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ORIGINAL ARTICLE

Nitric Oxide Decreases the Enzymatic Activity of Insulin Degrading Enzyme in APP/PS1 Mice

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Abstract Nitric oxide has been implicated in the regulation of enzyme activity, particularly the activity of metalloproteinases. Since the inducible form of the nitric oxide synthase (NOS2), is upregulated in Alzheimer's disease, we investigated the activity of two amyloid β degrading enzymes, IDE and neprilysin. In vitro we demonstrated that the activity of IDE was inhibited by *NO donor Sin-1, whereas activity of neprilysin remained unaffected. In vivo the activity of insulin-degrading enzyme was lowered in APP/PS1 mice, but not in APP/PS1/NOS2(−/−) mice. These data suggest that NOS2 upregulation impairs amyloid β degradation through negative regulation of IDE activity and thus loss of NOS2 activity will positively influence amyloid β clearance.

Keywords IDE · Insulin degrading enzyme · Nitric oxide · Aβ · Nitration

Abbreviations

AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
PN	Peroxynitrate
Sin-1	3-morpholino-sydnonimine
DetaNONOate	(Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
GSNO	S-Nitrosoglutathione

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Introduction

Alzheimer's disease is histopathologically characterized by extracellular deposition of amyloid β (Aβ) fragments of the amyloid precursor protein, intracellular deposits of hyperphosphorylated tau protein in neurofibrillary tangles (Querfurth and LaFerla 2010) and chronic neuroinflammation (Heneka et al. 2010) indicated by the activation of microglia and astrocytes in particular in the vicinity of amyloid plaques (Glass et al. 2010).

Besides the well investigated production pathways of Aβ, the degradation of these peptides is not entirely understood. There are two main proteases involved in this process, insulin-degrading enzyme (IDE) (Farris et al. 2003) and neprilysin (Iwata et al. 2000). IDE (EC 3.4.24.56) is a 110 kDa neutral thiol metalloproteinase that froms a homodimer (Fig. 1a,b) (reviewed in Malito et al. 2008). It is present in the cytosol and peroxisomes, but has been shown to be unconventionally secreted by exosomes (Bulloj et al. 2010; Tamboli et al. 2010). Neprilysin (EC 3.4.24.11) is a 90–110 kDa zinc-dependent, *phosphoramidon*-sensitive, single-pass type II membrane metallopeptidase with an ectodomain containing the catalytic side (reviewed in Malito et al. 2008).

As an effector of neuroinflammation an increase in oxidative/nitrosative species has been observed during AD (Smith et al. 1997; Castegna et al. 2003; Butterfield et al. 2007; Fernández-Vizarra et al. 2004), in particular the generation of highly reactive peroxynitrite resulting from the

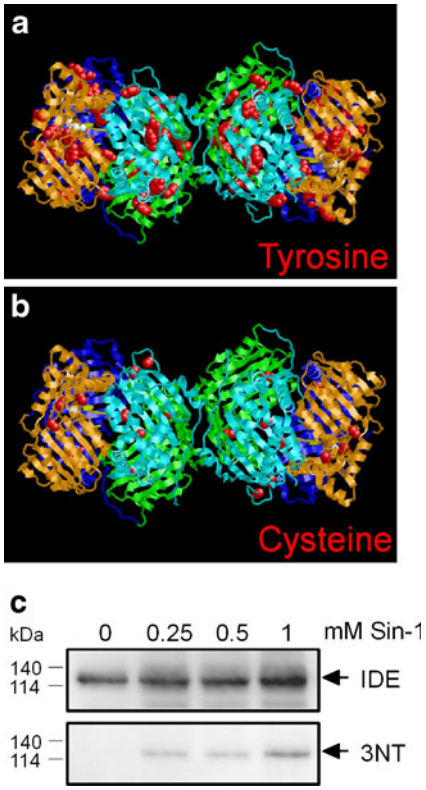

Fig. 1 *NO-mediated post-translational modifications of IDE. **a** Crystallographic structure of IDE dimer (atomic coordinates are from the Protein Data Bank). Tyrosine side chains are colored red in **b** Same as A but cysteine side chains are depicted in red **c** IDE was incubated in the presence or absence of the *NO-releaser Sin-1 for 4 h and samples were incubated with IDE antibody PC720 and with antibody 1A6 to detect 3′-nitrotyrosine

conversion of nitric oxide, a molecule produced by nitric oxide synthases.

There are three NOS proteins that generate *NO in the brain. Neuronal NOS and endothelial NOS are expressed constitutively (Stamler et al. 1997), whereas the inducible nitric oxide synthase (NOS2), inactive under non-pathological conditions, is upregulated in neurons, microglia and astrocytes in response to inflammatory stimuli in AD, potentially aggravating disease progression (Vodovotz et al. 1996; Heneka et al. 2001). Indeed AD lesions reveal the pathological pattern of oxidative and nitrosative injury, especially the posttranslational modifications of cysteine and tyrosine residues (Butterfield et al. 2007; Castegna et al. 2003; Fernández-Vizarra et al. 2004; Tohgi et al. 1999; Lüth et al. 2002). One of the modifications is S-nitrosylation, or covalent reaction of *NO with specific protein thiol groups, leading to protein misfolding (Nakamura and Lipton 2009; Radi 2004; Gow et al. 2004). In addition, conversion of protein tyrosine residues to 3′-nitrotyrosine has been found under pathological conditions and results in irreversible changes in enzyme activity and protein degradation (Souza et al. 2008).

Here we assessed the effect of *NO-donors on the activity of the Aβ degrading enzymes IDE and neprilysin as well as the impact of NOS2 gene deficiency in APP/PS1 transgenic mice on IDE activity.

Material and methods

Animals

APP/PS1 transgenic animals (# 004462, The Jackson Laboratory) (Jankowsky et al. 2001) and NOS2 deficient animals (# 002609, The Jackson Laboratory) (Laubach et al. 1995) were both of the BC57/Bl6 genetic background. Mice were housed in groups of 4 under standard conditions at 22°C and a 12 h light-dark cycle with free access to food and water. At 12 month of age animals were anesthetized, transcardially perfused with heparinized sodium chloride (0.9%), and brains were removed. Animal care and handling was performed according to the declaration of Helsinki and approved by the local ethical committees.

Brain protein extraction

Snap-frozen brain hemispheres were homogenized in PBS with protease inhibitor mixture (Sigma, Munich, Germany). In case of the enzymatic assays the protease inhibitor mixture was replaced with 1 mM AEBSF. The homogenate was extracted in 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40 for 30 min on ice. Protein concentration in the soluble fraction was determined using the BCA protein assay (Thermo, Bonn, Germany).

Western blotting

Protein samples (25 μg) were separated by 4–12% NuPAGE (Invitrogen, Karlsruhe, Germany) using MES or MOPS buffer and transferred to nitrocellulose membranes. For detection of Aβ, blots were boiled for 5 min in water. Aβ was detected using antibody 6E10 (1:2000; Covance), IDE using antibody PC730 (1:5000; Merck, Darmstadt, Germany), Grp75 using antibody Mortalin/GRP75 (1:1000; Antibodies Incorporated, Davis, CA) and 3′-nitrotyrosine using antibody 1A6 (Millipore, Schwalbach, Gemany) followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected by enhanced chemiluminescence reaction (Millipore, Schwalbach, Germany) and luminescence intensities were analyzed using Chemidoc XRS documentation system (Biorad, München, Germany).

Enzymatic assays

Fluorogenic Substrate V (7-methoxycoumarin-4-yl)acetyl-RPPGFSAFK-2,4-dinitrophenyl; R&D, Wiesbaden, Germany) a substrate for IDE and neprilysin, was used to monitor the effect of the nitric oxide releaser Sin-1, DetaNONOate, and GSNO (all Cayman, Ann Arbor, MI, USA). For that 20 ng recombinant human IDE (R&D) in 100 mM Tris pH 7.5, 1 M NaCl or 50 ng recombinant human neprilysin (R&D) in 100 mM Tris pH 7, 150 mM NaCl were preincubated with different concentrations of Sin-1 for 30 min at 22°C or 60 min at 37°C for DetaNONOate and GSNO. Afterwards the substrate was added to a final concentration of 10 μM. The hydrolysis of substrate V was measured based on the increase of fluorescence (excitation 320 nm/emission 405 nm) using a SpectrafluorPlus (Tecan, Austria) plate reader for a time period of up to 60 min in 1 min intervals at 37°C. Determination of IDE activity in mouse brain was performed using the fluorogenic peptide substrate 2-aminobenzoyl-GGFLRKHGQ-ethylenediamine-2,4-dinitrophenyl (Bachem, Switzerland) (Song et al. 2003). For that, mice brains were homogenized in PBS containing 1 mM AEBSF, 10% sucrose, 1% Tx-100 and centrifuged at 100000×g for 30 min. 10 μg of supernatants were incubated with 25 μM phosphoramidon to inhibit neprilysin activity. The fluorogenic substrate was added to a final concentration of 20 μM and the resulting fluorescence signal was measured (exitation 320 nm/emmission 413 nm) every 3 min at 37°C using a infinite 200 plate reader (Tecan, Austria). Evaluation was performed within the linear range of the reaction after 60 min.

Aβ degradation assay

To determine the effect of Sin-1 on the degradation of Aβ, 160 ng Aβ1-42 (Sigma, Munich, Germany) were incubated with 60 ng recombinant IDE (R&D Systems, Wiesbaden, Germany) in the presence of indicated concentrations of Sin-1 in 100 mM Tris pH 7, 150 mM NaCl for 2 h at 37 C. Samples were separated by 4–12% Nupage and immunoblotted. Aβ was detected using antibody 6E10 and IDE was detected using antibody PC730 (Merck, Darmstadt, Germany) against IDE.

Results

Nitration of IDE in vitro

Nitration of proteins can either occur on tyrosine residues resulting in the formation of 3-nitrotyrosine or by nitrosylation of cysteines leading to the formation of nitrosothiol. Human IDE possesses 45 tyrosine and 13 cysteine residues that may be modified by nitric oxide or its reaction products (Fig. 1a,b). Since nitrosylation of IDE has already been described (Cordes et al. 2009; Ralat et al. 2009), we investigated if the *NO-donor Sin-1 is able to induce the nitration of tyrosine. Incubation of purified IDE and Sin-1 resulted in an concentration dependent increase of 3′-nitrotyrosines as detected by the specific antibody 1A6 (Fig. 1c)

Nitric oxide selectively impairs IDE activity in vitro and in vivo

The discovery of nitric oxide as a signaling molecule also fed the idea of nitric oxide as a modulator of enzyme activity. We therefore asked whether the activity of two Aβ degrading enzymes, insulin degrading enzyme (IDE) and neprilysin, is affected by nitric oxide. Using an fluorogenic substrate assay we determined the activity of both enzyme after incubation with increasing concentration of the *NO-releaser Sin-1. We observed an inhibitory effect of Sin-1 on IDE activity, which resulted in almost complete inhibition at 1 mM Sin-1 (Fig. 2a). Of note, the steady state concentration of peroxynitrite is only up to 3.6% of the added Sin-1 concentration (Martin-Romero et al. 2004). We could not detect a significant effect of *NO on the activity of neprilysin (Fig. 2b). irregardless whether peroxynitrite or Sin-1 was applied (Fig. 2c,d). Since nitration of tyrosines requires the formation of peroxynitrite as an intermediate reaction product, we used instead of Sin-1, which in addition to *NO also produces superoxide and thereby peroxynitrite, the *NO donors detaNONOAte and GSNO (Fig. 2e,f). In contrast to Sin-1 or peroxynitrite, we observed a slight increase in IDE activity, excluding that $^{\bullet}NO$ alone can mediate the inhibitory effects. To exclude that the inhibition observed with Sin-1 is due to the oxidation of IDE we tested the effect of H_2O_2. We observed a 20% decrease in IDE activity suggesting that oxidation itself has a negative effect on the activity of IDE (Fig. 2g).

Aβ degradation by IDE is inhibited by Sin-1

To determine the effect of Sin-1 on the degradation of Aβ, we preincubated IDE with Sin-1 for 30 min. followed by addition of Aβ1-42. Analysis after 2 h of incubation showed decreased degradation of Aβ with increasing concentration of Sin-1 (Fig. 3a) demonstrating that the observed effect in the fluorogenic substrate assay also applies to Aβ.

IDE activity in APP/PS1 NOS2 (−/−) mice

To measure the sensitivity of insulin degrading enzyme activity in mouse brain lysates we incubated different amounts of RIPA lysate with the synthetic substrate 2-aminobenzoyl-GGFLRKHGQ-ethylenediamine-2,4-dinitrophenyl in the presence of 25 μM phosphoramidon to prevent neprilysin

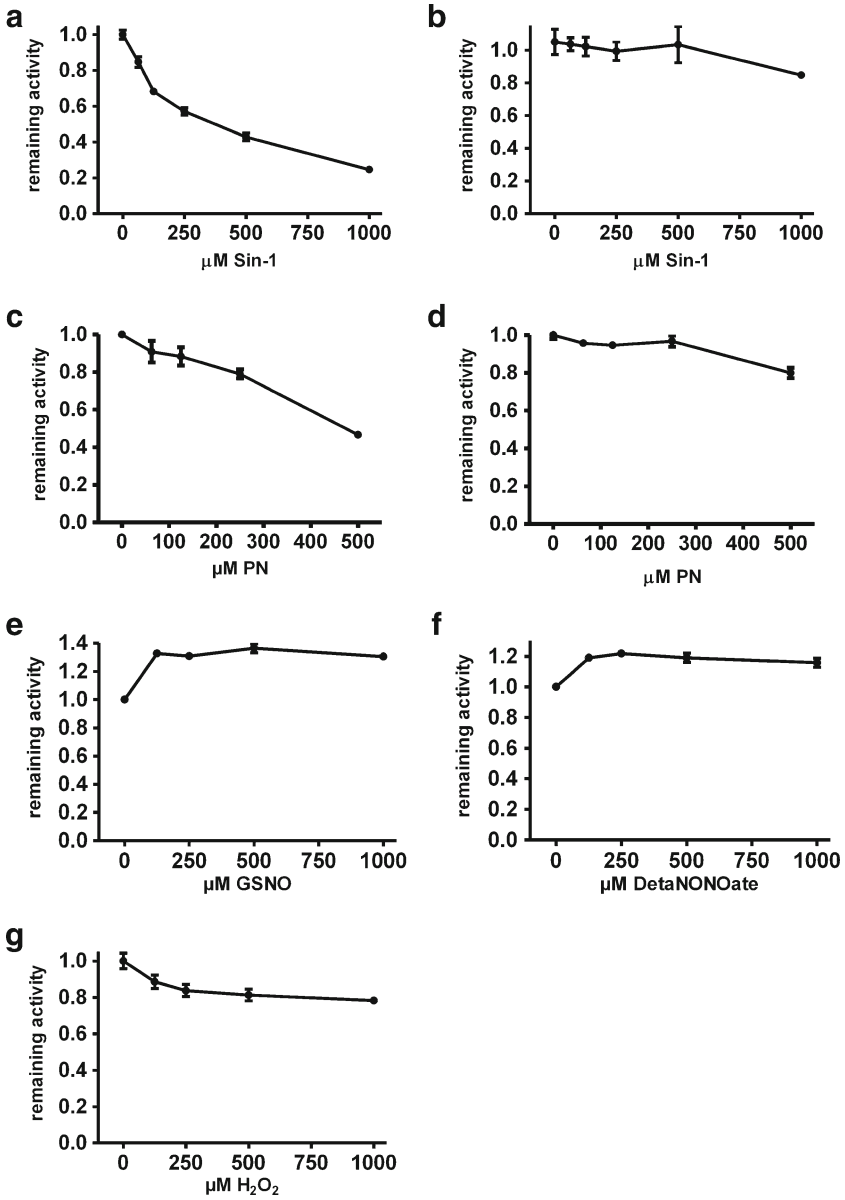

Fig. 2 Enzymatic activity of IDE, but not neprilysin, is negatively affected by nitrosative agents. **a** IDE was incubated with the increasing concentrations of the *NO-donor Sin-1 for 20 min before adding the fluorogenic substrate. Samples were measured for 30 min in 1 min intervals. The remaining IDE activity was plotted against the Sin-1 concentration **b** Same experiment as in A but with neprilysin **c** IDE was incubated with the increasing concentrations of peroxynitrite for 1 min before adding the fluorogenic substrate. Samples were measured for up to 30 min in 1 min intervals. The remaining IDE activity was plotted against the peroxynitrate concentration **d** Same experiment as in A but with neprilysin. Experiments were conducted in duplicates. **e** Incubation of IDE with increasing concentration of H_2O_2 for 1 h before adding the fluorogenic substrate . Experiments were conducted in triplicates. **f** Same as E but IDE was incubated with the *NO donor detaNONOate **g** Same as E but IDE was incubated with the *NO donor GSNO

activity on the cleavage of the substrate. We observed a protein-dependent increase in IDE activity over time (Fig. 4a). Using the metalloprotease inhibitor phenanthroline, we were able to completely inhibit the cleavage of the fluorogenic substrate (Fig. 4b). In addition, incubation of the RIPA lysate with 1 mM Sin-1 resulted in a strong reduction of IDE activity (Fig. 4c).

To verify this finding in vivo we measured the activity of IDE in lysates of 12 month old wild type, APP/PS1, NOS2 (−/−) and APP/PS1 NOS2 (−/−) animals using the IDE specific fluorogenic substrate (Song et al. 2003). To eliminate any residual activity of neprilysin, we performed the assay in the presence of the aspartyl protease inhibitor phosphoramidon. In accordance with our in vitro finding we observed a decrease in IDE activity by the APP/PS1-transgene that was rescued by the deletion of NOS2 (Fig. 4d). In addition, we did not observe a loss in IDE expression in these mice that could explain the loss of IDE activity simply by a decreased presence of the enzyme (Fig. 4e).

Discussion

Chronic neuroinflammation represents an important component of Alzheimer's disease (AD) pathology, characterized and executed by micro- and astroglial activation and the production of several pro- and anti-inflammatory mediators, including complement factors and cytokines which in concert are able to induce the synthesis and activity of inflammatory enzyme systems such cerebral NOS2 expression (Glass et al. 2010). As a consequence, increased levels of *NO and its reaction product peroxynitrite cause nitrosative stress and can

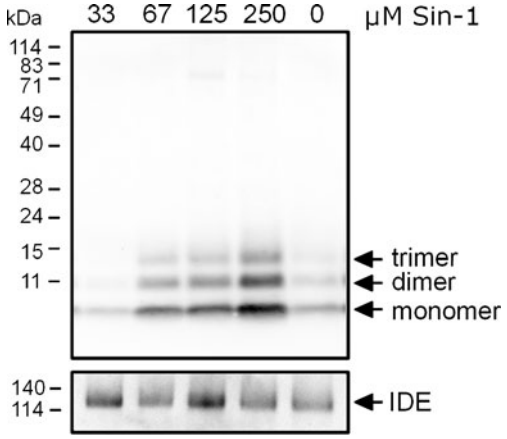

Fig. 3 Sin-1 decreases degradation of Aβ in vitro. Recombinant IDE was incubated with 1 mM Sin-1 for 30 min and degradation of Aβ1-42 was analyzed after 2 h by Western blot using antibody 6E10. IDE was detected using antibody PC720

lead to post-translational protein modifications of tyrosine and cysteine residues. These *NO-mediated protein modifications may in turn compromise protein function or lead to an entirely new effect on the respective target. In the case of metalloproteinases, which are a pivotal *NO target, up- and downregulation of enzyme activity has been described.

The presence of NOS2 expression in AD (Vodovotz et al. 1996; Heneka et al. 2001; Lee et al. 1999) and in related murine models (Rodrigo et al. 2004) correlates well with signatures of stress in AD relevant brain areas together suggesting that NOS2 derived *NO may also affect the function of enzymes which are involved in Aβ degradation including IDE and NEP (Castegna et al. 2003; Fernández-Vizarra et al. 2004; Lüth et al. 2001; Hensley et al. 1998).

Supporting this hypothesis, we demonstrate in the APP/PS1 mouse model of AD that the production and deposition of Aβ decreases IDE activity and that this can be rescued by deletion of NOS2. In vitro, the inhibitory effect of *NO could be mimicked by *NO-donors or peroxynitrite suggesting either cysteine nitrosylation or tyrosine nitration as a potential molecular mechanism. The impact of nitroslyation on IDE activity and oligomerization, in particular on cysteine 178, has been recently studied in detail (Cordes et al. 2009; Ralat et al. 2009). Here we show that besides this

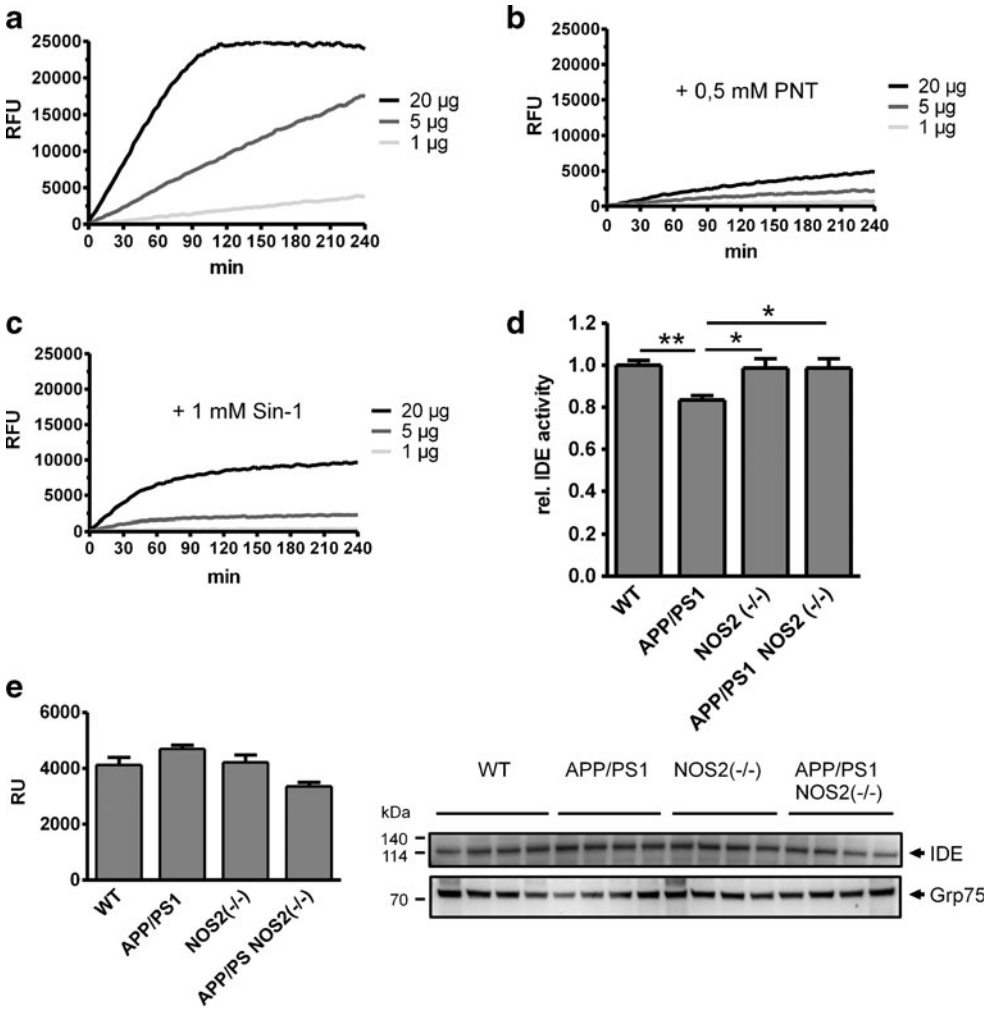

Fig. 4 IDE activity in the APP/PS1 mouse model. **a** Determination of IDE activity using a fluorogenic peptide substrate assay of different protein amounts from a brain lysate of a 12 month old wild type mouse in the presence of 25 µM phosporamidon to inhibit neprilysin activity **b** Same experiment as in A but in the presence of 0,5 mM phenanthroline **c** Same experiment as in A but in the presence of 1 mM Sin-1 **d** Brain lysate of wild type, APP/PS1, NOS2 (−/−), and APP/PS1 NOS2 (−/−) mice were incubated for 40 min with the fluorogenic peptide substrate in the presence of 25 µM phosporamidon (n=4 +/− SEM, one-way ANOVA, Tukey post-hoc test, *p<0.05, **p<0.01). **e** Samples from E were immunoblotted using an antibody for murine IDE and signals were quantified densiometrically. Immunoblotting Grp75 was used as a loading control

post-translational modification, IDE is also being nitrated in vitro, opening an additional pathway for enzymatic regulation (Fig. 5). In contrast to nitroslyation, nitration of a tyrosine residue is irreversible and therefore results in a persistent decrease of enzyme activity. Hence, nitration of IDE is not a physiological sensor for reactive nitrosative species as it has been suggested for IDE nitrosylation (Ralat et al. 2009), but may be considered as a pathological feature contributing to reduced degradation and clearance of Aβ and therefore aggravating and fueling disease progression.

More strikingly, Sin-1 and peroxynitrite specifically inhibited the activity of IDE whereas the other main protease implicated in Aβ degradation, neprilysin remained unaffected. This suggests a specific regulation of IDE activity by nitric oxide and not a random destruction of protein conformation. Since neprilysin possesses tyrosine and cysteine residues as well, one might speculate about the different sensitivity towards nitrosative agents. Besides different production pathways of the two enzymes that may prevent or promote *NO-modifications, one might also consider the absence of critical cysteines and tyrosines affecting catalysis within neprilysin.

We did not observe effects of the *NO donors DetaNONOate and GSNO on the activity of IDE as reported before (Cordes et al. 2009; Ralat et al. 2009). Even though we cannot explain these differences so far, we note that under the given experimental conditions (pH and temperature) the half life of GSNO (Nikitovic and Holmgren 1996) and DetaNONOate (Pervin et al. 2001) are in the range of 10–20 h whereas the decomposition of Sin-1 take place at a rate of 1% per min (Crow and Ischiropoulos 1996), suggesting that the effects mediated by Sin-1 should be more rapid especially within the time range of the experiment. In addition, 18 h incubation of IDE with either GSNO or DetaNONOate at 37°C resulted in complete loss of enzyme activity in all samples (data not shown).

The reduction of IDE activity by niric oxide in brains of APP/PS1 mice might not be the most important effect leading to deposition of Aβ (Kummer et al. 2011), however a persistent loss of 20% enzyme activity will over time accelerate disease pathology and contribute to the overall cerebral Aβ load. More importantly, we could show that loss of NOS2 restores IDE activity back to basal levels suggesting a mechanism by which neuroinflammation via NOS2 activation directly contribute to Aβ deposition.

In contrast to this study, Wilcock and colleagues reported about a beneficial role of NOS2 in an AD mouse model expressing a single APP transgene (Wilcock et al. 2008). These mice lacking a PS1 transgene, which may account for the opposite effects observed in this study, but so far there is no mechanistic explanation for this observation. On the opposite, one mechanism for the detrimental effect of NOS2 during

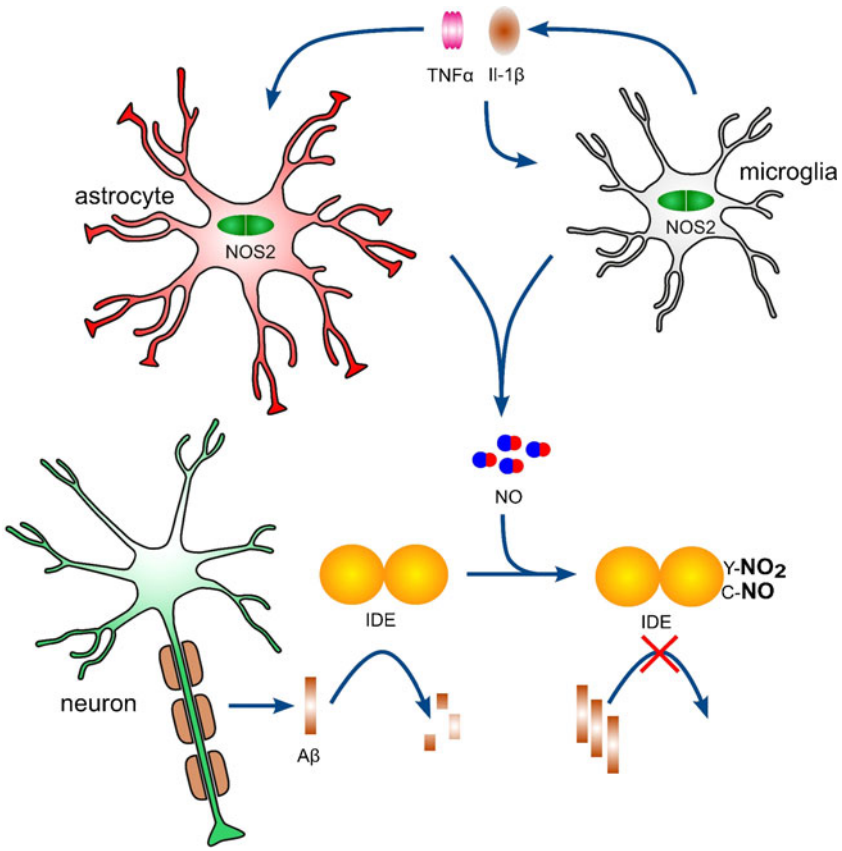

Fig. 5 Scheme of inflammation induced inactivation of IDE by nitric oxide. Chronic inflammation in AD results in the release of proinflammatory cytokines by microglia like TNFα and Il-1β which in turn induce expression of NOS2 in astrocytes and to lower extent in microglia. The resulting increase in *NO and its derivatives modifies critical cysteine and tyrosine residues of IDE thereby decreasing its activity. As a consequence Aβ is not efficiently degraded anymore and accumulates resulting in the formation of amyloid plaques

AD pathology was recently revealed as the nitration of the amyloid β peptide itself (Kummer et al. 2011).

Additional investigations are necessary to determine the nature and the positions of the *NO-mediated IDE modifications including the purification of modified IDE from AD mouse models and human AD tissue to fully characterize the changes that modulate IDE activity.

Together, chronic neuroinflammation causes persistent nitrosative stress and thereby *NO-mediated post-translational protein modifications, that can be diminished by NOS2 inhibition. As IDE is critically involved in Aβ degradation, NOS2 inhibition may open an additional treatment strategy for delaying the accumulation of Aβ by maintaining or restoring cerebral IDE activity.

Conflict of interest The authors declare no conflict of interest.

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