**β-Amyloid toxicity in organotypic hippocampal cultures:** Protection by EUK-8, a synthetic catalytic free radical scavenger

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**ABSTRACT** Oxygen free radicals have been proposed to mediate amyloid peptide (BAP)-induced neurotoxicity. To test this hypothesis, we evaluated the effects of EUK-8, a synthetic catalytic superoxide and hydrogen peroxide scavenger, on neuronal injury produced by BAP in organotypic hippocampal slice cultures. Cultures of equivalent postnatal day 35 (defined as mature) and 14 (defined as immature) were exposed to various concentrations of BAP (1–42 or 1–40) in the absence or presence of 25 μM EUK-8 for up to 72 hours. Neuronal injury was assessed by lactate dehydrogenase release and semiquantitative analysis of propidium iodide uptake at various times after the initiation of BAP exposure. Free radical production was inferred from the relative increase in dichlorofluorescein fluorescence, and the degree of lipid peroxidation was determined by assaying thiobarbituric acid-reactive substances. Treatment of mature cultures with BAP (50–250 μg/ml) in serum-free conditions resulted in a reproducible pattern of damage, causing a time-dependent increase in neuronal injury accompanied with formation of reactive oxygen species. However, immature cultures were entirely resistant to BAP-induced neurotoxicity and also demonstrated no dichlorofluorescein fluorescence or increased lipid peroxidation after BAP treatment. Moreover, mature slices exposed to BAP in the presence of 25 μM EUK-8 were significantly protected from BAP-induced neurotoxicity. EUK-8 also completely blocked BAP-induced free radical accumulation and lipid peroxidation. These results not only support a role for oxygen free radicals in BAP toxicity but also highlight the therapeutic potential of synthetic radical scavengers in Alzheimer disease.

The insoluble extracellular deposits that constitute senile plaques, which are hallmark features of Alzheimer disease (AD), are primarily composed of aggregated β-amloid protein (BAP) fragments (1). The observation that these plaques are associated with areas of selective neuronal loss (2), combined with recent findings indicating that some forms of AD are related to mutations of the amyloid precursor protein (3–5), has sparked extensive research into the association between BAP accumulation and neuronal degeneration. The recent report that transgenic mice overexpressing the human amyloid precursor protein develop several pathological manifestations of AD also strongly supports the involvement of BAP in AD (6). Although BAP has generally been found to produce neurotoxicity in vitro and in vivo (7, 8), there is little agreement in the literature concerning the cellular pathways (i.e., apoptosis or necrosis) involved in BAP-induced neuronal death (9–11) or the mechanisms by which BAP exerts its toxic effect (12, 13). One major issue in studying BAP-induced neurotoxicity lies in the choice of experimental models. In vivo BAP injection is not always neurotoxic (14), and most reports describing neuronal BAP toxicity utilize dissociated primary neuronal or PC-12 cultures. However, dissociated neurons retain none of their original connectivity and exist in a nonphysiological monolayer, whereas PC-12 cells exhibit only select neuronal properties. In the present study, we used an organotypic hippocampal culture system to evaluate the neurotoxicity of BAP. Organotypic cultures combine the accessibility and maintenance of in vitro culture systems while preserving intact the hippocampal synaptic circuitry and anatomy. This culture system has recently been used quite extensively to study neuronal death associated with excitotoxicity (15–17), hypoxia and hypoglycemia (18, 19), and knife-cut lesions (20).

Although the mechanisms underlying BAP toxicity are not yet understood, it has been proposed that reactive oxygen species (ROS) play a critical role in producing neuronal damage (21, 22). This idea was based in part on the protective effect of ROS scavengers against BAP-induced neuronal death (12, 21–25). We previously showed that the synthetic catalytic scavenger of oxygen free radicals, the saleno-manganese complex EUK-8, provided a significant degree of protection against hypoxia- and acidosis-induced neuronal damage in hippocampal slices (26). We therefore tested the protective effects of EUK-8 on BAP-induced toxicity in hippocampal slice cultures. Neuronal injury was quantified by determining the extent of neuronal uptake of the fluorescent dye propidium iodide (PI) and of lactate dehydrogenase (LDH) release into the medium. Additionally, as it has been reported that dissociated neurons maintained in cultures for a few days are resistant to BAP toxicity (27), we determined the effects of BAP on hippocampal slices maintained in cultures for a few days or a few weeks. The role of ROS in BAP-induced injury was further investigated by determining the levels of free radical accumulation [as indicated by increased 2',7'-dichlorofluorescein (DCF) fluorescence] and lipid peroxidation (as reflected by the assay of thiobarbituric acid reactive substances) in BAP-treated slices from both immature and mature cultures.

**MATERIALS AND METHODS**

Materials. Minimum essential medium and horse serum were purchased from Gibco. EUK-8 was provided by Eukarion (Bedford, MA), and BAP (1–40 and 1–42) was obtained from C. Glabe and prepared as described in Burdick et al. (28) and stored lyophilized until use. 2',7'-Dichlorofluorescein diacetate (DCF-DA) was purchased from Molecular Probes. All other chemical products were purchased from Sigma.

Culture Techniques. Organotypic hippocampal cultures were prepared using the technique of Stoppini et al. (29). Briefly, hippocampi were harvested from 5- to 7-day-old

Abbreviations: BAP, β-amloid peptide; AD, Alzheimer disease; LDH, lactate dehydrogenase; PI, propidium iodide; DCF, 2',7'-dichlorofluorescein; DCF-DA, 2',7'-dichlorofluorescein diacetate; ROS, reactive oxygen species; MDA, malondialdehyde.

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Sprague-Dawley rat pups under sterile conditions and cut transversely at 400 μm using a McIlwain tissue chopper. Tissue sections were then plated onto 30-mm cell culture inserts (Millicell-CM; Millipore; three slices per insert) and maintained in a humidified CO₂ incubator. Culture medium was changed once weekly, and sections were allowed to equilibrate to in vitro conditions for at least 5–7 days before treatment. Immature cultures refer to hippocampal slices that were maintained in vitro for only 7 days, whereas mature cultures correspond to slices that were kept for 4 weeks before treatment.

**βAP Treatment.** βAP (1–40 or 1–42) was initially solubilized in sterile H₂O, incubated overnight at room temperature, and then applied to slice cultures at various concentrations (50, 100, or 250 μg/ml) in serum-free exposure medium. Similar results were obtained with either peptide, and unless otherwise noted, βAP in the text refers to either one. A 100-μl aliquot of βAP containing medium was applied directly to the top of slices at the onset of the exposure to ensure sufficient neuronal exposure to the aggregated peptide (the slices were covered only for a few minutes, as the medium rapidly diffused through the slices). Selected sections were also treated with EUK-8, which was applied to the medium at the same time as βAP. Control sections were subjected to the exact same procedure but did not receive βAP or EUK-8. At 24, 48, and 72 hr after exposure onset, the extent of neuronal injury was determined as described below. To eliminate the possibility that EUK-8 directly interfered with the formation of insoluble aggregates of βAP, we determined the amount of aggregated βAP after incubation of βAP with EUK-8 in H₂O for up to 72 hr or in the medium at the end of the incubation with slices. Aggregated βAP was collected by centrifugation, and the concentration of proteins in the pellet was determined with the Bradford assay (30). Under both conditions, EUK-8 was found not to reduce the amount of aggregated βAP.

**Assessment of Neuronal Injury.** The extent of neuronal injury was evaluated at the termination of βAP exposure and was based on two different parameters: (i) the extent of neuronal uptake of the fluorescent dye PI, and (ii) the amount of LDH released into the medium. PI uptake into damaged neurons was observed with a fluorescent microscope, using a standard rhodamine filter set. Neuronal PI uptake was semiquantitatively analyzed in sections by assigning a score of 0, 1, 2, 3, or 4 to each section corresponding to 0%, 25%, 50%, 75%, or 100% of neurons exhibiting distinct fluorescence. LDH activity in the medium was measured as described by Koh and Choi (31).

After PI and LDH evaluations, the tissue was removed from the membranes and homogenized in ice-cold phosphate-buffered saline. The formation of ROS and the degree of lipid peroxidation were determined by analyzing DCF fluorescence and malondialdehyde (MDA) release, respectively. DCF-DA (25 μM) was applied to the culture medium at the onset of βAP exposure. DCF-DA is freely permeable, and once inside cells it is readily converted to 2',7'-dichlorofluorescin, which is then able to interact with intracellular free radicals and peroxides to form the highly fluorescent DCF. DCF fluorescence was detected using a spectrofluorometer with excitation wavelength set at 502 nm and emission wavelength set at 523 nm. Standard curves were prepared using known amounts of DCF, and results were calculated as nanomoles of DCF formed per milligram of protein and then converted to a percentage of the control. As EUK-8 alone caused a mild increase in DCF fluorescence even when incubated in the absence of cells (data not shown), DCF fluorescence in βAP plus EUK-8-treated sections was expressed as a percentage of the value measured in EUK-8 alone treated sections. MDA concentration was determined by using the thiobarbituric acid-reactive substances assay, as described by Kovachich and Mishra (32). Briefly, hippocampal slices were homogenized in ice-cold phosphate-buffered saline, the homogenates were precipitated in 5% trichloroacetic acid, and an aliquot of the trichloroacetic acid precipitate was incubated with 0.335% thiobarbituric acid in glacial acetic acid for 1 hr at 90–95°C. Lipids were then extracted in butanol, and the optical density was measured spectrophotometrically at 540 nm. A standard curve was established using known amounts of MDA equivalents under identical assay conditions, and the results were calculated as nanomoles of MDA released per milligram of protein and then converted to a percentage of the control.

**Statistical Analyses.** In each experiment, four inserts (i.e., 12 slices) were subjected to the same treatments, and each experiment was reproduced two or three times. Therefore data are the means of at least 24 values (slice data) or 8 values (LDH data). All data were analyzed using one-way ANOVA, followed by least significant difference post-hoc analysis to determine statistical significance. P values <0.05 were considered statistically significant.

**RESULTS**

**βAP Toxicity in Organotypic Hippocampal Cultures.** The application of βAP (100 μg/ml) to mature hippocampal slice cultures resulted in widespread neuronal injury, as indicated by both PI uptake and LDH release (Fig. 1). After 48 hr of continuous βAP exposure, about 25% of neurons were positively fluorescent, and this value continued to increase to reach

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Time course of βAP-induced PI uptake and LDH release in the absence or presence of EUK-8. Mature organotypic hippocampal cultures were exposed to βAP at 100 μg/ml for 24, 48, or 72 hr in the absence or presence of 25 μM EUK-8, and the extent of toxicity was then determined by quantification of PI uptake (A) and LDH release (B) as described in Material and Methods. Data are means ± SEM of at least 24 (A) or 8 values (B). *, P < 0.05 as compared to control levels (the difference between control values at 0 and 72 hr is not statistically significant in A, but it is in B).
near 40% by 72 hr (Fig. 1A). Hippocampal slices exposed to 
βAP in the presence of 25 μM EUK-8 were completely 
protected against the βAP-induced increase in PI uptake (Fig. 1A), 
whereas incubation of cultures with EUK-8 alone had no 
effect on PI uptake (data not shown). The semiquantitative 
analysis employed to quantify PI uptake is based on overall 
fluorescence of the entire slice, but a qualitative evaluation of 
βAP-induced PI uptake revealed considerably higher fluorescence 
in pyramidal neurons as compared to dentate granule 
neurons, with no obvious difference in PI uptake between 
CA1, CA3, or hilar neurons (data not shown). βAP exposure 
also resulted in significant increases in LDH release (Fig. 1B), 
and the increase in LDH release was of the same order of 
magnitude that previously observed after excitotoxin treat-
mant (17). LDH release from βAP-treated sections was sta-
tistically significantly different from control slices at 72 hr, 
at which time there was a 70% increase in LDH release as 
compared to untreated controls (Fig. 1B). As was observed 
with PI uptake, slices treated with βAP in the presence of 25 
μM EUK-8 were completely protected against βAP-induced 
LDH release (Fig. 1B), whereas incubation with EUK-8 alone 
had no effect on LDH release (data not shown). Biochemical 
and histological analyses indicated that control cultures were 
able to tolerate the 72-hr period of serum deprivation with no 
obvious signs of distress (A.J.B. and M.B., unpublished obser-
vations). On the basis of these observations, we assume that the 
steady increase in LDH release in control cultures reflects 
accumulation of LDH in the medium through basal, consti-
tutive release mechanisms, rather than a general decline in 
culture viability.

The effects of increasing concentrations of βAP on neuronal 
PI uptake and LDH release were also determined (Fig. 2). 
βAP (72-hr exposure) at 50 μg/ml did not significantly affect 
neuronal PI uptake (Fig. 2A), but βAP at 100 and 250 μg/ml 
causèd significant increases in PI uptake into 30% and 45% of 
total neuronal population, respectively (Fig. 2A). Cotreatment 
with 25 μM EUK-8 significantly attenuated PI uptake induced 
by βAP at either 100 to 250 μg/ml (Fig. 2A). Similar results 
were obtained with LDH release as increasing βAP doses 
(50–250 μg/ml) produced significant, dose-dependent 
increases (85–150% increase), which were nearly completely 
blocked by 25 μM EUK-8 (Fig. 2B).

βAP Toxicity and Oxidative Stress in Immature and Ma-
ture Cultures. It has been reported that dissociated neurons 
maintained in vitro for less than 4–5 days are resistant to βAP 
toxicity (27). We were therefore interested in investigating the 
effects of βAP application to immature hippocampal slice 
cultures. Neurons in hippocampal slice cultures have been 
shown to develop in a parallel manner to in vivo hippocam-
pal neurons in terms of excitotoxic susceptibility (17), long-term 
potentiation (29), and synaptic reorganization after lesion 
(20). Exposure of immature cultures to βAP at 100 μg/ml for 
72 hr had no effect on PI uptake, whereas mature cultures 
treated with the same βAP solution showed the characteristic 
increase in PI fluorescent neurons (Fig. 3A). EUK-8 treatment

![Fig. 2](image_url) Effects of various concentrations of βAP on PI uptake and LDH release in hippocampal cultures in the absence or presence of EUK-8. Mature organotypic hippocampal cultures were exposed to βAP at 50, 100, or 250 μg/ml for 72 hr in the presence or absence of 25 μM EUK-8, and the extent of toxicity was determined by quantification of PI uptake (A) and LDH release (B) as described in Materials and Methods. Data are means ± SEM of at least 24 (A) or 8 values (B). *, P < 0.05 as compared to βAP alone.

![Fig. 3](image_url) Effects of EUK-8 on βAP-induced PI uptake and LDH release in immature and mature hippocampal slice cultures. Immature and mature hippocampal slice cultures were exposed to βAP at 100 μg/ml for 72 hr in the presence or absence of 25 μM EUK-8, and the extent of toxicity was then determined by quantification of PI uptake (A) and LDH release (B) as described in Materials and Methods. Data are means ± SEM of at least 24 (A) or 8 values (B). *, P < 0.05 as compared to control levels.
nearly completely blocked the βAP-induced PI uptake in mature cultures, but had no effect on basal PI uptake in either immature or mature cultures (Fig. 3A). Additionally, immature cultures did not show any increase in LDH release after 72 hr of continuous βAP exposure, whereas mature cultures exhibited a 75% increase in release, which was significantly attenuated by 25 μM EUK-8 (Fig. 3B). Basal LDH release in immature cultures was somewhat higher than in mature cultures, but as there were no other biological or morphological indications of neuronal injury, we assume this reflects a greater level of basal release from immature cultures. Indeed, there is some degree of constitutive cell loss during in vitro maintenance (33), and, if the LDH values were presented as unit LDH activity per milligram of protein rather than per milliliter of medium, the basal levels of release in immature and mature cultures would be identical.

The degree of oxidative stress (ROS accumulation and lipid peroxidation) after βAP exposure was also determined to further characterize the role of ROS in βAP toxicity. The accumulation of oxygen free radicals and hydroperoxides was estimated by the conversion of dichlorofluorescin to the highly fluorescent DCF, as described in Materials and Methods. Exposure of hippocampal slices to βAP at 100 μg/ml for 72 hr caused DCF fluorescence to significantly increase by 35% above control levels in mature cultures, and this effect was completely inhibited by cotreatment with 25 μM EUK-8 (Fig. 4A). In contrast, immature cultures showed no increase in DCF fluorescence when exposed to the same βAP solution (Fig. 4A). The degree of lipid peroxidation produced by βAP treatment of immature and mature cultures was estimated by assaying thiobarbituric acid-reactive substances (32). Treatment of mature cultures with βAP at 100 μg/ml (72-hr exposure) resulted in a significant increase (+26%) in MDA levels as compared to control values, and this effect was completely blocked by 25 μM EUK-8 (Fig. 4B). As was observed with DCF fluorescence, immature cultures showed no increase in MDA release after βAP treatment (Fig. 4B). EUK-8 alone did not alter MDA release from immature or mature cultures (Fig. 4B).

**DISCUSSION**

The present study provides strong evidence in support of the hypothesis that ROS mediate βAP-induced neuronal injury and indicates that organotypic hippocampal cultures represent a model with which to study βAP toxicity. This model is especially appropriate as there is discrepancy between the reported in vitro and in vivo effects of βAP. Although βAP application in vitro (to either neurons or neuronal cell lines) has been shown to be neurotoxic (7, 8), application of βAP in vivo has produced mixed results in the hands of different investigators (8, 34, 35) and may be toxic only if applied after treatment with streptozotocin or some other glucose-destabilizing agent (36). Using organotypic hippocampal slice cultures, we observed a reliable pattern of neuronal damage when βAP was applied under serum-free medium conditions. This is in contrast to the report that βAP toxicity was not observed unless the peptides (1–40 or 25–35) were microinjected directly into the neurons (37). Possible reasons for the different results include the method of preparation and maintenance of the cultured slices (roller-tube vs. membrane cultures) and, more importantly, the presence or absence of serum. It is possible that extended periods of serum deprivation stress neurons in such a way as to make them more susceptible to βAP toxicity. However, no discernible biochemical or histological indications of significant neuronal injury were ever present in control cultures, suggesting that neurons were able to tolerate these conditions.

The extent of PI uptake and LDH release has been used previously by us (17), as well as others (16, 31, 38), to quantify neuronal injury and death in culture systems. Although there is generally a good correlation between these two indices, βAP exposure had a more robust effect on PI uptake than on LDH release in the present study. It is generally accepted that βAP must be in an aggregated form to exert neurotoxic effects (7). A larger effect of βAP treatment on PI uptake as compared to LDH release might therefore reflect the accumulation of aggregated βAP on the superficial layers of the slice, which is the main region of the slices observed when measuring PI fluorescence (this might also account for the relatively high concentrations of βAP required in our culture system, as aggregated βAP is likely to poorly diffuse through the membranes and into the cultures). This also suggests that the lack of toxic effect of βAP in immature cultures is not due to the decreased penetration of aggregated βAP as they are thicker than mature cultures, since PI fluorescence should have still been able to detect such an effect. Furthermore, we previously reported that immature cultures are in fact more sensitive to the toxic effects of N-methyl-d-aspartate than mature cultures (17), which excludes the possibility that the differential effect of βAP reflects a generalized difference in sensitivity between immature and mature cultures or a differential ability to tolerate serum-free conditions.

The measurement of DCF fluorescence as an indicator of ROS accumulation has previously been used to demonstrate ROS production during βAP exposure in dissociated neurons (12, 25, 39). The assay of thiobarbituric-reactive substances, which measures MDA released from oxidized lipid bilayers,

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**FIG. 4.** Effects of EUK-8 on βAP-induced oxidative stress in immature and mature hippocampal slice cultures. Immature and mature hippocampal slice cultures were exposed to βAP at 100 μg/ml for 72 hr in the presence or absence of 25 μM EUK-8, and the extent of oxidative stress was then determined by quantification of DCF fluorescence (A) and MDA release (B) as described in Materials and Methods. Data are means ± SEM of at least eight values. *, P < 0.05 as compared to control levels.
has also been widely used to document lipid peroxidation after a variety of neuronal insults (26, 40, 41). Although the later assay has its flaws, the combination of these two assays provides an accurate representation of the degree of oxidative stress in neuronal slice cultures after βAP application. Both assays clearly indicate that βAP treatment of hippocampal cultures, cut into slices, in cultures that produce oxidative injury. Thus, our results are in good agreement with several studies indicating the involvement of free radicals in βAP toxicity and the protective effects of free radical scavengers (12, 25, 39, 42). In our report, we present three lines of evidence in support of a pivotal role for ROS in βAP toxicity: (i) βAP-induced PI uptake and LDH release were nearly completely blocked by coincubation with EUK-8, a synthetic catalytic free radical scavenger; (ii) βAP application to mature cultures resulted in significant increases in DCF fluorescence and lipid peroxidation, both of which were completely inhibited by EUK-8; and (iii) immature cultures, which were not susceptible to βAP toxicity, did not show any increase in DCF fluorescence or lipid peroxidation. EUK-8 has been well characterized in terms of both its free radical scavenging ability (43) and its effectiveness in in vitro models of oxidative neuronal injury (26). Furthermore, EUK-8 did not affect βAP aggregation in test tubes or in the slice culture medium (data not shown). The inhibition of DCF fluorescence and MDA release by EUK-8 also supports its specific action as an oxygen free radical and/or peroxide scavenger. The use of immature cultures as a negative control is a novel approach to explore βAP toxicity. Although cultures of this age are also resistant to kainic acid lesioning, they are highly sensitive to N-methyl-d-aspartate toxicity (17). Thus, their resistance to βAP-induced toxicity is not a consequence of some artifact of plating that confers a general, but temporary, resistance to exogenously applied toxins. The demonstration that βAP application to immature cultures does not result in increased DCF fluorescence and MDA release indicates that these parameters are integral to the manifestations of damage and therefore are not nonspecific consequences of peptide aggregation in the presence of neurons. Additionally, the inhibition of the increases in these parameters (in susceptible neurons) by the antioxidant EUK-8 indicates that both DCF fluorescence and MDA release occur downstream to free radical production. The mechanisms by which βAP induces the production of ROS in susceptible neuronal populations are not illuminated by these studies. It has been proposed that βAP toxicity is related to the secondary and tertiary structures of the peptides (44) and is contingent upon disruption in calcium homeostasis and subsequent calcium overload (42). The ease of preparation of the hippocampal slice cultures should make it an interesting model preparation to study the cellular and molecular mechanisms of βAP neurotoxicity.

In summary, our results provide a model with which to study βAP-induced neuronal injury and highlight some interesting characteristics of βAP toxicity. The confirmation that ROS play a critical role in βAP toxicity offers further support for clinical developments of antioxidants in the treatment of AD and expand the widening field of oxygen free radical research in aging. The elucidation of the factors that confer resistance to βAP-induced oxidative stress and toxicity in immature cultures would also have far reaching implications for the study of the mechanisms of AD susceptibility, as well as for therapeutic interventions in AD patients.

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