Hemin and related porphyrins inhibit β-amyloid aggregation

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Abstract Porphyrins related to the naturally occurring pigment heme were found to effectively interfere with the aggregation of β-amyloid peptides as determined by an immunoassay configured for the detection of β-amyloid oligomers. Oligomerisation of β-amyloid is believed to be a key event in the progression of Alzheimer's disease. Inhibition of this aggregation is thus an important strategy in combating this commonest form of senile dementia. Evidence was also generated for hemin and hematin mediated protection of cultured cells against the neurotoxic effects of β-amyloid. These data are discussed with reference to the known pathology of Alzheimer's disease and the chemistry of porphyrins.

Key words: β-Amyloid; Alzheimer's disease; Hemin; Hematin

1. Introduction

Heme is an iron protoporphyrin IX complex which is essential to the function of a number of proteins. For instance, haemoglobin, one of the most abundant proteins in the human system, contains four non-covalently bound molecules of heme; these play a vital role in the transport of oxygen by this protein. Cytochrome, peroxidase, catalase and nitric oxide synthase also contain a heme moiety which, unlike the case of haemoglobin, is covalently bound to the protein. Physiological processes involving these proteins, and mediated by heme, include electron transport and other redox mechanisms [1].

As in the case of all proteinaceous material, heme-containing proteins have a definite lifetime. When of age they are eliminated from the organism by a series of degradation processes. Haemoglobin derived peptides have been reported to exhibit some opioid-like neurological activity (the so-called haemorphins) [2]. Thus heme released via protein degradation has been found to mediate neurite outgrowth [3] and cell development and differentiation [4].

Alzheimer's disease is a progressive neurodegenerative disorder characterised by neuronal cell death, synaptic loss and proteinaceous amyloid deposits at vascular and neuronal sites. It has been known for some time that neuronal dysfunction in Alzheimer's disease is initiated by the aggregation of amyloid-β peptides, which contain 40 or 42 amino acid residues. Levels of β-amyloid peptide and the aggregation of β-amyloid have been linked to the neurodegenerative process [5,6]. These peptides are a metabolic product of the amyloid precursor protein (APP) [7,8].

Previous reports have suggested that the pathology of Alzheimer's disease may be linked to oxidative stress [9]. Iron is known to be a facilitator of oxidative stress due to the production of hydroxyl radicals from hydrogen peroxide via the Fenton reaction [10–12]. Iron in the brain is mainly associated with ferritin and relatively very low levels are free and in reactive form. Nevertheless the ratio of ferritin to iron decreases in Alzheimer's disease with the localisation of the effect correlating with the severity of the neurological damage [12]. Free iron has been reported to promote β-amyloid aggregation [13] whereas iron chelation has been reported to be protective [10]. The direct effect on amyloid production remains unclear but there is evidence that the processing of APP shows a direct relationship with free iron levels and an indirect relationship with hemin and iron chelators such as desferrioxamine [11].

In our studies related to the disruption of β-amyloid aggregation, we found that hemin, hematin and zinc protoporphyrin IX inhibited this process to a varying degree. Hemin and hematin were also potent inhibitors of aggregation dependent β-amyloid cell toxicity. These results suggest another possible physiological role of heme related porphyrins in reducing the cytotoxicity of β-amyloid aggregation and fibril formation.

2. Materials and methods

2.1. Materials

Hemin, hematin, zinc protoporphyrin IX, protoporphyrin IX and (3-[4,5-dimethylthiaxol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Poole, UK). β-Amyloid peptides 1–40 and 1–42 were obtained from California Peptide Research Inc. (Napa, CA) and US Peptide Inc. (Fullerton, CA), respectively. All porphyrins were dissolved in dimethyl sulphoxide at 20 mM. Peptides were dissolved in 0.1% acetic acid at 400 μM. Subsequent dilutions of porphyrins and peptides were in phosphate buffered saline containing 0.02% (w/v) Tween-20. Appropriate vehicle controls were run in all experiments.

2.2. Immunoassay for β-amyloid aggregation

The aggregation of β-amyloid peptides was assessed by immunoassay. Briefly, β-amyloid 1–40 or 1–42 was incubated at 50 μg/ml in PBS/0.02% (w/v) Tween-20, overnight at 37°C, in the presence or absence of potential inhibitor. Peptide was then captured onto a microtitre plate pre-coated with a monoclonal antibody (2F12) raised to the 1–16 sequence of β-amyloid 1–40/42. Detection was via a biotinylated version of the 2F12 monoclonal and binding of streptavidin-europium (Wallac, Milton Keynes, UK) allowing quantitation by DELFIA (delayed enhanced lanthanide fluorescent immunoassay – Wallac). By comparison with standard electron microscopy techniques, and by using rifampicin as an inhibitor of oligomerisation [14], we have shown (unpublished data) that this antibody configuration only recognises oligomeric β-amyloid peptide. In a typical experiment, non-aggregated peptide gave a signal of approximately 20 000 light units; the signal for aggregated peptide (24 h incubation) was between 600 000 and 800 000 light units.

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Abbreviations: APP, amyloid precursor protein; MTT, (3-[4,5-dimethylthiaxol-2-yl]-2,5-diphenyltetrazolium bromide

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2.3. Cell toxicity measurements

To assess the cell toxicity potential of the aggregate(s) generated by overnight incubation of β-amyloid 1–40 (11.55 μM), IMR32 cells (human neuroblastoma, ECACC, Porton Down, UK) were plated at 6×10^4 cells/cm^2 in a 96 well microtitre plate (Nunc). After 2 h attachment, potentially aggregated peptide solution (containing β-amyloid 1–40 plus porphyrin, or peptide alone) was added at concentrations of 0.1–200 nM β-amyloid. After a further 24 h incubation, cell viability was assessed by an MTT assay, which depends upon the integrity of mitochondrial respiration for the conversion of the metabolic dye MTT (3.0 mM) to a blue formazan product [15].

3. Results and discussion

These results show that the iron porphyrins, hemin and hematin are powerful inhibitors of the β-amyloid aggregation process. Fig. 1 shows the dramatic fall in the maximum aggregation of β-amyloid 1–40 peptide in the presence of these porphyrins, compared to vehicle. Replacement of iron at the centre of the tetrapyrrole macrocycle by zinc still inhibits the aggregation process. On the other hand protoporphyrin IX, where the iron is now replaced by two protons, is at least two orders of magnitude less inhibitory than hemin. We intend to perform further spectroscopic studies to establish the mechanism of interaction of the porphyrins and β-amyloid, as this may provide more information about the nature of β-amyloid aggregation.

We do not know the nature of this metal porphyrin-β-amyloid peptide complexation. However, it is interesting to note that β-amyloid peptides contain three histidine residues. Thus it is possible that the π-electrons of one of the imidazole rings of these amino acids are involved in bonding to the metal ion at the centre of the porphyrin ring, in the same way that heme iron in oxygen-carrying proteins complexes with an imidazole group of a histidine side chain [16]. Fig. 2 shows that hemin also inhibits the aggregation of the more hydrophobic 1–42 β-amyloid peptide to a similar extent as that of the shorter 1–40 peptide. The IC_{50} for the inhibition of aggregation of either peptide by hemin is about 1 μM porphyrin, approximately one tenth of the β-amyloid peptide concentration (11.6 μM) used in these experiments. The results can be interpreted as demonstrating that 1 mole of hemin or hematin binds either (i) to approximately 10 mole of β-amyloid or (ii) to an aggregate progenitor comprising some 10% of the total peptide mass, in either case preventing the formation of a toxic species of peptide. However, as the level of heme and related porphyrins in the brain has yet to be reported it is not possible to relate the activity described above to physiological heme levels.

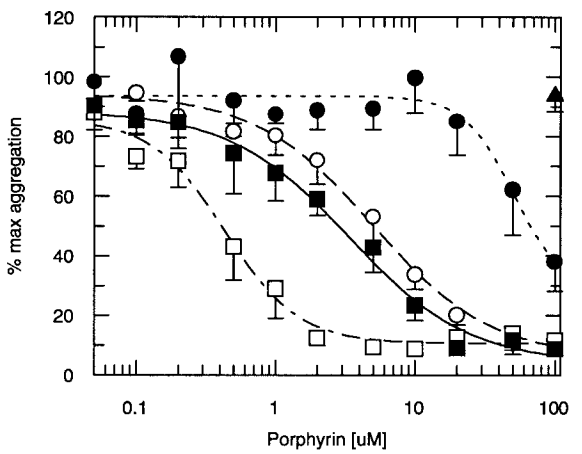

Fig. 1. Inhibition of aggregation of β-amyloid 1–40 by porphyrins: hemin (□), hematin (■), zinc protoporphyrin IX (○) and protoporphyrin IX (●). Vehicle corresponding to 100 μM porphyrin only is shown (▲). Data points are the means of three experiments, determined in duplicate (error bars are ±S.E.M.).

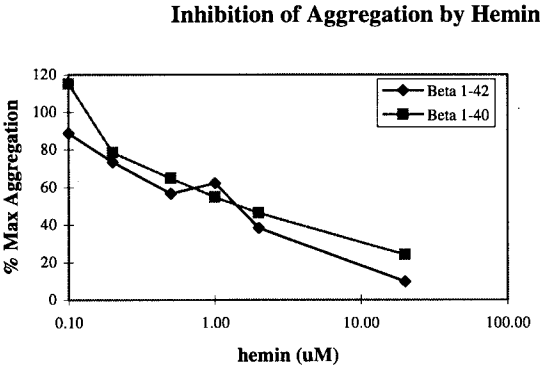

Fig. 2. Inhibition of aggregation of β-amyloid 1–40 and 1–42 by hemin.

A major test of the efficacy of hemin and other porphyrins to inhibit the aggregation of β-amyloid peptides was to add the porphyrin-β-amyloid incubates to human IMR32 neuroblastoma cells in culture. These cells are regarded as a suitable model for studying neurotoxic agents [17]. In the presence of vehicle, β-amyloid produced approximately 30% decrease in MTT reduction. This was attenuated in the presence of metallo-porphyrin. As shown in Fig. 3, both hemin and hematin showed similar potency in inhibiting the production of toxic aggregate of β-amyloid. Hence, the data obtained in the toxicity assay are in excellent agreement with, and fully supportive of, the immunoassay results. The addition of hemin or hematin to pre-fibrillised β-amyloid had no effect on the toxicity response suggesting that the porphyrins had no direct effect on β-amyloid cell toxicity (data not shown). It is unlikely that the reduction in cell toxicity is a result of a direct interaction between MTT and hemin or hematin; the concentration of MTT is more than five orders of magnitude higher than that of the porphyrins. Similar data could not be collected for protoporphyrin IX and zinc protoporphyrin IX as these molecules were toxic to the IMR32 cells.

The accumulation of insoluble deposits of amyloid in the brain parenchyma is thought to be initiated to a large extent by the aggregation of hydrophobic β-amyloid peptides, containing 40 or 42 amino acid residues. The latter are metabolic products formed from the protease cleavage of a transmembrane protein, the amyloid precursor protein, encoded by the APP gene [7,8,18]. Diffuse plaque, mostly made up of aggregated β-amyloid, degenerates neurones and leads to the irreversible progression of Alzheimer's disease. Inhibiting either the aggregation or deposition of β-amyloid peptides is one of the therapeutic strategies that have been followed by a number of workers in attempts to combat this terrible disease [14,19,20].

From the present data it is difficult to identify whether hemin and hematin have any physiological role in the protection against β-amyloid toxicity in Alzheimer's disease. The low level of β-amyloid released from the precursor protein β-APP is in the soluble monomeric form, and circulates as such in the blood [21]. As heme-containing proteins exist

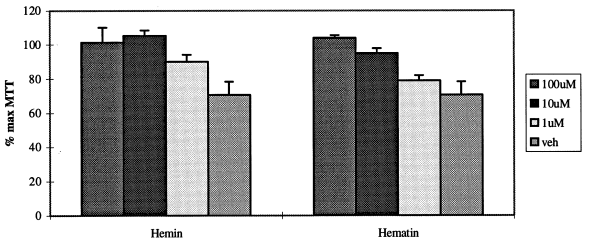

Fig. 3. Effects of hematin and hemin on β-amyloid 1–40 aggregation-dependent toxicity of IMR32 cells. Data points shown are the mean values from three experiments, carried out in triplicate (error bars are ±S.E.M.).

both within and outside the brain, different levels of this and related porphyrins must be released during the metabolism of these proteins. β-Amyloid in the cerebrospinal fluid is also rapidly cleared into blood [22]. Moreover, the physiological concentration of heme appears to be age dependent. For instance, it has been reported that the changes in total haemoglobin (the biggest source of heme) concentration during activation of brain (frontal cortex) function decreases with age [23]; the mean changes in elderly patients were found to be about 45% lower than those recorded for young subjects. The activity of heme oxygenase-1, the enzyme which catalyses the degradation of heme to biliverdin and iron, also increases during the progression of Alzheimer's disease [24]. The latter phenomenon has been related to the role of oxidative stress in the neurofibrillary pathology of Alzheimer's disease. However, as a result of our present findings it may be possible to speculate that an increase in activity of heme oxygenase-1 may have another role of decreasing the concentration of heme and its oxidation products, thus enhancing the β-amyloid aggregation process.

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