

**Biological & Biomedical Sciences****Theorized Mechanism of Non-Steroidal Anti-Inflammatory Drugs Against Alzheimer's Disease Onset and Progression****Sanjay Basu**

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General Hospital**Abstract**

Clinical studies testing possible Alzheimer's disease (AD) treatments have shown that non-steroidal anti-inflammatory drugs (NSAIDs) appear to delay the onset and slow the progression of AD. The primary objective of the current study is to elucidate the involvement of NSAIDs in a key inflammatory mechanism: the production of hydroxyl free radicals in the AD brain. The combined data in this experiment from a thiobarbituric acid (TBA) assay and a metal reduction test indicate that NSAIDs neutralize destructive hydroxyl free radicals. In the human body, this could prevent radical-mediated neurotoxic cell death and hinder the formation of neuritic plaques in the brain. This reaction mechanism offers one possible explanation for the protective effect of NSAIDs, while posing new roles for the drugs as effective treatments for AD and other diseases progressed by hydroxyl radical damage to the body.

Introduction

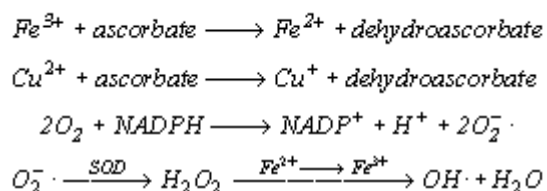
In 1995, scientists in the "Rotterdam Study" concluded that an unknown mechanism allowed non-steroidal anti-inflammatory drugs (NSAIDs) to delay the onset and slow the progression of Alzheimer's disease (AD) in clinical trials (Andersen *et al.* 1995). Since then, others have observed the amelioration of AD in patients using NSAIDs, although no model has explained this observation (Breitner 1996; Rich *et al.* 1995).

Current theories suggest that NSAIDs may neutralize products of the inflammatory response in AD lesions of the brain, although the interaction points are unclear (Appendix A; Eikelenboom *et al.* 1994; Rich *et al.* 1995). The inflammatory mediators within such a response may be sufficient to cause AD neurotoxicity, so NSAID neutralization of these inflammatory products may hinder AD pathogenesis (Wasco *et al.* 1984).

This study focuses on one inflammatory mechanism: the production of hydroxyl free radicals

by microglia in AD lesions of the brain. Though microglia primarily function as phagocytes, consuming and eliminating foreign bodies, they additionally produce hydroxyl radicals (Breitner 1996). These radicals induce neurotoxic cell death, lipid peroxidation, metal-catalyzed protein oxidation, and DNA single strand breaks. (McGeer *et al.* 1994; Bensasson *et al.* 1985).

The current study seeks to determine the existence of any correlation between hydroxyl free radicals, NSAIDs, and the interdiction of AD onset and progression. We hypothesize that the protective effect observed in the Rotterdam study is the result of NSAID neutralization of hydroxyl radicals. We use the identification of this mechanism to construct of a model for AD amelioration by NSAIDs, which can lead to novel therapies for the disease. This study first establishes the existence of a reaction between NSAIDs and the hydroxyl radical. Reactions of free radicals with NSAIDs are detectable in a thiobarbituric acid (TBA) assay, a hydroxyl radical-producing system (Gutteridge 1981). The TBA assay is a highly sensitive test capable of measuring the capability of various compounds to neutralize hydroxyl radicals. The detection of hydroxyl radicals can be initiated through a sequence of reactions. The first of these is the reduction of copper and iron, which allows the reduced form of each metal to react with hydrogen peroxide and form hydroxyl radicals. The abundance of copper and iron in the human brain qualify the use of these two metals in this assay. The entire mechanism used in the TBA assay has been designed to parallel free radical production in the brain. The free radicals produced react with deoxyribose in the solution, generating products that combine with thiobarbituric acid (TBA) to form a pink chromagen. The pink color of the assay solution directly correlates to the production of hydroxyl radicals, so any neutralization of radicals upon the addition of NSAIDs would decrease chromagen concentrations in the solution, which would be detectable by spectrophotometry. However, an ambiguity arises when using the TBA assay. Both the neutralization of hydroxyl radicals and the prevention of metal reduction in the TBA solution appear to decrease chromagen levels.



To resolve this ambiguity, we perform a novel metal reduction test. This test involves the reduction of both copper and iron in the presence of NSAIDs to determine whether the drugs prevent the reduction of metal through chelation. Chelation is the uptake of a reduced form of metal, which could cause a decline in absorption levels in the TBA assay. The key reactions used in the test are: The ability of NSAIDs to prevent metal reduction can be evaluated by determining if the drugs hinder the above reactions. The indicators Bathocuproinedisulfonic acid (BC) and Bathophenanthrolinedisulfonic acid (BP) activate in the presence of the reduced form of the metal. Higher concentrations of reduced metal in the solution correspond to greater indicator activation. Indicator activation levels, measured by [spectrophotometric analysis](#), can thus show if NSAIDs prevent metal reduction.

Methods & Materials

Thiobarbituric Acid Assay

The TBA test was used to evaluate three NSAIDs, based on their availability and range of potency: Acetylsalicylic acid (aspirin), 4-isobutyl-a -methylphenylacetic acid (ibuprofen), and (S)-2-naphthaleneacetic acid,6-methoxy-a -methyl-sodium salt (naproxen sodium). Of the

three drugs, potency is highest for naproxen and lowest for aspirin in clinical trials of the drugs (Arrigoni-Martelli 1977).

In the assay, each drug (10mM) was individually incubated with Cu(II) or Fe(III) (1mM) and deoxyribose (0.9375 mM, Sigma) in 500 mL Dulbecco's phosphate buffered saline (PBS: CaCl₂ 1.19 mM, MgCl₂ 0.6 mM, KCl 2.7 mM, KH₂PO₄ 1.4 mM, NaCl 137 mM, Na₂HPO₄ 7.68 mM, pH 7.4) at 37°C for one hour. After incubation, trichloroacetic acid (250 mL x 17 M in double-distilled H₂O) and 2-thiobarbituric acid (250 mL x 1%, w/v, in 0.05 M NaOH) were added to each sample. The samples were then incubated for 10 minutes at 100°C and placed on ice for 1-3 minutes before distribution in 3 x 300 mL samples. Absorption levels were read by a plate reader (SPECTRAMax Plus, Molecular Devices, CA) at 532 nm and averaged. The net absorption value for each sample was obtained by deducting from the given sample absorption value the mean absorption value of a negative control sample consisting of all elements of the assay except for NSAIDs. Two other control samples, consisting of D-Mannitol (5 mM) and dimethyl sulfoxide (DMSO, 5 mM) coincubated with vitamin C/peroxide (10 mM + 500 mM H₂O₂), were used to determine the effects of hydroxyl radical scavengers on the generation of thiobarbituric acid reactivity.

Metal Reduction Test

A metal reduction test was performed in a 96-well microtiter plate (Costar, MA) containing solutions of each drug (10 mM), Fe (III) or Cu (II) (25mM), ascorbate (25 mM), and one of the reduced metal ion indicators Bathocuproinedisulfonic acid (BC, copper solutions, 250mM) or Bathophenanthrolinedisulfonic acid (BP, iron solutions, 250 mM). Solutions were coincubated in Dulbecco's PBS at 37°C and subjected to spectrophotometric analysis (SPECTRAMax Plus, Molecular Devices, CA) at either 483nm (copper solutions) or 536nm (iron solutions). Negative control (background signal) samples consisted only of metal ions and indicators. Net absorbances (DA) were calculated by deducting the absorption values of these negative controls from the absorption values generated by the NSAIDs in solution with each metal ion and the respective indicator. Concentrations of both metal ions were calculated using the formula: $(DA \times 10^6)/ML$, where M represents the known molar absorption coefficient ($M^{-1} \text{ cm}^{-1}$) and L is the vertical path length automatically set by the platereader to 1 cm. For Fe(II)-BP, $M = 22,140$ at 536nm, while $M = 12,250$ at 483 nm for Cu(I)-BC. Solutions containing metal ions, ascorbate, and indicators without NSAIDs were used as positive controls.

Results

The addition of NSAID drugs to the TBA solutions significantly decreased absorption levels of each sample ($p < 0.05$, two-tailed t -test; Table 1; Figure 1). Similarly, the addition of the hydroxyl radical scavengers DMSO and D-Mannitol caused a significant decrease in absorption levels ($p < 0.05$, two-tailed t -test). All absorption readings of background control solutions were statistically insignificant, ranging in value from 0.046 to 0.061.

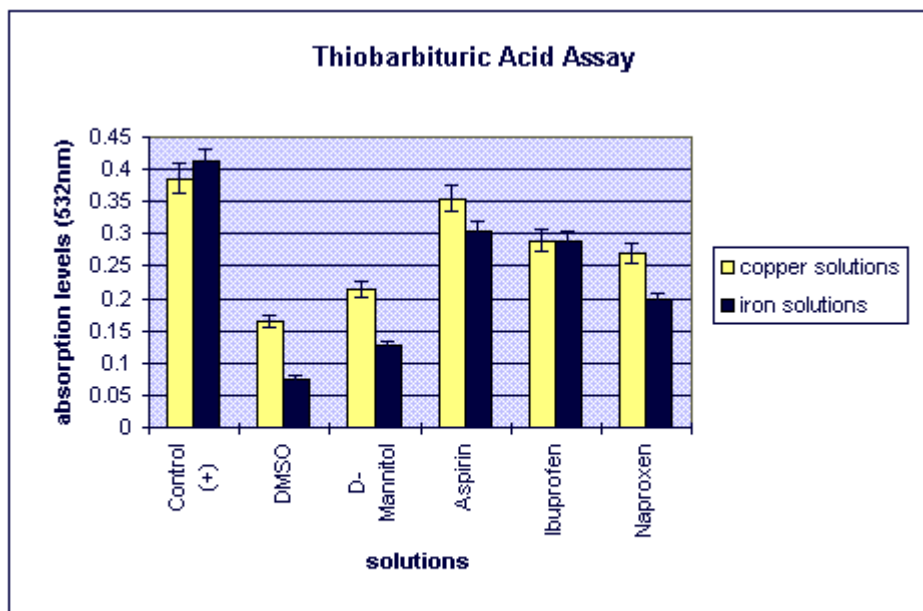


Figure 1: Thiobarbituric Acid Assay

In contrast, absorption levels of NSAID samples varied negligibly from the absorption levels of positive control samples in the metal reduction test (Table 2, Figure 2). Background control solutions showed statistically insignificant absorption values, ranging from 0.030 to 0.037.

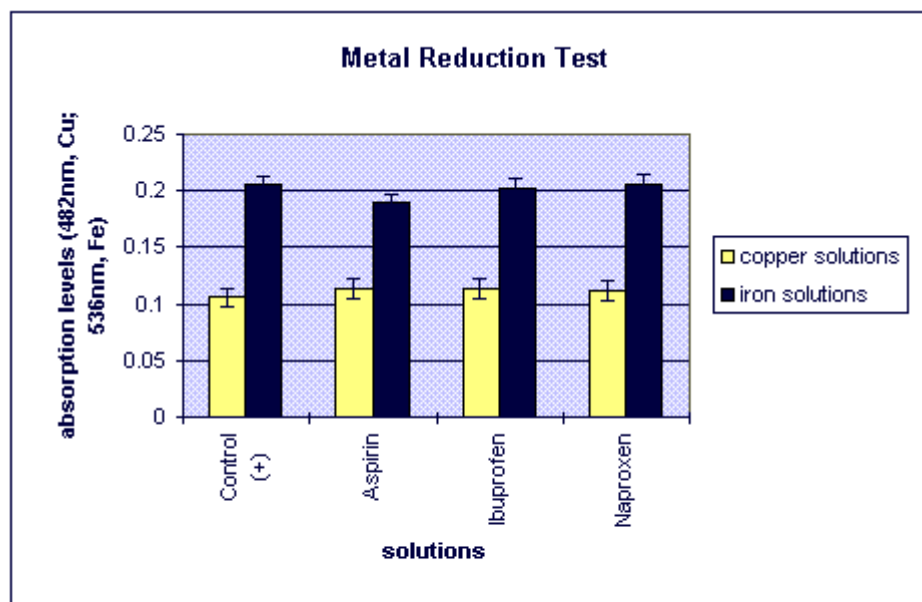


Figure 2: Metal Reduction Test

Discussion

In the TBA assay, hydroxyl radicals were the only components in solution that could have

reacted with either DMSO or D-Mannitol (Bensasson *et al.* 1985). The significant reduction in absorption values in these control solutions suggests that the decline in pink chromagen levels in these samples was caused solely by hydroxyl radical neutralization. The significant decrease in absorption levels upon the addition of NSAIDs to the TBA solutions can be explained by one of two reactions. Either the NSAIDs mimicked hydroxyl radical scavengers in their neutralization of hydroxyl radicals or the NSAIDs chelated the metals in solution.

The results of the metal reduction test indicated that the former reaction was responsible for the decline in NSAID solution absorption levels. We detected no significant differences between the NSAID and control solutions in the metal reduction test, suggesting that equal amounts of the reduced metal were present in both systems. The NSAIDs, therefore, did not chelate either metal.

Since this data was taken from *in vitro* experiments, the reaction mechanisms involved may not have occurred exactly as they would have in the human body. To decrease the significance of this factor, most parts of the experiment were carried out at a neutral pH and involved reactants normally present in the brain. The TBA assay, however, required temperatures higher than a normal human body temperature. It should be noted that the high-temperature incubation step of the TBA assay was only necessary to turn the solution pink, a step necessary for spectrophotometry analysis. Any other reactions, including the reactions between NSAIDs and reactants in the solution, would have already taken place prior to this high-temperature incubation. The earlier reactions were performed inside an incubator held at body temperature. Therefore, the reactions relevant to the NSAID neutralization of hydroxyl radicals could potentially take place in the body.

The data sets from both the TBA assay and the metal reduction test indicate that NSAIDs neutralize hydroxyl radicals under the conditions used. It may be argued that the high reactivity of the hydroxyl free radical allows it to react with other molecules before its interaction with NSAIDs in the human body. Any reaction involving hydroxyl free radicals and neuritic molecules, however, would result in the formation of a new radical (Bensasson *et al.* 1985). The ability of NSAIDs to neutralize the hydroxyl radical, one of the most reactive radicals, indicates that the drugs are likely to neutralize less reactive radicals through the same process as that undergone with the hydroxyl radical (Halliwell *et al.* 1985). Therefore, the high reactivity of the hydroxyl radical with other molecules is unlikely to affect the ultimate NSAID neutralization of radicals in general.

The physiological relevance of this neutralization can be explained after a review of radical production in the human body. The mechanisms producing radicals in the body can then be related to AD pathogenesis to explain the observed NSAID protective effect in AD patients. It is widely known that respiration processes involve the aerobic reduction of molecular oxygen to water by a cytochrome C oxidase-catalyzed reaction. This mechanism includes the transfer of four electrons to molecular oxygen, a process that produces superoxide radicals and hydrogen peroxide as side products. The metal-catalyzed Fenton reaction then creates the hydroxyl radical from the superoxide radical and hydrogen peroxide. The hydroxyl radical is produced only intermittently in this way, but the process nevertheless leads to the instantaneous oxidation of molecules.

The human body enhances its defense mechanisms to combat this effect, as it does against most pathogens. Such defense mechanisms include the superoxide dismutase (SOD) catalyzed dismutation of the superoxide radical using catalase or glutathione peroxidase (GPx) to convert the radical to water and molecular oxygen. Radical-scavenging anti-oxidants like Vitamin E also interrupt the chain reactions that normally lead to cell damage. With age, the hereditary-controlled body defense mechanisms decline, initiating a condition known as oxidative stress. Oxidative stress involves an outnumbering of defense mechanisms by reactive oxygen species (ROS), including the hydroxyl radical (Hanin *et al.* 1995).

In the AD brain, microglial cells activate in "respiratory burst pathways" (Appendix B). The uptake of molecular oxygen by microglial activation results in the formation of numerous hydroxyl radicals, contributing to overall radical accumulation caused by oxidative stress. The peptide $A\beta$, found in the neuritic plaques characteristic of AD, compounds this effect through its creation of hydroxyl radicals through a metal-catalyzed reaction. The $A\beta$ peptide is generated from the amyloid precursor protein (APP) and rapidly reacts with the hydroxyl radical it produces. This reaction leads to the cross-linkage and polymerization of $A\beta$, resulting in the formation of neuritic plaques characteristic of AD. The plaques activate more microglia, increasing hydroxyl radical production and producing a potential feedback mechanism of neuron destruction (Huang *et al.* in press; Tanzi *et al.* in press). Although these plaques are normally present in the brains of elderly people, the observation of high plaque concentration allows for a diagnosis of Alzheimer's disease (Wasco *et al.* 1984).

This "cascade effect" beginning with $A\beta$ and leading to neuritic plaques relates the neutralization of the hydroxyl radical to the observed protective effect of NSAIDs against AD. NSAIDs have been shown to readily cross the blood-brain barrier (Arrigoni-Martelli 1977). The neutralization capability of such drugs then indicates that NSAIDs could prevent radical-mediated neurotoxic cell death while aiding natural free-radical defense mechanisms to delay the onset and slow the progression of AD. These drugs may also simultaneously prevent the cross-linkage and polymerization of $A\beta$ by removing hydroxyl radicals from the presence of the peptide before cross-linkage. By diagnostic standards, this prevention of neuritic plaque formation delays AD onset. Since plaques activate more microglial cells, the NSAID effect also hinders the vicious cycle that produces hydroxyl radicals and leads to further cell death (Figure 3).

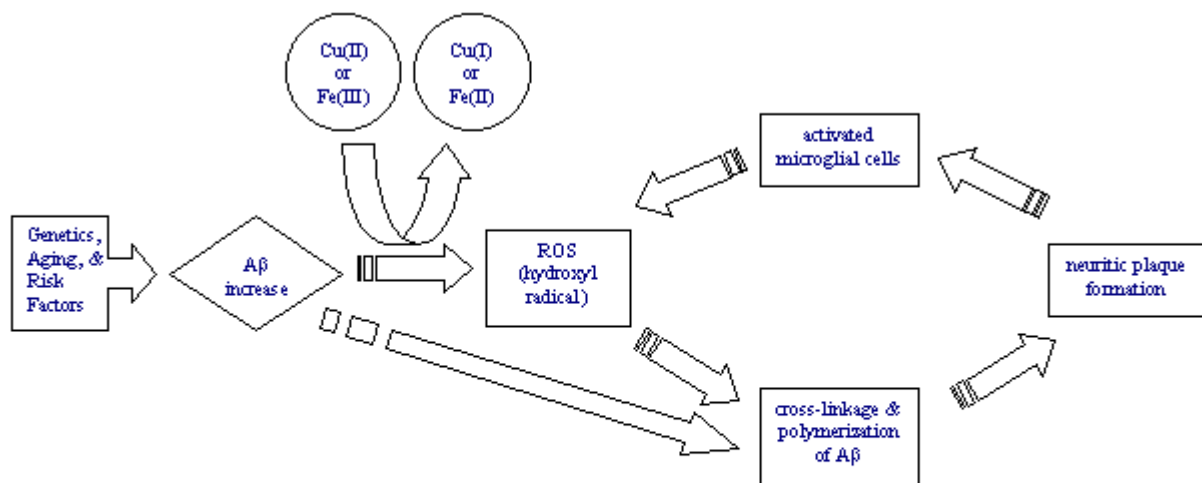


Figure 3: NSAID neutralization of the hydroxyl radical can partially inhibit, but not totally eradicate, the cascade pathway leading to neuritic plaque formation.

Further support for this mechanism has come from other drug studies on AD. Although NSAIDs appear to be effective against AD, glucocorticoids and steroidal anti-inflammatory drugs have had little observed efficacy against the disease (Breitner 1985). This discrepancy between steroidal and non-steroidal drugs may result from the inability of steroidal drugs to act on the products of the inflammatory process (Arrigoni-Martelli 1977). Unlike NSAIDs, steroidal drugs act on cells that are active during the human inflammatory response. The

peptide Ab would likely be unaffected by such steroids and would therefore contribute to continued hydroxyl radical production, neurotoxic cell death, and neuritic plaque formation. This hypothesis, however, requires further testing.

In vivo studies of NSAIDs against AD should also be performed following the current study. Future testing should include *in vivo* studies of the NSAID group against a variety of diseases involving hydroxyl free radicals and oxidative stress. This could establish if the repression of oxidative stress in these diseases assists in ameliorating disease conditions. Known diseases involving oxidative stress include cancer and Parkinson's disease (Bensasson et al. 1985; Halliwell *et al.* 1985; Hanin *et al.* 1995). The introduction of an NSAID drug therapy into the treatment plans of Alzheimer's disease patients should also be considered, given the present data.

Conclusion

The data in this study support the hypothesis that NSAIDs exhibit a protective effect against AD onset and progression by reacting with hydroxyl radicals known to be contributory to AD, yielding innocuous products. It appears that this reaction could delay the onset and slow the progression of AD through the prevention of neurotoxic cell death and the inhibition of neuritic plaque accumulation. This study's data support previous observations that NSAIDs can delay the onset and slow the progression of Alzheimer's disease (Andersen 1995). Further understanding of the mechanism involved in the NSAID-induced interdiction of AD may provide new therapeutic possibilities for diseases involving oxidative stress.

Acknowledgments

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