule results in the activation of signaling cascades leading to inflammation (Park, 2013). 1L-1β is a pro-inflammatory interleukin signaling molecule produced in a variety of cell types, most notably cells of the innate immune system such as macrophages. It can be secreted in response to toxins, bacterial infection, and inflammasome activation (Lopez-Castejon & Brough, 2011). TNF-α is another cytokine involved with immunity and inflammation that is produced in a variety of immune cells, particularly microglia in the central nervous system (Caminero et al., 2011).

HYPOTHESIS

In *in vivo* studies, the P2D compound has been shown to reduce inflammation and improve cognition in mice; there is the hope that the drug will be able to do the same in human models to alleviate some of the known symptoms of Alzheimer's Disease. However, the compound's mechanism for achieving those effects remains unclear. Prior studies examining the effect of P2D2244 on NF-κB activation have reported a decrease in the secretion of TNF-α, one of the target genes, as well as a decrease in target gene transcription in the presence of the drug (Abdalla & Paugh, personal communication). Together, these studies suggest that P2D2244 inhibits NF-κB activation at some point in the pathway.

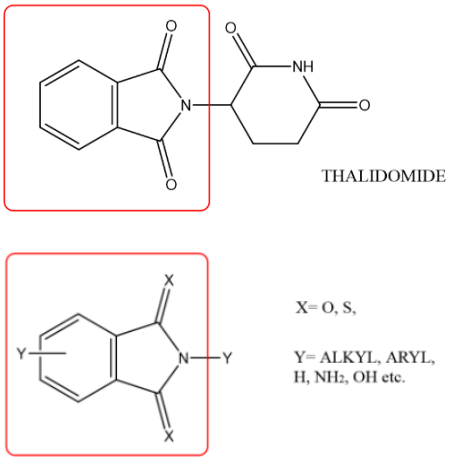


Fig. 2: The structure of the P2D compound is an analog of the isoindolinone moiety of thalidomide.

Given the complexity of the IκBα/NF-κB pathway, there are multiple potential points in the pathway the drug could be modulating in order to reduce the inflammatory response. For example, it could exert its influence by inhibiting the gene expression of pro-inflammatory cytokines, or by inactivating the receptor for those signals. This particular project focuses on the possibility of the drug interfering with the IκBα/NF-κB interaction, and tests the hypothesis that the P2D compounds prevent IκBα degradation to reduce NF-κB activation and subsequently block inflammation.

If our hypothesis is correct, we should see an overall decrease in IκBα degradation in cells treated with the drug. However, interpreting the protein quantification results requires a preface regarding the typical pattern of IκBα degradation: when the inflammation pathway is stimulated in normal, untreated cells, there is an initial decrease in IκBα levels followed by a natural rebound. This corresponds with the fact that IκBα is one of the genes upregulated by NF-κB— as discussed before, upon activation of the pathway, IκBα is degraded to free NF-κB and creates that observed decrease in IκBα levels. However, as NF-κB activity continues and begins to express its target genes, that also includes the production of IκBα, which then causes the observed recovery of IκBα levels. It stands, then, that if the hypothesis is correct and the P2D drug works to prevent chronic NF-κB activation by blocking IκBα degradation, one would expect to see comparatively higher IκBα levels at *all* time points, even taking into account the natural decrease/resynthesis pattern, in the cells treated with the P2D drug.

METHODS

Antibody Selection

Three different anti-IκBα antibodies were tested for activity via a Western blot with various lysates: SC1643, CST92425, and Sigma SAB1305978. From there, a suitable anti-IKB antibody (Sigma SAB1305978) was identified and selected for further use in these experiments.

Cell Treatment/Harvesting

BV-2 cells were treated with 30 μM P2D (2244 or 340) (P2D Biosciences), as previous studies have shown that concentrations higher than 30 μM have led to cellular cytotoxicity. Then the cells were allowed to incubate for 30 minutes before being exposed to 100 μg/ml of LPS for 0, 5, 10, 20, 40, or 60 minutes.

The cells were harvested by aspirating the cell media containing the LPS, rinsing with PBS, then manually scraping the wells. The cells were then resuspended in SDS lysis buffer, incubated on ice for 15 minutes, and centrifuged at 14,000 rpm for 10 minutes at 4°C. After that, they were mixed with SDS loading buffer and boiled at 95°C for 5 minutes.

Bradford Assay

A Bradford assay was conducted using Bradford reagent (Sigma, B6916) according to standard protocol in a 96-well plate, and a standard BSA curve of 0, 0.5, 1, 2, and 4 μg/ml was generated. The resulting line equation was used to determine the protein concentration of each sample, then calculations were performed to determine the amount of each sample necessary to create an identical amount of protein across all samples.

Western Blot

The samples were separated using SDS PAGE, followed by transfer onto PVDF membranes at 100v for 1 hour. The membranes were blocked for an hour in 5% BLOTTO (5% w/v non-fat dry milk in TBST), followed by incubation with the primary antibody mixture for

either 2 hours at room temperature, or overnight at 4°C. Next, they were rinsed thrice by shaking, 10 minutes each, in BLOTTO (5% non-fat dry milk in TBST), then incubated with the secondary antibody mixture for one hour at room temperature on a rocker. The membranes were again rinsed in BLOTTO for 3 10-minute intervals, then bands were visualized using NBT/BCIP (Promega S3771) following the manufacturer instructions. At that point, they were allowed to briefly rinse in water then air-dried.

Analysis

Once the membranes had dried, their picture was taken with the FastGel Fas-V gel imager system and each relative band intensity was analyzed using ImageJ software. IκBα band intensity was normalized by dividing the values by their corresponding β-Actin band intensity value.

RESULTS

Identifying a Suitable IκBα-Antibody

Prior to starting experiments with P2D compounds, a suitable anti-IκBα antibody was identified. Three different potential anti-IκBα antibodies— SC1643, CST92425, and Sigma SAB1305978 were tested for activity with various cell lysates. The former two antibodies did not produce any bands, and although bands failed to show for both controls of β-Actin and tubulin, bands appeared for the samples treated with SAB1305978.

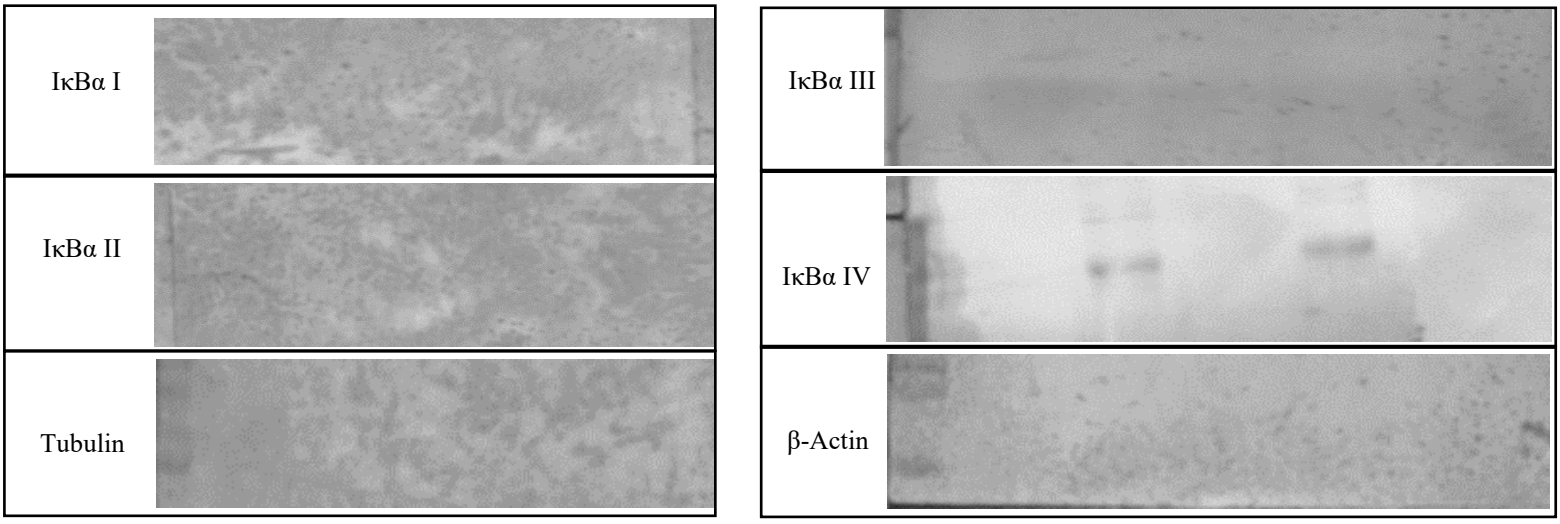
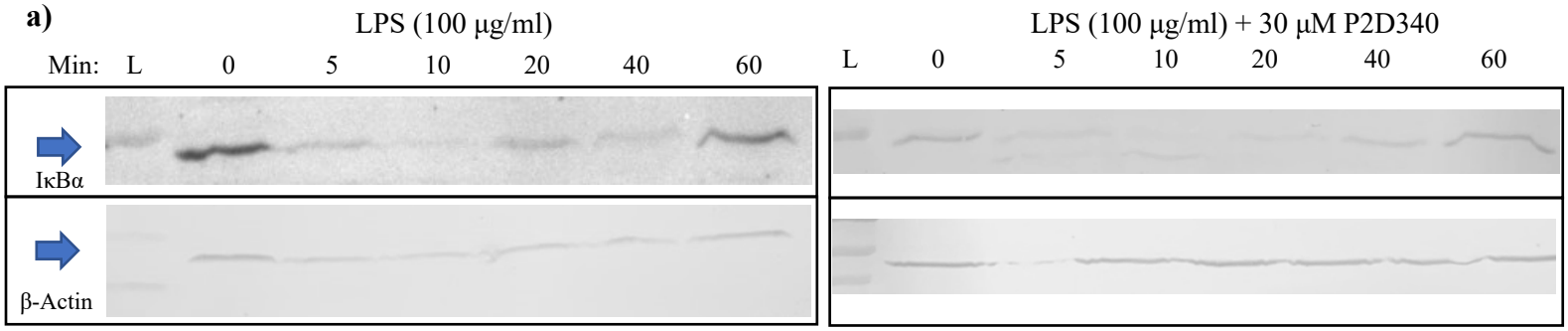


Fig. 3: Western blot results from various IκBα antibodies and batches SC1643 (I and II), CST92425 (III), and Sigma SAB1305978 (IV).

P2D340

P2D340 has been shown to improve memory and cognition in *in vivo* mouse disease models, but the mechanism has yet to be elucidated. This experiment with the P2D340 compound was conducted to determine if the drug inhibits NF-κB activation by inhibiting IκBα degradation. BV-2 cells were plated, incubated with 30 µM P2D340, exposed to 100 µg/ml LPS for time periods ranging from 0-60 minutes, then harvested according to protocol. This concentration was selected because previous MTT cytotoxicity studies have shown that concentrations above 30 µM exhibited significant cytotoxicity. A Western blot was performed on the samples and the bands corresponding to levels of IκBα were quantified.



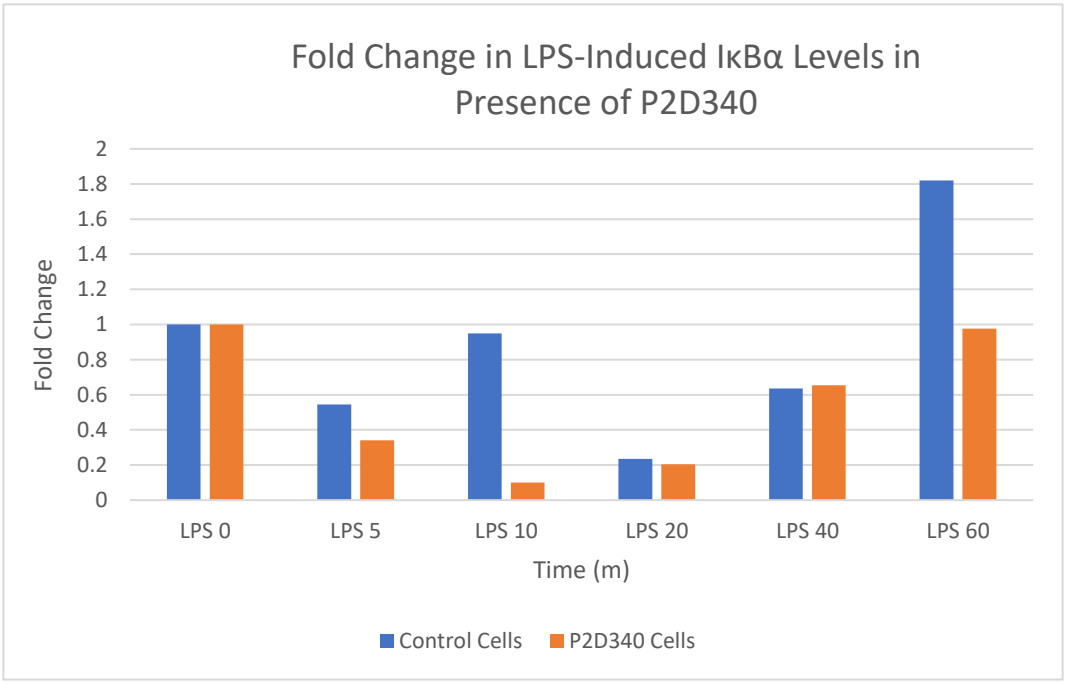


Fig. 4: Effect of P2D340 on LPS-mediated degradation of IκBα: **(a)** Western blot results **(b)** ImageJ analysis and quantification.

P2D2244

P2D2244 has been shown to improve memory and cognition in *in vivo* mouse disease models, but the mechanism has yet to be elucidated. This experiment with the P2D2244 compound was conducted to determine if the drug inhibits NF-κB activation by inhibiting IκBα degradation. BV-2 cells were plated, incubated with 30 µM P2D2244, exposed to 100 µg/ml LPS for time periods ranging from 0-60 minutes, then harvested according to protocol. This concentration was selected because previous MTT cytotoxicity studies have shown that concentrations above 30 µM exhibited significant cytotoxicity. A Western blot was performed on the samples and the bands corresponding to levels of IκBα were quantified. This experiment was repeated 5 times and the average IκBα fold expression data was compiled.

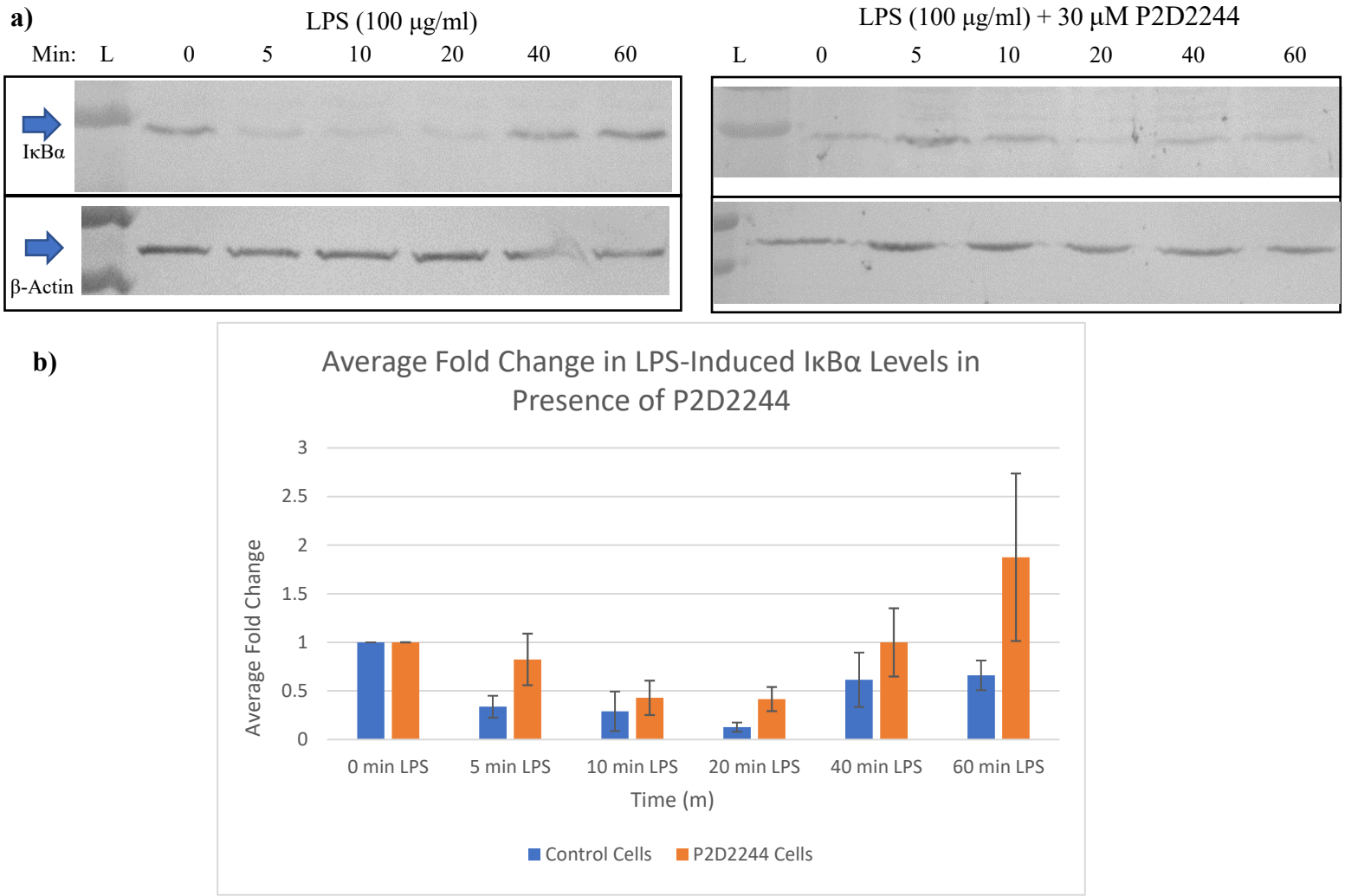


Fig. 5: Effect of P2D2244 on LPS-mediated degradation of IκBα: **(a)** Western blot results **(b)** ImageJ analysis and quantification. The sample is an average of 5 experiments.

DISCUSSION AND CONCLUSIONS

P2D compounds have demonstrated the ability to reduce inflammation and improve memory and cognition in *in vivo* mouse models, which indicate potential to alleviate those same symptoms in Alzheimer's Disease. This project examined the effect of the P2D compound (both P2D340 and P2D2244) on LPS-stimulated IκBα degradation: in each experiment, we were able to confirm the typical pattern of IκBα degradation in the control cells, then compare IκBα levels in the cells treated with the drug to the control cells.

The effect of P2D340 was tested, but this compound was ultimately not pursued further due to the relatively unconvincing results from our experiment that the drug induced a change in IκBα levels. According to the chart depicted in Fig. 4b, the cells exposed to P2D340 experienced more of a decrease (or, more of a fold change) in IκBα levels compared to the control cells. Thus, IκBα was degraded and NF-κB was freed to express its target genes, including the pro-inflammatory cytokines, *more* in the treated cells than the control cells. These results suggest that the drug does not prevent NF-κB activation by inhibiting IκBα degradation.

Five experiments in total were conducted to examine the effect of P2D2244 on LPS-stimulated IκBα degradation. Compilation and analysis of the data (Fig. 5b) showed that the cells treated with P2D2244 experienced, on average, a smaller decrease in IκBα levels (or, less of a fold change) at all time points of LPS exposure compared to the control cells. Given the inflammation pathway, this would imply that in the treated cells, IκBα was not being degraded as much, and thus NF-κB was not able to express its target genes, which includes pro-inflammatory cytokines. These results suggest P2D2244 inhibits degradation of IκBα, and when bolstered by the previous studies that reported decreased TNF-α secretions and NF-κB target gene

transcription (Abdalla & Paugh, personal communication) in the presence of the compound, may indicate a possible mechanism of action for P2D2244.

The results of this project suggest that P2D2244 may inhibit IκBα degradation at some upstream point in the pathway, but the exact point of interference remains unknown. Further research is needed to ascertain a more specific interaction by which the drug may be working to prevent IκBα degradation, so more can be known about its ability to reduce inflammation and ultimately alleviate aspects of the core Alzheimer's pathology in humans.

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