Irreversible inactivation of magnesium-dependent neutral sphingomyelinase 1 (NSM1) by peroxynitrite, a nitric oxide-derived oxidant

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Abstract Previous results have indicated that the generation of ceramide by hydrolysis of sphingomyelin by magnesium-dependent neutral sphingomyelinase 1 (NSM1) is reversibly inhibited by hydrogen peroxide (H_2O_2) and oxidized glutathione (GSSG). This redox-dependent reversible regulation of NSM1 activity has been shown to involve the reversible formation and breakage of disulfide bonds. In this paper, we show that peroxynitrite, a nitric oxide-derived oxidant generated by SIN1, inactivates dose-dependently the NSM1 activity in an irreversible manner. In addition, we show that, in contrast to the reversible inhibition of NSM1 by H_2O_2 or GSSG which involves the formation of disulfide bonds, irreversible inactivation of this enzyme by peroxynitrite generated from SIN1 is likely due to definitive oxidative thiol modification. These results suggest that depending on the nature of the oxidative stress, the enzymatic activity of NSM1 could be reversibly or irreversibly inactivated.

Key words: Neutral sphingomyelinase; Ceramide; Nitric oxide; Peroxynitrite; Inhibition; Oxidation

1. Introduction

Generation of ceramide through hydrolysis of sphingomyelin (SM) by sphingomyelinases (SMases) is a signalling pathway implicated in multiple cellular responses [1–3]. Different SMases have been described in eukaryotes and prokaryotes and are distinguished by their localization, pH optima and requirement for metal ions [3]. However, in eukaryotes, only a few SMases have been characterized at the molecular level. The best characterized of these enzymes is the acidic SMase, which is deficient in patients with Niemann–Pick disease [4] and the Mg^{2+}-dependent neutral SMases 1 and 2 (NSM1 and NSM2) [5–10]. While NSM2 is a brain-specific enzyme associated with Golgi membranes [9], NSM1 is ubiquitously expressed and found in endoplasmic reticulum (ER) membranes [5–7,11]. Although NSM1 has been shown to be involved in apoptosis triggered by T-cell receptor activation [12], overexpression of this enzyme showed minor effects on apoptosis triggered by tumor necrosis factor (TNF) [5,13] or H_2O_2 [14]. In addition, recent results also suggested that a NSM1-like enzyme from the avian DT-40 B-cell line is not involved in the apoptotic response to ER stress [15].

Mouse and human NSM1 have been described in vitro as enzymes with neutral SMase and lysophospholipid phospholipase C activities [8,14]. However, SM appeared in vitro as a better substrate when compared to lysophospholipids [8,10]. In addition, NSM1 knock-out mice showed no cellular accumulation or changes in the metabolism of SM or lysophospholipids [10]. Thus, no clear role for NSM1 in SM metabolism has been proposed so far [10].

Biochemical analysis of recombinant and partially purified mouse NSM1 [6,8] showed that the enzyme has reactive cysteine residues and that its enzymatic activity is redox-regulated. Indeed, NSM1 is reversibly inhibited by H_2O_2 or GSSG and reversibly stimulated by DTT or GSH [6,8,15]. The reversible redox regulation relies upon the reversible formation and breakage of disulfide bonds [8].

Nitric oxide (NO) and NO-derived oxidants are important biological messenger that play a role in physiological and pathophysiological processes such as apoptosis, vasorelaxation and inflammation [16–19]. The interaction of NO and NO reactive species with reactive cysteine-containing proteins has gained considerable importance [19]. Indeed, in many systems, the oxidative or nitrosative modification of protein thiol groups by NO and NO-related oxidants affects protein function [19,20]. Many fundamental enzymes with essential reactive thiol groups have been shown to be inactivated by NO-derived oxidants [19]. Targets of this type of modification, among others, are caspase 3 [21], HIV-1 reverse transcriptase [22] or parasite cysteine proteases [23,24]. Many effects of NO are based on its reaction with other important species. For instance, NO reacts with superoxide to make peroxynitrite ($ONOO^-$) [25] and in vivo peroxynitrite formation has been described during sepsis, autoimmune and inflammatory conditions [26]. Peroxynitrite is one of the most reactive NO-derived oxidants implicated in oxidative modification of proteins [27,28] and this reactive molecule has been shown to irreversibly inactivate creatine kinase [26], tryptophan hydroxylase [28] or α1-antiproteinase [25].

It is now established that ceramide generation is regulated either positively or negatively by NO and NO reactive species [29]. For instance, NO has been shown to inhibit TNFα-induced apoptosis by reducing the generation of ceramide [30].

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These data suggested that SMases could be targets of NO-derived oxidants. Since NSM1 is a redox-regulated SMase containing reactive cysteine residues [6,8,15], we studied whether this enzyme could be inactivated by NO-derived oxidants and more specifically by peroxynitrite using the well-known peroxynitrite generator SIN1 (3-morpholinosydnonimine). We show here that in contrast to H_2O_2 or GSSG, peroxynitrite generated by SIN1 irreversibly inhibits NSM1 activity in a dose-dependent manner. We also show that this inactivation is likely due to the irreversible modifications of reactive cysteine residues of the NSM1 enzyme. Taken together, these data and our previous results [6,8,15] suggest that the generation of ceramide from SM by NSM1 could be reversibly or irreversibly inhibited depending on the nature of the oxidative stress.

2. Materials and methods

2.1. Materials

[^{14}C-methyl]SM and pGEX-2T vector were supplied by Amersham Pharmacia. The vector pRSET was from Invitrogen. Anti-polyHis antibody was from Qiagen. Probond nickel resin was from Invitrogen. Anti-fluorescein Fab′ fragments conjugated to peroxidase, fluorescein-conjugated iodoacetamide and Complete protease inhibitor tablets were from Roche. Sodium nitroprusside (SNP, a NO donor) and 3-morpholinosydnonimine (SIN1, a peroxynitrite generator) and all other chemicals were obtained from Sigma.

2.2. Heterologous expression and purification of proteins

The mouse NSM1 cDNA, previously cloned in our laboratory [6], was subcloned into pGEX-2T and pRSET vectors, which encode the enzyme as a glutathione *S*-transferase fusion protein (GST-NSM1) or as a polyhistidine-tagged fusion protein (polyHis-NSM1), respectively. Transformed bacteria were induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside and grown at 18°C for 18 h. GST fusion proteins were prepared as described previously [8]. Eluted GST-NSM1 was buffer-exchanged into 25 mM Tris–HCl, pH 7, 1 mM dithiothreitol (DTT), 0.1% Triton X-100, 10% glycerol and stored at −20°C.

PolyHis-NSM1 protein was prepared essentially as described for GST-NSM1 but no DTT was included in the buffers. The supernatant was added to 2 ml of nickel resin incubated with rotation at 4°C for 90 min. Subsequently, the resin was washed with 20 mM sodium phosphate, 500 mM NaCl, 0.2% Triton X-100, pH 6 and proteins eluted with 250 mM imidazole in the same buffer. The eluate was buffer-exchanged into 25 mM Tris–HCl, pH 7, 1 mM DTT, 0.1% Triton X-100, 10% glycerol and stored at −20°C.

Both GST-NMS1 and polyHis-NSM1 have been shown to be fully active and used for biochemical characterization of the NSM1 enzyme [6–8].

2.3. Measurement of neutral SMase activity

The activities of recombinant enzymes were measured using radiolabelled substrate as previously described [6,8]. Briefly, enzyme preparations were incubated with [^{14}C]SM for 1 h at 37°C. Following quenching and phase separation, a sample of the aqueous phase was analyzed by scintillation counting. To assess the effect of NO reactive species or H_2O_2 on NSM activity, GST-NSM1 or polyHis-NSM1 was first reduced by 5 mM DTT for 15 min at room temperature and buffer-exchanged to Tris–HCl 25 mM, pH 7, 0.1% Triton X-100. Recombinant proteins were then preincubated with the NO reactive species for 15 min at 37°C in Tris–HCl 25 mM, pH 7, 0.1% Triton X-100 and the remaining activity was measured using the standard assay in the presence of 10 mM DTT (unless otherwise noted) as described previously [6,8].

2.4. Disulfide bond-sensitive electrophoretic shift assay

Breakage or formation of disulfide bonds in polyHis-tagged NSM1 (polyHis-NSM1) were monitored by SDS–PAGE as described by Mahoney et al. [31] followed by Western blotting with anti-polyHis antibody. PolyHis-NSM1 was first reduced by 5 mM DTT for 15 min at room temperature and buffer-exchanged to Tris–HCl 25 mM, pH 7, 0.1% Triton X-100. To assess the effect of H_2O_2 and SIN1 on polyHis-NSM1, aliquots were preincubated with H_2O_2 (500 μM final) or SIN1 (250 μM final) for 15 min at 37°C. Mobility of the proteins was then analyzed by SDS–PAGE in the presence or in the absence of DTT (10 mM final) followed by Western blotting.

2.5. Fluorescein-conjugated iodoacetamide labelling of proteins

Purified GST-NSM1 was preincubated with or without SIN1 for 15 min at 37°C and then incubated with fluorescein-conjugated iodoacetamide (200 μM final) for 10 min at 37°C as described by [32]. Samples were then analyzed by SDS–PAGE (in the presence of 10 mM DTT) and Western blotting using anti-fluorescein Fab′ fragments conjugated to peroxidase.

2.6. Protein determination, SDS–PAGE and Western blotting

Protein concentrations were determined using a Bradford assay (Bio-Rad). Samples for gel electrophoresis were combined with non-reducing 4×SDS sample buffer and separated by SDS–PAGE. Gels were stained by Coomassie brilliant blue R-250. For Western blotting, following separation by SDS–PAGE, proteins were electro-transferred to polyvinylidene difluoride membrane. The membrane was blocked with Tris-buffered saline/Tween 20 (TBS) containing 5% dried milk powder for 1 h. Anti-polyHis (1:1000) or anti-fluorescein Fab fragments conjugated to horseradish peroxidase (1:3000) was added for 1 h in TBS followed by washing. In the case of anti-polyHis, secondary antibody (anti-mouse, 1:2000) was added for 30 min. Following washing, ECL (Amersham Pharmacia Biotech) was used for detection.

3. Results and discussion

SMase activity of mouse or human NSM1 has been shown to be redox state-dependent. Recently, using NO-releasing compounds, De Nadai et al. [30] suggested that the generation of ceramide during apoptosis could be greatly reduced by NO.

These data prompted us to analyze whether the generation of ceramide from SM by the NSM1 enzyme could be modified by NO-derived oxidants. We first tested whether SNP, a com-

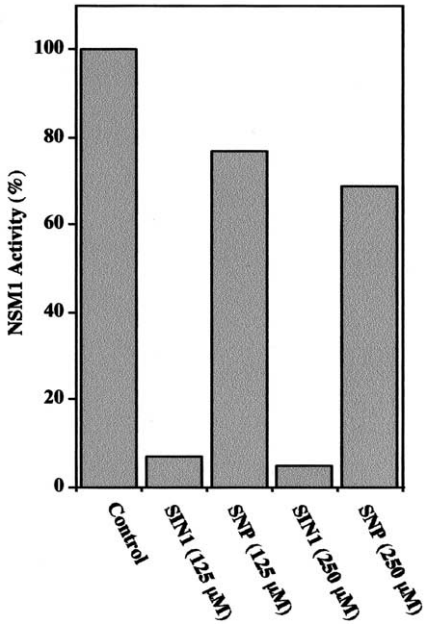

Fig. 1. Effect of SNP and SIN1 on NSM1 enzyme activity. Reduced GST-NSM1 (5 μM) was preincubated with SNP or SIN1 (at specified final concentrations) for 15 min at 37°C in Tris–HCl 25 mM, pH 7, 0.1% Triton X-100. Remaining NSM1 activity was measured using the standard assay in the presence of 10 mM DTT. A control was prepared without SNP or SIN1 (control bar). These results are representative of three independent experiments.

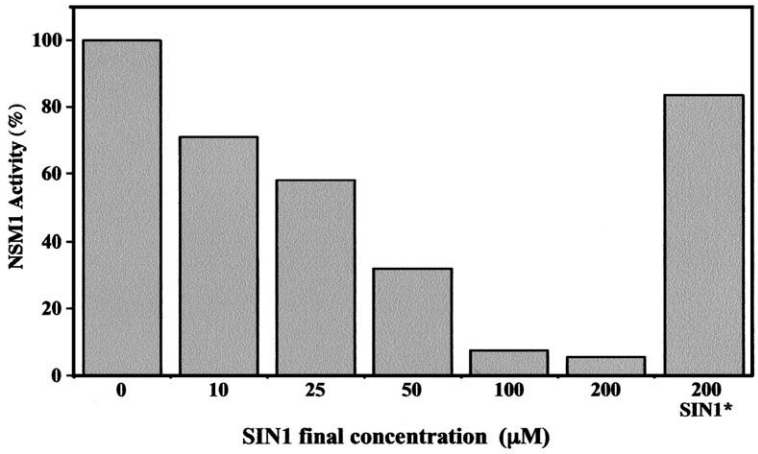

Fig. 2. Dose-dependent inhibition of NSM1 enzyme activity by peroxynitrite generated by SIN1. Reduced GST-NSM1 (5 μM) was preincubated with increasing concentrations of SIN1 (10–200 μM final) for 15 min at 37°C in Tris–HCl 25 mM, pH 7, 0.1% Triton X-100. Remaining NSM1 activity was measured using the standard assay in the presence of 10 mM DTT. NSM1 preincubated without SIN1 (control bar) and NSM1 preincubated with decomposed SIN1 (SIN1* bar) were used as controls. These results are representative of three independent experiments.

pound generally used as a model of NO donor [22], and SIN1, a molecule used as a model for the continuous generation of peroxynitrite [26], had an effect on NSM1 activity. SNP and SIN1 have been described as having similar half-times for NO and peroxynitrite release [34]. Preincubation of GST-NSM1 (5 μM) with SIN1 (125 and 250 μM final) for 10 min at 37°C was able to inhibit more than 90% of NSM1 activity (Fig. 1) whilst in the same conditions around 30% inhibition was obtained with SNP (Fig. 1). These data suggested that the NSM1 enzyme could be inhibited by NO and by peroxynitrite, this latter giving a stronger inhibition. We further analyzed the inhibition of NSM1 by SIN1 at different concentrations. As shown in Fig. 2, SIN1 produced a dose-dependent inhibition of NSM1 activity. The IC_{50} for inhibition of the enzyme activity by SIN1 was estimated to be around 40 μM when the enzyme concentration was 5 μM. Decomposed SIN1 (obtained by allowing decomposition at room temperature in the dark in Tris–HCl 25 mM, pH 7, 0.1% Triton X-100) did not have an effect on NSM1 activity (Fig. 2). These results were similar to data obtained with tryptophan hydroxylase, a brain enzyme known to be sensitive to NO reactive species and more specifically to peroxynitrite [28].

Inhibition of NSM1 by high concentrations of H_2O_2 (500 μM) and GSSG (1 mM) has been shown to be reversible since fully active NSM1 can be obtained after incubation of oxidized enzyme with reducing agents (such as DTT) or by assaying the oxidized enzyme in the presence of reducing agents [8]. In contrast to what has been described with H_2O_2 or GSSG [8], Figs. 1 and 2 suggested that the high concentration of DTT (10 mM) present in the reaction mixture during the assay was unable to restore NSM1 activity, indicating that inhibition by peroxynitrite generated by SIN1 could be irreversible. Reducing agents such as GSH and DTT were tested for the ability to prevent peroxynitrite-induced inactivation of

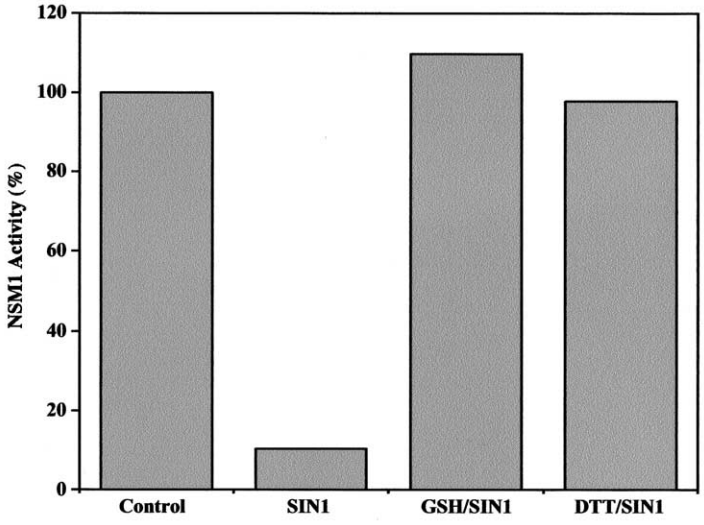

Fig. 3. Protection of NSM1 from peroxynitrite-induced inactivation by reducing agents. Reduced GST-NSM1 (5 μM) was preincubated with SIN1 (200 μM final) in the presence of 5 mM GSH (GSH/SIN1 bar) or in the presence of 5 mM DTT (DTT/SIN1 bar) for 15 min at 37°C in Tris–HCl 25 mM, pH 7, 0.1% Triton X-100 and NSM1 activity was measured using the standard assay in the presence of 10 mM DTT. Controls with SIN1 only (SIN1 bar) or without SIN1 (control bar) were done. These results are representative of three independent experiments.

NSM1 and the results are presented in Fig. 3. It can be seen that preincubation of the enzyme with SIN1 in the presence of reducing agents (5 mM GSH or 5 mM DTT) that react directly with peroxynitrite provided protection of NSM1 from inactivation. However, these reducing agents were unable to restore the NSM1 activity after inactivation of the enzyme by peroxynitrite (Fig. 3, bar SIN1 and Figs. 1 and 2). Similar results have been reported for tryptophan hydroxylase [28] or creatine kinase [26], which enzymatic activities are irreversibly inhibited by peroxynitrite, unless preincubation with this NO-related oxidant is done in the presence of reducing agents.

As stated above, the catalytic activity of NSM1 is reversibly inhibited by H_2O_2 or by GSSG and this redox-dependent reversible regulation of NSM1 activity has been shown to involve the reversible formation and breakage of disulfide bonds [8]. As shown in Fig. 4 (upper panel, −DTT experiment), preincubation of NSM1 with H_2O_2 (500 μM final) induced a strong (90%) inhibition of the enzyme. This inhibition was concomitant with the formation of one or several intramolecular disulfide bonds as shown by the shift in gel electrophoresis mobility of NSM1 (Fig. 4, lower panel, −DTT experiment). A similar level of inactivation (90%) was obtained with SIN1 (200 μM final) (Fig. 4, upper panel, −DTT experiment), but no gel shift was observed suggesting that inhibition by peroxynitrite did not induce the formation of intramolecular disulfide bonds as observed with H_2O_2 (Fig. 4, lower panel, −DTT experiment). The addition of DTT (10 mM) to H_2O_2-inactivated NSM1 was able to restore its catalytic activity (Fig. 4, upper panel, +DTT experiment) with the concomitant reduction of the intramolecular disulfide bonds as shown by the gel shift (Fig. 4, lower panel, +DTT experiment). Conversely, as seen previously (Figs. 1–3), no catalytic activity could be restored by the DTT with peroxynitrite-inactivated NSM1 enzyme (Fig. 4, upper panel, +DTT experiment) and no gel shift could be detected (Fig. 4, lower panel, +DTT experiment). These results clearly showed that peroxynitrite irreversibly inactivated NSM1 by a mechanism that is different from the H_2O_2-reversible inhibition.

Peroxynitrite is a powerful sulfhydryl oxidant which can also modify tyrosine residues in proteins [19]. Nevertheless, chemical modification of tyrosine by peroxynitrite is less likely to occur given its much lower reactivity with respect to cysteine residues [22]. In addition, treatment of mouse NSM1 with *N*-acetylimidazole (20 mM), a tyrosine-modifying agent, did not alter the enzymatic activity (data not shown), suggesting that tyrosine residues in NSM1 did not appear to play an influential role in its catalytic activity. Thus, it was likely that inhibition of NSM1 by peroxynitrite was due to modification

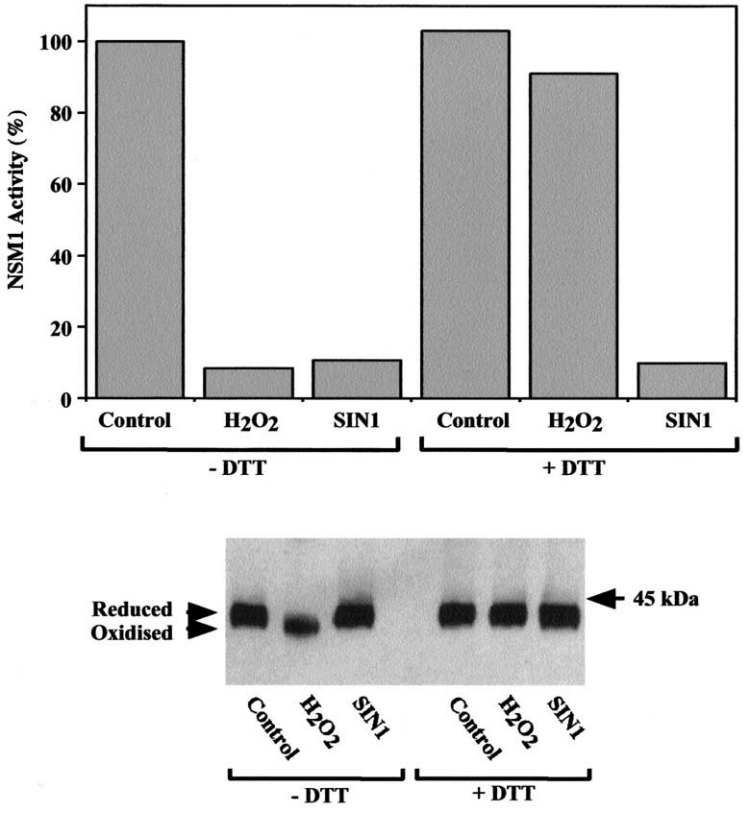

Fig. 4. Peroxynitrite inhibits NSM1 activity by a mechanism different from H_2O_2-induced inhibition. Upper panel: reduced polyHis-NSM1 (200 μg/ml) was preincubated with H_2O_2 (500 μM final) or with SIN1 (200 μM) for 15 min at 37°C in Tris–HCl 25 mM, pH 7, 0.1% Triton X-100 and remaining NSM1 activity was measured using the standard assay without DTT. A control without H_2O_2 or SIN1 was done. The same experiment was done but this time the remaining NSM1 activity was measured using the standard assay in the presence of 10 mM DTT. Lower panel: in parallel to the experiments described above, reduced polyHis-NSM1 (200 μg/ml) was preincubated with H_2O_2 (500 μM final) or with SIN1 (200 μM) for 15 min at 37°C in Tris–HCl 25 mM, pH 7, 0.1% Triton X-100 and subjected to SDS–PAGE under non-reducing conditions and Western blotting with anti-polyHis antibody. PolyHis-NSM1 reduced by 10 mM DTT was used as a control. The same experiment was done but this time samples were incubated with 10 mM DTT prior to SDS–PAGE and Western blotting. Left arrows indicate the positions of reduced polyHis-NSM1 (reduced) and of H_2O_2-oxidized polyHis-NSM1 which contains intramolecular disulfide bonds as described in [8]. These results are representative of three independent experiments.

of cysteine residues given that NSM1 has been shown to be a redox-regulated enzyme sensitive to inactivation by a variety of sulfhydryl reagents [8]. Therefore, the effects of peroxynitrite on sulfhydryl groups in NSM1 were determined using fluorescein-conjugated iodoacetamide which binds to free, unoxidized sulfhydryl groups as described previously [32]. As shown in Fig. 5, preincubation of GST-NSM1 with increasing concentrations of SIN1 led to an increasing modification of cysteine residues as indicated by the disappearance of fluorescein-iodoacetamide labelling. In the same conditions, GST alone was not labelled (data not shown). These results clearly showed evidence of modification of NSM1 cysteine residues by peroxynitrite generated by SIN1. Taken together, our data suggested that the irreversible inactivation of NSM1 by peroxynitrite was due to the oxidation of cysteine residues beyond sulfenic acid, since the activity of SIN1-treated enzyme could not be restored by DTT reduction. Irreversible thiol oxidation has also been described as a critical event leading to the inactivation of caspase 3 [35] and creatine kinase [26] by peroxynitrite generated by SIN1.

Besides its role as a second messenger in cell death [17,29], NO and its derived reactive species can also protect cells against apoptosis under different conditions [33]. This anti-apoptotic mechanism can be understood via expression of protective genes such as heat shock proteins as well as direct inhibition of enzymes such as the apoptotic caspase family proteases by modification of reactive cysteine residues [19,33]. More broadly, in other biological processes, effects of NO and NO-related oxidants can also be due to the modification of cysteine residues of pivotal proteins such as transcriptional activators and protein-phosphotyrosine phosphatases [19,36,37]. The results presented herein suggest that oxidation of reactive cysteine residues of NSM1 by peroxynitrite, a NO-related oxidant, leads to the irreversible inactivation of the enzyme. In contrast, NSM1 activity is reversibly inactivated by H_2O_2 or GSSG [6,8,15]. Therefore, NSM1 appears as a redox-sensitive enzyme which catalytic activity can

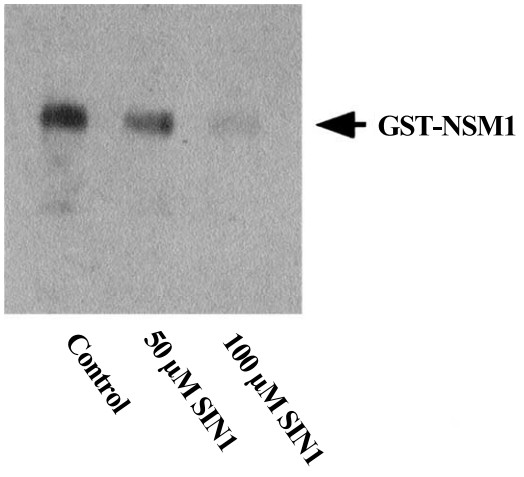

Fig. 5. Oxidation of cysteine residues in NSM1 by peroxynitrite. Reduced GST-NSM1 (1 μM) was preincubated with SIN1 (50 μM and 100 μM final) for 15 min at 37°C in Tris–HCl 25 mM, pH 7, 0.1% Triton X-100. Fluorescein-conjugated iodoacetamide was added (200 μM final) and the mixture was incubated for 10 min at 37°C prior to SDS–PAGE under reducing conditions and Western blotting using anti-fluorescein antibody. A control was done with GST-NSM1 without SIN1.

be reversibly or irreversibly inactivated depending on the nature of the oxidative stress.

Although NSM1 has been shown to be involved in apoptosis triggered by T-cell receptor activation [12], the role of this SMase in other apoptotic pathways seems to be minor [5,13–15]. Involvement of NSM1 in regulation of the SM/cholesterol ratio in membranes and their metabolism has been speculated [10].

With regard to the identity of the SMases involved in the ceramide generation during apoptosis, most findings have described the implications of neutral SMases that can be regulated by the cellular redox state [29]. In addition, numerous studies have demonstrated that ceramide generation is regulated positively or negatively by NO and NO-related oxidants [29]. The data reported in our present study suggest that NSM1, a neutral SMase, can be the target of NO reactive species and open the question as to whether other neutral SMase isoforms, such as NSM2, could also be inhibited by NO reactive species.

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