IN VIVO PROTECTIVE EFFECTS OF FERULIC ACID ETHYL ESTER AGAINST AMYLOID β-PEPTIDE (1-42)-INDUCED OXIDATIVE STRESS

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Running Head: In Vivo Neuroprotection Against Aβ1-42 by FAEE
Abstract

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by the deposition of amyloid beta-peptide (Aβ), a peptide that as both oligomers and fibrils is believed to play a central role in the development and progress of AD by inducing oxidative stress in brain. Therefore, treatment with antioxidants might, in principle, prevent propagation of tissue damage and neurological dysfunction. The aim of the present study was to investigate the in vivo protective effect of the antioxidant compound ferulic acid ethyl ester (FAEE) against Aβ-induced oxidative damage on isolated synaptosomes. Gerbils were injected intraperitoneally (i.p.) with FAEE or with DMSO, and synaptosomes were isolated from the brain. Synaptosomes isolated from FAEE injected gerbils and then treated ex vivo with Aβ(1-42) showed a significant decrease of oxidative stress parameters: ROS levels, protein oxidation (protein carbonyl and 3-nitrotyrosine levels) and lipid peroxidation (4-hydroxy-2-nonenal levels). Consistent with these results, both FAEE and Aβ(1-42) increased levels of antioxidant defense systems, evidenced by increased levels of heme oxygenase 1 and heat shock protein 72. FAEE led to decreased levels of inducible nitric oxide synthase. These results are discussed with potential therapeutic implications of FAEE, a brain accessible, multi-functional antioxidant compound, for AD involving modulation of free radicals generated by amyloid β-peptide.

Keywords: Ferulic acid ethyl ester, Amyloid beta peptide, Alzheimer’s disease, heme oxygenase-1, heat shock proteins, oxidative stress.
Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive cognitive decline resulting from selective neuronal dysfunction, synaptic loss, and neuronal cell death. AD is accompanied by the presence of extracellular amyloid plaques containing aggregated amyloid β-peptide (Aβ), a polypeptide of 39–43 amino acids that is thought to play a major role in the pathogenesis of this disorder (Butterfield 2002; Mattson and Mattson 2002).

Loss of synaptic terminals in AD brain demonstrates a higher correlation with decreased cognitive function than do cell death or plaque development, which has led to the hypothesis that disappearance of synapses is a key event in early cognitive decline (Terry et al. 1991). The cellular location of initial amyloid-related damage is controversial, but a growing body of evidence suggests that intracellular accumulation of Aβ precedes plaque formation. However, the mechanisms of synapse loss in AD remain uncertain. Nevertheless, the damage in these regions is directly correlated with severity of dementia, oxidative stress, and deposition of Aβ (1–42). Therefore, the investigation of oxidative damage occurring in synaptosomes isolated from murine brain has been shown to be a suitable experimental model to study the extent of Aβ-induced toxicity (Lauderback et al. 2001; Mattson et al. 1998).

Increased production of reactive oxygen and nitrogen species such as superoxide radical anion and nitric oxide together with an imbalance of antioxidant defenses was observed in neuronal systems after Aβ(1-42) treatment (Butterfield 2002; Keller et al. 1997; Varadarajan et al. 2000; Yatin et al. 1999). Previous studies from our laboratory and others reported that Aβ(1-42) induces in vitro and in vivo reactive oxygen species
ROS production, protein oxidation, DNA and RNA oxidation and lipid peroxidation (Boyd-Kimball et al. 2005a; Butterfield 2002; Butterfield et al. 2002b; Butterfield and Lauderback 2002; Drake et al., 2003; Mohmmad Abduel et al., 2004; 2006; Keller et al. 2000; Keller et al. 1997).

Indeed, several studies performed to date examined whether dietary intake of several antioxidants, such as flavonoids, carotenoids and vitamins, might prevent or reduce the progression of AD (Butterfield et al. 2002a). Among these class of molecules, we focused our attention on the phenol compound ferulic acid ethyl ester. Ferulic acid (FA) is a substance found in most plants, especially in the brans of grasses such as wheat, rice, and oats. Due to its phenolic nucleus and an extended side chain conjugation, FA readily forms a resonance stabilized phenoxy radical which accounts for its antioxidant potential (Kanski et al. 2002). The esterification of the acid group (ferulic acid ethyl ester, FAEE) confers to the molecule lipophilic properties, thus increasing its antioxidant potential (Kikuzaki et al. 2002; Scapagnini et al. 2004; Schroeter et al. 2000; Sultana et al. 2005d). Previous studies from our laboratory and others demonstrated in vitro its scavenging activities toward hydroxyl radical, peroxy-nitrite and oxidized low-density lipoprotein (oxLDL) (Pannala et al. 1998; Schroeter et al. 2000; Sultana et al. 2005d). Recent findings have shown the ability of ferulic acid ethyl ester to potently induce hemeoxygenase1 (HO-1) and heat shock protein 72 (HSP72) expression in neuronal cell culture (Joshi et al. 2005; Scapagnini et al. 2004) and synaptosomal systems (Joshi et al. 2006).

Given the neuroprotective success of FAEE against Aβ(1-42) in vitro (Sultana et al. 2005d) and based upon the mechanisms by which FAEE scavenges free radicals, the
aim of the present study was to investigate the ability of FAEE to provide in vivo neuroprotection against Aβ-induced oxidative stress. For this purpose, different parameters of oxidative stress have been evaluated: ROS levels, protein oxidation and lipid peroxidation and role of heat shock response. The results are consistent with the hypothesis that FAEE is a potent brain-accessible antioxidant that potentially could be beneficial in the treatment of AD and other oxidative stress-related disorders.

**Materials and Methods:**

**Materials**

FAEE and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorescent indicator for ROS measurement, 2,7-dichlorofluorescin diacetate (DCFH-DA), was obtained from Molecular Probes (Eugene, OR, U.S.A.), and a fresh 10 mM stock solution was prepared in ethanol. Fresh FAEE (50 µM) was prepared by dissolution in DMSO. The OxyBlot™ oxidized protein kit was obtained from Intergen, Inc. (Purchase, NY). Amyloid-β peptide (1-42) (HPLC- and MS- certified purity) was purchased from Anaspec, Inc. (San Jose, CA). For all experiments, Aβ was incubated for 24 h in PBS at 37 °C before application to synaptosomes. Primary antibodies for 4-hydroxynonenal (HNE) and 3-nitrotyrosine were obtained from Chemicon. Anti-HO-1, anti-iNOS and anti HSP72 primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Animals**
For the present study, three month-old male Mongolian gerbils, approximately 70 g in weight, were used to isolate synaptosomes. All the experimental protocols were approved by the University of Kentucky Animal Care and Use Committee. All the animals were kept under 12 h light/dark condition at University of Kentucky Animal Facility, and fed with standard Purina rodent laboratory chow ad libidum. The gerbils were injected i.p. with freshly prepared FAEE dissolved in dimethyl sulfoxide (DMSO) (150 mg/Kg body weight) 1 h before sacrifice. The dose and the time of FAEE were chosen according to previous data obtained in our laboratory (data not shown), in order to achieve brain accessibility of the compound and lack of any toxic effects (Joshi et al. 2006). Control animals were injected with DMSO for the same time. The animals were euthanized with sodium pentobarbital before sacrifice.

**Synaptosomal preparation**

Synaptosomes were isolated from gerbils injected intraperitoneally (i.p.) with DMSO (control, CTR) or with FAEE in DMSO, 1h after injection. The isolation of synaptosomes from whole brain was carried out according to the procedure described by Keller et al. (2000). The brain was isolated immediately after decapitation and placed in a 0.32M sucrose isolation buffer containing 4 µg/mL leupeptin, 4 µg/mL pepstatin, 5 µg/mL aprotinin, 20 µg/mL trypsin inhibitor, 0.2 mM PMSF, 2 mM EDTA, 2mM EGTA, 20 mM HEPES pH 7.4. Samples were homogenized with a Wheaton tissue homogenizer and centrifuged at 1,500 x g for 10 minutes. The pellet was discarded and the supernatant was retained and centrifuged at 20,000 x g for 10 min. The resulting pellet was resuspended in 1mL of 0.32 sucrose buffer and layered onto discontinuous sucrose
density gradients of 10 mL each of 0.85M, pH 8.0; 1.0M, pH 8.0; 1.18M, pH 8.5 sucrose solution each containing 10mM Hepes, 2mM EDTA and 2mM EGTA. The gradients were spun in a Beckman L7-55 ultracentrifuge at 82,550 × g for 1 h at 4°C. The purified synaptosomes were collected at the 1/1.18M sucrose interface, washed twice with PBS for 10 min at 32000g, yielding synaptosomes. Protein concentrations of the purified synaptosomal membranes were determined by the BCA assay (Pierce, Rockford, IL).

Synaptosomes from DMSO- and FAEE-injected gerbils were incubated with and without 10 µM Aβ(1-42) for 6 h at 37°C (time chosen based on prior studies) (Boyd-Kimball et al. 2005a). Therefore, the present study entails four groups for comparison: synaptosomes isolated from DMSO- and FAEE-injected gerbils not treated with 10 µM Aβ(1-42), both used as controls, and synaptosomes from DMSO- and FAEE-injected gerbils treated with 10 µM Aβ(1-42).

**Reactive oxygen species (ROS) measurements**

The dichlorofluorescein (DCF) assay was used to measure the levels of ROS, according to the procedure previously described by Wang et al. (Wang and Joseph 1999). The cell permeable dichlorofluorescein diacetate (DCFH-DA) crosses inside the synaptosomal vesicle where it is de-esterified by cellular esterases resulting in DCFH. DCFH in turn is converted upon oxidation to the highly fluorescent dichlorofluorescein (DCF). By measuring the fluorescence, we were able to quantify the levels of ROS. After incubation with Aβ(1-42) for 6 hrs, synaptosomes (1mg/mL) were washed with PBS and incubated with 10 µM of non-fluorescent DCFH-DA for 30 min. Previous studies showed that fluorescence was due to intrasynaptosomal oxidative process rather than to DCF.
exiting the synaptosomes to react with oxidant (Joshi et al. 2005). Synaptosomes were spun at 3,000 x g in a tabletop Eppendorf centrifuge for 5 min at 4°C. Synaptosomes were resuspended in 500 µL of PBS and run in triplicate (100 µL per well) in a black microtiter plate. The measurements were performed on a Molecular Devices SpectraMax microtiter plate reader with λ_{ex} = 495 nm and λ_{em} = 530 nm. Data are given as percentage of corresponding controls and are the mean of at least six independent experiments.

**Protein carbonyl measurement**

Protein oxidation was determined by an oxidized protein detection kit (Oxyblot, Chemicon). Briefly, 5 µL of synaptosomes (4 mg/mL) were incubated for 20 min with 12% SDS and 2,4-dinitrophenylhydrazine (DNPH) in 10% trifluoroacetic acid with vortexing every 5 min, and then neutralized with Oxyblot Neutralization solution. We blotted 250 ng of protein onto nitrocellulose paper by the slot blotting technique. Membranes were incubated with blocking buffer for 60 min at 27 °C, incubated with rabbit antibodies to DNPH (diluted 1:150) for 90 min, and then by anti-rabbit IgG coupled to alkaline phosphatase (1:10,000) for 1 h at 27 °C. After being washed and developed with SigmaFast chromogen (Sigma), blots were scanned into Adobe Photoshop (Adobe System, Inc., Mountain View, CA) and quantitated with Scion Image (PC version of Macintosh compatible NIH Image).

**3-Nitrotyrosine levels (3-NT)**

Nitrotyrosine content was determined by incubating the samples with Laemmli buffer (0.125 M Trizma base, pH 6.8, 4% SDS, 20% glycerol) for 20 min. Samples (250
ng of protein) were blotted onto nitrocellulose membranes and immunochemical methods were performed. The rabbit anti–3-NT primary antibody was incubated 1:200 in blocking buffer (BSA 3% in TBS-T) for 2 h. The membranes were washed three times with TBS-T and incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1: 10,000). Densitometric analysis of bands in images of the blots was used to calculate levels of 3-NT.

**Lipid peroxidation measurement**

4-hydroxy-2-nonenal (HNE) levels were measured as markers of lipid peroxidation. The samples (5 µL) were incubated with 10 µL Laemmli buffer for 20 min at room temperature. 250 ng of protein samples were loaded in each well on nitrocellulose membrane in a slot blot apparatus under vacuum. The membranes were incubated with anti–HNE rabbit polyclonal antibody (1: 5000) for 2 h, washed three times with TBS-T and then incubated with an anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody (1:10,000). Blots were developed using SigmaFast tablets (BCIP/NBT), dried and quantified with Scion Image.

**Western Blot**

Synaptosome samples (100 µg) were added with sample loading buffer, denaturated for 5 min at 100 °C and then loaded on 10% SDS-polyacrylamide gels. Proteins were transferred on nitrocellulose at 80 mA/gel for 2 h. The blots were blocked for 2 h in 3% nofat dry milk in phosphate-buffered saline (PBS). Membranes were then probed with primary antibody, anti HSP72, anti-HO-1 and anti-iNOS (1:1000) for 2 hrs
at room temperature. After three washes with PBS, membranes were incubated for 1 h
with horseradish peroxidase-conjugated secondary antibodies in PBS. The membranes
were washed again for three times with PBS and the bands were visualized using a
chemiluminescence kit (Amersham, Piscataway, NJ, USA).

Statistical analysis

Analysis of variance (ANOVA) was used for comparison among the groups
followed by Student’s t-tests for analysis of significance. P values < 0.05 were considered
significant for comparison between control and experimental results.

Results

FAEE protects in vivo against Aβ(1-42)-induced ROS production

ROS levels generated by Aβ(1-42) in our experimental model were measured by
dCF fluorescence. In the absence of Aβ(1-42), levels of ROS in synaptosomes from
DMSO-injected gerbils did not show any significant difference compared with the levels
measured in synaptosomes from FAEE-injected gerbils. Hence, both these groups can be
referred as control. Thus, FAEE itself at the concentration used does not reduce basal
oxidation levels. Synaptosomes isolated from DMSO-injected gerbils and treated with 10
µM Aβ(1-42) for 6 h displayed an increased fluorescence, about 20% compared to
control synaptosomes (untreated) (p < 0.05). Synaptosomes isolated from FAEE-injected
ergibs did not lead to Aβ(1-42)-induced ROS accumulation (p < 0.01). Thus, FAEE in
vivo significantly prevents free radical formation in synaptosomes by Aβ(1-42).
FAEE in vivo protects against Aβ(1-42)-induced protein oxidation and lipid peroxidation

Protein carbonyls and 3-NT levels were measured as markers of protein oxidation (Berlett and Stadtman 1997; Lauderback et al. 2001; Sultana et al. 2005d). Protein carbonyl groups are incorporated into proteins by direct oxidation of certain amino acid side chains, peptide backbone scission, or by Michael addition reactions with products of lipid peroxidation or glycoxidation (Berlett and Stadtman 1997; Butterfield and Lauderback 2002). Oxidative stress could also stimulate additional damage via the overexpression of inducible nitric oxide synthase (iNOS) and the action of constitutive neuronal NOS (nNOS) that leads to increased levels of 3-NT. Fig. 2 shows the carbonyl levels in synaptosomes isolated from DMSO- and from FAEE-injected gerbils that were subsequently treated with 10 µM Aβ(1-42). The level of carbonyls was found to be significantly higher (p < 0.01) in Aβ(1-42)-treated synaptosomes previously isolated from DMSO-injected gerbils. FAEE in vivo treatment protects subsequently isolated synaptosomes against Aβ(1-42)-induced oxidative protein damage (p < 0.005). The antioxidant properties of FAEE were further confirmed by measuring 3-NT levels, formed by reaction of reactive nitrogen species (RNS) with proteins (Castegna et al. 2003; Sultana et al., 2005c). Fig. 3 shows the protective effects of FAEE on Aβ(1-42)-induced formation of 3-NT. Synaptosomes isolated from DMSO-injected gerbils showed increased levels of 3-NT (p < 0.05) when treated in vitro with 10 µM Aβ(1-42), while synaptosomes isolated from FAEE-injected gerbils and treated with Aβ(1-42) were completely protected (p < 0.01).

Free radical attack on phospholipids PUFA leads to the formation of reactive aldehydes, among which one of the most neurotoxic is HNE (Esterbauer et al. 1991;
Lauderback et al. 2001). This alkenal reacts with proteins forming stable covalent adducts to histidine, lysine and cysteine residues via Michael addition (Berlett and Stadtman 1997; Butterfield 2002; Butterfield et al. 2002b; Butterfield and Lauderback 2002). The extent of this reaction can be measured immunochemically by quantifying the levels of HNE-bound proteins. Fig. 4 shows the HNE-bound protein levels in synaptosomes isolated from gerbils previously injected with FAEE or with DMSO and incubated in vitro with 10 μM Aβ(1-42) for 6 h. Consistent with the protein oxidation results shown above, we observed in vivo protection by FAEE against 10 μM Aβ-induced lipid peroxidation. HNE levels were found to be higher in Aβ(1-42)-treated synaptosomes isolated from DMSO injected gerbils (p < 0.01), while Aβ(1-42)-treated synaptosomes isolated from FAEE-injected gerbils showed reduced levels of HNE-bound proteins (p < 0.005). These results are consistent with recent in vitro data obtained on primary neuronal cultures (Sultana et al., 2005d), indicating that FAEE acts as a potent antioxidant thus preventing protein oxidation and lipid peroxidation.

**FAEE leads to elevated HO-1 and HSP72 protein levels**

It has been well documented that oxidative stress conditions induce expression of the so-called stress response proteins, such as the heat shock protein family (HSPs) and HO-system (Calabrese et al. 2004b; Fauconneau et al. 2002; Polla et al. 1996; Poon et al. 2004). Figures 5a and b show the increased levels of both HO-1 and HSP72 protein levels in synaptosomes isolated from DMSO-injected gerbils and then treated with Aβ(1-42) for 6 h (p < 0.05). This effect becomes more pronounced in synaptosomes isolated from FAEE-injected gerbils that were subsequently treated with Aβ(1-42) (p < 0.01), thus suggesting that both oxidative stress and FAEE facilitate increased HO-1 and HSP72
levels. Consistent with this suggestion, an increased level of HSP72 in synaptosomes isolated from FAEE-injected gerbils not treated with Aβ(1-42) was observed (Figure 6a). Based on this finding and on our previous data (Joshi et al. 2006; Scapagnini et al. 2004; Sultana et al. 2005d), we confirmed the ability of FAEE independently to lead to elevated levels of the stress response proteins HO-1 and HSP72, a process that might represent an efficient antioxidant system against Aβ-induced neurotoxicity.

*FAEE leads to lower levels of iNOS*

Considerable evidences demonstrated the involvement of neuroinflammatory processes in AD brain (McGeer et al. 2000; Togo et al. 2004; Tuppo and Arias 2005). Neurotoxic amounts of reactive nitrogen species (RNS) are formed by the activity of iNOS (Haas et al. 2002; Heneka and Feinstein 2001). In the current study, we show that iNOS protein levels are sharply increased in synaptosomes isolated from DMSO-injected gerbils treated with Aβ(1-42) compared with control synaptosomes (DMSO-injected gerbils) (p < 0.05). *In vivo* FAEE treatment decreased iNOS protein levels in synaptosomes isolated from FAEE-injected gerbils treated with Aβ(1-42) (Figure 7a). Interestingly, we also observed that treatment of FAEE alone is able to decrease the protein level of iNOS (Figure 6b).

**Discussion**

Oxidative stress induced by amyloid-β peptide *in vivo* plays a prominent role in the neurodegeneration associated with AD (Boyd-Kimball et al. 2005b; Butterfield 2002; Butterfield et al. 2002b; Butterfield and Lauderback 2002; Drake et al. 2003; Friedlich
and Butcher 1994; Smith et al. 1998). Therefore, a safe and effective, brain-accessible drug, one that possesses both antioxidant properties and has the property to lead to elevated levels of neuroprotective proteins while leading to decreased levels of a potentially harmful protein, might prove to be beneficial in treating the symptoms of AD or slowing its onset. Based on the notion that ethyl ferulate showed increased antioxidant properties compared with ferulic acid, and that its higher lipophilicity might improve its brain accessibility (Kikuzaki et al. 2002; Scapagnini et al. 2004), in the current study, we provide evidence that in vivo FAEE treatment exerts protective effects against Aβ(1-42)-induced oxidative stress in our experimental model, while leading to elevated levels of HO-1 and HSP-72 and decreased levels of i-NOS.

Synapse loss is believed to be an early pathological event in AD (Mattson et al., 1998), and synaptosomes have been shown to be oxidized by treatment with Aβ(1–42) (Butterfield 2002; Butterfield et al. 2002b; Butterfield and Lauderback 2002; Lauderback et al. 2001; 2002). In the present study, we showed that the levels of ROS decreased in synaptosomes isolated from FAEE-injected gerbils and treated ex vivo with Aβ(1-42) when compared with CTR synaptosomes (DMSO-injected gerbils). Our data demonstrate the ability of FAEE to act in vivo as a potent free radical scavenger. Due to its phenolic nucleus and an extended side chain conjugation (Kanski et al. 2002), FAEE readily traps free radical species such as hydroxyl and peroxyl radicals by forming a resonance stabilized phenoxy radical (Kanski et al. 2002; Sultana et al. 2005d). FA attenuates iron-induced oxidative damage and apoptosis in cultured neurons (Kanski et al. 2002; Zhang et al. 2003) and inhibits LPS-induced production of tumor necrosis factor-α and macrophage inflammatory protein-2 in a murine macrophage cell line (Sakai et al. 1997).
In addition to the radical scavenging activity of an antioxidant, both its polarity and three-dimensional interaction with lipid bilayers may contribute to its antioxidant activity. Synaptic membranes are particularly vulnerable to oxidative stress, thus the ability of an antioxidant to act at membrane sites due to its high lipophilicity results in a higher antioxidant potential. Since the synapse is one of the primary targets of Aβ-mediated neurotoxicity, the affinity of FAEE with lipid substrates might be an important factor to modulate Aβ-induced oxidative damage.

Many studies showed that the oxidative damage associated with AD is represented by lipid peroxidation (Lauderback et al. 2001; Markesbery and Lovell 1998; Sayre et al. 1997), nitration (Castegna et al. 2003; Smith et al. 1997; Sultana et al. 2005c), reactive carbonyls production (Aksenov et al. 2001; Butterfield 2002; Butterfield et al. 2002b; Butterfield and Lauderback 2002; Sultana et al. 2005a; Sultana et al. 2005b), and nucleic acid oxidation (Mecocci et al., 1993; Wang et al. 2005) that are all increased in vulnerable neurons of diseased brain.

In the current study, carbonyl levels and 3-NT levels were found to be decreased in Aβ(1-42)-treated synaptosomes isolated from FAEE-injected gerbils compared with control synaptosomes. In addition, \textit{in vivo} FAEE treatment resulted in protective effects against Aβ(1-42)-induced lipid peroxidation. HNE is one of the most reactive and toxic endproduct of lipid peroxidation, that is thought to interfere with normal cellular functions in AD brain tissues (Butterfield and Lauderback 2002; Esterbauer et al. 1991; Markesbery and Lovell 1998; Sayre et al. 1997).

There are a variety of genes encoding proteins that possess anti-oxidant properties. Of particular interest in the CNS is HO-1, which has been reported to operate
as a fundamental defensive mechanism for neurons exposed to an oxidant challenge (Calabrese et al. 2004b; Maines 2000; Mancuso 2004; Poon et al. 2004). A growing body of evidence reveals that HO-1 and one of the reaction products catalyzed by HO-1, biliverdin, which is rapidly converted into bilirubin in mammalian cells, are potent antioxidants at lower levels (Calabrese et al. 2003; Dore et al. 1999; Mancuso 2004; Takata et al. 2002). HO-1, also known as HSP32, is a member of the HSP, a family of chaperone proteins that are crucial for recovery from stress-induced protein damage (Calabrese et al. 2002; Mancuso 2004; Poon et al. 2004). In AD cortex and hippocampus HO-1 has been shown to be over-expressed and co-localizes to senile plaques and neurofibrillary tangles (Schipper 2000).

The 72-kDa HSP (HSP72) is a stress-inducible protein that belongs to the HSP70 chaperone family. HSP72 shows very low expression in brain under physiological conditions but it is induced after certain oxidative stress (Bergeron et al. 1996; Poon et al. 2004). Evidence indicates that HSP72 may contribute to cellular protection against a variety of stresses by preventing protein aggregation, by assisting in the refolding of damaged proteins, and by serving as a chaperone for nascent polypeptides along ribosomes (Mayer and Bukau 2005).

HSP induction is not only a signal for detection of physiological stress but is utilized by the cells in the repair process following a wide range of injuries, to prevent damage resulting from the accumulation of non-native proteins (Kelly and Yenari 2002). In the present study we showed that the levels of both HO-1 and HSP72 were elevated by Aβ treatment as a cellular response to the oxidative injury cascade activated by the peptide.
Our findings of even more increased levels of these stress response proteins in Aβ(1-42)-treated synaptosomes isolated from FAEE-injected gerbils suggests that the activation of HSPs (HSP72 and HO-1) is a protective mechanism exerted by FAEE against Aβ-induced oxidative stress. Several studies indicated that synaptosomes have the capacity for protein synthesis (Jimenez et al. 2002; Steward et al. 1991; Witzmann et al. 2005), and recent findings have shown, by using two-dimensional gel electrophoresis, that synaptosomes display differential expression in protein levels under various conditions (Boyd-Kimball et al., 2005a). We have previously demonstrated that HO-1 expression is increased in FAEE-treated astrocytes (Scapagnini et al. 2004) and neurons (Sultana et al., 2005d) as a protective mechanism against oxidative stress with relevance to preconditioning. Consistent with these in vitro results, we demonstrated in the current study that FAEE alone is able to lead to elevated HSP72 levels in synaptosomes, thus confirming the likely ability of FAEE to provide neuroprotection in part by stimulating the stress response. The heat shock response contributes to establishing a cytoprotective state in a variety of metabolic disturbances and injuries, including hypoxia, stroke, epilepsy, cell and tissue trauma, neurodegenerative disease, and aging (Calabrese et al. 2004b; Latchman 2004; Mancuso 2004). This notion has opened new perspectives in medicine and pharmacology, as molecules activating this defense mechanism appear to be possible candidates for novel cytoprotective strategies. In agreement with this observation, we suggest that FAEE could provide neuroprotection against Aβ toxicity by modulating oxidative stress directly and by inducing protective cellular response, particularly induction of HO-1 and HSP72.
Deposition of the β-amyloid plaques and neurofibrillary tangles of Alzheimer's disease is associated with glial activation, loss of neurons and decline of cognitive function (Combs et al. 2001; Hu et al. 1998; Saez et al. 2004). Long-term or excessive activation of glia increases production of chemokines and cytokines, such as interleukin-1 beta (IL-1β), and oxidative stress-related enzymes, such as a highly active form of iNOS. The excessive production of inflammation-related substances can, in turn, contribute to further exacerbation of the disease process. The iNOS induced as a result of glial activation generates NO, which can combine with superoxide to damage neurons (Mander and Brown 2005). Therefore, development of new compounds that can modulate these disease-linked biological processes might provide insight into alternative therapeutic approaches and future identification of new drug targets (Ishii et al. 2000; Yan et al. 2003). Consistent with this notion, we found increased levels of iNOS, and nitrated proteins (3-NT), in synaptosomes isolated from DMSO-injected gerbils and then treated with A. We observed in the current study a significant reduction of iNOS levels and 3-NT levels in synaptosomes isolated from FAEE-injected gerbils and treated ex vivo with Aβ(1-42). We have also shown that i.p. injection of FAEE alone is able to decrease levels of iNOS in synaptosomes. We suggest that beside the effect of FAEE alone on iNOS, the protective effect of FAEE relies on its ability to block the activation of iNOS induced by Aβ(1-42). Thus, FAEE is not only able to decrease the basal level of iNOS but it has also a marked ability to prevent the Aβ(1-42)-dependent increase of iNOS thus modulating the inflammatory process and the oxidative burden cascade activated by nitric oxide.
In conclusion, the present study demonstrates the ability of FAEE to act as a potent antioxidant in vivo, thus providing neuroprotection against Aβ-induced oxidative stress. Our data suggest that the ester derivative of ferulic acid, FAEE, shows higher lipophilicity with increased ability to penetrate the blood-brain barrier. We hypothesize a multifaceted mechanism of in vivo neuroprotection by this compound: i) FAEE is a potent free radical scavenger by significantly attenuating ROS production, protein oxidation and lipid peroxidation; ii) FAEE is also neuroprotective by leading to elevated levels of stress response proteins, such as HO-1 and HSP72; iii) FAEE modulates neuroinflammatory processes mediated by iNOS.

Further studies are required to gain insight into the potential use of FAEE in the treatment of AD and other oxidative stress-related disorders. Investigations on the use of FAEE on animal models of AD are in progress in our laboratory.

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Figure legends:

**Fig. 1:** Protective effects of FAEE against Aβ(1-42)-induced ROS production. ROS levels were determined by the DCF fluorescence assay. Ctr = synaptosomes isolated from DMSO injected gerbils with no further treatment (n=6); FAEE = synaptosomes isolated from FAEE injected gerbils with no further treatment (n=6); Aβ(1-42) = synaptosomes isolated from DMSO injected gerbils and treated with 10 µM Aβ(1-42) for 6 h (n=6); Aβ(1-42) + FAEE = synaptosomes isolated from FAEE injected gerbils and treated with 10 µM Aβ(1-42) for 6h (n=6). The data are the mean ± S.E.M of six independent experiments, expressed as percentage of control values. Statistical comparison was made using ANOVA test (n=6 for each group). (*) p < 0.05, Aβ(1-42) vs Control; (**) p < 0.01, Aβ(1-42) vs Aβ(1-42) + FAEE.

**Fig. 2:** Protective effects of FAEE on Aβ(1-42)-induced protein oxidation. Protein carbonyl content of synaptosomes was measured as described in “Materials and Methods.” Synaptosomes isolated from DMSO-injected gerbils and treated with 10 µM Aβ(1-42) demonstrate a higher level of protein carbonyls than that of untreated controls (Ctr and FAEE). (*) p < 0.01, Aβ(1-42) vs Control. Synaptosomes isolated from FAEE injected gerbils were completely protected from Aβ-induced oxidative modifications [Aβ(1-42) + FAEE]. (**) p < 0.005, Aβ(1-42) vs Aβ(1-42) + FAEE.

**Fig. 3:** Protective effect of FAEE on Aβ(1-42)-induced 3-NT formation. 3-NT levels were determined as described in the “Materials and Methods” section. Ctr =
synaptosomes isolated from saline or FAEE injected gerbils with no further treatment; FAEE = synaptosomes isolated from FAEE injected gerbils with no further treatment; Aβ(1-42) = synaptosomes isolated from DMSO injected gerbils and treated with 10 µM Aβ(1-42) for 6 h; Aβ(1-42) + FAEE = synaptosomes isolated from FAEE injected gerbils and treated with 10 µM Aβ(1-42) for 6 h. The data are the mean ± S.E.M expressed as percentage of control values (n=6). (*) p < 0.05, Aβ(1-42) vs Control; (**) p < 0.01, Aβ(1-42) vs Aβ (1-42) + FAEE.

Fig. 4: Protective effect of FAEE on Aβ-induced lipid peroxidation (HNE levels). HNE levels were determined as described in the “Material and Methods” section. Ctr = synaptosomes isolated from DMSO/FAEE injected gerbils; FAEE = synaptosomes isolated from FAEE injected gerbils with no further treatment; Aβ(1-42) = synaptosomes isolated from saline injected gerbils and treated with 10 µM Aβ(1-42) for 6 hrs; Aβ(1-42) + FAEE = synaptosomes isolated from FAEE injected gerbils and treated with 10 µM Aβ(1-42) for 6 hrs. The data are the mean ± S.E.M expressed as percentage of control values (n=6). (*) p < 0.01, Aβ(1-42) vs Control; (**) p < 0.005, Aβ(1-42) vs Aβ (1-42) + FAEE.

Fig. 5: Western immunoblot analysis of synaptosomes for HO-1 (a), HSP72 (b) and iNOS (c) protein levels. Samples containing 50 µg of protein were loaded onto 10% SDS-PAGE gels, and the blots were probed with the polyclonal anti-HO-1 (1:2000), anti-HSP70 (1:500) and anti-iNOS antibodies (1:1000), respectively, for 2 h. Immunoblots were scanned by densitometry and all values were normalized to β-actin. Densitometric
values represent the mean ± S.E.M obtained from three independent experiments. The figures show a representative experiment (one of three), with each lane in duplicate. Significant differences were assessed by ANOVA. * p < 0.05, control vs Aβ(1-42); ** p < 0.01, Aβ(1-42) + FAEE.

**Fig. 6:** Representative Western blots showing *in vivo* effects of FAEE alone in synaptosomes isolated from FAEE injected gerbils (150 mg/kg body weight). Samples containing 50 µg of protein we analyzed by SDS gel electrophoresis and immunoblotting as described in Materials and Methods section.

a) HSP72; b) iNOS.
Figure 1

![Bar graph showing DCF fluorescence (% CTR) for different treatments: CTR, FAEE, Aβ(1-42), and Aβ(1-42) + FAEE. The graph indicates a significant difference (* and **) between the Aβ(1-42) + FAEE group compared to the other groups.]
Figure 2

![Bar graph showing protein carbonyls (% CTR)]

- CTR
- FAEE
- Aβ(1-42)
- A((1-42) +)

Y-axis: Protein Carbonyls (% CTR)
X-axis: CTR, FAEE, Aβ(1-42), A((1-42) +)
Figure 3

![Graph showing 3-Nitrotyrosine (% CTR) for different conditions: CTR, FAEE, Aβ(1-42), Aβ(1-42) + FAEE.](image)
Figure 4

![Bar chart showing HNE levels (% CTR) for CTR, FAEE, A(1-42), and A(1-42) + treatments. The bars are labeled with standard error bars. CTR, FAEE, and A(1-42) treatments have similar levels of HNE, while A(1-42) + shows a statistically significant increase marked by an asterisk.]
Figure 5

(a)

(b)

CTR Abeta Abeta + FAEE

% of control

CTR Abeta Abeta + FAEE

% of control

* **
Figure 6

a)

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b)

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