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Antibody-catalyzed water-oxidation pathway*

Paul Wentworth, Jr.[1,2,3,‡] and Daniel P. Witter[3]

[1]Departments of Chemistry and [2]The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037, USA; [3]The Scripps-Oxford Laboratory, Department of Biochemistry, Glycobiology Institute, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

Abstract: The intrinsic ability of all antibodies to generate hydrogen peroxide (H_2O_2) from singlet dioxygen ($^1O_2$*) via the antibody-catalyzed water-oxidation pathway (ACWOP) has triggered a rethink of the potential role of antibodies both in immune defense, inflammation, and disease. It has been shown that photochemical activation of this pathway is highly bactericidal. More recently, cholesterol oxidation by-products that may arise from the ACWOP have been discovered in vivo and are receiving a great deal of attention as possible key players in atherosclerosis and diseases of protein misfolding, such as Alzheimer's disease and Parkinson's disease.

Keywords: immune defense; inflammation; ACWOP; protein misfolding; atherosclerosis; Parkinson's disease; water oxidation.

INTRODUCTION

A central concept within immunology is that antibodies are the key molecular link between recognition and ultimate destruction of antigens/pathogens via phagocytosis and/or complement fixation [1]. The antibody catalysis field introduced by Lerner [2,3] and Schultz [4] has demonstrated that the antibody molecule can be programmed to perform a wide range of chemical reactions [5,6], but there had been no compelling evidence that antibodies use this catalytic potential in their normal immune function. Recently, however, it has been discovered that all antibodies can catalyze a reaction between singlet oxygen ($^1O_2$) and water to generate hydrogen peroxide (H_2O_2) (eq. 1) [7]. This antibody-mediated process is independent of antigenic specificity and is triggered upon binding of $^1O_2$ to conserved binding sites within the antibody fold [8,9].

$$x^1O_2^* + H_2O \rightarrow H_2O_2 + (x - 1)^3O_2 \quad (1)$$

Preliminary kinetic studies revealed that the rate of formation of H_2O_2 by antibodies is increased in deuterium oxide (D_2O), reduced in sodium azide (NaN_3) [7] and proportional to the UV absorbance profile of the protein [8], suggesting that $^1O_2$* was a substrate in this reaction. Subsequent studies revealed that the $^1O_2$* could be generated by either direct UV irradiation of the antibody molecule, by visible light and a $^3O_2$ sensitizer such as hematoporphyrin IX, or by thermal decomposition of endoperoxides, and in each case antibody-catalyzed formation of H_2O_2 commences.

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‡Corresponding author: E-mail: paulw@scripps.edu

The efficiency of H_2O_2 formation by antibodies, upon long-term UV irradiation, is unparalleled by non-immunoglobulin proteins. Typically, other proteins display a short burst of H_2O_2 production followed by a rapid quenching in rate as photooxidation occurs. Antibodies typically exhibit linear formation of H_2O_2 for up to 40 mol equiv of H_2O_2 before the rates declines. It appears that H_2O_2 reversibly inhibits its own formation (apparent IC_{50} ~225 μM) and antibodies can resume photoproduction of H_2O_2 at the same initial rate if catalase is added to destroy the H_2O_2. If a cycle is established that involves photoirradiation of an immunoglobulin solution in aqueous buffer, followed by removal of the generated H_2O_2 by catalase, and further photoirradiation, >500 equiv of H_2O_2 can be generated by each antibody molecule.

The photoproduction of >500 equiv of H_2O_2 from $^1O_2$*, per antibody molecule, creates a critical electron inventory problem. Preliminary studies to identify the electron source ruled out metal-mediated redox process, amino acid oxidation, and chloride ion. At this point, the accumulated observations pointed to the involvement of a non-obvious electron source that does not deactivate the protein catalyst, and that could account for the high turnover numbers, hence that is quasi-unlimited.

Isotopic labeling experiments were then undertaken to determine the oxygen source for H_2O_2. These studies revealed that water is oxidized by $^1O_2$*, demonstrated by the incorporation of oxygen from water into H_2O_2 upon UV irradiation of antibodies [8]. The isotope incorporation experiments suggested that a molecule of water, in the presence of an antibody, may hypothetically add as a nucleophile to $^1O_2$* and form dihydrogentrioxide (H_2O_3) as an intermediate on a pathway that ultimately leads to H_2O_2. Thus, water in becoming oxidized to H_2O_2, fulfils the role of the electron source.

BACTERICIDAL ACTION OF IMMUNOGLOBULINS

The discovery of this pathway raised an important question: Can antibodies behave as bactericidal agents when presented with a chemical or biological source of $^1O_2$*? Experiments demonstrated that under conditions where the antibody-catalyzed water-oxidation pathway (ACWOP) was activated, with $^1O_2$* generated by irradiation of hematoporphyrin IX with white light, a 1–3 log reduction in cold-shocked gram-negative bacterial viability can be achieved [10]. Bactericidal activity was observed at antibody concentrations of >nanomolar, and was independent of binding of antibodies to cell-surface antigens. In a typical in vitro experiment, a >99 % reduction of viability required ~20 μM of (specific as well as non-specific) antibody. The bactericidal activity is a function of antibody concentration, as well as irradiation time and sensitizer concentration (hematoporphyrin IX) and is inactivated by addition of catalase. These observations support the key role of both $^1O_2$* and the water-oxidation pathway in antibody-mediated bactericidal activity.

Bactericidal studies with H_2O_2 revealed that the protective effect of catalase was not due to simply protecting the bacteria from the H_2O_2 generated by antibodies, but rather because it prevented the reaction between H_2O_2 and ozone that produces hydroxyl radical, a potent antibacterial agent [10].

The morphology of the killed bacteria was examined by electron microscopy. Distinct stages in the bactericidal pathway in which increasing cell wall and plasma membrane permeability is assumed to result from oxidative damage. Given that the bacterium is under an internal pressure of ca. 30 atm, weakening of the membrane by any mechanism leads to catastrophic rupture of the cell well and plasma membrane. In this regard, it is interesting that the observed morphologies induced by antibody-mediated killing are similar to those seen when bacteria are destroyed by phagocytosis [11]. Thus, the water-oxidation pathway may represent an ancient activity by which immunoglobulins, which have existed for over 500 million years [12], inactivated microbes and supplied host defense.

Given that $^1O_2$* is known to be toxic in eukaryotic systems, an additional and protective role for antibodies may be in host defense against this reactive oxygen species. This postulate of course requires the further processing of H_2O_2 into water and triplet oxygen by catalase or glutathione peroxidase. Given that catalase is known to be an ancient protein arising as far back as archaebacteria [13], the question can be raised as to whether the structural element that is responsible for the catalytic destruction of

$^1O_2$* is equally ancient and considerably precedes what we know today as antibodies. In this regard, it makes sense to search among ancient organisms for proteins that can intercept $^1O_2$* at rates approaching diffusion control and are not destroyed in doing so. One may even contemplate whether $^1O_2$* has played a decisive role in the initiation of the evolution of the immunoglobulin fold.

BIOLOGICAL RELEVANCE AND EFFECTOR FUNCTION?

If antibody-mediated oxidation plays any significant role in vivo, what is the origin of the high-energy singlet ($^1\Delta g$) oxygen molecule, required for initiation of the pathway? The primary source of $^1O_2$* in vivo is thought to be activated phagocytes, including monocytes [14] and polymorphonuclear neutrophils (PMNs) [15]. The amount of $^1O_2$* generated during the neutrophil respiratory burst has been estimated to be up to a staggering 19 % of the total cell oxygen consumption [16]. Current thinking supports the most plausible chemical route for the intracellular generation of $^1O_2$* in these activated phagocytes arising from the oxidation of dihydrogen peroxide (HOOH) by hypochlorous acid (eq. 2) [17].

$$HOOH + HOCl \rightarrow {}^1O_2* + HCl \quad (2)$$

It has been reported that IgG-coated PMNs, activated with phorbol myristate acetate (PMA), generate an oxidant with the chemical signature of ozone [10,18], offering preliminary evidence that this pathway may be relevant in phagocytes. It was further demonstrated that this same oxidant was generated in inflamed tissue in a reversed Arthus model [10]. However, it should be noted that the evidence that ozone may be formed in phagocytes arises from indirect evidence using chemical probes. The case for ozone formation will be strengthened if and when it can be detected as directly as possible. However, the discovery that activated neutrophils generate oxidants that are traceable to the water-oxidation pathway of antibodies may transform how oxidative destruction and signaling within the immune system is considered.

An important question, which remains to be answered, is whether the oxidative antibody effector mechanism contributes to in vivo destruction of microorganisms in higher organisms, which have evolved powerful and well-regulated antibody effector mechanisms. Complement activation and Fc receptor-mediated effects, such as phagocytosis, are strictly regulated such that they are only initiated upon binding of specific antibodies to pathogen surfaces. Random activation of this water-oxidation effector activity would result in significant host damage, considering that immunoglobulin is present at >10 mg/ml concentrations in human serum. This issue is exacerbated by the fact that all antibodies can catalyze the water-oxidation pathway, whether or not they are bound to antigen. However, it is envisaged that the regulation of this pathway is controlled de facto in the same way as phagocytosis is regulated, antibody-antigen binding. The origin of the regulation of this pathway, however, is the short lifetime ($\tau_\Delta < 0.6$ μs) [19] and short diffusion length ($\delta \leq 0.07$ μm) [20] of $^1O_2$*. As described vide supra, under normal conditions, $^1O_2$* is only generated in significant amounts within activated phagocytes, and in vivo, phagocyte activation is strictly regulated. The short diffusion length of $^1O_2$* thus prevents it reaching nonspecific immunoglobulins beyond the site of antigen-destruction and minimizes peripheral damage.

The products of the water-oxidation pathway have also to be considered in this context, that is, while they are generated locally at sites of inflammation what is their propensity to diffuse away? H_2O_2 is a stable molecule that can diffuse away from the site of inflammation and could potentially inflict widespread toxic effects. However, the ubiquitous nature of catalase in vivo essentially removes this concern. Ozone is highly toxic, but short-lived in biological systems. These properties make it an ideal effector molecule since any damage would be localized to the site of inflammation. Thus, akin to other immune effectors, its synthesis is triggered by antigen-antibody union that occurs when activated neutrophils at the site of an inflammation generate $^1O_2$*. Ozone shares another key hallmark of immune ef-

fectors in that it not only kills but also functions as a signaling molecule that serves to amplify cell-based immune responses and the production of inflammatory cytokines including NFκ-b, IL-6, and TNF-α [21–25].

PATHOLOGICAL EFFECTS AND INFLAMMATORY DISEASES

Recent investigations have revealed that there may be pathological effects of the ACWOP in inflammation [26]. Inflammation, both acute and chronic, is a central player in the pathophysiology of a wide range of human diseases ranging from atherosclerosis, to neurological diseases, to autoimmunity. It is now generally accepted that inflammation associated with the presence of leukocytes and immunoglobulins is a critical component in human atherosclerosis [27–31]. Given that all the components necessary to activate the ACWOP are thought to be present in atherosclerotic arteries, it was investigated whether this pathway is traceable in human atherosclerosis and whether excised advanced plaque material could be induced to produce ozone in vitro. Evidence for the ACWOP within atherosclerotic human carotid arteries was obtained by analysis of the cholesterol oxidation products within carotid plaques that had been surgically removed by carotid endarterectomy [26]. Carotid plaques were removed from both symptomatic and asymptomatic stenoses. Cholesterol is a major lipid component of atherosclerotic plaques [32] being present at such high concentrations that, in certain cases, it can form a crystalline phase within the lipid core of the diseased artery. Previous studies have shown that amongst a panel of oxidants such as triplet oxygen ($^3O_2$), $^1O_2$*, superoxide anion, hydroxyl radical (from the reaction between H_2O_2 and Fe^{2+}), and ozone, only ozone cleaves the $\Delta^{5,6}$ double bond of cholesterol (**CHOL**) to yield the 5,6-*seco*sterol (atheronal-A, **KA**) as the principle product [33–36] (Fig. 1). As part of our ongoing studies into the potential that trioxygen species may be generated in inflammatory cells [5,8,9,18], we recently discovered the cholesterol 5,6-*seco*sterols, 3β-hydroxy-5-*oxo*-5,6-*seco*-cholestan-6-al (**KA**) and its aldol product, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (**ALD**), that we term atheronal-A and atheronal-B, respectively, are indeed present in atherosclerotic plaque material and are significantly elevated in the plaque material if the residual leukocytes are activated with PMA (Fig. 2) [18]. We have since shown that they are present within the human central nervous system (CNS) [37] and play a role in atherosclerosis progression [38].

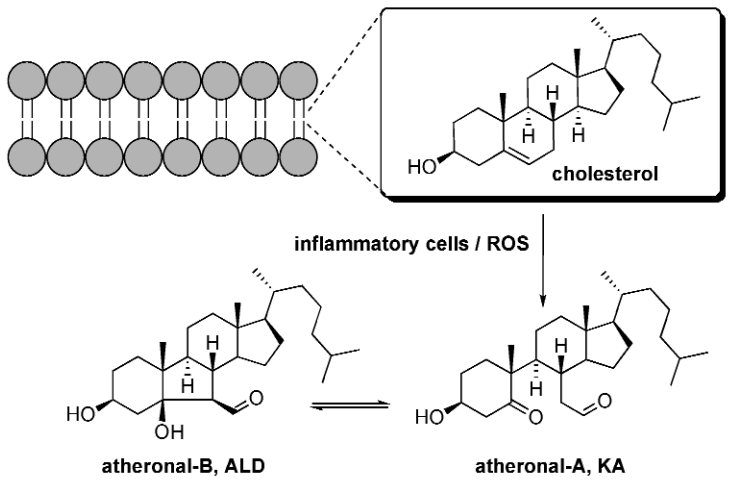

Fig. 1 Cholesterol *seco*-sterols, **KA** and **ALD**, are present in inflamed artery walls.

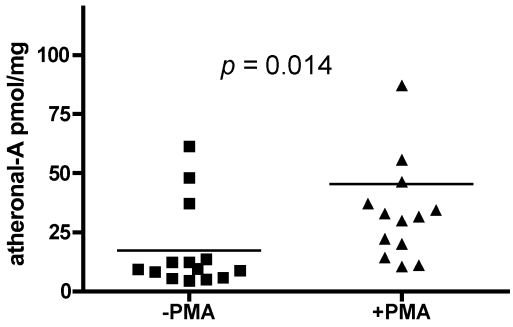

Fig. 2 KA is present in vivo. (A) Measured levels of **KA** in atherosclerotic plaque without PMA treatment. (B) Measured levels of **KA** in atherosclerotic plaque after PMA treatment.

An early pathological change within the vasculature during atherosclerosis is the fatty streak. This histopathological lesion is characterized by the formation of lipid-laden macrophages within the intima of large-sized arterial vessels. These cells are known as foam cells because of the appearance of foamy vacuoles within the cytoplasm consisting of oxidized low-density lipoprotein (LDL) molecules. Modifications of LDL that increase its atherogenicity are considered pivotal events in the development of cardiovascular disease [29]. Specifically, oxidative modifications to LDL, or apoprotein B_{100} (apoB-100, the protein component of LDL), that render it into a high-uptake form for macrophages via CD36 and other macrophage scavenger receptors are considered critical causative pathological events in the onset of atherosclerosis. We have shown that when LDL (100 μg/mL) is incubated with **KA** and **ALD** (each at 25 μM) in the presence of unactivated murine macrophages (J774.1), lipid-loading of these cells occurs, leading to foam cell formation, hinting at a potential atherogenic effect of these molecules [38].

PROTEIN MISFOLDING DISEASES AND CHOLESTEROL *SECO*-STEROL ALDEHYDES

apoB_{100}

Ursini and Sevanian [39] have demonstrated that oxidized LDL has reduced secondary structure compared to native LDL. This change in secondary structure, which is thought to lead to proteolysis and exposure of epitopes that bind to macrophage scavenger receptors, such as CD36 and SR-A, has been surmised to be responsible in part for foam cell formation and has led to the implication that atherosclerosis is a protein misfolding disease [40]. Such modifications of LDL that increase its atherogenicity are considered pivotal events in the development of cardiovascular disease [29]. When we discovered **KA** and **ALD** in atherosclerotic plaque and observed that these molecules, when complexed to LDL, triggered foam cell formation [38], we hypothesized that they may be causing a conformational change in apoB_{100}, the protein component of LDL [26]. When LDL was incubated with **KA** or **ALD**, a time-dependent change in the far-UV circular dichroism (CD) spectra of the component peptide is observed (Fig. 3). CD analysis reveals there is a significant loss of secondary structure, mainly accounted for by a lower content of α helix (**KA** ~23 ± 5 %; **ALD** ~20 ± 2 %) and a higher percentage of random coil (**KA** ~39 ± 2 %; **ALD** 32 ± 4 %). This experimental observation was the first clear evidence that biologically relevant aldehydes, when adducted to misfolding prone proteins, could accelerate the misfolding event [26].

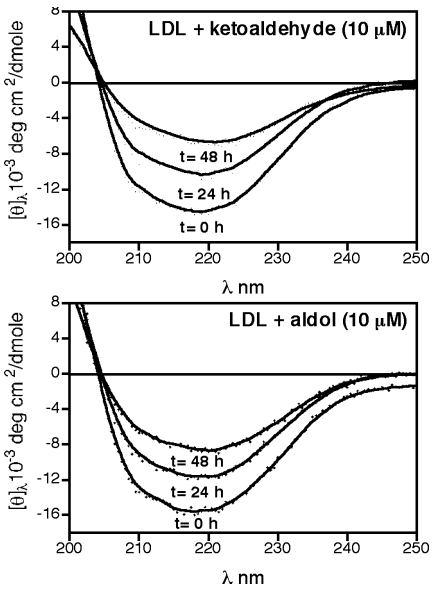

Fig. 3 Far-UV CD spectra of LDL (100 μg/ml) and either **KA** (10 μM, UPPER) or **ALD** (10 μM, LOWER) in PBS (pH 7.4 with 1 % IPA) at 37 °C. The CD spectra of LDL alone showed no changes over the time frame of this experiment.

α-Amyloid (Aβ) and α-synuclein (α-SYN)

In collaboration with Prof. J. Kelly's group at The Scripps Research Institute we then discovered that aggregation of both Aβ and α-SYN is substantially accelerated in the presence of the oxidative lipid metabolites **KA** and **ALD** [37,41]. Thioflavin T (ThT) positive aggregates of both Aβ and α-SYN form at an accelerated rate in the presence of the oxidative lipid metabolites **KA**, **ALD** but not with another cholesterol aldehyde [37]. Thus, incubation of seed-free Aβ 1-40 peptide at neutral pH (pH 7.4, 300 mM NaCl, 37 °C) with *seco*-sterols **ALD** leads to formation of Aβ aggregates which bind the amyloidophilic dye ThT within less than 10 h. Under the same conditions, no ThT signal was observed in the absence of the cholesterol *seco*-sterols, emphasizing this is not simply a nonspecific process that involves adduction of a hydrophobic aldehyde molecule to the surface of a charged protein. Atomic force microscopy (AFM) revealed that no aggregates of Aβ 1-40 (100 μM) were formed in the absence of **KA** (Fig. 4A). However, in the presence of **KA**, spherical Aβ 1-40 aggregates with a diameter of 5–8 nm are formed within 1 h (Fig. 4B). To observe fibrillization on a reasonable timescale, Aβ aggregation reactions have to be agitated. AFM confirms the aggregates are fibrillar, both in the presence and absence of **KA**, when shaken (Figs. 4C,D) [41]. Similarly, **KA** and **ALD** accelerate α-SYN aggregation in both stagnant and agitated assays. ThT signal increases over 72 h in samples of α-SYN (25 μM) in the presence of **KA** and **ALD** (25 μM).

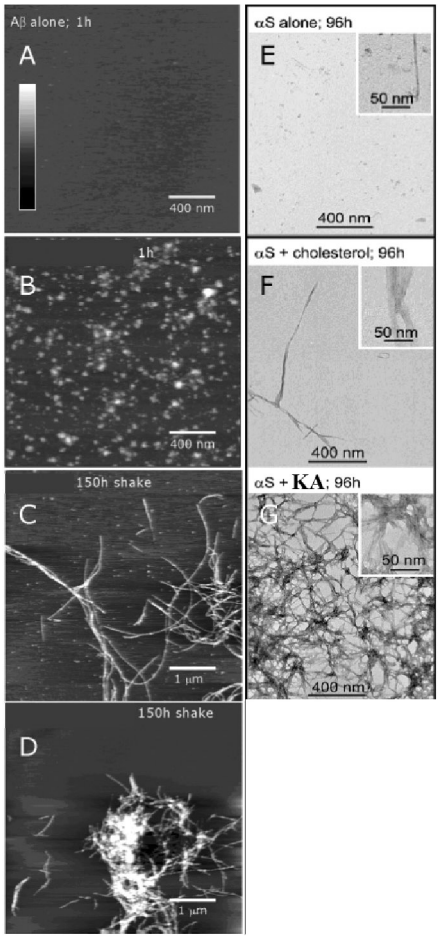

Fig. 4 AFM and TEM images of Aβ1-40 and α-SYN aggregates, respectively, formed with **KA**. (A) Aβ1-40 (stagnant). (B) Aβ1-40 + **KA** (50 μM) (stagnant). (C) Aβ1-40 (agitated). (D) Aβ1-40 + **KA** (50 μM) (agitated). (E) α-SYN alone. (F) α-SYN and cholesterol. (G) α-SYN and **KA**.

The adduction of **KA** and **ALD** to synthetic Aβ peptide was found to lower the critical concentration for fibril formation (K_c) of the protein [41]. We hypothesize that the acceleration in aggregation is triggered when increasing the hydrophobicity at the surface of the fibrillogenic peptide adjacent to where the adduction occurs destabilizes the protein structure [42,43]. This would translate into a decrease in K_c, which would allow metabolite-modified peptides to form fibrils at lower concentrations than their unmodified counterparts and could explain how Aβ forms fibrils at physiological concentrations (~1 to 10 nM) [44], which are much lower than the critical concentrations typically reported for Aβ aggregation in vitro (~1 to 10 μM) [45,46]. The small fraction of soluble Aβ-**KA** adduct that could be detected in solution put a lower limit on the critical concentration of 70 ± 20 nM. Thus, **KA** lowered the critical concentration of aggregation of the Aβ 1-40 peptide by at least two orders of magnitude. An important and largely unanswered question regarding lipid–aldehyde-induced protein misfolding is the nature of the interaction between the aldehyde and the protein and how this facilitates protein aggregation. We have recently shown, by kinetic analyses of the **ALD**-induced oligomerization and fibrillization of a panel of synthetic mono-, bis- and tris-*N*,*N*-dimethylamine-containing Aβ(1-40) protein sequences, that the aggregation of Aβ(1-40) peptide is accelerated by **ALD** only when the aldehyde adducts to the ε-amino group of Lys16; no initiation in oligomerization of Aβ is observed when the

aldehyde is adducted to either the ε-amino group of Lys28 or the α-amino group amine of Asp1 (Fig. 5) [47]. In addition, the **ALD**-induced aggregation of peptide Aβ(1-40) is inhibited by cholesterol. Both data combine to suggest that the atheronal-B-induced aggregation of Aβ(1-40) involves a high degree of structural recognition between the lipid and the peptide that involves, in part, binding of **ALD** into the putative cholesterol-binding domain of Aβ, a truly startling observation.

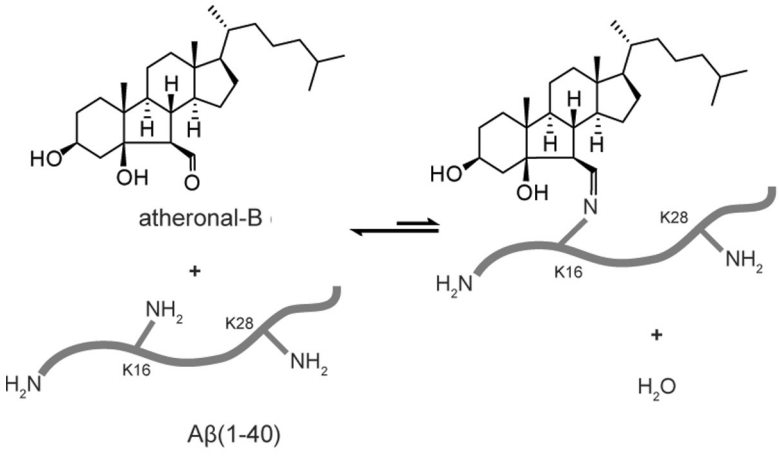

Fig. 5 ALD-induced aggregation of Aβ(1-40) peptide. Schiff base equilibrium between Lys16 of Aβ(1-40) and aldehyde is the only one that induces aggregation of the Aβ(1-40) peptide.

The ACWOP is being considered as a new effector function of the immune system, and may become known as a new chemical arm to immune defense. As commented recently by one observer, it may be that this innate ability of antibodies is an ancient effector function that has no present-day role in immune defense [48]. However, a tremendous amount of further research at the interface of chemistry and biology will be required before this critical issue is resolved. One thing has become clear during our ongoing studies—there are molecular consequences of this pathway in biology. The atheronals are such molecular consequences and the impact of these small lipid oxidation products and others yet to be discovered, may ultimately prove to have even more impact than the ACWOP itself.

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