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Attenuation of chronic neuroinflammation by a nitric oxide-releasing derivative of the antioxidant ferulic acid

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Abstract

Chronic neuroinflammation and oxidative stress contribute to the neurodegeneration associated with Alzheimer's disease and represent targets for therapy. Ferulic acid is a natural compound that expresses antioxidant and anti-inflammatory activities. Nitric oxide is also a key modulator of inflammatory responses. Grafting a nitric oxide-releasing moiety onto anti-inflammatory drugs results in enhanced anti-inflammatory activity. We compared the effectiveness of ferulic acid with a novel nitric oxide-releasing derivative of ferulic acid in an animal model of chronic neuroinflammation that reproduces many interesting features of Alzheimer's disease. Lipopolysaccharide was infused into the 4th ventricle of young rats for 14 days. Various doses of ferulic acid or its nitric oxide-releasing derivative were administered daily. Both drugs produced a dose-dependent reduction in microglia activation within the temporal lobe. However, the nitric oxide-releasing ferulic acid derivative was significantly more potent. If we delayed the initiation of therapy for 14 days, we found no reduction in microglial activation. In addition, both drugs demonstrated antioxidant and hydroxyl radical scavenging abilities in *in vitro* studies. Overall, our results predict that a treatment using nitric oxide-releasing ferulic acid may attenuate the processes that drive the pathology associated with Alzheimer's disease if the treatment is initiated before the neuroinflammatory processes can develop.

Keywords: Alzheimer's disease, ferulic acid, NCX 2057, neuroinflammation, nitric oxide.

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Neuroinflammation and oxidative stress play prominent roles in the progression of Alzheimer's disease (AD: Friedlich and Butcher 1994; Smith *et al*. 1998; Gahtan and Overmier 1999; McGeer and McGeer 1999; Akiyama *et al*. 2000; Schubert *et al*. 2000). Brain regions that demonstrate the greatest degree of microglial activation early in the disease ultimately show the highest rate of atrophy and pathology (Cagnin *et al*. 2001). Epidemiological studies indicate that conventional anti-inflammatory therapies using non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk of developing AD, slow the progression and decrease the severity of the dementia (In'T Veld *et al*. 2002; Etminan *et al*. 2003). However, NSAIDs or antioxidants such as α-tocopherol have provided little or no therapeutic benefit for AD patients (Sano *et al*. 1997; Scharf *et al*. 1999; Van Gool *et al*. 2001; Aisen *et al*. 2003). Long-term treatment with the most typical anti-inflammatory drugs has been limited by gastrointestinal or renal toxicity (Brune and Neubert 2001) or the ability of the patient to tolerate the drugs (Scharf *et al*. 1999; Aisen *et al*. 2003), and dietary antioxidants did not attenuate cognitive decline (Sano *et al*. 1997).

Nitric oxide (NO) is a key modulator of the immune and inflammatory responses (Bogdan 2001; Coleman 2002). Compounds containing NO-releasing moieties have been shown to possess increased anti-inflammatory properties

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Abbreviations used: aCSF, artificial cerebrospinal fluid; AD, Alzheimer's disease; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EPR, electronic paramagnetic spectroscopy; FA, ferulic acid; ISMN, isosorbide mononitrate; NO-FA, nitric oxide-donating ferulic acid (NCX 2057); LPS, lipopolysaccharide; MHC II, major histocompatibility complex, class II; NO, nitric oxide; NSAID, non-steroidal anti-inflammatory drug; PBS, phosphate-buffered saline; SNAP, S-nitroso-*N*-acetyl-penicillamine.

(Hauss-Wegrzyniak *et al*. 1998b, 1999; Wenk *et al*. 2000, 2002). We have developed an animal model of chronic neuroinflammation, using a chronic infusion of lipopolysaccharide (LPS) into the 4th ventricle of young rats, that reproduces many of the behavioral, neurochemical and neuropathological changes associated with AD (Hauss-Wegrzyniak *et al*. 1998a, 2000, 2002; Wenk and Hauss-Wegrzyniak 2001). We have used this model to demonstrate that NO-releasing derivatives of anti-inflammatory drugs reduce biomarkers of brain inflammation and provide neuroprotection from the consequences of the inflammation more potently than the parent compound (Hauss-Wegrzyniak *et al*. 1998b, 1999; Wenk *et al*. 2000, 2002).

Ferulic acid (FA) is a phytochemical which exhibits both antioxidant and anti-inflammatory properties that may be of interest in the therapy for AD. For example, long-term, low-dose FA administration via the drinking water induced a resistance of the brain's toxicity to exposure to β-amyloid (Yan *et al*. 2001) similar to that reported following oral administration of the phytochemical curcumin (Frautschy *et al*. 2001; Lim *et al*. 2001), a molecule chemically related to FA (Wang *et al*. 1997). 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). Furthermore, a series of hydrophobic FA derivatives has been found to reduce the expression of inducible NO synthase and cyclooxygenase activity following exposure to LPS (Murakami *et al*. 2000; Hosoda *et al*. 2002). Given the potential beneficial actions of FA in these *in vivo* and *in vitro* models of oxidative stress and inflammation, and also given our previous findings of increased potency of anti-inflammatory drugs that release NO, the current study compared FA and a novel NO-releasing derivative of FA (NO-FA) using an *in vivo* model of brain inflammation induced by the chronic infusion of LPS into the 4th ventricle of young rats.

Materials and methods

Subjects

Male 3-month-old F-344 rats were housed singly in a colony room with a 12 : 12 dark : light cycle with lights off at 20.00 h. The rats had free access to food and water in a temperature-controlled room (22°C). All rats were given health checks, were handled on arrival and given at least 1 week to adapt to their new environment prior to surgery.

Materials

LPS (*E. coli*, serotype 055:B5) was obtained from Sigma Chemicals (St. Louis, MO, USA). FA and its NO-releasing derivative NCX 2057 [3-((4-hydroxy-3-methoxyphenyl)-2-propenoic acid 4nitroxy)butyl ester; NO-FA] were obtained from Nicox Research Institute, Milan, Italy. [^{3}H]PK11195 was obtained from Perkin Elmer (Boston, MA, USA).

In vitro comparative studies of NO-FA and FA

The following *in vitro* studies were conducted in order to evaluate and compare specific physicochemical properties and antioxidant actions of FA and NO-FA.

Measurements of NO release

We used an electronic paramagnetic spectroscopy (EPR) approach to measure bioactive NO as formation of the paramagnetic nitrosylhemoglobin [HbFe(II)NO] complex in whole rat blood. Venous rat blood was taken by withdrawal with a heparinized syringe from the abdominal vein and deoxygenated with a gas mixture of nitrogen (95%) carbon dioxide (5%) for 30 min in a sealing glass purge system equipped with an inlet and outlet valve and a water warming jacket to maintain controlled temperature (37°C). The system was kept under gentle stirring. S-nitroso-*N*-acetyl-penicillamine (SNAP) and isosorbide mononitrate (ISMN) were used as reference NO donors and dissolved, as well as NO-FA, in dimethylsulfoxide (DMSO). The drugs were added to whole blood at a final concentration of 100 μM. The blank was prepared by adding the vehicle (final content 1%, v/v). At fixed times (0–4 h), blood aliquots were anaerobiocally taken by degassed syringes and transferred into EPR quartz tubes (4 mm i.d.). In order to increase sensitivity and reproducibility, plasma erythrocytes were isolated by immediate tube centrifugation (4°C, 3200 r.p.m., 10 min) and samples kept in liquid nitrogen until analyses.

EPR measurements of frozen samples were carried out at 100 K with a Bruker EMX spectrometer (X band) equipped with a high sensitivity cylindrical cavity (ER4119HS; Bruker). The spectrometer was operated at a microwave frequency of 9.33 GHz, microwave power of 20.1 mW, modulation frequency of 100 kHz, modulation amplitude of 5.0 Gauss, number of scans of 20, resolution of 1024 points, conversion time of 20.48 ms, time constant of 10.24 ms, sweep time of 20.97 s, center field at 3300 Gauss and sweep width of 1200 Gauss. Recorded EPR data were manipulated and plotted using the Bruker WINEPR system, Version 2.11 and Origin for Windows, Version 7.0 (Microcal., Northampton, MA, USA). All spectra were subtracted for the vehicle. The concentration of erythrocyte HbFe(II)NO was determined by double integration of the signal using $CuSO_4$-EDTA as reference standard and was expressed as micromoles per liter.

Assessment of antioxidant activity

The antioxidant properties of NO-FA were determined according to the method of Vaananen *et al*. (1992). This *in vitro* assay was performed in order to determine whether the NO-FA maintains the full antioxidant properties of FA. Briefly, 180 μL of fresh 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution (100 μM in 95% ethanol) were added to a clear microtiter plate together with 20 μL of various concentrations (0.1–100 μM final) of FA, NO-FA or ascorbic acid diluted in 95% ethanol. The absorbance was monitored at 510 ηm in a plate reader for 15 min. The radical scavenging activity of tested compounds is proportional to the decrease in absorbance of the DPPH. The plots of the absorbance at 515 ηm versus the concentration of sample in the final solution were analyzed by non-linear regression (ascorbic acid, $r^2 = 0.953$; FA,

$r^2 = 0.988$; NO-FA, $r^2 = 0.996$). The mean slope coefficient of the curves ± SE represents the anti-radical activity expressed in micromoles of DPPH per micromole of compound (Fukumoto and Mazza 2000; Kanski *et al*. 2002).

Determination of hydroxyl radical scavenging activity

Both FA and NO are hydroxyl radicals scavengers (Xu 2000; Ogiwara *et al*. 2002). Thus, we investigated whether FA and NO-FA differ in this scavenging activity *in vitro*. The ability of FA and NO-FA to scavenge hydroxyl radicals was measured by a Pholasin-dependent chemiluminescence test (ABEL Anti-oxidant Test Kit, Knight Scientific, Plymouth, UK). Hydroxyl radicals are detected using the photoprotein Pholasin that emits light upon reaction with these radicals. The hydroxyl radical-scavenging activity of the compounds is proportional to the decrease in light emission. Preliminary experiments were performed using D-mannitol, a well-established scavenger for hydroxyl radicals (Shen *et al*. 1997). In this setting, D-mannitol scavenging activity was detectable at concentrations ranging in the micromolar and millimolar range, e.g. inhibition of about 80% and above was observed at 3–10 mM D-mannitol (data not shown).

In vivo comparative studies of NO-FA and FA

The following *in vivo* studies were conducted in order to evaluate and compare the anti-inflammatory actions of FA and NO-releasing FA molecules.

Surgical procedures

Each rat was anesthetized with isoflurane gas and placed in a stereotaxic instrument with the incisor bar set 3.0 mm below the earbars (Hauss-Wegrzyniak *et al*. 1998a,b, 2002). The scalp was incised and retracted and a hole was made at the appropriate location in the skull with a dental drill. Co-ordinates for the 4th ventricle infusions were as follows: 2.5 mm posterior to Lambda, on the midline, and 7.0 mm ventral to the dura. An osmotic minipump (Alzet, Palo Alto, CA, USA, model 2004, to deliver 250 ηL/h) was attached via a catheter to a chronic indwelling cannula that had been positioned stereotaxically so that the tip extended to the co-ordinates given above. Each minipump was prepared to inject either artificial CSF (aCSF) or 250 ηg LPS/h (prepared in aCSF). The composition of the aCSF (in mM/L) was 140 NaCl; 3.0 KCl; 2.5 $CaCl_2$; 1.2 Na_2HPO_4, pH 7.4. Eight rats were assigned to each of seven LPS-infused groups and were injected (s.c.) daily with either FA (3 or 30 mg/kg/day) or NO-FA (2.5, 5, 10 or 50 mg/kg/day), or the vehicle (1.0 mL/kg). An additional group was infused with aCSF into the 4th ventricle and treated with the vehicle. The dosages of FA and NO-FA were chosen so that equivalent amounts of FA would be administered, e.g. 30 versus 50 and 3 versus 5 mg/kg/day for FA and NO-FA, respectively. The NO molecule and its ester linkage account for 20% of the molecular weight of NO-FA. Drug treatment was initiated immediately after surgery and lasted for 14 consecutive days. The FA and NO-FA were prepared fresh each day in glass containers in the vehicle (0.5% carboxymethyl-cellulose/DMSO, 50 : 50 ratio) and administered every morning. An additional pair of groups, i.e. LPS- and aCSF-infused, was also prepared ($n = 10$); for these rats drug treatment (NO-FA, 50 mg/kg/day, s.c.) began 14 days after surgery and continued for 14 days. This group was prepared to determine whether the highest dose of NO-FA could attenuate the neuroinflammation once it was already fully initiated by the LPS infusion.

The following post-operative care was provided to all rats. Betadine was applied to the exposed skull and scalp prior to closure to limit local infection and 5 mL sterile isotonic saline were injected (s.c.) to prevent dehydration during recovery. The rats were closely monitored during recovery and kept in an incubator (Ohio Medical Products, Air Reduction Co. Inc.) at temperatures ranging from 30 to 33°C. Body weights were determined daily and general behavior was monitored for seizures.

Immunohistochemistry

At the end of the drug treatment period, rats from each group were prepared for histological analysis by *in situ* perfusion of the brain with cold saline containing 1 U/mL heparin, followed by 4% paraformaldehyde in 0.1 M/L sodium phosphate buffer, pH 7.4. The brains were post-fixed 1 h in the same fixative and then stored (4°C) in phosphate-buffered saline (PBS), pH 7.4. Free-floating, serial coronal sections (40 μm) were taken by vibratome and washed in PBS. The monoclonal antibody OX-6 (final dilution 1 : 400, Pharmingen, San Diego, USA) was used to visualize activated microglia cells as previously described (Hauss-Wegrzyniak *et al*. 1998a). This antibody is directed against Class II major histocompatibility complex (MHC II) antigen. The sections were incubated overnight at room temperature with primary antibodies directed against the specific epitopes. Thereafter, the sections were washed in PBS and incubated for 1 h at room temperature with the monoclonal antibody, a rat adsorbed biotinylated horse anti-mouse immunoglobulin G (final dilution 1 : 200; Vector Laboratories, Burlingame, CA, USA), diluted in PBS containing 0.5% Triton X-100. After washing in PBS, the sections were incubated for 1 h at room temperature with avidin-biotinylated horseradish peroxydase macromolecule complex (Vectastain, Elite ABC kit, Vector Laboratories). After washing again in PBS, the sections were incubated for 1–5 min with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) as chromogen (DAB kit, Vector Laboratories). The reaction was stopped by washing the sections with PBS. No staining was detected in the absence of the primary or secondary antibodies. Sections were mounted on gelatin–chrome–alum-coated glass slides, air-dried and coverslipped with Cytoseal mounting medium. The location of immunohistochemically defined cells was examined by light microscopy.

Double immunofluorescence staining

Two biotinylated secondary antibodies and avidin-D fluorochrome conjugates were used to stain free-floating slices for MHC II sites on activated microglia (Fluorescein green) and neurons (Texas Red). This additional histological study was performed in order to confirm the effects of the drug therapy on resident microglia, and also to investigate potential widespread neuronal loss within the affected brain regions. After washing in PBS, the sections were incubated with PBS, 5% normal rabbit serum and Avidin D solution (Avidin Biotin Bloking Kit, Vector Laboratories) for 30 min, and then incubated for 2 h with the primary OX-6 antibody (final dilution 1 : 400; Pharmingen). After washing in PBS, the sections were incubated for 30 min with the secondary biotinylated anti-goat antibody (final dilution 1 : 200; Vector Laboratories). After washing, the sections were incubated for 30 min with the fluorochrome

Fluorescein Avidin D FITC (Vector Laboratories); they were then incubated for 1 h with the primary antibody NeuN (NeuN-mouse anti-neuronal nuclei monoclonal antibody; Chemicon International, Temecula, CA, USA), followed by the secondary anti-mouse biotinylated antibody. The sections were incubated with Texas Red Avidin D (Vector Laboratories) for 20 min; after washing, the sections were mounted. Images were acquired from the double-labeled sections using a confocal laser-scanning microscope (Carl Zeiss, model 510NLO-META, Thornwood, NY, USA) equipped with a krypton-argon laser.

Biochemistry

[^{3}H]PK11195 binding

Rats taken for biochemical studies were first deeply anesthetized and then killed. Sections of frontal sensorimotor cortex were dissected and stored (at − 70°C) for assay of [^{3}H]PK11195 binding sites as a quantitative estimate of activated microglia. Two main classes of benzodiazepine receptors exist in mammals: a high affinity receptor found in the brain that is a component of the GABA receptor complex, and a peripheral receptor site that resides on the outer mitochondrial membrane (Stephenson *et al*. 1995) known as the peripheral-type benzodiazepine receptor (Rao *et al*. 2001). This receptor is a hetero-oligomeric complex of three proteins; their major function may be the translocation of cholesterol across the mitochondrial membranes for processing into neurosteroids (Rao *et al*. 2001). The isoquinoline carboxamide PK11195 selectively binds to this peripheral receptor site and has no affinity for the central receptor type. Within the brain, PK11195 binds predominantly to activated microglia at this site (Stephenson *et al*. 1995; Cagnin *et al*. 2001; Rao *et al*. 2001). For the [^{3}H]PK11195 filtration binding assay (Rao *et al*. 2001), samples of frontal sensorimotor neocortex were homogenized in 20 volumes of ice-cold 50 mM/L Tris-HCl (pH 7.4) buffer. Membranes were prepared for binding assays by repeated centrifugation (40 000 *g* for 20 min at 4°C), repeated freeze–thawing (three times) and resuspension in 10 volumes of buffer. Protein content of the final suspension was determined according to the method of Lowry *et al*. (1951). Binding assays were performed at 4°C for 2 h in a final volume of 500 μL containing 1 ηM [^{3}H]PK11195 (specific activity, 85.5 Ci/mM). The assay was terminated by addition of 4 mL ice-cold buffer followed by vacuum filtration through Whatman GF/B glass microfiber filters (pre-soaked in 0.3% polyethylenimine for at least 30 min). The filters were washed twice with ice-cold buffer, air-dried by vacuum and the radioactivity on the filters determined by liquid scintillation spectrometry. All assays were performed in triplicate; specific binding was defined by addition of 20 μM/L diazepam to the incubation solution. The results were analyzed by ANOVA.

Results

In vitro comparative results of NO-FA and FA

NO-FA released bioactive NO with a linear time-dependent course as determined by the formation of HbFe(II)NO, which was comparable with that of the slow-releasing reference NO donor ISM (see Fig. 1). For NO-FA, the HbFe(II)NO levels

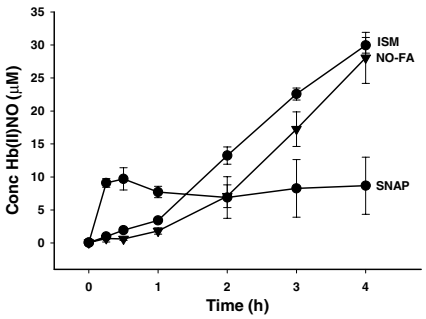


Fig. 1 Kinetics of nitrosylhemoglobin [HbFe(II)NO] formation in whole rat blood following treatment with S-nitroso-*N*-acetyl-penicillamine (SNAP), isosorbide mononitrate (ISMN) and NO-FA. SNAP quickly increased HbFe(II)NO levels that were stable over 4 h. HbFe(II)NO formation from ISMN and NO-FA was slower than that from SNAP. Values are means ± SEM of three independent experiments.

ranged from 0.7 ± 0.5 μM to 28.0 ± 3.9 μM after 15 min and 4 h incubation, respectively. For ISMN, HbFe(II)NO levels ranged from 1.0 ± 0.2 μM to 30.0 ± 1.2 μM at 15 min and 4 h, respectively. SNAP, a fast NO releaser, showed a rapid increase of HbFe(II)NO levels which were then maintained throughout the experimental period (9.1 ± 0.6 μM and 8.7 ± 4.3 μM at 15 min and 4 h, respectively).

Antioxidant activity was measured by the ability of the test drug to scavenge a stable free radical, i.e. DPPH. Ascorbic acid (Vitamin C) was used as positive control. In this *in vitro* assay, FA scavenged DPPH less efficiently than ascorbic acid. NO-FA and FA demonstrated a similar degree of antioxidant activity in this assay (see Table 1). The hydroxyl radical scavenging properties of FA and NO-FA did not differ significantly from each other, as indicated by the comparable slopes of the concentration curve (see Fig. 2).

In vivo comparative results of NO-FA and FA

Immunohistochemistry

Infusion of aCSF produced a minimal inflammatory response by the resident microglia (see Fig. 3a). Infusion of LPS produced a significant and widespread degree of microglial

Table 1 Anti-radical activity of ascorbic acid, ferulic acid and NCX 2057

	Anti-radical activity	
Compound	Current study[a]	Literature
Ascorbic acid	− 1.91 ± 0.55	− 1.83[b]
Ferulic acid	− 1.36 ± 0.17	− 1.34[b]; − 1.39[c]
NCX 2057	− 1.35 ± 0.31	n.a.

[a]Values represent slope coefficients calculated by non-linear regression ± SE in μM of DPPH/μM of compound. [b]Fukumoto *et al.* (2000). [c]Kanski *et al.* (2002). n.a., not available.

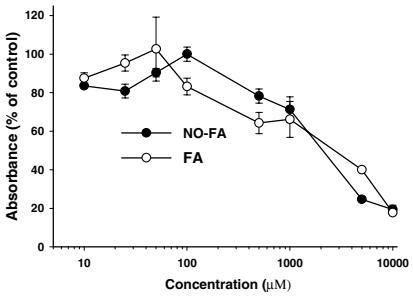


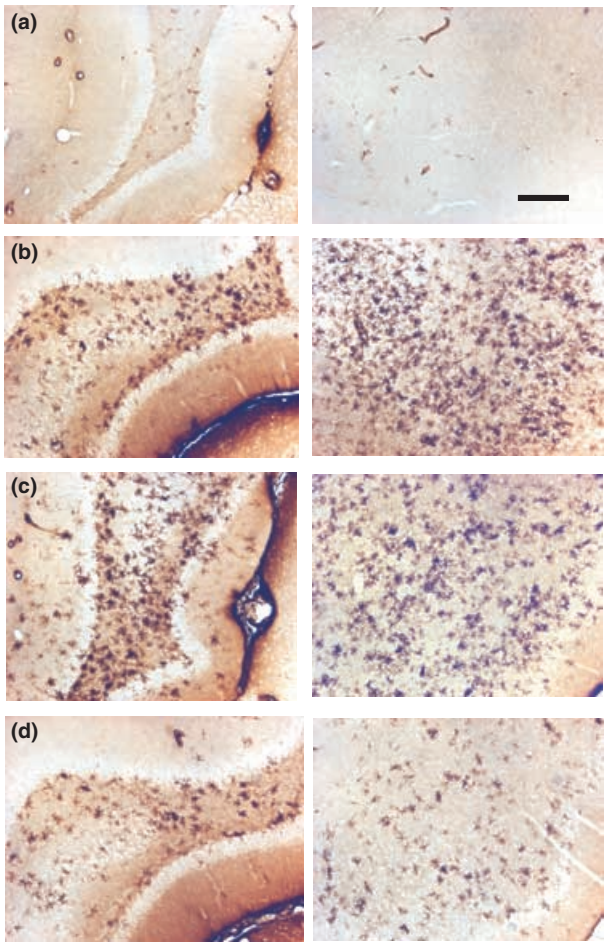
Fig. 2 A Pholasin-dependent chemiluminescence assay compared the ability of FA and NO-FA to scavenge hydroxyl radicals. The hydroxyl radical-scavenging activity of FA and NO-FA was proportional to the decrease in light emission at increasing concentration of the compounds. This activity was not affected by the presence or absence of the NO moiety on FA. Values are means ± SEM of three independent experiments.

activation throughout the brain, with the majority of the activated microglia concentrated in the cingulate gyrus (not shown), hippocampus and entorhinal cortex (Fig. 3b). The activated microglia were characterized by a contraction of their highly ramified processes that appeared bushy in morphology. These findings were consistent with our previous reports (Hauss-Wegrzyniak *et al*. 1998a,b, 1999, 2000, 2002). Chronic treatment with a low dose (3 mg/kg/day) of FA did not reduce the apparent number of activated microglia (Fig. 3c). In contrast, rats treated for 14 days with a higher dose (30 mg/kg/day) of FA demonstrated clear reduction in the number of activated microglia (Fig. 3d). The addition of the NO-releasing moiety significantly enhanced the ability of FA to reduce the degree of microglial activation. Treatment with NO-FA for 14 days beginning immediately after the initiation of the infusion of LPS produced a dose-dependent reduction in the level of microglial activation in the hippocampus and entorhinal cortex (see Fig. 4). Comparison of the immunocytochemical photomicrographs indicates that the lowest dose of NO-FA (Fig. 4a, 2.5 mg/kg/day) was as effective as the highest dose of FA (Fig. 4d, 30 mg/kg/day) tested.

The double immunofluorescence staining (see Fig. 4, lower box) provided additional confirmation of the effectiveness of the NO-FA to reduce the degree of microglial activation within the dentate gyrus dose-dependently. However, when the administration of a high dose (50 mg/kg/day) of NO-FA was delayed for 2 weeks prior to beginning 14 days of drug treatment, we found no reduction in the level of microglial activation in the hippocampus and entorhinal cortex (not shown).

Biochemistry

Analysis of the number of [^{3}H]PK11195 binding sites on activated microglia in the frontal sensorimotor cortex showed a significant main effect of group ($F_{3,19} = 12.5$, $p < 0.001$).



COLOUR FIG.

Fig. 3 Immunohistochemistry for activated microglia using the OX-6 antibody in the dentate gyrus of the hippocampus (left panels) and entorhinal cortex (right panels). Rats infused with aCSF (a) had only a few activated microglia scattered throughout the hippocampus and entorhinal cortex. Chronic infusion of LPS into the 4th ventricle produced highly activated microglia distributed throughout the cingulate gyrus, dentate gyrus of the hippocampus and entorhinal cortex (b). The activated microglia were characterized by a contraction of their highly ramified processes that appeared bushy in morphology in this thick section. Chronic treatment with a low dose of FA (c, 3 mg/kg/day) did not reduce the apparent number of activated microglia. Chronic treatment with a higher dose of FA (d, 30 mg/kg/day) reduced the number and level of activation of the microglia in the hippocampus and entorhinal cortex. Bar = 150 μm.

Post hoc multiple pair-wise comparisons found that rats infused with LPS and treated with vehicle (see Fig. 4, right box) had significantly more ($p < 0.001$) bindings sites, i.e. activated microglia, than the rats infused with aCSF, or the LPS-infused rats that were treated chronically with both doses of NO-FA. The higher dose of NO-FA was not significantly more effective than the lower dose in terms of reducing the number of [^{3}H]PK11195 binding sites. We did not quantify [^{3}H]PK11195 binding sites in rats that were treated only with FA.

COLOUR FIG.

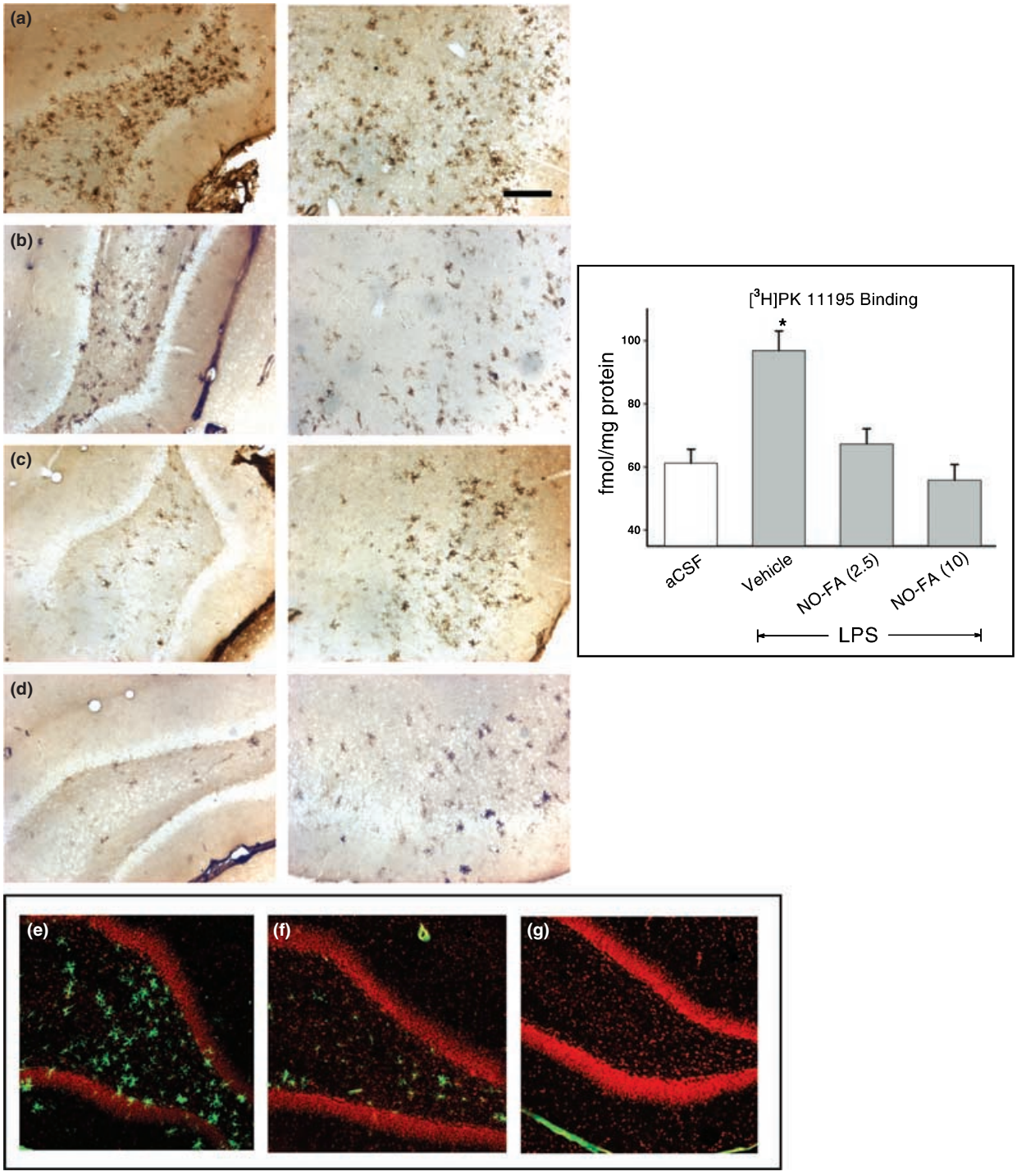


Fig. 4 Immunohistochemistry for activated microglia. Upper panel: OX-6 antibody immunohistochemistry in the dentate gyrus of the hippocampus (left side) and entorhinal cortex (right side). Chronic treatment with NO-FA dose-dependently reduced the number and level of activation of the microglia: (a) 2.5; (b) 5; (c) 10; (d) 50 mg/kg/day. Bar = 150 μm. Right Box: Quantitative determination of the number of [^{3}H]PK11195 binding sites upon activated microglia within the frontal sensorimotor neocortex. Rats infused for 14 days with LPS, and treated with vehicle, had significantly more bindings sites, i.e. activated microglia, than rats infused chronically with LPS and treated with NO-FA (2.5 and 10 mg/kg/day) or infused with aCSF. Values are means ± SEM. *$p < 0.001$. Lower Box: Double immunofluorescence for MHC II receptors on activated microglia and neurons. MHC II receptors are stained with Fluorescein green and neurons are stained with Texas Red in the hippocampal dentate gyrus of LPS-infused, vehicle-treated (a), LPS-infused, NO-FA (5 mg/kg/day)-treated (b), and LPS-infused NO-FA (50 mg/kg/day)-treated (c) rats. Magnification bar = 120 μm.

Discussion

Oxidative stress induced by the deposition of β-amyloid and the presence of inflammation play prominent roles in the neurodegeneration associated with AD (Friedlich and Butcher 1994; Smith *et al*. 1998; Akiyama *et al*. 2000). Therefore, a safe and effective drug that possesses both anti-inflammatory and antioxidant properties might prove to be beneficial in treating the symptoms of AD or slowing their onset (Behl 1999; Akiyama *et al*. 2000). FA is a phenolic molecule that displays antioxidant and free radical scavenger activities (Graf 1992; Scott *et al*. 1993; Kikuzaki *et al*. 2002; Ogiwara *et al*. 2002) and inhibits the formation of leukotrienes via lipoxygenases (Huang *et al*. 1991). In the current study, daily peripheral administration of FA produced a dose-dependent reduction in the level of microglial activation in the brain. A fairly high dose of FA (30 mg/kg/day) was required to achieve a modest reduction in the number of activated cells. Therefore, because we had previously shown that NO-releasing derivatives of anti-inflammatory drugs are more potent than their native compounds (Hauss-Wegrzyniak 24 *et al*. 1998b, 1999; Wenk *et al*. 2000, 2002), we investigated the ability of the NO-releasing derivative of FA to reduce brain inflammation. Interestingly, an approximately 10-fold lower dose (e.g. 2.5 mg/kg/day) of the novel NO-donating derivative of FA was able to reduce the degree of microglial activation to a level equivalent to that achieved using the high dose of FA (30 mg/kg/day). Using an equimolar dose of NO-FA (50 mg/kg/day), the number of activated microglia in the brain was reduced to a level similar to that seen in the aCSF-infused control rats. This finding is consistent with our previous report that the effectiveness of an NO-donating derivative of the anti-inflammatory drug flurbiprofen was significantly greater than the administration of flurbiprofen alone (Wenk *et al*. 2002). Similar to the histologically determined changes in the degree of microglial activation, 25 treatment with NO-FA also significantly reduced the number of [^{3}H]PK11195 binding sites, which is a biochemical marker of microglia activation.

The mechanism(s) underlying the increased potency and effectiveness of the NO-releasing derivative of FA are unknown. When we compared these two drugs using an *in vitro* assay, NO-FA and FA demonstrated similar degrees of antioxidant and free radical scavenging abilities. One hypothesis is that the greater effectiveness of NO-FA in the intact whole animal, as compared with FA, might be due to the release of NO within the blood. We therefore speculate that once the NO molecule is released into the blood, it significantly alters the integrity and permeability of the blood brain barrier (Hooper *et al*. 2001; Thiel and Audus 2001; Chen *et al*. 2003), shifts cerebral microcirculation (Faraci and Sobey 1999) and thereby indirectly augments the uptake of FA into the brain. Once inside the brain, the anti-inflammatory and antioxidant properties of FA are likely due to its ability to inhibit the leukotriene production and reduce oxidative stress (Murakami *et al*. 2000; Yan *et al*. 2001; Hosoda *et al*. 2002). Similar dietary phenolic molecules demonstrate anti-inflammatory properties due to their ability to inhibit cytokine expression and the induction of tumor necrosis factor-α by LPS by blocking the DNA binding of nuclear binding factor κB (Ma *et al*. 2003). Whether NO-FA actually enters the blood–brain barrier intact and releases NO directly in the brain remains to be explored.

Consistent with other drugs previously synthesized and linked to the nitrate moiety through an ester bond, NO-FA releases NO in tissues and bloodstream in a slow and controlled way, thus producing functional NO-mediated effects (Aldini *et al*. 2002; Keeble and Moore 2002). Esterases are the most likely enzymes involved in the formation of bioactive NO and free FA from NO-FA. Once released, bioactive NO may be stored via formation of reversible complexes with hemoglobin (Gladwin *et al*. 2000; Carini *et al*. 2001; Napoli *et al*. 2002) or biological thiols and then transferred from the blood to target tissues (e.g. to brain neuroglobins) when hemoglobin saturation and pO2 are low. This process would result in a local delivery of NO to the tissue (Cannon *et al*. 2001). Free FA and the bioactive storage forms of NO may thus contribute, together, to the anti-inflammatory and antioxidant activity of NO-FA in brain. For example, NO released from NO-FA could also contribute to the antioxidant and anti-inflammatory actions of the drug by lowering free radical production secondary to elevated intracellular calcium, and by attenuating the inflammatory cascade through inhibition of the interleukin-1β-converting enzyme (ICE/caspase-1) involved in cytokine processing (Fiorucci 2001; Law *et al*. 2001).

Recent investigations using animal models of AD indicate that an anti-inflammatory therapy might be able to alter the progression of AD pathology (Jantzen *et al*. 2002; Yan *et al*. 2003). Furthermore, considerable epidemiological evidence also predicts that long-term use of anti-inflammatory drugs reduces the incidence and slows the progression of AD (Stewart *et al*. 1997; In 'T Veld *et al*. 2001; Zandi *et al*. 2002). However, a series of recent interventional studies using a variety of anti-inflammatory and antioxidant drugs has failed to discover significant clinical benefit (Sano *et al*. 1997; Scharf *et al*. 1999; Van Gool *et al*. 2001; Aisen *et al*. 2003). The patients in these studies were often in the early or middle stages of their disease, suggesting that it was already too late to influence the progression of a disease process that was fully established in the brain. We have shown that the neuroinflammation associated with normal aging is not reduced by anti-inflammatory drug therapy (Hauss-Wegrzyniak *et al*. 1999). We demonstrated that an NO-releasing derivative of flurbiprofen could significantly attenuate the neuroinflammation and cognitive impairments generated by infusion of LPS into the 4th ventricle of young and adult rats, but was without effect in old rats (Hauss-Wegrzyniak *et al*.

1999). Healthy aged rats have extensive and rather widespread activation of their resident microglia (Hauss-Wegrzyniak *et al*. 1999). These findings suggest that the overall effectiveness of an anti-inflammatory drug therapy can be significantly attenuated by the degree of inflammation that exists in the brain at the time treatment is initiated. Due to the clinical importance of resolving this issue, in the current study we waited for two weeks after the infusion of LPS had begun before initiating treatment with a high dose of the NO-releasing derivative of FA. Under these conditions, NO-FA was unable to reduce the level of brain inflammation in young rats. This is the first demonstration that delaying the administration of an otherwise effective NO-releasing NSAID undermines its effectiveness in an experimental model of brain inflammation. These findings are consistent with the results from current clinical studies (Scharf *et al*. 1999; Aisen *et al*. 2003) and also with the hypothesis that once the activation of microglia is fully established initiated in the human brain, due to either aging or AD, that NSAID therapy treatment with an anti-inflammatory/antioxidant agent would not be effective. The loss of anti-inflammatory effectiveness with aging may be related to changes in the responsiveness of glia to inflammogens. One recent study demonstrated that LPS-induced production of NO by aged microglia was less responsive to inhibition by transforming growth factor-beta-1 than glia from younger animals (Rozovsky *et al*. 1998). These results provide additional evidence to our previous report (Hauss-Wegrzyniak *et al*. 1999) that anti-inflammatory drug therapy should be initiated prior to the appearance of brain inflammation or the neuropathological processes that lead to this condition.

Given our current appreciation of the significance of chronic neuroinflammation, oxidative stress, glutamate receptor-related excitotoxicity and forebrain cholinergic system dysfunction in the progression of the dementia associated with AD (Albin and Greenamyre 1992; Smith *et al*. 1998; Akiyama *et al*. 2000; Coyle and Kershaw 2001), our results suggest that treatment with a drug combining anti-inflammatory and antioxidant properties might significantly attenuate the processes that drive the pathology associated with AD if the therapy is initiated in the early stages of the age-associated processes occurring within the brain.

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