RESEARCH COMMUNICATION

Amyloid-β binds catalase with high affinity and inhibits hydrogen peroxide breakdown

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Amyloid-β (Aβ) specifically bound purified catalase with high affinity and inhibited catalase breakdown of H_2O_2. The Aβ-induced catalase inhibition involved formation of the inactive catalase Compound II and was reversible. Catalase $\leftrightarrow$ Aβ interactions provide rapid functional assays for the cytotoxic domain of Aβ and suggest a mechanism for some of the observed actions of Aβ plus catalase *in vitro*.

Key words: Alzheimer's, antibodies, cytotoxicity, myeloma, peroxidase.

INTRODUCTION

The amyloid-β (Aβ) peptide is a major component of the senile plaques in Alzheimer's disease, and these are associated with areas of neurodegeneration [1]. The cytotoxicity of Aβ is mediated via a functional domain between Aβ residues 25–35 which is active in a range of cell types [2–4]. Binding sites for Aβ have been identified [5,6] on the P75 neurotrophin receptor (P75ntr) and the intracellular endoplasmic-reticulum Aβ-binding protein (ERAB). Inhibition of Aβ binding to both the P75ntr and ERAB reduces the cytotoxicity of Aβ, suggesting that these proteins play a permissive role in Aβ cytotoxicity [5,6].

The peroxidase enzyme catalase is also associated with senile plaques [7,8] and protects cells in culture from the actions of Aβ [9–12]. The ability of catalase to breakdown hydrogen proxide (H_2O_2) generated in response to Aβ was suggested as a mechanism for catalase protection [9]. The existence of cytoplasmic catalase [13] and Aβ uptake into cells [14] suggests that interactions between these compounds could occur within cells. Addition of inactivated catalase to cell cultures still provides protection [12], suggesting that a direct interaction between Aβ and catalase may be involved.

The present study set out to determine whether catalase binds to Aβ and to assess the effects of Aβ on catalase breakdown of H_2O_2.

METHODS

Binding studies

Biotinylated Aβ-(1–42)-peptide (Aβ 1–42), Aβ 12–28 and Aβ 25–35 were prepared using a LinKit–Biolink Kit (ISL, Paignton, Devon, U.K.). ELISA plates were coated with catalase (1 μg/ml) in carbonate buffer, pH 9.6, and unoccupied sites blocked with 5% (w/v) Marvel dried milk. Biotinylated peptides (200 pM) were incubated alone, with control peptides or with unlabelled Aβ peptides in PBS (containing 0.1% BSA and 0.05% Tween-20) at 37 °C for 4 h. After washing to remove unbound material, an alkaline phosphatase polymer–streptavidin conjugate (Sigma, Poole, Dorset, U.K.) was added and incubated at 37 °C for 2 h. After washing to remove unbound material, *p*-nitrophenyl phosphate substrate was added and absorbance at 405 nm determined. Affinity constants were determined by Scatchard analysis [15] using the following equation:

$$A_0/(A_0-A) = 1+K_d/a_0$$

and plotting $v[(A_0-A)/A_0]$ against v/a, where A_0 is the absorbance in the absence of unlabelled peptide, A is the absorbance in the presence of unlabelled peptide, a_0 is the total concentration of unlabelled peptide and a is the concentration of unlabelled peptide added. The dissociation constant (K_d) was equal to -1/slope of v against v/a.

Catalase activity

Catalase (EC 1.11.1.6) from human erythrocytes (Sigma) was used for all incubation experiments. The activity of catalase (50 k-units/litre) incubated with test peptides (2 μM) was determined [16] after incubation in 60 mM sodium/potassium phosphate buffer, pH 7.4, at 37 °C in a total volume of 100 μl. After incubation, catalase activity was determined by mixing 50 μl of sample with 50 μl of substrate (6.5 μmol of H_2O_2 in phosphate buffer) for 60 s, adding 100 μl of 32.4 mM ammonium molybdate and measurement of absorbance change at 405 nm. Catalase activity was calculated from a standard curve (0–100 k-units/litre) using purified human catalase of known activity.

Cell cytotoxicity

SP2/0-Ag-14 mouse myeloma cells (2×10^5 cells/ml) were plated in PBS containing 0.1% BSA and test peptides (20 μM) and incubated at 37 °C in a 5%-CO_2 humidified atmosphere. Cells were harvested and viability was determined by Trypan Blue dye exclusion [9] with at least 100 cells counted per well.

Monoclonal-antibody production

Mouse spleen cells were immunized *in vitro* with Aβ 17–42 using a Cel-Prime Kit (Insight Biotechnology, Wembley, Middx.,

Abbreviations used: Aβ, amyloid-β; P75ntr, P75 neurotrophin receptor; ERAB, endoplasmic-reticulum Aβ-binding protein; Aβ *x–y*, Aβ-(*x–y*)-peptide; AIP, a β-sheet breaker peptide with the amino acid sequence His-Pro-Gln-Pro-Leu-Asp-Phe-Phe-Gln-Pro; CRH, corticotrophin-releasing hormone.

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U.K.). After immunization, cells were fused with mouse myeloma cells using poly(ethylene glycol). Hybridomas selected in hypoxanthine/aminopterin/thymidine ('HAT') medium were screened for antibodies against Aβ 25–35 by ELISA [17] and cell line ALI-01, producing an IgM isotype anti-(Aβ 25–35) antibody was selected.

Data analysis

Data are expressed as means $\pm$ S.E.M. Catalase levels are expressed as percentage inhibition of control (untreated) catalase. For cytotoxicity experiments data are expressed as percentage of dead (Trypan Blue-stained) cells. The significance of differences between data was evaluated by one-way analysis of variance. A P value of < 0.05 was considered statistically significant.

RESULTS

Catalase binding to Aβ

Biotinylated Aβ peptides were used to test for Aβ binding to human catalase. Results showed that Aβ 1–42 and Aβ 25–35, but not Aβ 12–28, bound specifically to catalase. Fragments of Aβ containing residues 31–35 could inhibit the binding of Aβ 1–42 to catalase (Figure 1A). This region of Aβ has previously been suggested to contain the binding domain responsible for Aβ cytotoxicity [18]. Scatchard analysis of binding data demonstrated a K_d of 3.3 ± 0.2 nM ($n = 5$) for Aβ 1–42 binding to catalase and showed no difference between the binding of Aβ 1–42 or Aβ 25–35 to catalase (Figure 1B). Binding to catalase-coated plates was also inhibited by catalase in solution, but was unaffected by the unrelated proteins (BSA, human fibrinogen, mouse IgGs and alkaline phosphatase).

Effect of Aβ on catalase breakdown of H_2O_2

Since Aβ binds to catalase, the effects of Aβ 1–42 and peptide fragments on catalase breakdown of H_2O_2 were examined. Incubation of human catalase with Aβ 1–42, Aβ 1–40, Aβ 17–42, Aβ 25–35 and Aβ 31–35 caused a significant inhibition of H_2O_2 breakdown (Figure 1C). The Aβ 31–35 region was thus contained in all the fragments of Aβ that bind to, and inhibit, catalase. The inhibition of catalase activity was time-dependent, with significant levels of inhibition detected after preincubation for 15 min with Aβ 31–35, preincubation for 30 min with Aβ 1–42, Aβ 17–42 or Aβ 25–35 and preincubation of 45 min with Aβ 1–40.

Monoclonal antibodies against Aβ [20,21] with specificities for the N-terminus (6F/3D) and C-terminus (G2-10 and G2-11) or a control antibody against corticotrophin-releasing hormone (CRH) [17] had no effect on Aβ-mediated inhibition of catalase. A specific monoclonal antibody (ALI-01) against the Aβ 25–35 region was therefore raised to confirm the role of the Aβ 25–35 component of Aβ 1–42 in catalase inhibition. The ALI-01 antibody was effective at preventing catalase inhibition by both Aβ 1–42 and Aβ 25–35 (Figure 1D) confirming that the Aβ 25–35 component of Aβ 1–42 was responsible for catalase inhibition.

The forms of Aβ which bind to, and inhibit, catalase all increase the percentage cell death in myeloma cell cultures incubated for 24 h (Figure 1E). This confirms that the effects of Aβ on catalase mirror the cytotoxicity of the peptide. The inhibition of catalase by Aβ was also concentration-dependent (Figure 1F). The concentrations of Aβ required to inhibit catalase activity and for cytotoxicity are in the micromolar range, in agreement with previous cytotoxicity studies [3] and within the tissue concentrations suggested for Alzheimer's-disease brains [19].

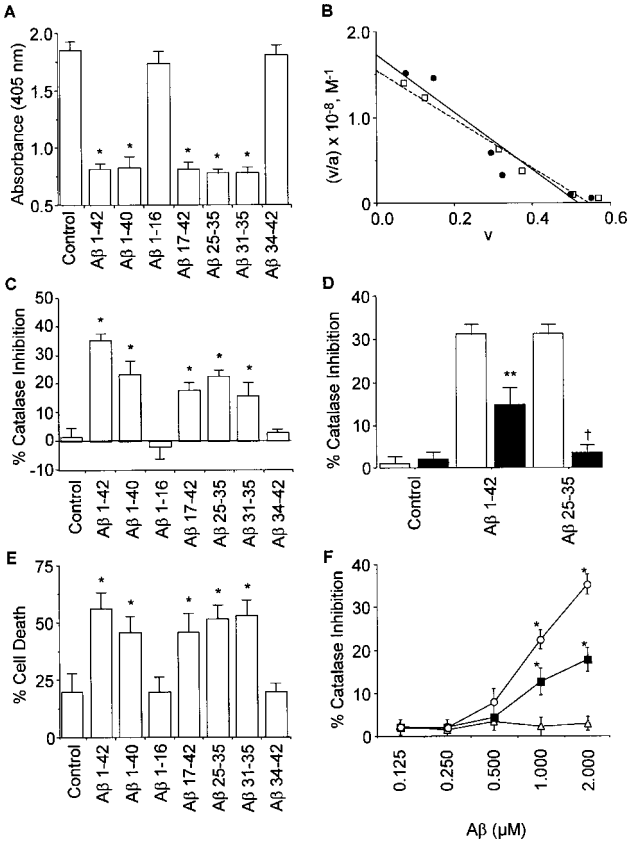

Figure 1 Binding of Aβ 1–42 and peptide fragments to catalase plus effects on activity and cell survival

(**A**) Binding of biotinylated Aβ 1–42 (200 pM) to catalase in the presence of 2 μM unlabelled peptide (open columns). (**B**) Scatchard plot of binding of Aβ 1–42 (□) and Aβ 25–35 (●) to catalase in the presence of unlabelled Aβ 1–42. (**C**) Human catalase inhibition in the presence of Aβ peptide fragments. (**D**) Effects of ALI-01, an anti-(Aβ 25–35) monoclonal antibody (closed columns) and control antibody (open columns) on human catalase inhibition. (**E**) Effect of Aβ peptide fragments on mouse myeloma cell viability. (**F**) Human catalase inhibition in the presence of increasing concentrations of Aβ 1–42 (○), Aβ 17–42 (■) and Aβ 34–42 (△). The control for all experiments was the CRH peptide. All results are means $\pm$ S.E.M., $n = 8$ for each group: $^*P < 0.05$ versus control peptide; $^{**}P < 0.05$ for ALI-01 + Aβ 1–42 versus control antibody + Aβ 1–42; †$P < 0.05$ for ALI-01 + Aβ 25–35 versus control antibody + Aβ 25–35.

Mechanism for Aβ inhibition of catalase

Catalase breakdown of H_2O_2 causes the formation of Compound I [22], which can then break down either H_2O_2 or ethanol. Compound I can be converted into an inactive form of catalase, Compound II [22], and this inactivation can be prevented by excess ethanol or reversed by NADPH (Figure 2A). Chemical inhibition of catalase by 3-amino-1,2,4-triazole or 3,3′-diaminobenzidine can be prevented by addition of ethanol and NADPH [22,23]. To determine whether Aβ acts in a similar manner, catalase-incubation experiments were carried out in the presence of ethanol or NADPH. Results showed that the inhibition of catalase by Aβ 1–42 was abolished in the presence of ethanol plus H_2O_2 and significantly decreased in the presence of NADPH; control incubations with H_2O_2 alone or $NADP^+$ had no effect (Figure 2B).

The Aβ 1–40 peptide contains a heavy-metal-binding domain [24] that can bind iron but which is absent from the N-terminally truncated form Aβ 17–40. Iron can increase Aβ cytotoxicity and

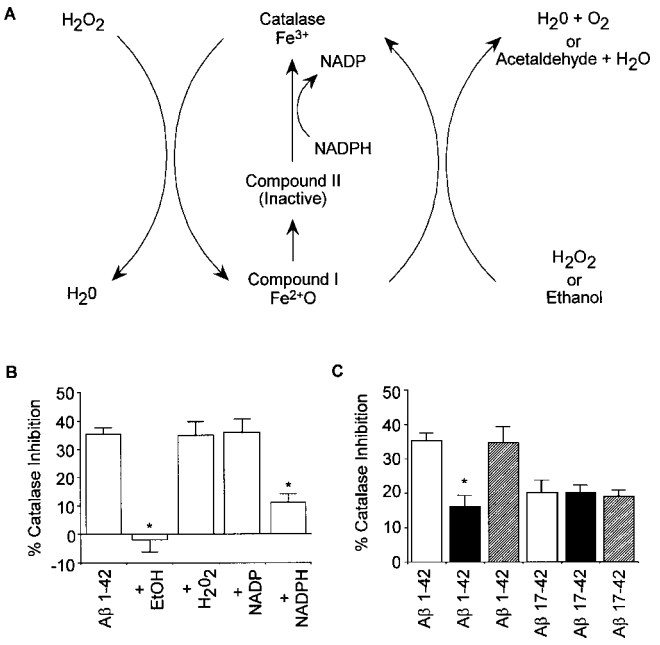

Figure 2 Mechanism for Aβ inhibition of catalase activity

(**A**) Proposed model for catalase breakdown of H_2O_2 and Compound II formation during human catalase inhibition [22]. (**B**) Effects of ethanol (EtOH; 20 mM) + H_2O_2 (1 mM), H_2O_2 (1 mM), $NADP^+$ (20 μM) and NADPH (20 μM) on human catalase inhibition by Aβ 1–42, (**C**) human catalase inhibition by Aβ 1–42 and Aβ 17–42 in phosphate buffer (open columns), phosphate buffer containing $FeCl_2$ (closed columns; 25 μM) and phosphate buffer containing $MgCl_2$ (hatched columns; 25 μM). All results are means $\pm$ S.E.M., $n = 8$ for each group; $^*P < 0.05$ versus Aβ 1–42.

aggregation *in vitro* [25,26] and is directly involved in Aβ generation of H_2O_2 [19]. Human catalase activity requires iron [22] bound to an active site at residue 358, and it is possible that an interaction between Aβ and iron could influence enzyme activity. Incubation of catalase with Aβ in buffer supplemented with 25 μM $FeCl_2$ caused a decrease in the Aβ 1–42, but not the Aβ 17–42, inhibition of catalase activity, whereas control incubations in buffer supplemented with 25 μM $MgCl_2$ had no effect (Figure 2C), suggesting that Aβ binding to the haem group of catalase is not the main cause of catalase inhibition. Under these conditions, Aβ 1–42 has previously been shown to aggregate [24], and the decreased ability of Aβ 1–42 to inhibit catalase in the presence of iron may be due to this effect. Incubation of Aβ 1–42 at 37 °C for 7 days prior to use has previously been shown to cause aggregation of Aβ [3,4]. A preparation of aggregated Aβ 1–42 was used to test whether aggregation prevented Aβ 1–42 inhibition of catalase. Results showed that aggregated Aβ 1–42 (2 μM) inhibited catalase activity by $15.4 \pm 2.9\%$ ($n = 8$), whereas freshly prepared Aβ 1–42 (2 μM) inhibited catalase activity by $34.7 \pm 4.9\%$ ($n = 8$), confirming that the decreased catalase inhibition by Aβ 1–42 in the presence of iron could be explained by aggregation of the peptide.

Use of a catalase-inhibition assay to identify compounds which block Aβ cytotoxicity

The ability of cytotoxic forms of Aβ to inhibit catalase activity suggests that measurement of catalase activity in the presence of Aβ could be adapted as an assay for molecules which interact with Aβ and prevent cytotoxicity. The β-sheet breaker peptides prevent aggregation of Aβ and are effective inhibitors of Aβ cytotoxicity [27]. A β-sheet breaker peptide (AIP) with the amino acid sequence His-Pro-Gln-Pro-Leu-Asp-Phe-Phe-Gln-Pro was tested in both a cytotoxicity assay and an Aβ–catalase inhibition assay. Results showed that the AIP peptide at equimolar concentrations prevented Aβ 1–42 inhibition of catalase activity and blocked Aβ 1–42 cytotoxicity (Table 1), further supporting the suggestion that an Aβ–catalase inhibition assay could be used to identify molecules which interact with Aβ and prevent cytotoxicity.

Table 1 Effect of a β-sheet breaker peptide, AIP, on Aβ 1–42 inhibition of catalase activity and cytotoxicity

The Table shows human catalase activity, expressed as percentage catalase inhibition, in the presence of 2 μM peptides, and mouse myeloma viability, expressed as percentage cell death, in the presence of 20 μM peptide. Control peptide 1 = CRH; control peptide 2 = somatostatin. All results are means $\pm$ S.E.M., $n = 8$ for each group: $^*P < 0.05$ versus control 1 + control 2; †$P < 0.05$ versus Aβ 1–42 + control 2.

	Catalase inhibition (%)	Cell death (%)
Control 1 + control 2	1.5 ± 3.1	20.1 ± 8.0
AIP + control 1	-0.8 ± 3.2	23.3 ± 7.1
Aβ 1–42 + control 2	33.2 ± 1.9 *	56.1 ± 7.4*
Aβ 1–42 + AIP	7.9 ± 1.6 †	23.5 ± 5.1†

DISCUSSION

The K_d for Aβ binding to catalase is in the same nanomolar range as the blood and cerebrospinal-fluid Aβ concentrations [28], and Aβ has biological activities at such nanomolar concentrations [2,29], suggesting that interactions between Aβ and catalase could have a physiological role. The binding of Aβ to catalase may be a component of the mechanism for Aβ inhibition of catalase breakdown of H_2O_2. The observation that blockade of catalase Compound II formation prevents Aβ inhibition of catalase suggests that Aβ may be involved in oxidizing the enzyme, a mechanism already proposed for Aβ inactivation of other enzymes [30], and this process appears to involve Aβ binding to catalase. The high-affinity binding of Aβ to catalase is probably responsible for the ability of inactivated catalase to neutralize Aβ cytotoxicity [12]. The specificity of the catalase for the cytotoxic domain of Aβ [2] provides a mechanism for catalase blockade of Aβ cytotoxicity.

The ability of Aβ to inhibit catalase activity may contribute to Aβ cytotoxicity. In the *in vitro* environment employed in cell-culture studies [2–4], the levels of Aβ are probably sufficient to inhibit catalase activity, and this would contribute to the cytotoxicity of Aβ. These Aβ concentrations are vastly in excess of the blood and cerebrospinal-fluid levels of soluble Aβ peptide [28]. However, the Alzheimer's-disease brain tissue total Aβ levels have been suggested to reach this value [19]. The insoluble nature of the Aβ in Alzheimer's-disease brain tissue [1] represents a form of Aβ that has a reduced ability to inhibit catalase activity. The key question for Alzheimer's pathology is whether sufficient Aβ would be in a form to bind to, and inhibit, catalase. The presence of catalase in Alzheimer's-disease plaques [7,8] may reflect *in vivo* binding of catalase to Aβ and, as a result of the total Aβ concentrations in this environment, the catalase activity could be inactivated.

The ability of Aβ to bind to, and inhibit the activity of, catalase with specificity for the cytotoxic Aβ 25–35 region provides potential rapid screening methods for compounds which interact with this region of Aβ. The catalase-inhibition assay can

be performed within 2 h and using the 96-well-microtitre-plate format has the potential to screen many samples simultaneously. The interactions between these compounds are likely to play roles both *in vivo* and *in vitro*.

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