Peripheral Treatment with Enoxaparin, a Low Molecular Weight Heparin, Reduces Plaques and β -Amyloid Accumulation in a Mouse Model of Alzheimer's Disease

Luigi Bergamaschini,^{1*} Emanuela Rossi,¹ Claudio Storini,² Simone Pizzimenti,² Maria Distaso,² Carlo Perego,² Ada De Luigi,² Carlo Vergani,¹ and Maria Grazia De Simoni^{2*}

¹Department of Internal Medicine, Ospedale Maggiore, Istituto di Ricovero e Cura a Carattere Scientifico, University of Milan, 20122 Milan, Italy, and

²Department of Neuroscience, Mario Negri Institute for Pharmacological Research, Milan d015, Italy

We investigated the effect of long-term, peripheral treatment with enoxaparin, a low molecular weight heparin, in transgenic mice overexpressing human amyloid precursor protein₇₅₁. Enoxaparin (6 IU per mouse intraperitoneally, three times a week for 6 months) significantly lowered the number and the area occupied by cortical β -amyloid deposits and the total β -amyloid (1–40) cortical concentration. Immunocytochemical analysis of glial fibrillary acid protein-positive cells showed that enoxaparin markedly reduced the number of activated astrocytes surrounding β -amyloid deposits. *In vitro*, the drug dose-dependently attenuated the toxic effect of β -amyloid on neuronal cells. Enoxaparin dose-dependently reduced the ability of β -amyloid to activate complement and contact systems, two powerful effectors of inflammatory response in AD brain. By reducing the β -amyloid load and cytotoxicity and proinflammatory activity, enoxaparin offers promise as a tool for slowing the progression of Alzheimer's disease.

Key words: Alzheimer; amyloid; A β ; binding agent; low molecular weight heparin; APP23 mouse; inflammation; complement system; kinin system; heparin

Introduction

The etiology of Alzheimer's disease (AD) is still not known, but evidence is accumulating that an innate immune response from inside the AD brain is a secondary response to the early accumulation of β -amyloid (A β) antibody in the brain. This inflammatory event associated with amyloid plaques is thought to be a self-attack by the host on neurons, leading to exacerbation of the fundamental pathology through activation of microglia and astrocytes, increased expression of cytokines and acute phase reactants, and complement and contact–kinin system activation (Yasuhara et al., 1994; Bergamaschini et al., 2001a; McGeer and McGeer, 2001, 2002; van Beek et al., 2003).

Evidence that A β accumulation probably contributes to AD provides the rationale for a therapy based on altering brain A β accumulation and reducing its cytotoxic and proinflammatory actions. Immunization with synthetic A β peptide (Schenk et al., 1999; Sigurdsson et al., 2001; Dodart et al., 2002) or administration of antibodies against A β (DeMattos et al., 2001; Chauhan and Siegel, 2002; Dodel et al., 2002; Mohajeri et al., 2002) reduces the pathology in an animal model of AD through a mechanism that might include crystallizable fragment of Ig (Fc)- and non-Fc-

mediated (Bard et al., 2000; Bacskai et al., 2002) clearance of A β antibody complexes by activated microglia. Brain A β levels may also be affected by the binding of A β in blood, as suggested by the observation that immunological and non-immunological molecules with high A β binding affinity can reduce the formation of amyloid plaques by altering the brain–periphery dynamics (DeMattos et al., 2001; Dodart et al., 2002; Dodel et al., 2002; Matsuoka et al., 2003). *In vitro*, charged residues within the region 1–11, and residues 13–16, the H-H-Q-K domain, are critical for the proinflammatory activity of A β (Velazquez et al., 1997; Webster et al., 1997a; Bergamaschini et al., 2001b) and microglia activation (Giulian et al., 1998), respectively. Thus, inhibition of these activities by pharmacological targeting of such regions might slow the neurodegeneration in AD brains. In this respect, heparins might be candidates for such therapy (Leveugle et al., 1994; Watson et al., 1997; Leveugle et al., 1998; Tyrrell et al., 1999; van Horssen et al., 2003). We have shown previously that native (unfractionated) heparin counteracts A β neurotoxicity and its proinflammatory activity by attenuating its ability to activate the complement and contact systems *in vitro* (Bergamaschini et al., 2002). Because unfractionated heparin is not suitable for long-term therapy because of the difficulty in maintaining optimal therapeutic concentrations and the high risk of bleeding, we investigated the effect of the low molecular weight heparin (LMWH) enoxaparin (ENO) in BG, D2-Tg (Th1App)23/15dz/black mice (APP23) transgenic mice (Sturchler-Pierrat et al., 1997; Bayer et al., 2003). LMWHs, including ENO, are currently used in humans for anticoagulant therapy. Notably, they have been reported to reduce kidney amyloidosis *in vivo* (Kisilevsky et al., 1995; Zhu et al., 2001).

In addition to exploring its *in vivo* actions, we investigated

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*L.B. and M.G.D. contributed equally to this work.

Correspondence should be addressed to Dr. Luigi Bergamaschini, Department of Internal Medicine, Ospedale Maggiore, Istituto di Ricovero e Cura a Carattere Scientifico, Via Pace 9, 20122 Milano, Italy. E-mail: luigi.bergamaschini@unimi.it.

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whether ENO could attenuate the proinflammatory activity and cytotoxicity of A β *in vitro* to gain an insight into its mechanism of action in APP23 mice.

Materials and Methods

Animals and ENO treatment. Six-month-old male APP23 mice (kindly provided by Dr. M. Staufenbiel, Novartis Pharma, Basel, Switzerland) (Sturchler-Pierrat et al., 1997; Bayer et al., 2003) were housed one mouse per cage in a controlled environment, with *ad libitum* access to food and water. Procedures involving animals and their care conformed to institutional guidelines that are in compliance with national (Decreto legge n.116, Gazzetta Ufficiale suppl. 40; February 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1; December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996).

APP23 mice and their age-matched wild type (WT) mice were injected intraperitoneally with 6 IU per mouse (60 μ g) of ENO (Aventis-Pharma, Milan, Italy) or saline, three times a week for 6 months. The dose of ENO corresponds to the clinically effective dose in humans (250 IU/kg). At the end of the treatment, mice were deeply anesthetized and transcardially perfused with 10 mM Tris buffer, pH 7.4, containing 10% sucrose, 1 mM EDTA, and 0.5 mM PMSF (all from Sigma Chemicals, St. Louis, MO). Brains were removed, and cortex and hippocampus were dissected from the right hemisphere and kept at -80°C for biochemical and molecular assays. The other brain hemisphere was fixed in 4% paraformaldehyde, processed in 20–30% sucrose, frozen, and stored at -80°C for immunocytochemistry.

Sandwich ELISA for A β (1–40). Total A β (soluble and insoluble species) was obtained from the cortex of APP23 and WT mice using a single-step extraction with formic acid, adapting the method proposed by Lim et al. (2000). A mixture of protease inhibitors [1 mM 4-(2-aminoethyl)benzenesulfonylfluoride, HCl, 0.8 μ M aprotinin, 50 μ M bestatin, 15 μ M E-64, 20 μ M leupeptin, 10 μ M pepstatin A; all from Calbiochem, La Jolla, CA and Novabiochem GmbH, San Diego, CA] was added to all solutions used for tissue extraction and ELISA measurements. Cortices were sonicated (10 W, 3×10 shots) in $3 \times$ wet weight 70% formic acid. Extracts were then neutralized with 2 M Tris, pH 8, containing 30% acetonitrile and 2 M NaOH and centrifuged at $100,000 \times g$ for 1 hr at 4°C . The concentration of A β was measured by human β -amyloid (1–40) ELISA kit according to the manufacturer's instructions (Biosource International, Camarillo, CA). A standard A β curve was plotted in neutralized formic acid and diluent buffer in the same ratio as for experimental samples.

Immunocytochemistry. Forty micrometer hemibrain cryostat coronal sections, prepared from perfused brains as described above, were used for assessment of A β deposits and astrocytic activation by glial fibrillary acidic protein (GFAP) immunostaining (De Simoni et al., 2000; Kalebica et al., 2000). For A β immunostaining, sections were rinsed for 15 min in 3% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT) in 0.1 M PBS and then incubated overnight with primary A β (mouse monoclonal human A β protein, clone 4G8, 1:250; Signet Pathology System, Dedham, MA). For GFAP immunostaining the sections were rinsed for 30 min in 0.4% Triton X-100 in PBS 0.1 M followed by 15 min in 0.1% Triton X-100 and 3% FCS in 0.1 M PBS. Sections were incubated overnight with GFAP (mouse monoclonal, 1:2500; Chemicon, Temecula, CA). For both stainings, sections were processed for conventional immunohistochemistry (De Simoni et al., 2000; Kalebica et al., 2000) the next day. We detected A β deposits in GFAP-immunostained sections with alkaline Congo Red (Sigma). Sections were dehydrated through graded alcohols, fixed in xylene, coverslipped using DPX mountant (BDH Laboratory Supplies), and examined with bright-field and cross-polarized light photomicroscopy.

A β deposits. After A β immunostaining, six 20 μ m sections at 40 μ m intervals from one cerebral hemisphere were selected for A β deposit count. The first section was at stereotaxic coordinates anteroposterior +3.20 from bregma. The number and total area (μm^2) of A β -immunopositive deposits in the cortex of each section were recorded and pooled for all six sections. Kontron Electronic KS300 Imaging System software interfaced with a Zeiss Axioskop microscope and a Grundig

Electronic video camera were used. The quantitative analysis was performed at $20\times$ magnification by an investigator blinded to the treatment.

RNA isolation, cDNA synthesis, and real-time PCR. Total RNA was isolated from hippocampi according to the acid guanidium–phenol–chloroform method, as described previously (De Simoni et al., 2000). cDNA was synthesized as follows: 1.5 μ g total RNA from each sample was reverse transcribed with random hexamer primers using Multi Scribe reverse transcriptase (TaqMan Reverse transcription reagents, Applied Biosystems, Foster City, CA). The following thermal cycling protocol for reverse transcription was used: 10 min at 25°C for incubation, 30 min at 48°C for reverse transcription, and then 5 min at 95°C for inactivation. Real-time PCR was performed using a GeneAmp 5700 Sequence detection System (Applied Biosystems). Fifty nanograms of cDNA and gene-specific primers (200 nM final concentration) were added to Master Mix SYBR Green I Dye, TaqMan DNA polymerase, dNTPs with dUTP and optimal buffer components (Applied Biosystems) and subjected to PCR amplification in a total volume of 25 μ l. The PCR protocol included one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min. For each experimental group, six RNA samples were used. Real-time PCR was conducted in triplicate with each RNA sample. The amplified transcripts were quantified using the comparative cycle threshold method (Applied Biosystems users bulletin 2). Primer optimizing procedures and validation experiments (data not shown) were performed according to the manufacturer's instructions to show that efficiencies of target and reference were equal. Primers were designed using Primer Express software (Applied Biosystems) based on the following GenBank accession numbers (given in the parenthesis): IL-1 β (NM008361), tumor necrosis factor- α (NM013693), ICAM-1 (NM010493), and β -actin, the housekeeping gene (NM007393).

Preparation of A β peptides. For all *in vitro* experiments, A β peptides were prepared by dissolving lyophilized synthetic analog of A β (1–40) and A β (1–42) (both from Bachem, Bubendorf, Germany) in distilled H $_2$ O at a concentration of 50 μ M and immediately diluted to 25 μ M (100 μ g/ml) with PBS, pH 7.4, or PBS containing ENO (10, 50, and 100 μ g/ml final concentrations). All A β preparations were used within 30 min from dissolution; therefore solubilized A β peptides after storage at room temperature for 30 min were centrifuged (10,000 rpm) for 10 min at room temperature, and the peptides, in supernatants and pellets, were morphologically characterized by light and electron microscopy. Congo Red staining was used for light microscopy examination of A β preparations. Congo Red (1 μ l) was added to each sample (20 μ l) for 20 min at room temperature. Samples were applied on precoated glass slides and covered with cover glass, and slides were examined with a light microscope equipped with a polarizing filter. For negatively stained specimens, samples (20 ml) were adsorbed to 200 mesh Formvar carbon-coated grids, air dried, and negatively stained with 2% uranyl acetate in water (w/v) for 2 min. The grids were examined and photographed in a Jeol JEM 1010 electron microscope operating at 80–100 kV.

Neuronal cell culture. Rat PC12 pheochromocytoma cells were maintained at 37°C with 5% CO $_2$ in gelatin-coated flasks with complete Roswell Park Memorial Institute (RPMI) 1640 medium (Seromed Biochrom, Berlin, Germany) containing complement-inactivated 10% horse serum (Life Technologies, Paisley, UK) and 5% fetal calf serum (Hyclone Laboratories). For neuronal differentiation, PC12 cells were removed from the flask with trypsin–EDTA (0.25%, Seromed Biochrom) and resuspended in the same medium at the density of 3×10^4 /ml. One hundred microliters of these cells were plated in collagen-coated wells (3×10^3 cells per well) and incubated at 37°C with 5% CO $_2$ for 24 hr. Medium was changed with complete RPMI 1640 supplemented with 1% horse serum and neuronal growth factor (100 ng/ml; Alomone Laboratories, Jerusalem, Israel) every 48 hr for 10–14 d until complete differentiation. Differentiated PC12 cells are commonly considered a reliable model of neuronal cells.

Effect of ENO on A β cytotoxicity. For A β cytotoxicity, differentiated PC12 cells were incubated for 72 hr in an equal volume of saline, ENO (1, 5, and 10 μ M), A β (1–40) or (1–42) (both 25 μ M), or a mixture of A β (1–40) or (1–42) and ENO at the three doses. At the end of incubation, cell viability was evaluated by measuring the conversion of tetrazolium bromide (MTT) (Sigma) to insoluble purple formazan. The reaction

Figure 1. ENO reduces the antibody load in APP23 mice. Representative photomicrographs of 40 μ m prefrontal cortex slices prepared from APP23 transgenic mice are shown. Immunostaining for A β revealed few small amyloid deposits in mice treated with ENO (A) but several deposits in mice given saline (B). Scale bar, 200 μ m. C–E, In cortex slices of APP23 mice treated with ENO or saline, the A β load was quantified by evaluating the number of A β deposits (horizontal bar indicates the median; * p < 0.02 vs saline; Student's t test) (C), the total area occupied by deposits (mean $\pm$ SE; * p < 0.03 vs saline; Student's t test) (D), and the total, soluble plus insoluble, A β (1–40) concentration (horizontal bar indicates the mean; *** p < 0.001 vs saline; Student's t test) (E).

product was analyzed spectrophotometrically at 540 nm with an automated microplate reader. MTT is a water-soluble tetrazolium salt that is converted to insoluble purple formazan by dehydrogenase enzymes. Because active mitochondrial dehydrogenases of living cells, but not of dead cells, will cause MTT conversion, this method is used as a measurement of cell viability (Martel et al., 1997).

Contact system activation. Contact system activation was assessed by measuring the degree of cleavage of high molecular weight kininogen (HK), a suitable marker of contact system activation (Berrettini et al., 1986), using densitometric analysis of immunostained blotting membranes after SDS-PAGE. This method simultaneously evaluates the native protein and its activation fragment. Plasma samples were loaded on 8% acrylamide gel and separated by SDS under nonreducing conditions. Proteins were transferred to polyvinylidene-difluoride membranes (Immobilon, Millipore, Bedford, MA) by electroblotting, and HK bands were visualized with polyclonal goat anti-light chain HK (Nordic, Thilburg, The Netherlands) and biotin-conjugated rabbit anti-goat IgG (Sigma) and an avidin–alkaline phosphatase substrate (Sigma). The blotting membranes were analyzed with a high-performance scanner and Image Master software (Amersham Biosciences, Uppsala, Sweden). The level of HK activation was expressed as the percentage of total protein (band II plus III versus bands I plus II and III). The apparent masses of native HK and its activation fragments were molecular weights 130,000 (band I), 107,000 (band II), and 98,000 (band III).

Complement activation. Complement activation was assessed by measuring the degree of C4 cleavage essentially as described for the contact system. The C4 bands were visualized with rabbit polyclonal anti-C4/C4c (Dako, Glostrup, Denmark) and biotin-conjugated goat anti-rabbit IgG (Sigma) and an avidin–alkaline phosphatase substrate (Sigma). C4 activation was expressed as the percentage of total protein (band II versus bands I plus II).

Effect of ENO on the A β -dependent activation of complement and contact systems. Freshly solubilized A β (1–40) or (1–42) (100 μ g/ml, 100 μ l), heat-aggregated human IgG (HAG) (20 mg/ml 10 μ l), or 1% kaolin (10 μ l) (Sigma) was incubated for 30 min at 37°C with normal Na-citrate plasma containing different amounts of ENO (0, 10, 50, and 100 μ g/ml, corresponding to 0, 0.5, 1, and 2 mM respectively). The reaction was quenched by adding 200 μ l of PBS containing soybean trypsin inhibitor (100 μ g/ml), EDTA (10 mM), and Polybrene (0.05% v/w) (all from Sigma). Normal Na-citrate plasma was the source of complement

and contact system factors, and HAG and kaolin were used as positive controls to activate the complement and contact systems, respectively, *in vitro*. HAG was prepared by incubating human IgG₁ myeloma protein (20 mg/ml) at 56°C for 30 min. The effect of heparin sulfate (HS) (Sigma) and dermatan sulfate (DS) (Sigma), other members of the glycosaminoglycan family, was also tested.

Results

Effect of peripheral ENO treatment in APP23 transgenic mice

To explore the effects of ENO *in vivo*, we chronically treated 6-month-old APP23 transgenic mice (overexpressing human APP₇₅₁). These mice develop A β deposits, a hallmark of AD neuropathology, in the cortex and hippocampus starting from 5 months of age. Most of the deposits are compact at their first appearance (Sturchler-Pierrat et al., 1997; Bornemann and Staufenbiel, 2000).

APP23 mice and their age-matched WT mice were injected with 6 IU of ENO (60 μ g, i.p.) or saline per mouse three times a week for 6 months. At the end of the treatment, quantitative imaging analysis of A β deposits showed a significant reduction in number and area occupied by amyloid deposits in the neocortex, compared with saline-treated APP23 mice (Fig. 1A–D). Quantification of the formic acid-extracted (i.e., total: soluble plus insoluble) A β (1–40) content in the cortex of these mice showed that the A β concentration was significantly lower in mice receiving ENO (Fig. 1E). In WT mice, A β was undetectable. Because activated glial cells are present around A β plaques as a result of the local inflammatory state elicited by A β , we checked for the presence of GFAP-immunopositive astrocytes in mice receiving ENO or saline (Fig. 2). Astrocytes showing an activated phenotype with enlarged cell bodies and long thick processes were found selectively around A β deposits in APP23 mice treated with saline. Only few, faintly stained, thin astrocytes were observed in the cortical tissue of ENO-treated mice, indicating a marked reduction in the astrocytic response.

The chronic treatment with ENO was well tolerated and did not elicit appreciable side effects as indicated by absence of hemorrhage in the brain and by the lack of significant differences in hippocampal mRNA expression of the inflammatory genes tested (IL-1 β : F = 3.46, p = 0.87; TNF- α : F = 3.26, p = 0.932; ICAM-1: F = 3.98, p = 0.743; two-way ANOVA) (supplemental Table 1, available at www.jneurosci.org).

Effect of ENO on A β aggregation state *in vitro*

Because A β toxicity *in vitro* might be related to its aggregation state, we checked whether ENO affected it under our experimental conditions, i.e., 30 min of incubation with A β at room temperature. Under light microscopy, A β and A β plus ENO (0.5–2 mM) preparations lacked any visible indications of precipitated aggregates, in both supernatants and pellets. Electron microscopy evaluation revealed that fibrils were poorly present in all preparations and that there was some increase in fibrillation at the highest concentration of ENO. Fibril morphology was similar in all A β and A β plus ENO preparations; the diameter of fibrils ranged between 40 and 52 Å (data not shown).

Figure 2. ENO reduces astrocyte activation in APP23 mice. Shown are representative photomicrographs of 40 μ m prefrontal cortex slices prepared from APP23 transgenic mice, showing GFAP-immunopositive astrocytes. Activated astrocytes with enlarged cell bodies and highly ramified, thick processes surround fibrillar amyloid deposits (stained with Congo Red) in APP23 mice treated with saline (A, C). Only a few, scattered GFAP-positive cells can be observed in APP23 mice treated with ENO (B, D), indicating that the treatment markedly reduced astrocyte activation. Scale bar, 100 μ m.

Figure 3. ENO dose-dependently attenuates A β (1–40) cytotoxicity. The viability of differentiated PC12 cells was measured by densitometric analysis of surviving cells stained with MTT. Cells ($15 \times 10^3/\text{cm}^2$) were exposed to A β (1–40) (25 μ M) or A β plus ENO at different concentrations (1, 5, and 10 mM) for 72 hr. Data are the mean $\pm$ SE optical density (O.D.). (** $p < 0.01$; *** $p < 0.001$ vs A β alone; ANOVA followed by Tukey's test).

ENO attenuates A β (1–40) and (1–42) cytotoxicity *in vitro*

We assessed whether ENO attenuated A β cytotoxicity using rat adrenal pheochromocytoma PC12 cells grown in the presence of neuronal growth factor to obtain complete differentiation into neuronal cells. ENO per se (up to 25 μ M; data not shown) had no effect on differentiated PC12 cell viability, but it reduced the cytotoxic effect of A β (1–40) in a dose-dependent manner (Fig. 3). Similar results were obtained using A β (1–42) (data not shown).

ENO attenuates A β (1–40) and (1–42) proinflammatory activities *in vitro*

We investigated whether ENO, like unfractionated heparin (Bergamaschini et al., 2002), attenuated A β toxicity by measuring the A β -driven activation of the complement and contact–kinin systems *in vitro*. These systems are powerful effectors of inflamma-

tory reactions and are thought to be involved in progressive AD neurodegeneration.

Enoxaparin dose-dependently reduced the ability of A β (1–40) to activate the complement and contact systems, as shown by the decrease in the activation bands of complement classical pathway (C4c) and of the contact system (HK bands II and III) (Fig. 4). Identical results were obtained using A β (1–42). The ability to prevent activation of these systems seems to be a characteristic of enoxaparin, because HS and DS, other members of the glycosaminoglycan family, have no such effect. Moreover, HS and DS, even at the lowest concentration used in our experiments (10 μ M), activated the complement and contact systems directly.

Discussion

This study found that prophylactic treatment with a clinically relevant dose of ENO, which has anti-inflammatory and cytoprotective effects *in vitro*, reduced reactive astrocytosis, the deposition of A β , and its total brain concentration in an animal model of AD. We did not address the effect of prophylactic treatment with ENO on APP23 behavioral changes because these mice (male, 12 months old) do not manifest significant cognitive impairment (Kelly et al., 2003). The absence of an inflammatory reaction (supplemental Table 1) and brain hemorrhages suggests that long-term treatment with ENO was well tolerated.

Whether the protective effect of ENO is caused by a decrease in amyloidogenesis (Zhu et al., 2001) or the prevention of A β brain deposition, or both, is not yet clear. There are at least two possible sites of action of ENO *in vivo*. First, ENO may act in the CNS, although it is still not understood whether LMWHs, including ENO, can cross the blood–brain barrier (BBB) (Dudas et al., 2002; Ma et al., 2002; Walzer et al., 2002). The BBB has never been clearly seen to be damaged in AD, but there is evidence that the pathological elevation of soluble A β in plasma may alter the permeability of brain capillaries (Zlokovic et al., 1993; Mackic et al., 1998; Strazielle et al., 2000; Ma et al., 2002), facilitating the passage of small molecules such as ENO. In the CNS, small amounts of the drug, which may affect the interactions between heparin sulfate and amyloid protein, might prevent A β deposition by impeding the structural changes necessary for fibril formation (Kisilevsky, 1997; Shuvaev and Siest, 2000; Zhu et al., 2001; Bazin et al., 2002). In addition, ENO may also protect neurons from the toxicity of soluble A β , as suggested by our data on cultured neuronal cells. *In vitro*, in our experimental conditions, ENO had no effect on A β fibrillarity, indicating that the neuroprotective effect of ENO was not caused by a change in A β conformation or aggregation state. Because heparin binds to the 13–16 residues of A β (HHQK domain), it is possible that ENO may have coated A β fibrils in such a way that they cannot interact efficiently with the neuronal or PC12 cell membrane. Alternatively, although the two mechanisms are not mutually exclusive, ENO could have eluted cell-bound A β (Mackic et al., 1998), preventing its toxic effects. Notably, targeting of interactions of the HHQK domain within A β with microglia attenuated the neurotoxic effects of microglia *in vitro* and reduced inflammation *in vivo* (Giulian et al., 1998). Although we could not evaluate possible ENO-mediated neuroprotective effect *in vivo* because APP23 mice do not develop noticeable cortical neurodegeneration (Calhoun et al., 1998), astrocyte activation was markedly reduced in ENO-treated mice.

A second possible site of action of ENO *in vivo* is blood. Apart from its pharmacological actions in the brain, ENO may also interact with circulating A β , thereby altering its brain–plasma

Figure 4. ENO inhibits $A\beta$ (1–40)-dependent activation of the complement and contact systems. Shown is representative SDS-PAGE and Western blotting analysis of the cleavage of C4 (A) and HK (B) as indicators of complement and contact system activation, respectively. At the bottom of each lane, the percentages of cleaved C4 (C4c vs C4 plus C4c) and HK (bands II and III vs I plus II and III) were determined by densitometric analysis of the immunostained membrane. ENO but not DS or HS caused dose-dependent inhibition of the cleavage of C4 and HK induced by $A\beta$ (25 μ M). Columns show the mean $\pm$ SE (%) of C4 (C) and HK (D) cleavage in three separate experiments run in triplicate (** $p > 0.01$; *** $p > 0.001$ vs $A\beta$ alone; Kruskal–Wallis followed by Dunn's test).

dynamics (Bard et al., 2000; Dodart et al., 2002; Dodel et al., 2002; Matsuoka et al., 2003).

Our *in vitro* findings that ENO reduces the ability of soluble $A\beta$ to activate the complement and contact systems in human plasma indicate that even in the presence of its physiologic ligands, specifically anti-thrombin III, ENO can attenuate $A\beta$ biological activity. The inhibitory mechanism of ENO probably involves an interaction with $A\beta$ and not with the plasma factors of those systems, because ENO, like natural heparin (Bergamaschini et al., 2002), has no effect on the activating ability of HAG and kaolin, activators of the complement classical pathway and contact system, respectively (data not shown).

The complement and contact systems, which are believed to be involved in AD pathology, are powerful effector mechanisms of the immune system. Although their activation contributes to the development of an inflammatory reaction to protect the host, chronic or unregulated activation of these systems can result in destructive inflammatory events and tissue damage. In AD brains, complement activation products may enhance the inflammatory response by synergizing with $A\beta$ to induce the production of proinflammatory cytokines and could participate in $A\beta$ neurotoxicity through the generation of anaphylatoxins (C3a, C5a) and of the cytolytic complex C5b-9 (Webster et al., 1997b; Terryberry et al., 1998; O'Barr and Cooper, 2000; McGeer and McGeer, 2002; Farkas et al., 2003; van Beek et al., 2003; Veerhuis et al., 2003). There is also evidence of a protective role for complement in AD pathology, because overexpression of the natural inhibitor C3 in APP transgenic mice results in an increase of $A\beta$ deposition and neuronal loss (Wyss-Coray et al., 2002). The situation in the brain compartment where $A\beta$ peptides accumulate is likely to be very complex, and it is possible that complement protective or toxic effects are threshold phenomena, so that above a given level of complement activation, cells, including neurons, are injured. The beneficial effects observed in our transgenic mice might also be achieved through a dampening of the local glial reactions, as indicated by the reduction of astrocyte activation around $A\beta$ deposits.

In conclusion, it would appear that a dose of ENO similar to the clinically effective dose in humans, administered in the early phase of the disease, attenuates brain $A\beta$ accumulation and deposition. Although $A\beta$ deposition is probably only one of the pathogenetic fac-

tors, our findings further support the hypothesis that LMWH might be beneficial against the progression of AD (Walzer et al., 2002). LMWHs, including ENO, are used extensively for anticoagulation, but anticoagulant therapy is usually too short to allow a retrospective study. The fact that the animal models available at present do not reproduce all the characteristics of AD, and that LMWHs, including ENO are easy to use, have few and well known side effects, and are suitable for long-term therapy (Lee et al., 2003), argues in favor of a clinical trial to check the beneficial effects of this drug in a therapeutic setting.

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