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Nitric oxide inhibits insulin-degrading enzyme activity and function through S-nitrosylation

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ABSTRACT

Insulin-degrading enzyme (IDE) is responsible for the degradation of a number of hormones and peptides, including insulin and amyloid β (Aβ). Genetic studies have linked IDE to both type 2 diabetes and Alzheimer's disease. Despite its potential importance in these diseases, relatively little is known about the factors that regulate the activity and function of IDE. Protein S-nitrosylation is now recognized as a redox-dependent, cGMP-independent signaling component that mediates a variety of actions of nitric oxide (NO). Here we describe a mechanism of inactivation of IDE by NO. NO donors decreased both insulin and Aβ degrading activities of IDE. Insulin-degrading activity appeared more sensitive to NO inhibition than Aβ degrading activity. IDE-mediated regulation of proteasome activity was affected similarly to insulin-degrading activity. We found IDE to be nitrosylated in the presence of NO donors compared to that of untreated enzyme and the control compound. S-nitrosylation of IDE enzyme did not affect the insulin degradation products produced by the enzyme, nor did NO affect insulin binding to IDE as determined by cross-linking studies. Kinetic analysis of NO inhibition of IDE confirmed that the inhibition was noncompetitive. These data suggest a possible reversible mechanism by which inhibition of IDE under conditions of nitrosative stress could contribute to pathological disease conditions such as Alzheimer's disease and type 2 diabetes.

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1. Introduction

Type 2 diabetes (T2DM) and Alzheimer's disease (AD) share a number of common features, including insulin resistance and the presence of subacute, chronic inflammation [1]. Chronic inflammation results in increased expression of the inducible isoform of nitric oxide synthase (iNOS), leading to heightened production of nitric oxide (NO) [2,3]. Increased iNOS is associated with a prolonged, exaggerated production of NO, and circulating levels of nitrite and nitrate (an indirect

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Abbreviations: Aβ, amyloid-beta; DSS, disuccinimydyl suberate; IDE, insulin-degrading enzyme; iNOS, inducible nitric oxide synthase; KFC, potassium ferricyanide; LLVY, succinyl-leu-leu-val-tyr-7-amido-4-methyl coumarin; LSTR, boc-leu-ser-thr-arg-7-amido-4-methyl coumarin; MMTS, methyl methanethiolsulfonate; NAP, *N*-acetylpenicillamine; NEM, *N*-ethylmalemide; NO, nitric oxide; PAPA-NONOate, (*Z*)-1-[*N*-3-aminopropyl]-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-dolate; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; sulfo-NONOate, disodium (*E*)-1-sulfonatodiazen-1-ium-1,2-diolate; TCA, trichloroacetic acid.

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measurement of NO production) are increased in patients with T2DM relative to healthy individuals [1,4].

The actions of NO can be classified as cGMP-dependent actions (such as those related to vascular dilation) or as reactive nitrogen species-mediated (independent of the actions of cGMP). The cGMP-independent effects are often mediated by nitrosative posttranslational modifications, such as S-nitrosylation, and are thought to play a role in pathological responses, including insulin resistance [5]. S-nitrosylation involves the covalent attachment of a NO moiety to protein sulfhydryls and is increased in the skeletal muscle of patients with T2DM [4]. Insulin signaling molecules, such as the insulin receptor, insulin receptor substrate-1 and Akt/PKB, are S-nitrosylated in the skeletal muscle of obese, diabetic mice [6]. Disruption of the iNOS gene has been shown to preserve insulin sensitivity and prevent hyperinsulinemia in mice with diet-induced insulin resistance [7,8]. iNOS gene disruption also results in decreased formation of amyloid β (Aβ) plaques in a mouse model of AD [9]. Therefore, a substantial body of research indicates the overproduction of NO plays a role in the development of both T2DM and AD.

Insulin-degrading enzyme (IDE, insulysin, insulinase, EC 3.4.24.56), a zinc metalloproteinase, is the primary enzymatic mechanism for intracellular insulin degradation, and appropriate IDE function is important for the preservation of insulin sensitivity [10]. The Goto-Kakizaki rat, an animal model of T2DM, contains mutations in the IDE gene and exhibits elevated blood glucose and insulin levels [11]. Studies conducted using the IDE knockout mouse have shown the mice to be glucose intolerant and hyperinsulinemic, supporting the concept that IDE is important in the maintenance of normal blood glucose and insulin levels [12]. Human genetic studies have linked genetic polymorphisms in the IDE gene to an increased risk for insulin resistance and T2DM [1,2]. In addition, IDE is involved in the degradation of Aβ in the brain, and IDE hypofunction has been shown to contribute to the accumulation of Aβ plaques in AD [3,7].

Despite the potential importance of IDE, relatively little has been published on its metabolic control. Ubiquitin, a peptide similar in size (8.5 kDa) to insulin, has been shown to inhibit insulin degradation by IDE, most likely in a competitive manner [13,14]. The small molecule ATP has also been shown to have effects on IDE. Camberos et al. showed ATP inhibited insulin degradation [15]. However, it has also been reported that ATP can increase degradation of small peptides by IDE, while having little effect on insulin metabolism [16,17]. Whatever the effect on insulin degradation, ATP does induce a conformational change in IDE [16]. We have shown that IDE activity is inhibited non-competitively by select free fatty acids and their coenzyme A thioesters [18]. IDE has been shown to be sensitive to hydrogen peroxide, which could act by affecting cysteine residues [19]. It has long been known that IDE is sensitive to sulfhydryl modifying reagents [20–22]. There are 13 cysteines in the predominately expressed form of IDE, including one at the active site. However, it was not known which of these was responsible for the sulfhydryl sensitivity. A recent paper has identified three cysteines, C178, C812 and C819 as the amino acid responsible for most of the sensitivity [23]. These cysteines may be susceptible to nitrosylation. Since, as noted above, increased NO production has been shown to have negative effects on insulin sensitivity, we chose to examine the effect of NO on IDE activity and function. In this study, we show that NO inhibits IDE-mediated degradation of two IDE substrates, insulin and Aβ, and that IDE-mediated regulation of the proteasome is affected. The inhibition is likely a result of S-nitrosylation. This demonstrates another potential pathway by which increased iNOS may contribute to the pathology of diabetes and Alzheimer's disease, and represents a potential therapeutic target for the treatment of these diseases.

2. Materials and methods

2.1. Chemicals

NO donors S-nitroso-N-acetylpenicillamine (SNAP), sodium nitroprusside (SNP), and (Z)-1-[N-3-aminopropyl]-N-(n-propyl)amino]diazen-1-ium-1,2-dolate (PAPA-NONOate), control compound, disodium (E)-1-sulfonatodiazen-1-ium-1,2-diolate (sulfo-NONOate), fluorogenic proteasome substrates succinyl-leu-leu-val-tyr-7-amido-4-methyl coumarin (LLVY) and boc-leu-ser-thr-arg-7-amido-4-methyl coumarin (LSTR)), methyl methanethiolsulfonate (MMTS), and disuccinimydyl suberate (DSS) were purchased from Sigma (St. Louis, MO). Potassium ferricyanide (KFC) and N-acetylpenicillamine (NAP) were purchased from Fisher Scientific (Pittsburg, PA). ^{125}I-human recombinant insulin and ^{125}I-β-amyloid were purchased from Phoenix Pharmaceuticals (Burlingame, CA). EZ-link biotin-HPDP (N-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)-propionamide and neutravidin-agarose beads were purchased from Pierce Biotechnology (Rockford, IL). Polyclonal anti-IDE antibody was purchased from Chemicon (Billerica, MA). All other chemicals were of at least reagent grade.

2.2. Enzyme preparation

Male Sprague-Dawley rats were maintained and used in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, under a protocol approved by the Subcommittee of Animal Studies and the Research and Development Committee of the Omaha Veterans Affairs Medical Center. Insulin-degrading enzyme/proteasome was prepared from rat muscle or liver by ultracentrifugation and ammonium sulfate precipitation, similar to that described previously [20].

2.3. Transfection, expression, and purification of recombinant 6-Histidine labeled insulin-degrading enzyme

Human embryonic kidney (HEK) 293T cells were grown and maintained in DMEM (high glucose)+10% FBS, 5% CO_2. Cells were transfected with pCMV 6-Histidine FLAG-labeled human IDE using a calcium phosphate-mediated method modified from Sambrook and Russell [24]. Cells were lysed in NETN buffer (20 mM Tris–Cl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, aprotinin, leupeptin, pepstatin, sodium orthovanadate, sodium fluoride, and PMSF), and clarified by centrifugation. Lysate was diluted in nickel binding buffer (20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, pH 7.4), applied to a nickel

affinity column (His GraviTrap, GE Healthcare), washed, and bound proteins eluted with buffer containing 500 mM imidazole. The fractions with the highest insulin-degrading activity were dialyzed against 50 mM HEPES-buffered saline + 1 mM $CaCl_2$ overnight and stored in 50% glycerol/HEPES-buffered saline + 1 mM $CaCl_2$ at −20 °C until assayed.

2.4. Insulin degradation

The degradation of ^{125}I-insulin and ^{125}I-β-amyloid was measured by the trichloroacetic acid (TCA) solubility method. SNAP was initially solubilized in DMSO and diluted with 100 mM Tris–Cl, pH 7.4, with a final concentration of DMSO of <0.2% DMSO in the assay (v/v). This concentration of DMSO had no effect on enzyme activity (data not shown). SNP and KFC were solubilized in the assay buffer (100 mM Tris–Cl). An aliquot of rat enzyme was preincubated with increasing concentrations of NO donor (SNP, SNAP, and PAPA-NONOate), control compound (KFC, SNAP(ox), NAP, or sulfo-NONOate), or ascorbate [1×10^{-3} M] for 1 h at 37 °C, after which ^{125}I-insulin or ^{125}I-β-amyloid was added and incubated an additional 15 min. The reaction was stopped by addition of 0.5% (final) BSA and 10% (final) TCA. The samples were centrifuged and the supernatant and pellet counted using a γ-counter, with data expressed as percent degradation relative to untreated enzyme. Substrate incubated without enzyme was subtracted as background solubility.

2.5. Proteasome activity

The degradation of fluorogenic peptide substrates LLVY and LSTR was used as measures of the chymotrypsin-like and trypsin-like activities of the proteasome, respectively. Aliquots of rat muscle enzyme preparation were incubated with varying concentrations of NO donors, control compounds, ascorbate [1×10^{-3} M] in a 96-well black plate at 37 °C for 1 h. LLVY or LSTR was added (13 μM final concentration), and fluorescence measurements (ex/em: 355/460 nm) were taken at 30, 60, 90, and 120 min. Data are expressed as the rate of change in fluorescence units and normalized with respect to the activity of untreated enzyme.

2.6. Biotinylation of NO-donor treated enzyme

Protein modifications were visualized using a biotinylation method adapted from Jaffrey et al. [25,26]. Enzyme from rat muscle or liver was treated with NO donors for 2 h at 37 °C to maximize adduction. After treatment, samples were acetone precipitated to remove any remaining NO donor and resuspendend in HEN buffer (250 mM HEPES (pH 7.7) 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 20 mM MMTS, 0.1 mM neocuproine, 1 mM PMSF, 80 uM carmustine). One volume of MMTS buffer (25 mM HEPES (pH 7.7) 5% SDS, 0.1 mM EDTA, 20 mM MMTS, 10 uM neocuproine) was added and the sample incubated at 50 °C for 30 min with frequent vortexing. Samples were acetone precipitated to remove any MMTS and resuspended in HPDP buffer (25 mM HEPES (pH 7.7) 1% SDS, 0.1 mM EDTA, 10 mM neocuproine, 5 mM sodium ascorbate, 0.2 mM EZ-link HPDP-biotin). Samples were incubated in HPDP buffer for 1 h at room temperature, and then acetone precipitated to remove the biotinylating agent. Samples were resuspended in HENS buffer (25 mM HEPES (pH 7.7) 1% SDS, 0.1 mM EDTA, 10 mM neocuproine) with two volumes of neutralization buffer (20 mM HEPES (pH 7.7), 100 mM NaCl, 1 mM EDTA 0.5% Triton X-100), and Neutravidin-agarose beads in order to select for biotinylated (i.e. 'nitrosylated') proteins. Samples were incubated with the Neutravidin-agarose beads overnight at 4 °C and the beads washed five times with neutralization buffer. Proteins bound to the beads were eluted by heating with Laemmli SDS-PAGE loading buffer (100 mM Tris, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, pH 6.8, β-mercaptoethanol, 10% (w/v)). Proteins were resolved on SDS-polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes, and probed for IDE using a polyclonal IDE antibody.

2.7. HPLC analysis of insulin degradation products

Rat liver IDE was incubated with the NO donors for 1 h at 37 °C in 100 mM Tris–Cl buffer. Treated and untreated enzymes were then incubated with approximately 5×10^5 cpm ^{125}I-insulin for 5 min at 37 °C and applied directly to a DuPont Zorbax C-8 (4.6 mm × 25 cm; 6 μm particle diameter) HPLC column, similar to previously described [27]. Fractions (0.5 mL) of the eluate were collected and analyzed using a γ-counter to determine the elution profile of radioactivity.

2.8. Covalent cross-linking of ^{125}I-insulin to insulin-degrading enzyme

Partially purified enzyme prepared from rat skeletal muscle or liver was incubated with HEPES-buffered saline (140 mM NaCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 50 mM HEPES, pH 7.4) and SNAP for 1 h at 37 °C. After the treatment, SNAP was removed by centrifugation in a Centricon Ultracel 50. Enzyme was incubated with 2 ng of ^{125}I-insulin (approximately 1×10^6 cpm) for 30 min at 4 °C. After the incubation, 1 mM DSS (in DMSO) was added and incubated an additional 40 min on ice, and the reaction stopped by the addition of 1 M Tris–Cl (pH 7.4). Samples were mixed with 2× Laemmli SDS-PAGE loading buffer, heated for 4 min at 100 °C, and electrophoresed on a 7.5% SDS-polyacrylamide gel. The gels were stained with Coomassie blue, dried, and scanned using an Amersham Molecular Dynamics STORM 820 Phosphorimager.

2.9. Measurement of NO release by donors

NO release by the donors used in the experiments was measured by quantitation of total nitrate/nitrite (final products of NO decomposition) using 2,3-diaminonaphthalene (DAN) (Nitrate/Nitrite Fluorometric Assay Kit; Cayman Chemical). Samples with enzyme were incubated with and without NO donors for 1 h at 37 °C and then filtered through a 10 kDa MWCO filter. Remaining nitrate in the solution was converted to nitrite, total nitrite was determined by reaction with DAN, and fluorescence was measured at 365/410–460 nm (excitation/emission).

2.10. Data analysis

Results are expressed as mean ± S.E.M. Data were analyzed using the Prism statistical software (v.4, GraphPad Software,

San Diego). Significance was determined by one-way ANOVA with Dunnett's multiple comparison tests.

3. Results

Fig. 1 shows the effect of NO donors and control compounds on the insulin-degrading activity of IDE. Fig. 1a shows the inhibition of insulin degradation with the addition of the NO donor, SNP. SNP inhibits insulin degradation by 50% at the highest concentration tested (1×10^{-3} M). KFC, a compound structurally similar to SNP, but not an NO donor, had relatively little effect. The treatment of the enzyme with both SNP and ascorbate [1×10^{-3} M] had an increased inhibitory effect on insulin degradation. Ascorbate alone [1×10^{-3} M] had no effect on insulin degradation (data not shown). Fig. 1b shows the concentration-dependent inhibitory effect of the NO donor, SNAP. However, SNAP combined with ascorbate [1×10^{-3} M] did not have an effect on insulin-degrading activity that was substantially different from that of SNAP alone, and the control compound, SNAP(ox) had no effect. Fig. 1c shows that the NO donor, PAPA-NONOate, significantly inhibited insulin degradation up to 70%, while sulfo-NONOate had no effect.

Since NO likely adducts IDE, we examined the kinetics of the reaction to classify the type of inhibition. SNP release of NO is dependent on the presence of light and/or reducing agents, so we chose SNAP as the NO donor because it likely releases NO spontaneously under our assay conditions. Analysis of the nature of IDE inhibition by NO donors by Michaelis–Menten plot confirmed that the inhibition by NO donors is noncompetitive. The data in Fig. 2 show the classic pattern of noncompetitive inhibition, with the K_m being similar for all the concentrations of SNAP tested (mean 7.6 ± 1.5 nM), while the velocity of the reaction decreased with increasing SNAP concentrations.

IDE degrades Aβ as well as insulin, and we examined the effect of the NO donors, SNAP, SNP, and PAPA-NONOate on IDE-mediated degradation of Aβ. First, we examined the specificity of our partially purified enzyme preparation toward Aβ degradation. Competitive inhibition of the substrate with insulin resulted in nearly complete inhibition of Aβ degradation (data not shown). We also studied the effect of IDE inhibitors that are known to inhibit insulin degradation. Fig. 3a shows that 1,10-phenanthroline, a zinc chelator, inhibited Aβ degradation significantly at 1×10^{-4} M, and this inhibition reached nearly 90% at 1×10^{-3} M. Irreversible sulfhydryl modification by *N*-ethylmalemide (NEM) also significantly inhibited Aβ. The serine protease inhibitor, PMSF, had no effect on Aβ degradation. We conclude that IDE was the major enzyme degrading Aβ in our preparation. We then examined the effect of NO on IDE-mediated degradation of its substrate, Aβ. Fig. 3b shows that SNAP significantly inhibited Aβ degradation by 25% at a concentration of 1×10^{-3} M. A control compound for SNAP, *N*-acetylpenicillamine (NAP), did not inhibit Aβ degradation. SNP and PAPA-NONOate also inhibited Aβ degradation significantly, but their respective control compounds (KFC and Sulfo-NONOate) did not exhibit an effect.

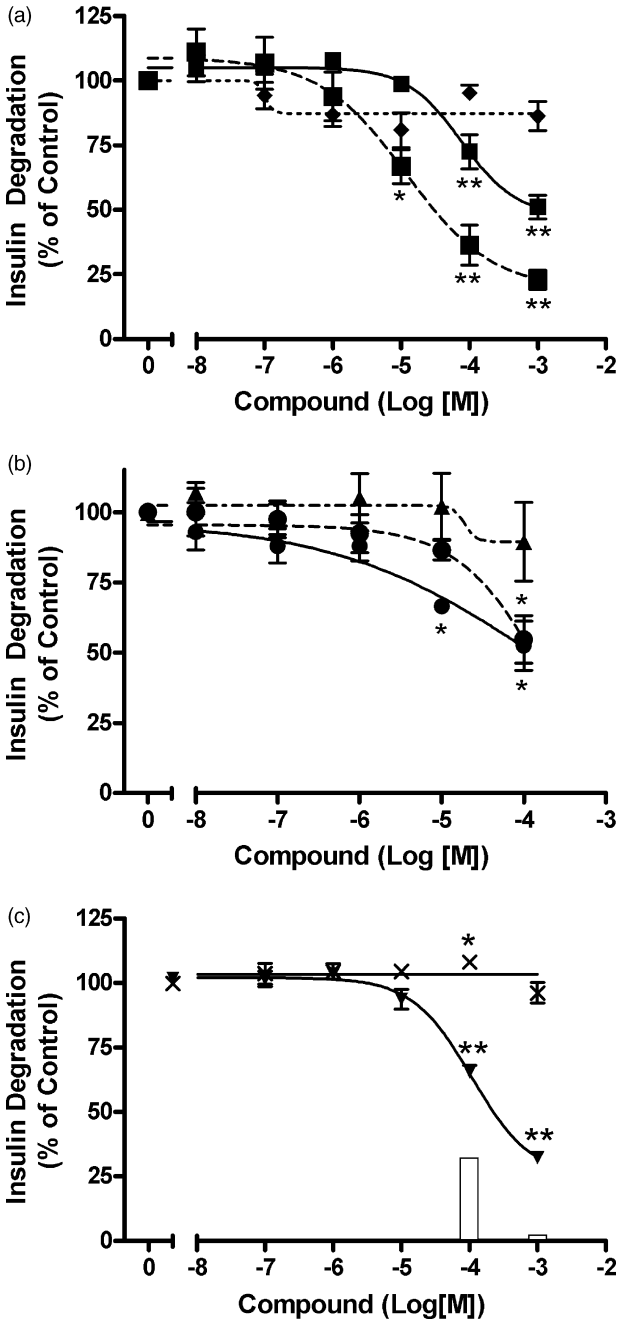

Fig. 1 – ^{125}I-Insulin degradation by IDE enzyme in the presence of NO donor compounds. Partially purified rat IDE enzyme was treated with increasing concentrations of NO donor compounds with and without ascorbate [1×10^{-3} M] and assessed for insulin-degrading activity. (a) Curve fit of insulin degradation with SNP (■), SNP + ascorbate (□), and control compound, KFC (◆). (b) Curve fit of enzyme treated with SNAP (●), SNAP + ascorbate (○), and the control compound, SNAP(ox) (▲). (c) Insulin degradation curve fit of PAPA-NONOate (▼) and sulfo-NONOate (×). NEM (grey bars) is shown for reference. All graphs represent the mean ± S.E.M. of four independent experiments. *$P < 0.05$, **$P < 0.01$.

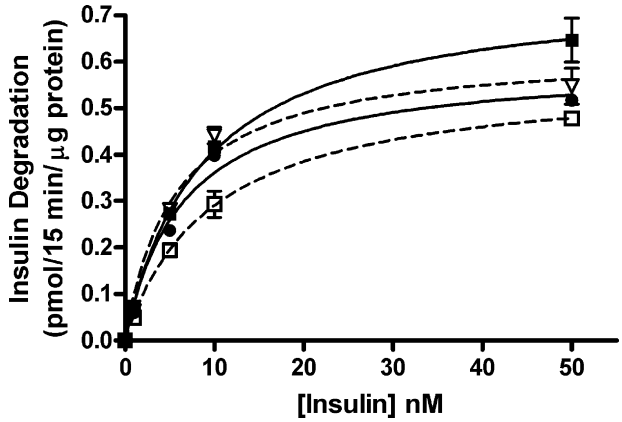

Fig. 2 – Kinetic analysis of the inhibition of insulin degradation by IDE enzyme in the presence of the NO donor, SNAP. Partially purified IDE enzyme was incubated with ^{125}I-labeled insulin without NO donor treatment (■), and treated with SNAP ([3×10^{-6} M] (▽), [3×10^{-5} M] (●), [3×10^{-4} M] (□)) and increasing concentrations of unlabeled insulin. Enzyme activity was measured by the generation of TCA-soluble ^{125}I-insulin fragments and expressed as picomoles of insulin degraded over 15 min per microgram of enzyme. IDE was noncompetitively inhibited by SNAP.

Our laboratory has shown that insulin will decrease the chymotrypsin- and trypsin-like activities of the proteasome when it is isolated in a complex with IDE. We tested whether NO donors would have an effect on this inhibition. Fig. 4a shows the inhibitory effect of insulin on chymotrypsin-like activity, which is similar to that previously published [28]. In the in vitro rat enzyme preparation containing the isolated IDE and proteasome complex, the addition of SNAP inhibited the chymotrypsin- and trypsin-like (Fig. 4a and b). The control, oxidized NO donor, SNAP(ox), had no effect. SNP inhibition of both proteasomal activities was only significant at 1×10^{-3} M, but the addition of ascorbate increased the inhibitory capacity of SNP toward the chymotrypsin-like activity (Fig. 4c and d). Unlike SNAP(ox), the control compound KFC inhibited the chymotrypsin- and trypsin-like activities 50% at a concentration of 1×10^{-3}M.

Table 1 – Nitric oxide release by NO donors.

Treatment	Donor compound (μM)	Nitrate/nitrite (μM)
SNAP	100	1.08 ± 0.12
PAPA-NONOate	1000	13.9 ± 2.10
SNP	1000	0.45 ± 0.15

Measurement of NO release by SNAP, PAPA-NONOate and SNP using fluorometric nitrate/nitrite quantitation. Background levels of total nitrate/nitrite were subtracted (mean ± S.E.M. of three independent experiments).

The concentration of NO release in our assay conditions was measured indirectly by fluorometric determination of total nitrate and nitrite in the solution (Table 1). For NO donors SNAP and PAPA-NONOate, the level of NO released was approximately 100 times less than the concentration of applied donor compound, with measurements in the μM range. A similar trend was seen with the NO donor, SNP, with 1000 μM of compound releasing less than 0.5 μM of NO. Thus, similar levels of enzyme inhibition are seen with similar NO concentrations, regardless of the donor used.

The ability of NO donors to induce S-nitrosylation of IDE was investigated using the biotin switch technique. Protein S-nitrosylation is a well-recognized reversible protein modification that occurs during oxidative stress [5]. In Fig. 5, we show that IDE is indeed nitrosylated in the presence of SNAP. When ascorbate or HPDP-biotin were eliminated from the assay, S-nitrosylated cysteine could not be switched for biotin, and the immunoblot signal detected was very minimal, with intensity similar to that of IDE in the absence of NO donor treatment (Fig. 5, lanes 2, 3, and 4).

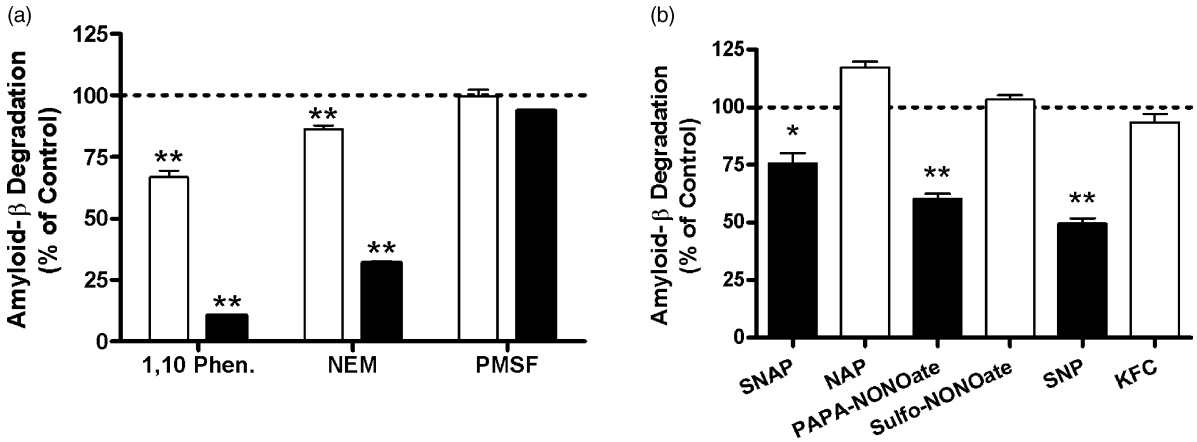

Fig. 3 – ^{125}I-Amyloid-β degradation by IDE in the presence of IDE inhibitors and with the addition of NO Donors. (a) Aβ degradation by IDE is significantly inhibited in the presence of known IDE inhibitors at [1×10^{-4}] (open bars) and [1×10^{-3} M] (filled bars) concentrations of 1,10 phenanthroline (1,10 Phen.) and NEM, but PMSF did not have an inhibitory effect. (b) Partially purified rat IDE enzyme was treated with NO donors (filled bars) or control compounds (open bars), at [1×10^{-3} M] (mean ± S.E.M. of at least three independent experiments). *P < 0.05, **P < 0.01.

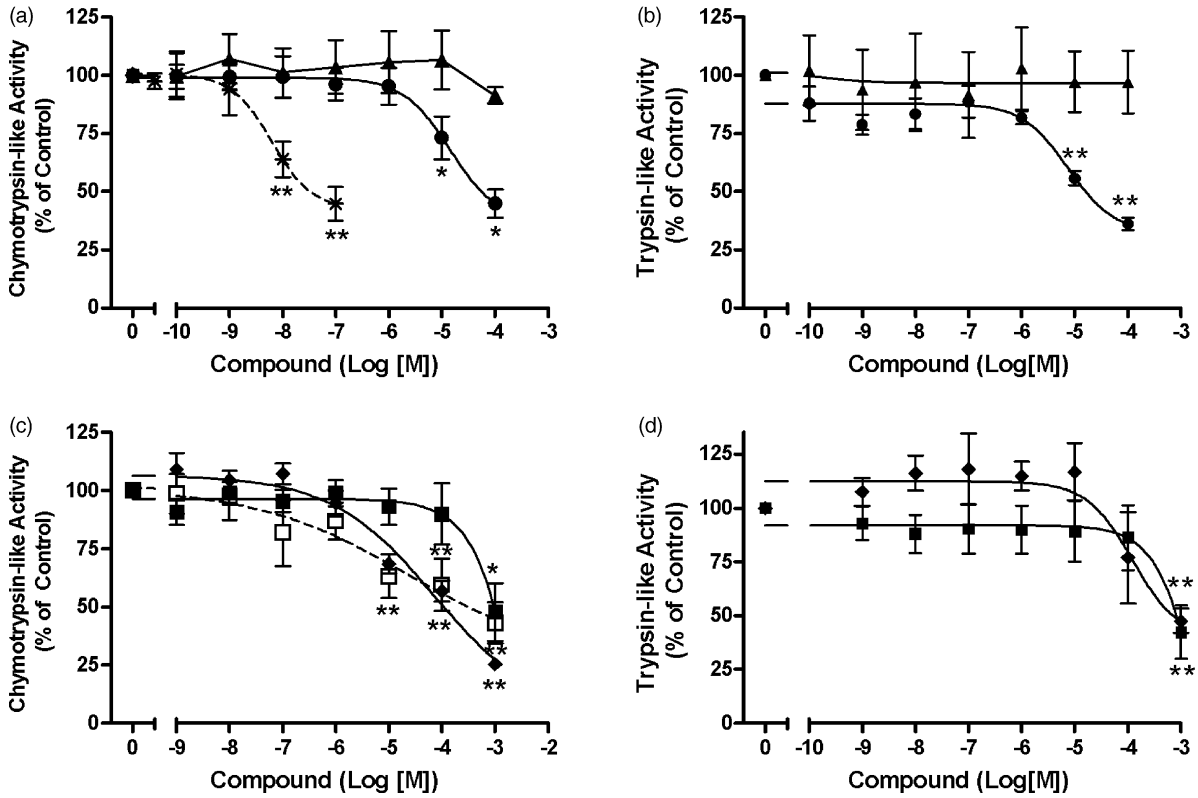

Fig. 4 – Proteasome trypsin- and chymotrypsin-like activity with NO donor treatment. Partially purified rat IDE enzyme with proteasome was treated with increasing concentrations of NO donor and assessed for proteasome trypsin- and chymotrypsin-like activity using fluorogenic proteasome substrates (mean ± S.E.M. of ≥3 independent experiments). (a) Insulin (✳) inhibited proteasome chymotrypsin-like activity by 50% at [1×10^{-7} M]. SNAP (●) inhibited activity significantly at [1×10^{-5} M] and [1×10^{-4} M] of compound, but the control, SNAP(ox) (▲) (curve fit did not converge), did not. (b) Similar results were seen with SNAP treatment on the proteasome trypsin-like activity. (c) SNP (■) significantly inhibited chymotrypsin-like activity at [1×10^{-3} M]. Treatment with both SNP + ascorbate [1×10^{-3} M] (□) and KFC (◆) significantly inhibited activity at [1×10^{-5} M]. (d) Trypsin-like activity was significantly inhibited by both SNP and KFC at [1×10^{-3} M]. *P < 0.05, **P < 0.01.

We also tested, by HPLC analysis, the qualitative effect of NO donors on the generation of insulin degradation products. Fig. 6a shows a representative elution profile of ^{125}I-insulin and fragments after incubation with partially purified recombinant human IDE. Fig. 6b shows the elution profile of insulin after incubation with the enzyme in the presence of SNAP and SNAP(ox). All product patterns were similar, with no major products missing, or additional peaks arising, with the addition of the NO donors. The only effect seen was a decrease in the production of small insulin fragments, which indicates that the enzyme was inhibited by SNAP, but not by the oxidized form. The same elution profiles were observed regardless of whether rat enzyme or the recombinant human enzyme was tested (partially purified rat enzyme data not shown).

Fig. 5 – Protein nitrosylation of IDE with the treatment of NO donor, SNAP. Partially purified rat IDE enzyme was incubated and processed using the biotin switch method (1) +SNAP [1×10^{-4} M], (2) untreated enzyme, (3) +SNAP [1×10^{-4} M] in the absence of the biotinylating agent, (4) +SNAP [1×10^{-4} M] but without ascorbate [1×10^{-3} M] treatment prior to the biotinylation step. Biotinylated proteins were removed from the samples by incubation with Neutravidin-agarose beads, and eluted proteins eluted were resolved by SDS-PAGE and immunoblotted using an anti-IDE antibody. IDE was nitrosylated in the presence of SNAP [1×10^{-4} M]. Blot is representative of three independent experiments.

To further examine the nature of the inhibition of NO on IDE, we cross-linked insulin to IDE in the presence and absence of NO donors. Fig. 7 shows a sample autoradiograph of the 110-kDa band (the molecular weight of IDE) and quantification by densitometry. SNAP at the highest concentration tested [1×10^{-4} M] did not suppress insulin cross-linking, nor did the SNAP(ox) control. We tested the effect of SNP and KFC on IDE binding of insulin, and found that these compounds also did not increase or decrease insulin binding to the enzyme (data not shown). This indicates that NO inhibits insulin degradation without disrupting insulin binding to IDE, which is suggestive of a noncompetitive type of inhibition.

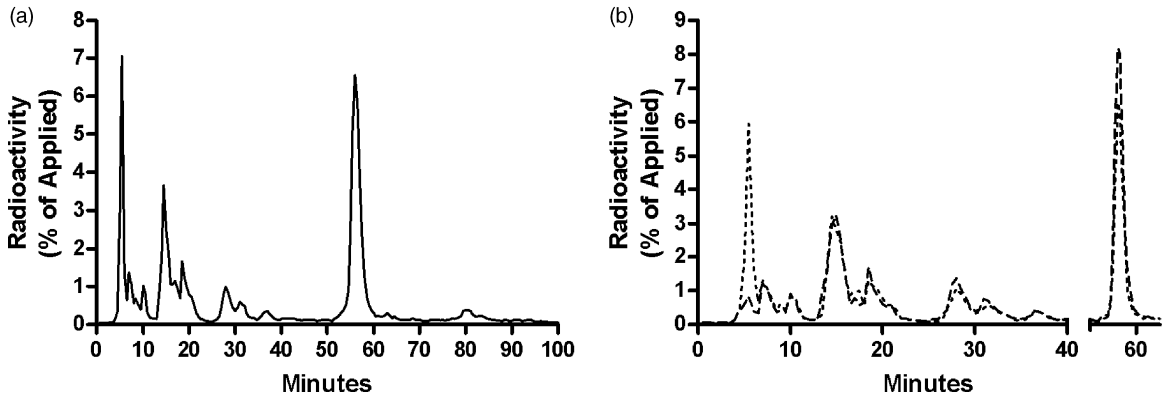

Fig. 6 – HPLC analysis of insulin degradation products. Insulin degradation products were measured in partially purified recombinant human IDE enzyme. (a) Untreated enzyme alone (b) Enzyme treated with 1×10^{-4} M SNAP (▪▪▪) and enzyme treated with 1×10^{-4} M SNAP(ox) (- - -). No qualitative differences were seen in the insulin degradation products produced by any of the treatments.

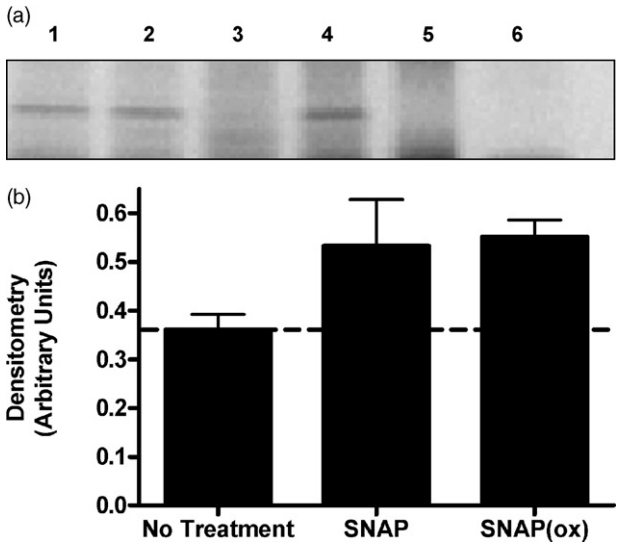

Fig. 7 – Covalent crosslink of partially purified IDE enzyme to ^{125}I-insulin in the presence of the NO donor, SNAP. (a) Representative autoradiograph of the effect of SNAP on ^{125}I-insulin binding to IDE. Lane (1) enzyme + SNAP [1×10^{-4} M], (2) enzyme + SNAP(ox) [1×10^{-4} M], (3) enzyme + non-radiolabeled insulin, (4) enzyme + radiolabeled insulin, (5) no enzyme and (6) enzyme without DSS cross-linking agent. (b) Densitometric analysis of three independent experiments. SNAP or control donor treatment did not significantly affect insulin binding to IDE.

4. Discussion

Our data show that NO can inhibit the actions of IDE toward two IDE substrates, insulin and Aβ. This inhibition has implications in conditions such as diabetes, insulin resistance, and Alzheimer's disease. Increased inflammation and the elevated production of NO have been shown to affect insulin clearance and processing, which contributes to overall insulin resistance [29,30]. Enhanced expression of iNOS as a result of inflammation is a factor in insulin resistance, and depletion of iNOS has been shown to enhance insulin sensitivity [7,8,29]. A large body of literature has shown that inflammation and insulin resistance may be underlying causes in the development of Alzheimer's disease as well [31–33]. Our studies show that NO donors noncompetitively inhibit IDE degradation of two substrates, insulin and Aβ, but that this inhibition does not change the insulin degradation products produced, nor does it affect IDE binding of insulin. Because IDE is responsible for initiating insulin degradation in endosomes, impairment of IDE degradation of insulin could contribute to decreased clearance of insulin and thereby contribute to the hyperinsulinemia that is observed under both insulin-resistant and diabetic conditions [34,35]. Likewise, inhibition of IDE by NO in the brain could contribute to an accumulation of Aβ, leading to the development of insoluble fibrous plaques.

Three different NO donors were used in this study, SNP, SNAP, and PAPA-NONOate and they each have different mechanisms of NO donation and chemistry. SNP has been used clinically as a NO donor and vasodilator for the treatment of severe hypertension and cardiovascular disease, and releases NO after reaction with reducing agents, such as ascorbate [26]. However, the ferrocyanide moiety present in the compound has the potential to generate cyanide and Fe(II), which can both act as oxidizing agents [36]. In order to differentiate the NO-attributed effects from the potential oxidative effects of SNP, the control compound, KFC, was used. SNP was shown to inhibit insulin degradation by IDE, and the addition of ascorbate to the reaction increased the inhibitory effect presumably because of the enhanced NO release from the compound. Additionally, ascorbate has been shown to convert S-nitrosylated cysteine residues to reduced cysteine residues, and this could potentially result in reactivation of the enzyme. However, the reactivating effect does not appear to be predominant in our experiments as the presence of ascorbate in the reaction increased the inhibitory effect of SNP. The control compound, KFC, did not inhibit insulin degradation, indicating that the inhibitory effects observed with SNP were attributable to the NO being released and not to potential

enzymatic oxidation of IDE. This same result was not observed in the analysis of IDE-mediated regulation of the proteasome activity. The proteasome is affected by oxidation, and that was apparent with the addition of KFC, which is an oxidizing agent [37,38]. However, the slopes of the inhibition curves for SNP and KFC were different. This suggests that the inhibition of proteasome activity by SNP and KFC were potentially operating through different mechanisms.

SNAP can directly release NO into solution by spontaneous cleavage of its NO moiety or can nitrosylate protein thiols by transfer of NO^+ [39,40]. The rate of NO release from this nitrosothiol compound is thought to be unaffected by ascorbate. We found this to be the case in our experiments, as SNAP inhibited insulin degradation by 50% with no effect on the potency of the compound with the addition of ascorbate. This agrees with the reported mechanism of action of SNAP [39]. In assessing the effect of SNAP on IDE-mediated regulation of proteasome activity, SNAP was found to inhibit proteasome trypsin- and chymotrypsin-like activities with an inhibition curve similar to SNAP inhibition of insulin degradation, which is indicative of NO inhibiting through a similar mechanism. The oxidized form of SNAP did not have an effect on the proteasome activity. These findings indicate that SNAP inhibition of the proteasome was functioning through the inhibition of IDE by NO in our enzyme preparation. We believe that the binding of NO to IDE, like the binding of insulin to IDE, results in a dissociation of IDE from the proteasome, resulting in a decrease in proteasome peptidolytic activity [41]. When NO levels are increased, such as in inflammation, protein degradation could be decreased, and, more importantly, the ability of insulin to alter protein degradation via the proteasome would be abrogated. NO inhibition of IDE may contribute to insulin resistance by disrupting insulin-mediated control of protein metabolism.

PAPA-NONOate spontaneously donates NO in a pH-dependent, first-order process, and has a half-life of 15 min under the assay conditions used in this study [42]. The control compound, sulfo-NONOate, is chemically similar to PAPA-NONOate, but it does not release NO; it dissociates in solution to form only sulfate and nitrous oxide (N_2O) [43]. PAPA-NONOate showed a significant inhibitory effect on both insulin and Aβ degradation, but the control compound sulfo-NONOate did not affect the degradation of either substrate, indicating that the inhibition seen was likely due to NO. The amount of NO released by PAPA-NONOate in the assay was similar to that which was released by SNAP, or about 100 fold less NO released than donor compound. The inhibitory effect of PAPA-NONOate on Aβ degradation was 25% greater at 1×10^{-3} M concentration of compound than the effect of SNAP at the same concentration. This could be attributed to the known half-life of this compound, which is on the order of minutes, compared to the half-life of SNAP, which is a few hours [39,44].

SNP, SNAP, and PAPA-NONOate were not equally efficacious in inhibiting insulin and Aβ degradation. This was due to differences in NO release from the compounds. SNP required a tenfold higher amount of donor compound to achieve the same level of inhibition in insulin degradation that was seen with 10^{-4} M SNAP. Even though the amount of SNP was greater, the actual amount of NO released from the compound was at the micromolar level, similar to that released by SNAP at 10^{-4} M (Table 1). The NO donors also appeared to inhibit insulin degradation at lower concentrations relative to Aβ degradation. This effect is likely due to differences in substrate size and substrate requirements for binding to the enzyme.

The inhibition of IDE by NO did not qualitatively change the insulin degradation products produced, but it did decrease the amount of the smallest products of insulin degradation. These results suggest that NO donors do not alter IDE preference for cleavage sites on insulin, but that NO does inhibit overall degradation. When we examined the ability of IDE to bind insulin in the presence of NO donors, we found that the NO donors did not inhibit insulin binding. This suggests that the NO donors are inhibiting IDE noncompetitively, and this was confirmed by kinetic analysis of the inhibition. This type of inhibition is consistent with what is known about the structure of IDE and the location of its cysteine residues.

IDE has long been shown to be sensitive to sulfhydryl reagents, but which of its cysteine residues was responsible had not been known [20–22]. Neant-Fery et al. have recently shown that cysteines C178, C812, and C819 are the residues largely conferring thiol sensitivity [23]. Thus, these are the residues most likely mediating the inhibitor effect of NO. Recently the three-dimensional structure of IDE has been elucidated revealing the spatial location of these cysteines, and demonstrating IDE to be a unique enzyme [45]. The N-terminal and C-terminal halves of the molecule form two bowl-like structures, linked with a short intermediate peptide chain. The two halves fit together like a clamshell, forming an interior pocket that contains the active site and substrate binding regions. The closed form, with the substrate buried within, appear to be the most catalytically active. Cysteine C178 is in the N-terminal half, and is proximate to the active site (aa 108–112). Nitrosylation of this residue could interfere with catalysis, similar to N-ethylmaleimide. The authors of that paper suggest adducting C178 could either alter the position of the active site residues, or interfere with the interaction of the C- and N-terminal regions (or ''closing'' the enzyme), since C178 is close to residue T825 when IDE is in the closed conformation. Similarly, C812 and C819 are at the interface of the N- and C-terminal halves. Thus, nitrosylation of these residues may prevent IDE from achieving the fully closed conformation. Neant-Fery et al. suggested this would block substrate binding, but our cross-link experiments indicate, at least for insulin, binding still occurs. We speculate that insulin can still associate with the enzyme in its open conformation, but the nitrosylated cysteines at C812 and C819 prevent IDE from bringing the active site into the proper position to catalyze hydrolysis. We cannot rule out that other cysteines are nitrosylated and contribute to the inactivation of IDE, but these residues seem the most likely candidates.

We have found that IDE is nitrosylated in the presence of NO donors, and this inhibition of IDE may represent a physiologic control mechanism for the protein. More than 100 proteins have been shown to be S-nitrosylated in vitro and in intact cells, including proteins involved in insulin signal transduction [46]. The concentrations of the artificial NO donors used in this study may seem superphysiologic; however, the amount of NO released from these compounds is actually at least 100-fold less than the concentration of compound used. This brings the amount of NO in these assays

into the micromolar and sub-micromolar range, well within the range of NO known to be released by iNOS intracellularly [47,48]. Therefore, S-nitrosylation of IDE is a potentially physiologically relevant mechanism of metabolic control for the actions of IDE. These findings have important implications for diabetes and insulin resistance, as well as Alzheimer's disease. Nitrosylative regulation of proteins is a complex and not fully understood process at this time, but what is known is that the redox state of the cell impacts the activity and function of a large number of proteins. How this type of protein regulation affects the cell is critical to providing a better understanding of the pathogenesis of disease that are affected by inflammation and nitrosative stress. Prevention of the effects of inflammation on cellular processes could lead to the development of novel therapeutic strategies to better treat or even prevent the onset of debilitating diseases such as diabetes and Alzheimer's.

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