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Review Article

Hypothesis: Soluble Aβ Oligomers in Association with Redox-Active Metal Ions Are the Optimal Generators of Reactive Oxygen Species in Alzheimer's Disease

Brian J. Tabner, Jennifer Mayes, and David Allsop

Division of Biomedical and Life Sciences, School of Health and Medicine, Lancaster University, Lancaster LA1 4YQ, UK

Correspondence should be addressed to David Allsop, d.allsop@lancaster.ac.uk

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Considerable evidence points to oxidative stress in the brain as an important event in the early stages of Alzheimer's disease (AD). The transition metal ions of Cu, Fe, and Zn are all enriched in the amyloid cores of senile plaques in AD. Those of Cu and Fe are redox active and bind to Aβ *in vitro*. When bound, they can facilitate the reduction of oxygen to hydrogen peroxide, and of the latter to the hydroxyl radical. This radical is very aggressive and can cause considerable oxidative damage. Recent research favours the involvement of small, soluble oligomers as the aggregating species responsible for Aβ neurotoxicity. We propose that the generation of reactive oxygen species (i.e., hydrogen peroxide and hydroxyl radicals) by these oligomers, in association with redox-active metal ions, is a key molecular mechanism underlying the pathogenesis of AD and some other neurodegenerative disorders.

1. Introduction

A large body of evidence supports an important role for oxidative stress in the pathogenesis of several different neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), the prion disorders, and motor neuron disease. Under normal physiological conditions, a balance would be anticipated between pro-oxidant and antioxidant levels in brain tissue. However, should there be an imbalance between the two, either as a result of excessive pro-oxidant levels or deterioration in antioxidant levels, a build up of reactive oxygen species (ROS) will occur, resulting in oxidative damage. Evidence of oxidative damage in AD and other neurodegenerative diseases has been found in a whole range of studies via a number of established markers (for recent reviews relevant to AD see, e.g., [1–4]).

Biometal ions, particularly those of Cu, Fe, and Zn, are also implicated in neurodegenerative disease, including AD (for some recent reviews see, e.g., [5–7], and references quoted therein). These ions were found to be enriched in the amyloid cores of senile plaques in AD sometime ago (see [8] for a summary) and are important for a number of reasons. Both Cu and Fe have two readily available oxidation states (i.e., Cu(II) and Cu(I) and Fe(III) and Fe(II)) which aid the transfer of electrons and are fundamental to redox chemistry. Consequently they are readily available for protein binding, can facilitate the Fenton reaction (illustrated in (2) with respect to Fe ions), and are able to transfer electrons to oxygen (to form H_2O_2). They are found as trace-level contaminants in laboratory buffers [8] and are present as impurities in synthetic Aβ [9]. It has been reported that even trace concentrations of Zn(II), Cu(II), and Fe(III) are all sufficient to enhance the Aβ(1–42) initiated seeding of Aβ(1–40) aggregation [8].

In this paper, we focus our attention on two ROS, that is, hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$). H_2O_2 is a cytotoxic oxidant which is freely permeable across cell membranes and consequently has effects that extend well beyond its site of generation. It is, therefore, likely to be responsible for global increases in oxidative damage. On the other hand, the hydroxyl radical is quite different. It is readily formed via Fenton chemistry from H_2O_2 (see (2)). It is very reactive, with a reaction rate largely controlled by its rate of diffusion and thus exerts its effects on a very local scale.

Largely because of its high reactivity, it is also very unselective in the site of attack and so will react with any biomolecule that is immediately adjacent to its site of formation. The most common reaction of $^{\bullet}$OH is hydrogen atom abstraction from the biomolecule, leaving the latter with a radical centre. The newly formed radical is then available to attack any other local biomolecule in a reaction typical of a classical chain reaction. These latter reactions will continue until such a time as termination occurs.

Until recently, Aβ amyloid fibrils themselves were believed to be an important toxic agent in AD. Support for this idea is now under question and presently there is growing interest in (a) the role of metal ions, and (b) the involvement of early stage Aβ assemblies (i.e., soluble oligomers) as the aggregating species potentially responsible for neurodegeneration in AD. A hypothesis that brings these two concepts together is the main focus of the remainder of this brief article.

2. Metal Ions: The Direct Generation of ROS and Aβ Oxidation

We now focus on the fascinating possibility that Aβ (and possibly several other amyloidogenic proteins) might have the ability to self-generate two important ROS, that is, H_2O_2 and $^{\bullet}$OH, during aggregation *in vitro*. In 1994, Behl et al. [10] reported results suggesting that Aβ could cause increased levels of H_2O_2 to accumulate in cells during incubation over an extended period although the source of the peroxide was not investigated. However, their results clearly indicate that, at least in the case of Aβ(25–35), H_2O_2 levels go through a maximum before subsequently declining. We believe this latter finding to be significant and this aspect is discussed further below.

Two key publications by Huang et al. followed in 1999 [11, 12]. These authors highlighted the direct generation of H_2O_2 and $^{\bullet}$OH during the incubation of solutions of both Aβ(1–40) and Aβ(1–42). They were able to establish that Aβ binds to Cu, Fe, and Zn ions and that the bound peptide can reduce Cu(II) to Cu(I) and Fe(III) to Fe(II). The binding of these metal ions is most significant since they are elevated in the amyloid deposits found in individuals with AD [13, 14]. Huang and colleagues were further able to show that the bound transition metal ions Cu(I) and Fe(II) were able to reduce molecular oxygen to H_2O_2 thus establishing the direct generation of the latter ROS during peptide incubation. Finally, and of prime importance, is that their results established that, when bound, both the Fe and Cu ions are redox active (note that Zn ions are not redox-active). This redox-active nature of the metal ion when bound to the peptide enables the ready formation of $^{\bullet}$OH via the well-known Fenton reaction. This latter observation has important implications, as discussed in more detail below. The essential reactions are

$$O_2 + 2\,Cu(I) + 2H^+ \longrightarrow H_2O_2 + 2\,Cu(II) \quad (1)$$

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + {}^{\bullet}OH + {}^{-}OH \quad (2)$$

These two reactions have important consequences. The reduction of oxygen to form H_2O_2 requires two electrons, whilst the formation of $^{\bullet}$OH from H_2O_2 requires one electron. It is clear that, in order for (1) and (2) to proceed, the bound metal ion needs to be in its lower oxidation state (i.e., either Cu(I) or Fe(II)). If the metal ions are in their higher oxidation state (i.e., Cu(II) or Fe(III)) the necessary conversion to their lower oxidation state requires an electron source. In the absence of any other electron donor it is possible that, (*in vitro*), the peptide itself could act as an electron donor resulting in the formation of the peptide radical cation:

$$A\beta/Cu(II) \longrightarrow (A\beta)^{\bullet+}/Cu(I) \quad (3)$$

The Aβ radical cation, thus formed, could then undergo a variety of rapid rearrangement reactions resulting in the formation of oxidation products consistent with those commonly observed [1–4]. This process could continue "long term" providing the bound metal ion: peptide complex remains redox active; that is, there is an interchange between the two oxidation states of the appropriate metal ion. However, these reactions could be limited by an inadequate supply of electrons and it has been questioned if the bound peptide has the ability to act as an electron donor to Cu(II) [15].

These results prompted us to carry out our own investigations, in which only impurity levels of Cu and Fe were present in samples of Aβ [9, 16]. Purified synthetic Aβ has significant metal ion content (estimated in our samples as 5.7×10^{-4} and 1.2×10^{-3} moles of Fe and Cu, resp., per mole of Aβ(1–40)) and these levels would be augmented by additional metal ions present in the buffers used [8, 9]. The experimental technique for H_2O_2 detection was quite different to that employed by Huang et al. and was based on electron spin resonance (ESR) spectroscopy. Here, H_2O_2 is converted to $^{\bullet}$OH via Fenton chemistry and the latter is detected by employing a well-established spin-trapping agent [9, 16]. Thus we were able to show, with many measurements undertaken over an extended incubation period, that Aβ(1–40), Aβ(1–42), and Aβ(25–35) can all self-generate H_2O_2 during incubation in solution. On the other hand, related nontoxic peptides, such as the reverse peptide Aβ(40–1) were inactive [9, 16]. Some typical ESR spectra obtained during the incubation of different Aβ peptides are shown in Figure 1.

At about the same time, a further publication from Opazo et al. [17] indicated that Huang's earlier data were flawed in the sense that a reagent required in their detection technique, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), (which was present during incubation) was found to act, in its capacity as nonbiological reducing agent, to significantly enhance the concentration of H_2O_2 generated. Also, when several biological reducing agents such as vitamin C, dopamine (DA), and L-3,4-dihydroxyphenylalanine (L-DOPA) were added (separately) they were found to also considerably enhance H_2O_2 levels, although, except in the case of DA, measurements were taken after only a single time point. These latter observations are consistent with other research, covering several different

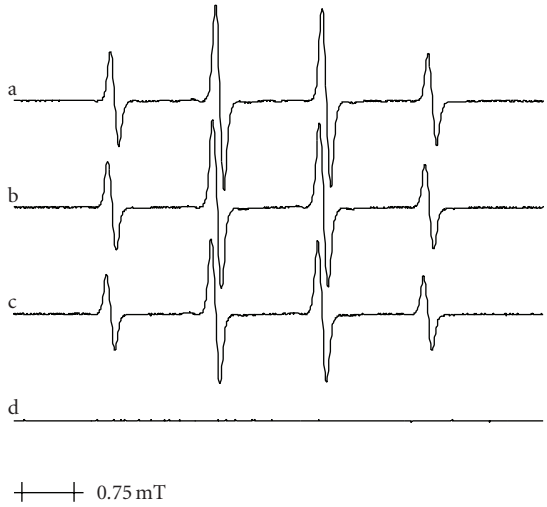

Figure 1: ESR spectra of the •OH adduct of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) recorded after 48 h incubation of (a) Aβ(1–40), (b) Aβ(1–42), (c) Aβ(25–35) (intensity ×1.5) and (d) Aβ(40–1) at 37°C and all at 100 μM. The intensity of the spectra observed in (a), (b), and (c) varied with incubation time as described in the text whereas in (d) no DMPO adduct spectrum was observed after any period of incubation. Control samples gave straight line spectra, such as that illustrated in (d), at every time point examined. The experimental methodology, reaction conditions, and ESR spectrometer settings are fully detailed elsewhere [9, 16].

biological reducing agents [18–20]. The ability of these biological reducing agents to act as an electron source for the reduction in the oxidation state of the bound metal ions reduces the necessity of the peptide itself to do so (3) and, consequently, enhances the long-term formation of ROS, especially the •OH radical, so increasing the potential for oxidative damage.

It is apparent, however, that the presence of biological (and other) reducing agents is not the only factor influencing the amount of H_2O_2 generated as the metal ion : peptide ratio is also significant [21]. For example, H_2O_2 production is very much enhanced when the Cu(II) : peptide ratio in Aβ(1–40) is increased from 0 : 1 to 1 : 1, and it is now apparent that great care must be taken in assigning enhanced generation of $H_2O_2$when both the reducing agent : Aβ and metal ion : Aβ ratios are not examined separately. Also measurements over an extended time period covering many time points are required as these give information on H_2O_2 levels as aggregation progresses.

We mentioned above that particular importance should be attached to H_2O_2 generated via the bound peptide. Not only is there a possibility of a direct reaction between H_2O_2 and the bound peptide (and any other accessible biomolecules) but also the conversion of H_2O_2 to •OH via electron transfer from the bound metal ion is significant as this would occur in the immediate vicinity of the peptide. As outlined above •OH is extremely aggressive and will react with adjacent molecules, including Aβ itself, with a reaction rate virtually controlled by diffusion. A common reaction is hydrogen atom abstraction, with the resulting peptidyl radical undergoing subsequent internal transformation reactions, resulting in oxidation products. If these reactions do not terminate the radical centre then these peptidyl radicals could themselves attack other peptide sites (again via hydrogen atom abstraction) leading to further oxidative damage until the radical centre is eventually terminated.

As noted above, there is now evidence that, *in vivo*, ROS could be involved in the pathogenesis of AD (and other neurodegenerative diseases) particularly under conditions where antioxidant defences have deteriorated to the point where they are unable to cope with the general ROS burden, possibly as part of the normal ageing process. Under these conditions not only could H_2O_2 undergo direct reaction with any biomolecules encountered but, when arriving at any appropriate site, such as a redox-active metal bound Aβ, conversion to •OH in the immediate vicinity of the peptide would lead to the aggressive reactions described immediately above.

3. Hypothesis: Soluble Oligomers Are the Optimal Generators of ROS

A key finding is that oxidative damage has been established to occur as an early event in AD [22, 23] indicating that ROS activity would be expected to be present during an early stage of the Aβ aggregation process. Soluble oligomers are likely to be the major type of Aβ aggregate present during this phase [24] and so these species become the prime candidate for involvement in early ROS generation.

Our own results, obtained during the early aggregation of Aβ(1–40), agree with the above inference [25]. They show that, *in vitro*, H_2O_2 is generated, during Aβ aggregation, as a "pulse" during the same time period that atomic force and electron microscopy images confirm the presence of small oligomers. Our results reveal a time delay before any H_2O_2 is observed, followed by a reasonably rapid increase in levels which reach a maximum before slowly declining back towards zero. The latter stage indicates that the rate of H_2O_2 removal (either through reacting directly with the peptide or via Fenton chemistry to form •OH) exceeds the rate of formation.

These observations raise a number of questions. In particular, what form of Aβ is optimal for the generation of ROS and why does the ability to generate H_2O_2 (and the associated toxicity) appear to decrease as the size of the Aβ aggregate increases beyond the oligomer stage?

Small soluble oligomers (such as dimers and trimers) will have a greater mobility than larger aggregates and so will be able to diffuse from their site of origin and insert into surrounding cell membranes, causing local damage, including reported changes in membrane permeability [27, 28]. The ability to generate ROS could also be a particular property of these small oligomers. A monomer bound to a single metal ion may not be particularly active in the formation of H_2O_2 if this reaction requires the synchronized transfer of two electrons [29]. Rapid diffusion would prevent the close

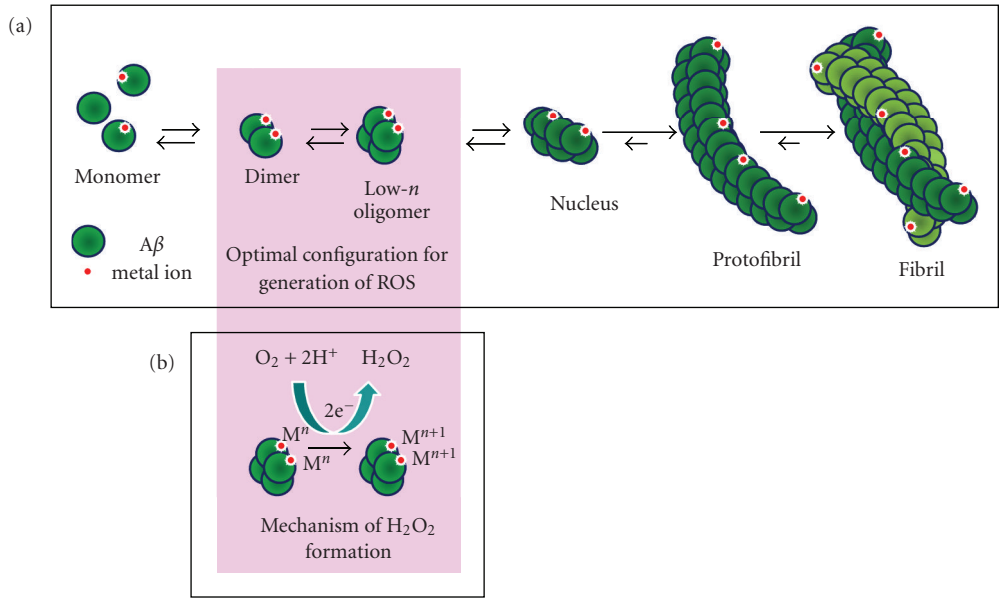

FIGURE 2: Soluble oligomers, together with their associated redox-active metal ions, are potentially responsible for the generation of H_2O_2. (a) Stages of Aβ aggregation, assuming that soluble oligomers are "on pathway" to fibril formation. The optimal configuration for generation of ROS may be at the small (low-n) oligomer stage. (b) Proposed mechanism for the generation of hydrogen peroxide. Here, M^n and M^{n+1} represent Cu(I) and Cu(II), or Fe(II) and Fe(III). The generation of H_2O_2 requires the synchronized transfer of two electrons from the bound metal ions and so reaction with the monomer is not favoured. Recent experimental evidence suggests that Cu ions remain bound to Aβ even in mature amyloid fibrils [26] and so the lack of activity of higher-order aggregates [25] is possibly due to some metal ions becoming sterically removed from any available O_2 molecules.

proximity of two such monomers to oxygen, thus impeding this reaction. Because the generation of H_2O_2 requires two electrons, the formation of early-stage oligomers would be expected to create a more favourable steric arrangement for this process to occur. One possible reason why higher-order aggregates, including mature amyloid fibrils, appear to lose the ability to generate H_2O_2 *in vitro* [25] could be that the supply of electrons becomes exhausted, although it is likely that available reducing agents would be plentiful *in vivo*. Another possible reason is that some of the metal ions associated with Aβ fibrils could lose their redox activity. The latter could be the result of a steric problem as the aggregate reaches its solubility limit (and beyond). Here, some metal ions could become sterically removed (i.e., hidden) from the O_2 molecule, with H_2O_2 generation becoming consequently more difficult. Under these circumstances, it is quite reasonable to suggest that the most suitable configuration for generating ROS lies within a range of optimum oligomer sizes. The latter could also explain the "time delay" observed in our aggregation experiments before levels rise, reach a maximum, and then decay [25]. These arguments lead us to hypothesize that ROS generation is greatest during the period when small (low-n) oligomers are the major species present. The many similarities between the properties of the amyloidogenic proteins/peptides implicated in other neurodegenerative diseases suggests that this type of mechanism could also apply to other disorders such as PD, the prion diseases and familial British dementia [9, 16, 25, 30–32].

Finally, it is becoming increasingly recognised that H_2O_2 acts as a diffusible signaling molecule in the CNS and is involved in the molecular mechanisms underlying synaptic plasticity and memory formation [33, 34]. Thus, the generation of ROS by soluble oligomers of Aβ could also, potentially, explain their potent effects on long-term potentiation (LTP), memory, and learning [35, 36].

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