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Role of S-nitrosoglutathione and Hydrogen Peroxide on Cathepsin D Chemical Modification and Function

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by deposits of β-amyloid peptide (Aβ) and insoluble hyperphosphorylated Tau protein. Cathepsin D (CTSD) is the main lysosomal protease and it has been suggested as hyperphosphorylated Tau-degradating enzyme. Oxidants such as hydrogen peroxide (H_2O_2) and nitric oxide (NO) and its derivatives, S-nitrosoglutathione (GSNO) are greatly generated during altered states, such as AD. Oxidants can react with proteins causing post-translational modifications, such as S-nitrosylation and S-glutathionylation. Therefore, we hypothesize that oxidants generated during several stages of AD, cause post-translational modifications on CTSD, leading to its inactivation. In this study we utilized mouse brain homogenates treated with H_2O_2 or GSNO and oxidative modifications on CTSD were analyzed by Western blotting. Overall, our results demonstrate that CTSD is a target for oxidation, but surprisingly, neither oxidant robustly changed CTSD activity. As a conclusion this study suggests that, different from other proteases such as caspases, CTSD can sustain functionality in elevated oxidant conditions. The understanding of mechanisms by which oxidants can impair proteins involved in AD can provide knowledge for potential antioxidant therapies against neurodegenerative diseases, since these diseases have been associated with increases in oxidant production and damage.

INTRODUCTION

Alzheimer's Disease (AD) represents the most common form of dementia, affecting over thirty million people (Querfurth and LaFerla 2010). In addition to accumulation of extracellular deposits of β-amyloid peptide, AD is also associated with intracellular accumulation of abnormally hyper-phosphorylated Tau protein, which forms insoluble deposits of pathological paired helical filaments, which subsequently contribute to the formation of insoluble neurofribrillary tangles, a hallmark of this disease (Binder, Guillozet-Bongaarts et al. 2005; Querfurth and LaFerla 2010; Mocanu, Nissen et al. 2008). This abnormal hyper-phosphorylated Tau protein no longer can exert its biological function--which is its involvement in axonal transport and neural polarity-- leading to neuronal cell death (Mocanu, Nissen, et al. 2008). How and why neurofibrillary tangles develop is not completely understood. Accumulation of hyper-phosphorylated Tau can be a consequence of an imbalance of its phosphorylation/de-phosphorylation (Chung 2009), as well as degradation of its insoluble form. Lysosomes exemplify one of the main protein degrading organelles, and lysosomal dysfunction has been shown to be implicated in a variety of neurological disorders including AD (Wang, Martinez-Vicente et al. 2009), Parkinson's disease, and Huntington's disease (Hamano, Gendron et al. 2008), suggesting its potential involvement in Tau and AD-associated protein breakdown. Proteases inside the lysosomes generally degrade dysfunctional proteins and any harmful agents or waste products. Tau has

been shown to be one of the substrates of the lysosomal proteases, including Cathepsin D (CTSD). Recently, CTSD was suggested suggest to play a role in Alzheimer's disease as a Tau-degradating enzyme (Kenessey, Nacharaju et al. 1997; Hamano, Gendron et al. 2008). Urbanelli and co-workers demonstrated that CTSD is decreased at both the transcriptional and translational levels in Alzheimer Disease fibroblasts (Urbanelli, Emiliani et al. 2008), therefore this enzyme can be a potential target for novel molecular therapies.

Oxidative and nitrosoactive stress is very well recognized as playing an important role in pathological states such as neurodegenerative diseases, including AD (Dalle-Donne, Milzani et al. 2008). Previous publications indicate that NO is associated with neurofibrillary tangles formation, since it induces Tau hyper-phosphorylation by activating glycogen synthase kinase-3beta (GSK-3beta), (Zhang, Xu et al. 2005). Furthermore, NO has also been implicated in neurodegeneration by compromising the activity of neuroprotective proteins and promoting apoptosis (Chung and David 2010). Despite the abundance of publications in the field, the mechanism by which NO and other oxidants regulate biological processes remain unclear. Several authors have demonstrated that NO and H_2O_2 regulate diverse biological processes by oxidative modification in proteins through a process called S-nitrosylation and S-glutathionylation (Mohr, Hallak et al. 1999; Martinez-Ruiz and Lamas 2007). These posttranslational modifications represent a covalent binding of NO or glutathione to thiol groups of cysteine residues (Mallis and Thomas 2000). Thiols are critical for redox signaling, and are crucial for multiple processes including signaling, transcription, and metabolism. As such, maintaining a careful balance between reactive oxygen species and antioxidants is imperative within any biological system, and a dysfunctional balance can lead to various pathological conditions by generating oxidative stress. These oxidative modifications (S-nitrosylation and S-glutathionylation) can be repaired by the enzymatic systems involving S-nitrosoglutathione reductase (GSNOR) (Liu, Hausladen et al. 2001) and glutaredoxin 1 (GRX1) (Lillig, Berndt et al. 2008), respectively.

Since cathepsins and other proteases (Mannick, Schonhoff et al. 2001; Godat, Herve-Grvepinet et al. 2008) have been reported to be affected by oxidants, one potential mechanism involving oxidants and hyper-phosphorylated Tau accumulation is the inactivation of CTSD (protease), by S-nitrosylation, S-glutathionylation and perhaps other types of oxidation. Therefore, we hypothesize that oxidants cause oxidative modifications on target proteins, such as CTSD, leading to their inactivation and impairment of de-phosphorylation and degradation of hyper-phosphorylated Tau proteins.

The goal of this study is to understand the molecular mechanisms by which oxidants can chemically modify protein structure and potentially affect their function, and the role this plays in pathophysiological states. In this study, we utilized brain from wild type (WT) and glutaredoxin 1 deficient mice, (GRX1-/-) as a source of Cathepsin D. Brain from GRX1-/- mice were used as a control for lacking the repair enzyme of S-glutathionylated proteins, glutaredoxin 1. We submitted the brain homogenates to treatment with the oxidants, GSNO or H_2O_2 , and found that both oxidants can cause oxidative modification in CTSD. However, these modifications only slightly affected its activity, especially in WT brain homogenates. The conclusion of this study is that CTSD is not as susceptible to activity alteration as other proteases, such as caspases (Mannick, Schonhoff et al. 2001).

MATERIALS AND METHODS

Materials

Glutathione reduced form (GSH) and DL-dithiothreitol (DTT) and N-ethylmaleimide (NEM) were from Sigma, N-(3-maleimidylpropionyl)biocytin (MPB) was from invitrogen, human recombinant cathepsin D was from Molecular Innovations, cathepsin D activity assay Kit and, cathepsin D polyclonal antibody were from BioVision, glutathione monoclonal antibody was from Virogen, Strepavidin-POD was from Roche, and IgG peroxidase-linked secondary mouse or rabbit antibody were from GE Healthcare.

All other reagents were of analytical grade from commercial sources.

Brain homogenates preparation

The brains utilized in this study were from wild type (WT) and Glutaredoxin 1 knockout (GRX1-/-) BalbC mice, kindly provided by Dr. Yvonne Janssen-Heininger, from the Department of Pathology, University of Vermont. The Institutional Animal Care and Use Committee of the University of Vermont granted approval for all procedures. The brains were frozen in liquid nitrogen. The frozen brains were initially pulverized in liquid nitrogen using a mortar and pestle followed by homogenization in 0.7% mL Lysis buffer (25 mM HEPES, 4 mM EDTA, 0.4 mM neocuproine, 1 mM DTPA, 0.5% Triton, 200 mM KCl, 10% Glycerol and 10 µL/mL freshly added protease inhibitor cocktail). Alternatively, the pulverized brains were homogenized in cathepsin activity assay lysis buffer, according to the manufacturer's instructions. Following homogenization, the samples were snap frozen in liquid nitrogen and then thawed on ice. The samples were then centrifuged at 14000 RPM at 4°C for 10 min. and the supernatant was utilized for the experiments described below. Protein concentration was determined using Bradford Bio-Rad protein Assay.

Immunoprecipitation

In general the proteins from the cell or tissue homogenates were precipitated in an appropriate lysis buffer containing primary antibody (1 µg) incubated overnight at 4 °C in a rotating platform. Twenty five µL of protein G agarose conjugate were added to each sample and incubated for 1h at 4 °C in a rotating platform. Proteins that bind to the antibody were centrifuged for 30 sec. at 14000 RPM, the supernatant was then carefully removed and the immunoprecipitate complexes were washed twice in 200 µL lysis buffer and once in PBS before being resuspended in 25 µL of PBS and 8 µL of 4x Laemmli sample buffer were added to the samples. All samples were then heated for 5 min. at 90-95 °C. Immunoprecipited proteins were further analyzed by SDS-PAGE and immunoblotting to determine their post-translational modifications and to determine the presence and quantity of proteins.

Treatment of brain homogenates

90 µL (approximately 200 g protein) of brain homogenates from wild type or GRX-/- mice were incubated with GSNO or H_2O_2 at the respective final concentrations (0.1, 0.5 or 1 mM) for 30 min, at room temperature, in the dark. GSNO (100 mM) was prepared according to procedures published previously (Mallis and Thomas 2000). Since DTT is a potent reducing agent, it was used as a control (50 mM final concentration) in the presence of 1 mM H_2O_2. After the treatment the brain homogenates were prepared for further analysis described next.

Detection of Cathepsin S-nitrosylation in brain homogenates.

After treatment with respective oxidants the proteins from homogenates were

precipitated by addition of 1:2 volume of cold acetone as previously published (Ckless, Lampert et al. 2008). The principle of the "biotin switch" technique to detect S-nitrosylated proteins is to replace S-nitroso moieties from proteins by more stable biotin

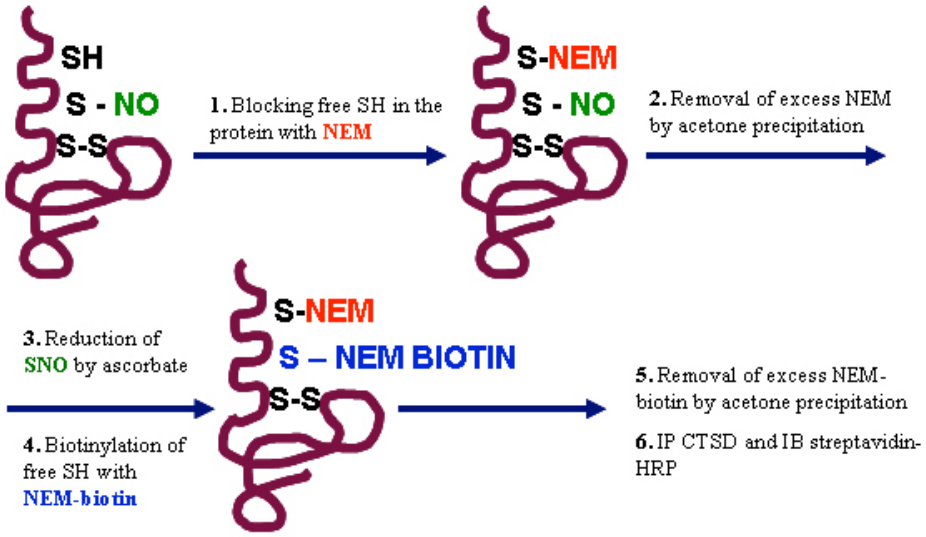

Figure 1: Schematic diagram for biotin chemical derivatization assay ("biotin switch assay") for S-nitrosylation. The "biotin switch" technique is based on replacement of S-nitroso moieties (SNO) from proteins by more stable biotin moieties (chemical derivatization). The presence or absence of ascorbate indicates that the technique is specific for detection S-NO in proteins, since the reducing of this moieties and consequent labeling is depending on ascorbate (reagent control).

moieties (chemical derivatization) and then proceed with immunopurification and detection by traditional Western blot techniques. The more S-nitrosylated proteins, the more intense the bands become. In order to verify whether the chemical derivatization is specific and complete, the cell lysates underwent the procedure in the presence or absence of ascorbate (reagent control) and in presence or absence of biotin label (labeling control). No or weak bands in these control experimental conditions indicates that the technique is functioning (Figure 1).

The samples were incubated for 20 min. at -20°C. After this procedure, the samples were centrifuged at 14000 RPM, at 4°C, for 10 min. The supernatant was discarded and the pellet was rinsed with 200 mL of cold acetone. Samples were resuspended in HEPS-SDS buffer and 20 mM of NEM was added to block free thiol groups (SH) in the proteins. The samples were incubated in the dark for 30 min and the excess NEM was removed by protein precipitation with acetone, as described previously. S-nitrosylated moieties in the proteins were reduced and biotinylated by the simultaneous addition of 10 mM sodium ascorbate and 0.1 mM of the sulfhydryl-specific biotinylating agent, MPB, for 30 min. at RT. Negative control experiments were performed in which sodium ascorbate was omitted, preventing the reduction of S-nitrothiols and consequent labeling with biotin. The extra biotin label was removed by another acetone precipitation and the proteins were resuspended in HEPES-SDS buffer for Cathepsin immunuprecipitation. Cathepsin D was immunoprecipitated from the samples by adding 1 µg of specific antibody. After immunoprecipitation the proteins were separated in SDS–PAGE and Western blotting analysis of biotinylated Cathepsin D was performed as described in section 2.8.

Assessment of total protein nitration and S-glutathionylation.

To assess total protein S-glutathionylation and nitration the proteins were separated in SDS–PAGE and Western blotting analysis was performed. After the treatment described previously the protein content was determined in the homogenates and at least 30 µg of protein was separated in

SDS–PAGE in non-reducing conditions followed by Western blot analysis using glutathione primary antibodies. As a negative control, a reducing agent, DTT, was utilized to decompose S-glutathionylated proteins. For total nitration the same procedure describe for determination of s-glutathionylation of proteins was performed, but with reducing SDS-PAGE conditions and a primary antibody against nitrotyrosine residues instead.

Assessment of Cathepsin D activity.

To assess Cathepsin D activity the brains were homogenated in lysis buffer provided by CTSD activity kit manufacturer. The homogenates were treated with 100, 500 and 1000 μM of GSNO or H_2O_2 for 30 min in the dark. Fifty micro liters of each sample were then incubated with the reaction mix as described in the manufacturer's instructions and Cathepsin D activity was determined in fluorimeter equipped with a 328nm excitation filter and 460nm emission filter. Cathepsin D activity was expressed by relative fluorescence units (RFU) per microgram protein in each sample.

Statistical analysis

All experiments were performed three times and the data are expressed as mean ± SEM. ANOVA followed by Dunnett's Multiple Comparison Test was performed for the CTSD enzymatic assay, using GraphPad Prism software.

RESULTS

S-nitrosylation of CTSD in brain homogenates

Homogenates were treated with different levels of oxidants followed by NEM-biotin labeling and CTSD immunoprecipitation to specific detect CTSD S-nitrosylation. WT brain homogenates contained a basal level of CTSD S-nitrosylation, which initially decreased with treatment of lower concentrations of GSNO 0.1 and 0.5 mM, and then increased at the highest concentration, 1 mM, especially at the smaller molecular weight (14kDa) (Figure 2A, top and middle panel). Consistent with expectations, decreases in labeling was observed when compared to GSNO 1 mM, reflecting specificity of the biotin labeling (Figure 2A, middle panel) We also observed that at low concentration (0.1 mM) GSNO slightly increases S-nitrosylation of CTSD in GRX1 -/- brain homogenates, and at 0.5 mM the S-nitrosylation is completely abolished (Figure 2A, bottom panel) We also demonstrated that S-nitrosylation of CTSD in brain homogenates from WT mice increased with higher concentrations of H_2O_2, As expected, DTT, a reducing agent, significantly diminished the S-nitrolylation of CTSD (Figure 2B, top and middle panel). However, brain homogenates from GRX1 -/- H_2O_2 treatment completely abolished S-nitrosylation of CTSD (Figure 2B, bottom panel).

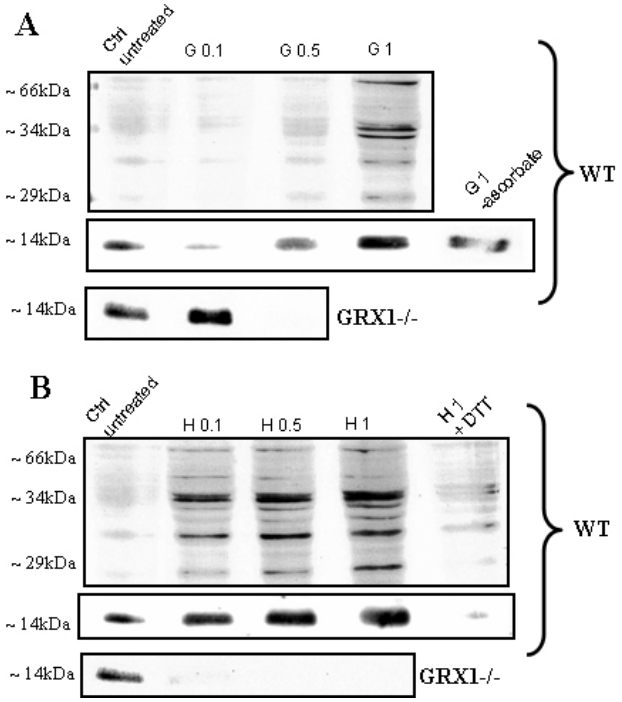

Figure 2: S-nitrosylation in brain homogenates. Brain homogenate were treated with different concentrations (mM) of GSNO **(A)** or H_2O_2 **(B)** for 30 min, at RT in the dark. The free thiols were blocked by adding 20 mM of NEM, for 30 min, at RT. After this period, protein were precipitated to

remove excess of NEM. One mM of sodium ascorbate was added in the presence 0.1 mM of NEM-biotin for simultaneous reducing and labeling of the SNO. Excess of NEM-biotin was removed by another acetone protein precipitation. CTSD was immunoprecipitated from the from the precipitated and proteins were separated in SDS–PAGE and of biotinylated CTSD was detected by Western blotting analysis using streptavidin-HRP. The experimental condition "–ascorbate" is the reagent control (negative control), since the SNO labeling is depending on ascorbate. The experimental condition "+ DTT" is also negative control, since it decomposes SNO and it is no longer can be labeled. This data is representative of at least three trials.

Total protein nitration and S-glutathionylation in brain homogenates

We also investigated the total protein S-glutathionylation and nitration in brain homogenates treated with different concentrations of GSNO and H_2O_2. As expected, total protein S-glutathionylation increased with treatment of GSNO at concentrations as low as 0.1 mM in brain homogenates from both WT and GRX1 -/- mice (Figure 3A, top and bottom panels). However, H_2O_2 decreased S-glutathionylation in brain homogenates from WT mice (Figure 3A, top panel), but contrastingly, increased in GRX1 -/- at the lowest concentration, and gradually decreased at 0.5 and 1 mM (Figure 3A, bottom panel).

Total protein nitration was also investigated to verify the redox changes caused by NO derivatives, such as peroxynitrite (ONOO-). As expected, higher GSNO concentrations (0.5 and 1 mM) caused minimal increases in total protein nitration in both WT and GRX 1-/- brain homogenates in comparison to the controls (Figure 3B, top and bottom panels).

Cathepsin D activity

Next we analyzed whether CTSD activity is affected by treatment with oxidants in WT, GRX 1-/- brain homogenates, and in purified recombinant Cathepsin D (rCTSD). Although statistically significant, the activity of this protease was surprisingly only mildly (20-14%, WT) affected, especially in the presence of H_2O_2 (Figure 4). And the changes in activity or level of S-nitrosylation of CTSD are not due to the differences in CTSD expression in either WT or GRX1 -/- brain homogenates (Figure 5).

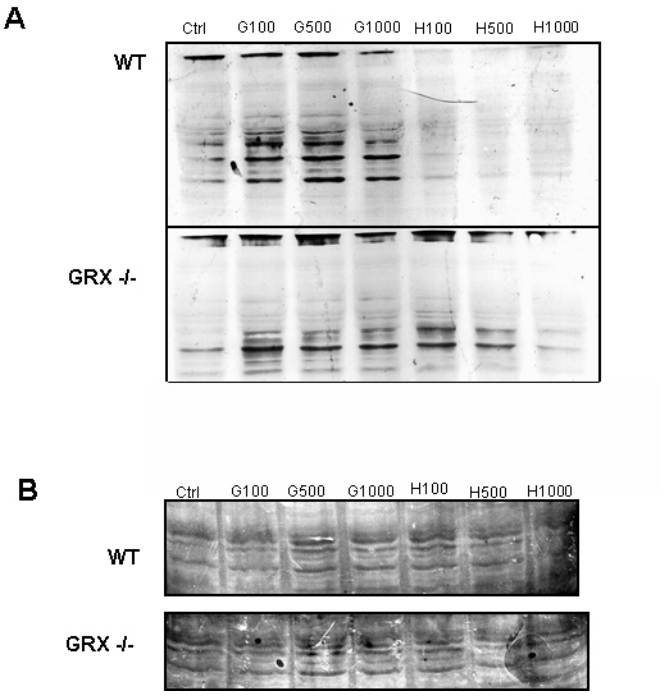

Figure 3: Total protein nitration and S-glutathionylation of brain homogenates. Homogenates were treated with different concentrations (0.1, 0.5 or 1 mM) GSNO (G) or H_2O_2 (H) followed by SDS-PAGE and total protein S-glutathionylation (A) or nitration (B) were detected using specific anti glutathione and nitrotyrosine antibodies. This data is representative of at least three trials.

DISCUSSION

It is widely known that oxidants such as S-nitrosoglutathione (GSNO) and hydrogen peroxide (H_2O_2) are capable of altering protein structure via oxidative mechanisms

such as S-nitrosylation and S-glutathionylation (Martinez-Ruiz and Lamas 2007). Such modifications are important to healthy cellular activity, but can harm cellular functioning when uncontrolled, causing physiological decline in various pathologies (Chung and David; Dalle-Donne, Milzani et al. 2008). The protease cathepsin D (CTSD) is very abundant in the brain (Kenessey, Nacharaju et al. 1997) and is susceptible to oxidation, potentially leading to its decreased activity in neurodegenerative diseases such as Alzheimer's disease (Urbanelli, Emiliani et al. 2008). Therefore, we hypothesized that

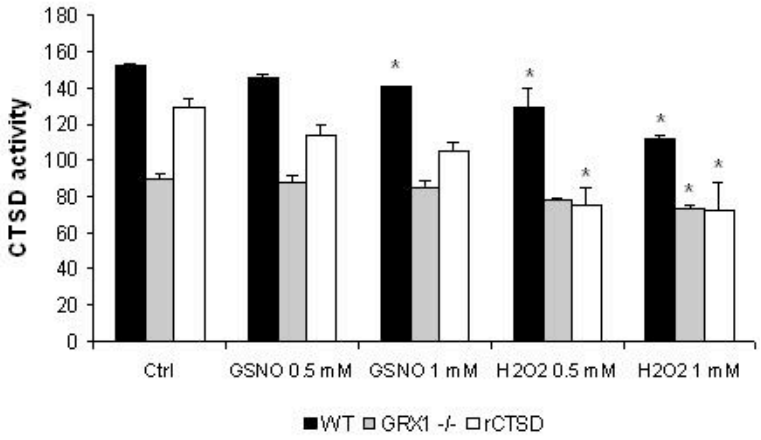

Figure 4: CTSD activity assay. WT and GRX1-/- brain homogenates were treated with different concentrations of oxidizing agents GSNO or H_2O_2 for 30 min. at RT. After 30 min. at RT. Recombinant human Cathepsin D (rCTSD) was submitted to the same treatment and utilized as control. After 30 min. CTSD activity was determinated in both human rCTSD and brain homogenates, by fluorescence assay according to the manufacturer's instruction and expressed as relative fluorescent units (RFU). The results are expressed as means ± SEM. (*p<0.001)

oxidant-induced S-nitrosylation and S-glutathionylation will impair CTSD function, leading to accumulation of dysfunctional proteins associated with Alzheimer's disease, such as tau protein. Thus, the aim of the present study was to detect S-glutathionylation and S-nitrosylation induced by GSNO and H_2O_2 and to evaluate its effects on CTSD activity.

In this study, we utilized CTSD from brain homogenates of wild type (WT) or Glutaredoxin 1 (GRX1-/-) knockout mice. Glutaredoxins, known as thioltransferases, belong to the thioredoxin superfamily.

The mammalian isoform, GRX1, specifically catalyzes the reduction of protein-glutathione mixed disulfides, also known as protein S-glutathionylation (PSSG) (Lillig, Berndt et al. 2008). In GRX1-/-, this enzyme is absent, leading to a deficiency in the PSSG repair mechanism.

The first set of experiments demonstrated that GSNO induces a denitrosylation of CTSD S-(14kDa) in WT brain homogenates. A possible reason for this finding could be that at low concentrations GSNO can undergoes chemical decomposition in the presence of other components of the homogenates, such as transition metals that then generate products that decompose basal level S-nitrosylation. Another possibility could be that at lower doses GSNO is S-glutahionylating rather S-nitrosylating.

However, at the highest concentration (1mM), GSNO might undergo a transnitrosation reaction generating NO^+ which is quickly transferred to the available thiol groups (Mallis and Thomas 2000) in the CTSD, reflecting in higher S-nitrosylation. A condition lacking ascorbate served as a control for S-nitrosylated CTSD, since ascorbate is needed for replacement of S-nitrosylated groups by NEM-biotin and thus

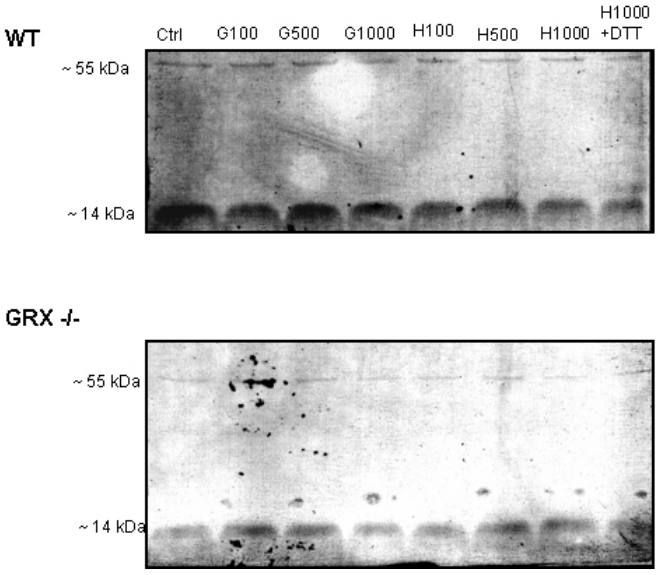

Figure 5: Assessment of total CTSD in brain homogenates from WT and GRX1-/- mice. Brain homogenates were treated with different concentration of oxidizing agents GSNO (G) or H_2O_2 (H) followed by SDS-PAGE and CTSD was detected utilizing specific primary antibody and HRP-conjugated secondary antibody. This data is representative of at least three trials.

successful labeling. These experimental conditions reveal that levels of CTSD S-nitrosylation in brain homogenates from WT mice also increased with higher concentrations of H_2O_2. More interestingly, a direct comparison of GSNO versus H_2O_2 treatment of brain homogenates suggests that H_2O_2 is more powerful inducer of S-nitrosylation. In fact, the lowest H_2O_2 concentration (0.1mM) yielded higher CTSD S-nitrosylation than the highest GSNO concentration (1mM). This came unexpected, since GSNO is known to be capable of inducing both S-nitrosylation and S-glutathionylation, while H_2O_2 primarily yields S-glutathionylation. Interactions between H_2O_2 and endogenous NO might account for this finding. As a neurotransmitter, NO and its derivatives are very abundant in the brain (Chung and David 2010), where they could potentially react with H_2O_2 forming $ONOO^-$ thereby increasing S-nitrosylation (Radi 2004).

CTSD S-nitrosylation was also investigated in brain homogenates from GRX1-/- mice. Since GRX1 only repairs glutathionylated thiols, we expected S-nitrosylation levels not differ from WT. However, deficiency in PSSG repair could potentially affect targets for PSNO. As observed in Figure 5 (bottom panel), CTSD S-nitrosylation increased slightly in homogenates treated with the lowest concentration of GSNO (0.1 mM), but when GSNO concentration was increased to 0.5 mM S-nitrosylation could no longer be detected. Interestingly, high GSNO levels produced highest CTSD S-nitrosylation levels in the WT (Figure 6A, top and middle panels) but failed in increasing CTSD S-nitrosylation from brain homogenate of GRX1-/- mice. These findings perhaps reflect the decreasing of targets for S-nitrosylation, since GRX1-/- mice are impaired in removing glutathione from its proteins, making these thiols unavailable to S-nitrosylation. Additionally, the complete lack of CTSD S-nitrosylation in high GSNO conditions suggests that higher GSNO concentrations in GRX1-/- might not only fail to induce S-nitrosylation but obstruct previously S-nitrosylated groups, leading to a decrease in S-nitrosylation even below basal levels, as observed in CTSD from brain homogenates of WT mice treated with the lowest concentration of GSNO. In addition, treatment with different H_2O_2 concentrations completely abolished CTSD S-nitrosylation in the brain homogenates from GRX1-/- mice, which is in contrast to the results observed in CTSD from the WT counter-parts. This result suggests that H_2O_2 or its products can react with existing nitrosyl groups of the protein. This might be possible due to the high oxidizing potency of H_2O_2 in comparison to GSNO, causing denitrosylation. These findings might be linked to basal level of total S-glutathionylation observed in the brain homogenates from these mice.

In the WT basal level, S-glutathionylation could be repaired by the native GRX1, freeing the cysteine groups and making them susceptible to other forms of oxidative modification such as S-nitrosylation. In contrast, in GRX1-/- basal level S-glutathionylation could not be removed and cysteine groups remained unavailable to S-nitrosylation. Various studies have confirmed that nitrosothiol can induce S-glutathionylation upon reaction with GSH (Martinez-Ruiz and Lamas 2007) and that S-glutathionylation and S-nitrosylation occur under the same conditions, supporting the notion that high S-glutathionylation could explain these trends. When GRX1-/- homogenates were treated with the two oxidants, similar trends in total protein S-glutathionylation were observed, but to a lesser magnitude. In brain homogenates from WT mice total protein S-glutathionylation increased with GSNO treatment at 0.1 and 0.5 mM, and a gradual decline was observed at 1

mM. However, upon increasing H_2O_2 concentrations the total protein S-glutathionylation decreased in comparison to untreated homogenates. A possible explanation for this difference in the effects of GSNO and H_2O_2 on S-glutathionylation levels might be due to a difference in potency between the two oxidants. Therefore, GSNO being the less powerful oxidant of the two might lead to gradual increases in S-glutathionylation levels, while the use of equal concentrations of H_2O_2 might lead to over oxidation instead. It is well known that total protein S-glutathionylation increases in response to a mild oxidative stress as a protective mechanism. However, under high doses of H_2O_2 this protective mechanism becomes overwhelmed. The cysteine residues then undergo further irreversible oxidations by groups such as sulfenic (SO_2^-) and sulfonic (SO_3^-) acids (Charles, Schroder et al. 2007) and no protein S-glutathionylation is observed as a result.

The total basal protein S-glutathionylation in brain homogenates from GRX1-/- mice is not enhanced in comparison to the WT counterparts, as expected. However with GSNO and H_2O_2 treatment, the brain homogenates from GRX1-/- mice appeared to have an increased total protein S-glutathionylation, especially at lower molecular weight.

As mentioned previously, the abundance of endogenous NO together with high concentrations of H_2O_2, could lead to the formation of ONOO-, which could either cause protein
S-nitosylation, S-glutathionylation, or nitration (Radi 2004). Protein nitration reflects further oxidation of NO and its derivatives to ONOO-, a powerful oxidant (Radi 2004). Therefore, total protein nitration in brain homogenates from WT and GRX1-/- mice was assessed. In both brain homogenates protein nitration levels increased slightly with higher GSNO and H_2O_2 concentration, suggesting that both oxidants can undergo further oxidation, thus generating ONOO-.

Since these modifications can affect protein activity, we next investigated CTSD activity in presence of GSNO or H_2O_2. Surprisingly, CTSD activity changed only mildly following oxidant treatment as compared to basal level activity, suggesting that this protease is not as susceptible to oxidative inhibition as other proteases such as caspase 3 (Mannick, Schonhoff et al. 2001).

Together the findings of this study suggest that while GSNO and H_2O_2 have differing S-glutathionylation and S-nitrosylation effects on CTSD in mice brain homogenates, it appears that these oxidants do not impair the enzyme activity dramatically in the conditions tested.

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