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COMMUNICATION

Interactions with amyloid beta peptide and acetylcholinesterase increase alkaline phosphatase activity

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Multiple studies have shown that the activity of alkaline phosphatase (AP) increases during Alzheimer's disease (AD). In this paper, using UV-Visible spectroscopy, we show that this increase in activity is due to its interaction with key components of AD such as amyloid β peptide and acetylcholinesterase. Activity increase also occurs due to high concentrations of acetylcholine and choline. These conditions are present in AD or could occur due to drugs used for treating AD.

Alzheimer's disease (AD) is a progressive, neurodegenerative disease that affects more than 6 million Americans (1). AD is characterized by the aggregation of two components in the brain, the extracellular amyloid-β peptides and intracellular tau protein (2). Amyloid-β peptides are produced in the brain when the precursor protein breaks down due to the activity of β and γ -secretases. Although peptides of different lengths are formed, the longer forms, specifically amyloid β-42, is deposited in the brain (3)(4). These peptides aggregate to form plaques. Recent evidence has shown that these amyloid peptides are associated with hyperphosphorylation of tau protein, which is present inside the neurons (5). This hyperphosphorylated tau forms neurofibrillary tangles which ultimately leads to neuron death and AD (6)(7).

In recent years, it has come to light that liver enzymes could play a role in AD. A recent study found that there is an association of increased liver alkaline phosphatase activity with poor cognition (8). Multiple studies have also shown that increased activity of plasma, serum, and tissue non-specific alkaline phosphatase are associated with cognitive decline or AD (9)(10). Alkaline phosphatase is an enzyme that removes a phosphate group from its substrate. It is known that alkaline phosphatase is present in the central nervous system and its function is to regulate the amount of phosphorylated compounds present (6). Upon neuron death, the hyperphosphorylated tau is released and alkaline phosphatase removes the phosphate groups from it. This could have a cascading effect since extracellular tau can be toxic for neurons. Extracellular dephosphorylated tau activates the muscarinic receptors and causes the influx of calcium into the cell which causes neuron death. This suggests that alkaline phosphatase plays a role in this positive feedback loop that causes neurodegeneration (11)(12)(13). However, the cause of its increased activity remains unclear.

In this work, we investigated changes in the activity of alkaline phosphatase (AkP) under different AD-relevant conditions. Upon reaction with p-nitrophenyl phosphate (PNPP), this enzyme forms p-nitrophenol (PNP) which absorbs at 405 nm. We conduct these experiments at pH 7 since it is close to the physiological pH and we use 50 mM HEPES buffer since it does not contain any phosphate salts that could affect alkaline phosphatase activity. We monitor this absorption to assay the enzyme activity. Our study reveals that alkaline phosphatase activity is affected by two species involved in Alzheimer's disease: 1) Amyloid β and 2) acetylcholinesterase.

As shown in Figure 1(a), the activity of alkaline phosphatase increases upon addition of amyloid β. In this work, we used custom synthesized amyloid β (42 amino acids long). The peptide sequence and characterization are given in the SI. We used concentrations close to 10 µM, since at that concentration, amyloid β causes neurotoxicity (14). This increase in activity is concentration dependent and increases with increasing concentration of amyloid β as shown in Figure 1(b).

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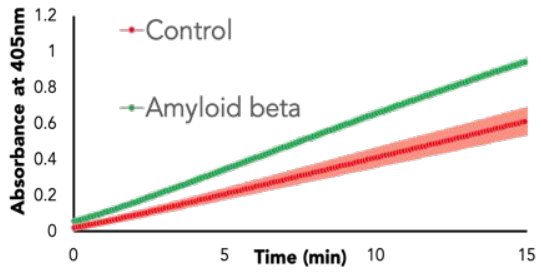
Electronic Supplementary Information (ESI) available: Materials, experimental methods, and supplementary figures. See DOI: 10.1039/x0xx00000x

(a)



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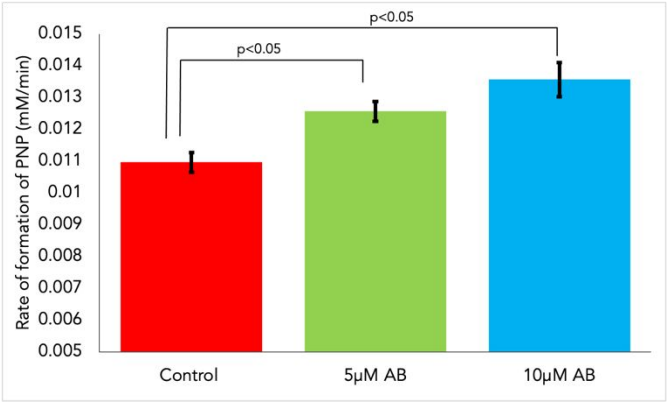


Figure 1: (a) Absorbance due to PNP formation at 405 nm measured using UV Visible spectroscopy in the presence and absence of 12.4 μM amyloid β (AB) for 0.2 μM alkaline phosphatase. (b) Effect of concentration of amyloid β on the rate of formation of PNP due to 0.2μM alkaline phosphatase. Results are the average of three trials and error bars represent the standard deviation from three trials.

The increase that we observed is comparable to the increases observed in cases of cognitive impairment or AD reported in the literature (Figure 2). This indicates that the increase in alkaline phosphatase activity observed in these studies could be due to the accumulation of amyloid β in the brain and its interaction with alkaline phosphatase.

In order to study this specific interaction, we performed a fluorescence resonance energy transfer (FRET) experiment to probe the aggregation state of alkaline phosphatase in the presence of amyloid β. A FRET experiment involves tagging one batch of alkaline phosphatase with the donor dye (Alexa Fluor 488) and one batch with the acceptor dye (Alexa Fluor 532). Upon aggregation (dyes separated by < 6 nm), energy transfer occurs between the two dyes. We excited the sample at 488nm and recorded emission between 500-600nm. Based on the emission of the acceptor and the total emission, we calculated FRET Efficiency, which is a measure of how much of the sample is aggregated. The FRET efficiency varies between 0 and 1 with 0 indicating no aggregation and 1 indicating complete aggregation. More details about the FRET experiment and analysis are given in the SI. We found that amyloid β de-aggregates alkaline phosphatase present in solution. (Figure 3)

Although amyloid β does not de-aggregate alkaline phosphatase by a large amount, the difference between the two FRET efficiencies after 5 minutes is statistically significant ($p<0.05$). This is a clear indication that amyloid β interacts with alkaline phosphatase.

To test if this phenomenon is general, we also repeated this experiment with alkaline phosphatase from *E. coli*, which is a different source than bovine intestinal mucosa, which was used previously. We found that alkaline phosphatase from *E. coli* showed a 17% increase in activity due to 10 μM amyloid β, suggesting that this phenomenon is general for alkaline phosphatase enzymes. (Figure 4) Interestingly, for alkaline phosphatase from *E. coli,* we don't see as much of a concentration dependence of the activity as the alkaline phosphatase from bovine intestinal mucosa.

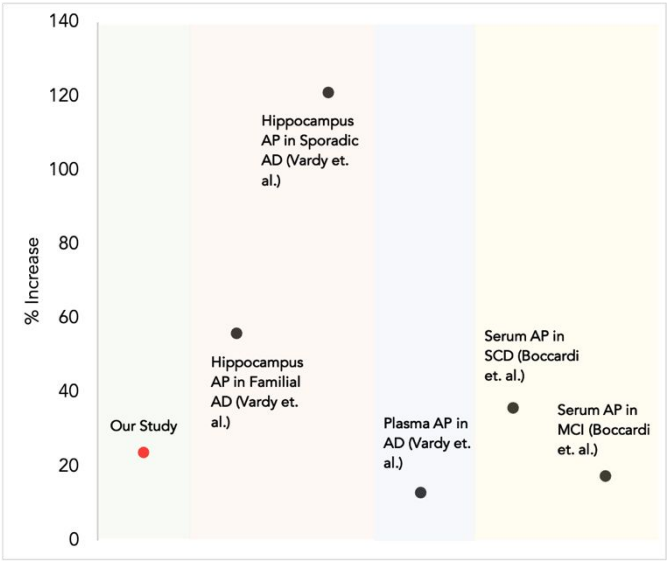


Figure 2: Comparison of increase in alkaline phosphatase activity observed in our study with familial and sporadic AD by Vardy et.al (10), and subjective cognitive decline (SCD) and mild cognitive impairment (MCI) by Boccardi et. Al (9). For Vardy et. al., % increases are between the TNAP activities in hippocampus and plasma AP as compared to age matched controls. For Boccardi et. al, % increases are between the adjusted means of ALP serum levels in healthy controls vs SCD and healthy controls vs MCI.

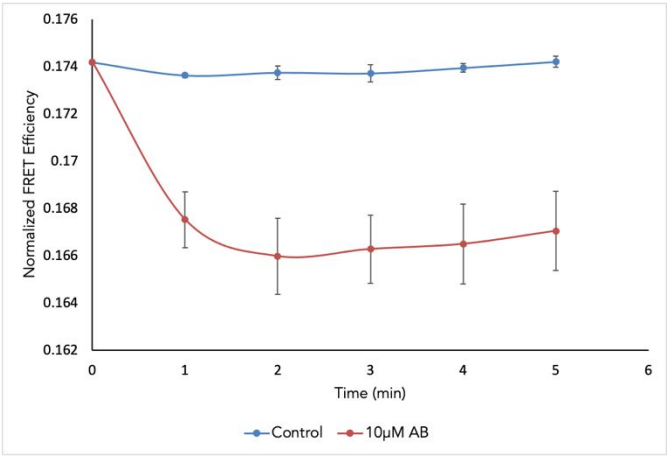


Figure 3: De-aggregation of 0.2 µM alkaline phosphatase observed with time in the presence of amyloid β (AB). Results are the average of three trials and error bars represent the standard deviation from three trials.

We tested if the activity of alkaline phosphatase increases due to other proteins such as bovine serum albumin (BSA). We found that the activity does not show a significant increase with the same concentration (10 µM) as well as the same amount by mass (0.7 µM) of BSA (Figure S1). This indicates that the interaction between amyloid β and alkaline phosphatase is specific and is not observed with all proteins.

Taken together, these results indicate that, due to a specific interaction between alkaline phosphatase and amyloid β, the activity of alkaline phosphatase increases.

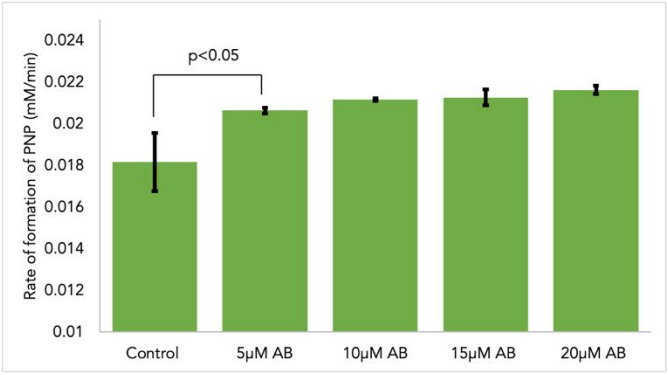


Figure 4: Rate of formation of PNP by 0.2µM alkaline phosphatase from *E. coli* in the presence of amyloid β (AB). Results are the average of three trials and error bars represent the standard deviation from three trials. Rate of formation of PNP in the presence of 5 µM AB is significantly different (p<0.05) from the control.

We also examined the effects of treatment procedures for AD. Cholinesterase inhibitors are commonly used for AD treatment (15)(16). Acetylcholinesterase (AchE) is an enzyme that converts the neurotransmitter acetylcholine to acetic acid and choline. It is present outside the neurons at synaptic junctions and is used to regulate the amount of acetylcholine (17)(18). There is a lowering of the amount of acetylcholine during AD. Thus, several approved drugs that are currently used, such as donepezil (Aricept®), rivastigmine (Exelon®), galantamine (Razadyne®), for treatment of AD are cholinesterase inhibitors. By inhibiting acetylcholinesterase, they ensure that the acetylcholine levels are maintained(15). In our study, we found that the activity of alkaline phosphatase also increases in the presence of acetylcholinesterase from *Electrophorus electricus* (electric eel) (Figure 5). This increase is also concentration-dependent.

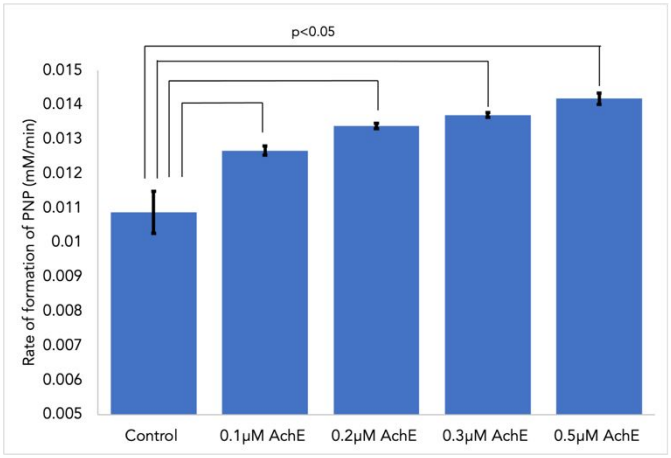
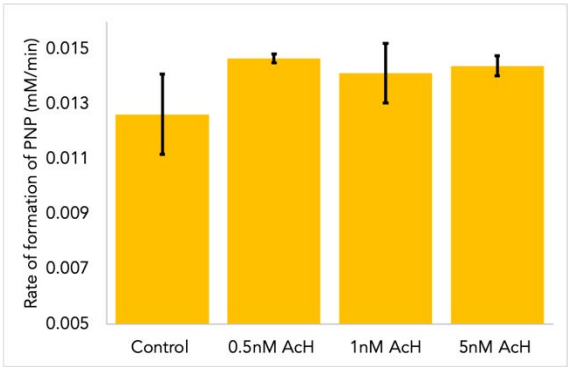


Figure 5: Rate of formation of PNP measured using UV Visible Spectroscopy in the presence of different concentrations of acetylcholinesterase (AchE) due to 0.2 µM alkaline phosphatase. Results are the average of three trials and error bars represent the standard deviation from three trials. 0.1, 0.2, 0.3 and 0.5µM AchE values are significantly different (p<0.05) from the control. Rates at the different concentrations of AchE are significantly different (p<0.05) from each other.

We also performed concentration dependent experiments to examine whether the activity of alkaline phosphatase also increases due to acetylcholine and choline at different physiological concentrations (Figures 6(a)(b)). For acetylcholine, the physiological concentration is very low. The concentration of acetylcholine in extracellular brain fluid is 0.1-6 nM (19). The concentration of choline in human cerebrospinal fluid determined by Haubrich et. al. ranged from 1.8-31.2 µM with a mean of 5.7 µM (20). We found that, at these concentrations, acetylcholine and choline do not affect the activity of alkaline phosphatase. However, at higher concentrations, both acetylcholine and choline increase the activity of alkaline phosphatase. (Figure S2). This is concerning because it means that if the concentration of acetylcholine increases due to cholinesterase inhibitor drugs, they could have a negative effect on AD. All these activity increases are summarized in Table 1. As expected, acetic acid did not increase alkaline phosphatase activity (Figure S3).

We also studied whether these interactions affect acetylcholinesterase activity. Using Ellman's assay, we monitored the formation of 5-thio 2-nitrobenzoic acid (TNB) which absorbs at 412 nm. We found that alkaline phosphatase and amyloid β do not have any effect on acetylcholinesterase activity. (Figure 7).

(a)



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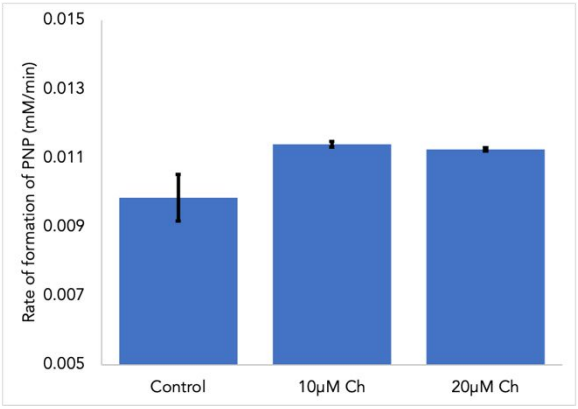

Figure 6: Rate of formation of PNP due to 0.2μM alkaline phosphatase measured using UV Visible spectroscopy in the presence of (a) nanomolar concentrations of acetylcholine chloride (AcH) and (b) micromolar concentrations of choline chloride (Ch). Results are the average of three trials and error bars represent the standard deviation from three trials. For both figures, none of the concentrations of AcH or Ch are significantly different ($p<0.05$) from the control.

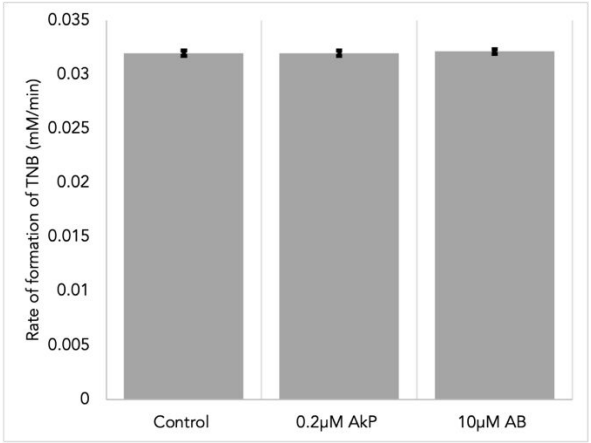

Figure 7: Rate of formation of TNB due to 0.01 μM acetylcholinesterase in the presence of 10 μM amyloid β (AB) and 0.2 μM alkaline phosphatase (AkP). Error bars represent the standard deviation from three trials. Activity in the presence of AkP and AB is not significantly different ($p<0.05$) from the control.

Conditions	% Increase
Amyloid β (10 μM)	24
Acetylcholinesterase (0.2 μM)	23
High concentration of choline (5 mM)	44
High concentration of acetylcholine (5 mM)	43

Table 1: Range of bovine intestinal mucosa alkaline phosphatase activity enhancements observed in our experiments under different conditions.

In conclusion, we have demonstrated that there is an interaction between amyloid β and alkaline phosphatase which causes an increase in the activity of alkaline phosphatase. We saw a 24% increase in activity due to 10 μM amyloid β which is comparable to the increases observed in previous studies in AD and cognitive impairment (Figure 2). We showed that this phenomenon is dependent on amyloid β concentration and not specific to a particular type of alkaline phosphatase. Thus, the increased activity of alkaline phosphatase associated with cognitive decline and/or AD diagnosis may not be due to higher *concentration* of the enzyme (8)(9)(10). We also showed that acetylcholinesterase enhances alkaline phosphatase activity and cholinesterase inhibitors used to treat AD could be affecting alkaline phosphatase activity. This increase in alkaline phosphatase activity could promote further neurodegeneration and has to be taken into account while studying AD.

Our observations suggest that molecular interactions can lead to conformational changes affecting alkaline phosphatase activity. Future studies should focus on the nature of the structural changes using alternative techniques and, in particular, attempt to identify the individual protein residues that interact with amyloid beta or acetylcholinesterase to cause the activity changes.

Author Contributions

Conceptualization - AB, AS; Experiments – AB; Writing - AB, AS; Supervision – AS; Funding Acquisition- AS

Conflicts of interest

There are no conflicts to declare.

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