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Original Contribution

# Thioredoxin-mimetic peptides as catalysts of S-denitrosylation and anti-nitrosative stress agents

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ABSTRACT

S-nitrosylation, the coupling of a nitric oxide moiety to a reactive cysteine residue to form an *S*-nitrosothiol (SNO), is an important posttranslational mechanism for regulating protein activity. Growing evidence indicates that hyper-S-nitrosylation may contribute to cellular dysfunction associated with various human diseases. It is also increasingly appreciated that thioredoxin and thioredoxin reductase play significant roles in the cellular catabolism of SNO and protection from nitrosative stress. Here, we investigated the SNO reductase activity and protective effects of thioredoxin-mimetic peptides (TXMs), Ac-Cys-Pro-Cys-amide (CB3) and Ac-Cys-Gly-Pro-Cys-amide (CB4), both under cell-free conditions and in nitrosatively stressed cultured cells. In vitro biochemical analyses revealed that the TXM peptides reduced small-molecule SNO compounds, such as *S*-nitrosoglutathione (GSNO), and acted as general and efficient protein-denitrosylating agents. In particular, CB3 was found to be a highly potent SNO-metabolizing agent. Notably, CB3 mimicked the activity of thioredoxin by coupling with thioredoxin reductase to enhance GSNO reduction. Moreover, in a cell-free lysate system, both CB3 and CB4 synergized with an NADPH-dependent activity to denitrosylate proteins. Further investigation revealed that the TXM peptides protect the peroxiredoxin–thioredoxin system from SNO-dependent inhibition. Indeed, SNO-inhibited Prx1 was efficiently denitrosylated and reactivated by CB3 or CB4. In addition, CB3 protected thioredoxin reductase from SNO-mediated inactivation both in vitro and in intact cells. Finally, CB3 and CB4 partially rescued human neuroblastoma SH-SY5Y cells and rat insulinoma INS-1 832/13 cells from GSNO-induced growth inhibition. Collectively, the present findings indicate the efficient denitrosylation activity and protective effects of TXM peptides and suggest their potential therapeutic value in treating pathological conditions related to nitrosative stress.



A large number of studies have demonstrated that the physiological and pathophysiological effects of nitric oxide (NO) are mediated in part by S-nitrosylation, the posttranslational modification of cysteine thiols to form *S*-nitrosothiols (SNOs) [1,2]. Under normal conditions, intracellular SNO levels are kept low and are transiently increased in response to particular extracellular stimuli. However, under certain cellular circumstances, constitutively and abnormally high levels of protein S-nitrosylation can develop. Persistently elevated S-nitrosylation may disrupt the normal function of various proteins and thereby lead to cellular dysfunction and ultimately cell death. Indeed, increasing evidence implicates excessive protein S-nitrosylation in the pathogenesis of chronic and degenerative diseases, including cardiovascular disease, stroke, and neurological disorders [3]. In this regard, aberrant S-nitrosylation, involving more than 30 proteins, has been linked to neurodegenerative conditions such as Alzheimer and Parkinson disease [4,5]. Likewise, hyper-S-nitrosylation of multiple proteins has been detected in metabolic diseases such as diabetes and obesity [6,7]. In most of the reported cases the cause of hyper-S-nitrosylation remains unknown. Nonetheless, it is reasonable to assume that interventions that enhance cellular denitrosylation activity may counteract the accumulation of excess SNO and preserve protein and cellular function.

Cell-free studies demonstrated long ago that dithiol compounds, such as dithiothreitol (DTT) and lipoic acid, efficiently

*Abbreviations:* CysNO, *S*-nitrosocysteine; SNO, *S*-nitrosothiol; Trx, thioredoxin; TrxR, thioredoxin reductase; TXM, thioredoxin mimetic; Prx, peroxiredoxin; CB3, Ac-Cys-Pro-Cys-amide; CB4, Ac-Cys-Gly-Pro-Cys-amide

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reduce peptide- and protein-SNO [8–10]. More recently, it became understood that cellular denitrosylation is largely mediated by enzymatic systems, among which the best characterized are the glutathione (GSH)/*S*-nitrosoglutathione reductase and the thioredoxin/thioredoxin reductase (Trx/TrxR) systems [11]. In particular, increasing evidence suggests a distinct function for the ubiquitous dithiol protein Trx, acting in conjunction with TrxR, in the denitrosylation of numerous and diverse proteins [11–16]. Thus, the emerging view is that the denitrosylation activity of Trx/TrxR plays a role both in NO-based signaling and in ameliorating nitrosative stress [11–18].

The above-mentioned findings prompted us to investigate the potential SNO reductase activity and related effects of Trx-mimetic (TXM) peptides, which are cell-permeable tri- or tetrapeptides, derived from the conserved Cys-Gly-Pro-Cys active-site motif of Trx [19,20]. The most studied TXM peptides are CB3 (Ac-Cys-Pro-Cys-amide) and CB4 (Ac-Cys-Gly-Pro-Cys-amide), which have been shown to protect primary bovine chromaffin cells, PC12 cells, and insulinoma cells from oxidative stress [19–21]. In vivo, CB3 has been found to attenuate allergic airway disease in mice subjected to ovalbumin inhalation by decreasing mitogen-activated protein kinase activity and NF-κB nuclear translocation [22]. Recently, it was demonstrated that CB3 and CB4 protect the neuronal-like SH-SY5Y cells from oxidative stress and that CB3 decreases neuroinflammatory stress signaling in diabetic rats [23].

In this study we investigated the TXM peptides CB3 and CB4 as denitrosylating agents. Using in vitro biochemical assays we show that these peptides efficiently reduce both low-molecular-weight SNO and protein SNO. Importantly, we demonstrate that the TXM peptides can synergize with TrxR or a cellular NADPH-dependent enzyme to efficiently reduce SNO substrates. Finally, we demonstrate that the TXM peptides ameliorate nitrosative stress in human neuroblastoma SH-SY5Y cells and rat insulinoma INS-1 832/13 cells. Our study establishes the TXM peptides as potent denitrosylating catalysts and opens a new avenue for exploring the applicability of these compounds for the treatment of pathological conditions associated with hyper-S-nitrosylation.

## Materials and methods

### *Reagents*

The TXM peptides CB3 and CB4 were custom synthesized by Genescript (Piscataway, NJ, USA). The peptides were dissolved in deoxygenated aqueous medium and stored at −20 °C. Rabbit polyclonal antibodies for peroxiredoxin 1 (Prx1) were obtained from Abcam (Cambridge, MA, USA; Catalog No. ab41906). Hydrogen peroxide ($H_2O_2$) was purchased from Carlo Erba Reagents (Rodano, Italy) and NADPH from Roche Diagnostics (Indianapolis, IN, USA). Auranofin was obtained from Enzo Life Sciences (Farmingdale, NY, USA). *S*-nitrosocysteine (CysNO), *S*-nitrosoglutathione (GSNO), and *S*-nitroso-*N*-acetyl-L-cysteine (NAC-SNO) were synthesized by reacting an equimolar concentration of L-cysteine, GSH, or *N*-acetyl-L-cysteine with sodium nitrite in 0.2 M HCl and used within 1 h. Recombinant Trx1 and recombinant Prx1 were prepared as described [24]. Recombinant rat TrxR1 (specific activity 28 units/mg) was kindly provided by Elias Arnér (Karolinska Institute, Stockholm, Sweden). Tissue culture media and reagents were from Biological Industries (Beit Haemek, Israel). Other materials were obtained from Sigma unless otherwise indicated.

### *Cell culture and treatment*

Human THP-1 monocytes were maintained in RPMI medium 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C under 5% $CO_2$. Rat INS-1 832/13 insulinoma cells and human SH-SY5Y neuroblastoma cells were maintained as previously described [21,23].

### *Biochemical analysis of nitrosylation/denitrosylation*

Measurement of low-molecular-weight (MW) SNOs was performed using the Saville–Griess assay or by copper–cysteine-based reductive chemiluminescence according to established protocols [24] (see also below). Assessment of nitrosylation/denitrosylation of cell lysate proteins was performed as previously described [13] with minor modification as follows. THP-1 cells were lysed in hypotonic buffer (10 mM Hepes, 3 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, pH 7.5) and then centrifuged at 17,000*g* for 1 h at 4 °C. The supernatant typically contained 3 mg/ml protein as determined by Bradford assay. These cytosolic fractions were treated with vehicle or CysNO (final concentration 50 μM) at room temperature for 30 min in the dark. After this step, low-MW species (including residual CysNO) were removed by repeated cycles of filtration through a 10-kDa-cutoff filter (Amicon Ultra-15; Millipore) and washing with HEN buffer (25 mM Hepes, 1 mM EDTA, 10 μM neocuproine, pH 7.5). The sample was subjected to denitrosylation by incubation with reducing agents, as indicated in the figure legends. To minimize spontaneous SNO decomposition, reactions were protected from light and carried out in the presence of the metal chelator EDTA. Where indicated, protein SNOs were separated from low-MW SNOs as follows: half of the sample was transferred to a separate microfuge tube. Then 3 volumes of cold acetone were added, and the sample was incubated at −20 °C for 30 min and centrifuged at 5000*g* for 5 min at 4 °C. The protein pellet was resuspended in HEN buffer and kept on ice until analysis. The other half of the sample was transferred to a 10-kDa-cutoff filter (Microcon, YM-10, centrifugal filter unit; Millipore). After centrifugation, the filtrate containing low-MW species was stored on ice until analysis. SNO content was determined by reductive chemiluminescence using an NO analyzer (CLD88; Eco Medics AG Switzerland). Values were derived by comparison with GSNO standards and were normalized to protein concentration in the extract.

### *Assessment of nitrosylation and activity of Prx1 in vitro*

Quantification of nitrosylated thiols in Prx1 was determined by Saville–Griess assay. Briefly, the nitrosylated protein was incubated in a final volume of 200 μl of assay buffer (1% sulfanilamide, 0.1% *N*-(1-naphthyl) ethylenediamine dehydrochloride, 1% HCl) in the absence or presence of 1 mM $HgCl_2$ for 30 min and absorbance readings were measured at 540 nm. Mercury-dependent absorbance was converted to SNO concentrations using GSNO standards treated identically. The concentration of GSNO was determined by UV–Vis spectrophotometry by measuring the absorbance at 335 nm (extinction coefficient of 920 $M^{-1}$ $cm^{-1}$). The peroxidase activity of Prx1 was measured based on a previously reported method [25] and as follows. Untreated or nitrosylated Prx1 (10 μM) diluted in 25 mM potassium phosphate, 1 mM EDTA, pH 7.0, was incubated with the Trx system (5 μM Trx, 0.1 μM TrxR, and 450 μM NADPH) and 500 μM $H_2O_2$ at 37 °C for 10 min. Twenty-microliter aliquots were taken every minute and transferred into 980 μl of Fox reagent (100 mM sorbitol, 125 μM xylenol orange, 250 μM ferrous ammonium sulfate, and 25 mM $H_2SO_4$). The absorbance at 560 nm was read after 30 min and compared with a $H_2O_2$ standard curve. The activity was derived from the linear regression of absorbance versus time.