Inhibition of Cathepsin K by Nitric Oxide Donors: Evidence for the Formation of Mixed Disulfides and a Sulfenic Acid

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ABSTRACT: The cysteine protease cathepsin K is believed to play a key role in bone resorption as it has collagenolytic activity and is expressed predominantly and in high levels in bone resorbing osteoclast cells. The addition of nitric oxide (NO) and NO donors to osteoclasts in vitro results in a reduction of bone resorption, although the mechanism of this effect is not fully understood. The *S*-nitroso derivatives of glutathione (GSNO) and *N*-acetylpenicillamine (SNAP) and the non-thiol NO donors NOR-1 and NOR-3 all inhibited the activity of purified cathepsin K in a time- and concentration-dependent manner (IC_{50} values after 15 min of preincubation at pH 7.5 of 28, 105, 0.4, and 10 μM, respectively). Cathepsin K activity in Chinese hamster ovary cells stably transfected with cathepsin K was also inhibited by the above NO donors with similar potencies. GSNO at 100 μM also completely inhibited the autocatalytic maturation at pH 4.0 of procathepsin K to cathepsin K. The inhibition of cathepsin K by GSNO was rapidly reversed by DTT, but inhibition by NOR-1 was not reversed by DTT, and analysis of the inhibited cathepsin K for S-nitrosylation using the Greiss reaction gave negative results in both cases. Analysis of the protein by electrospray liquid chromatography/mass spectrometry showed that the inhibition of cathepsin K by GSNO resulted in a mass increase of 306 ± 2 Da, consistent with the formation of a glutathione adduct. Prior inhibition of cathepsin K by the active site thiol-modifying inhibitor E-64 blocked the modification by GSNO, indicating that the glutathione adduct is likely formed at the active site cysteine. Treatment of cathepsin K with NOR-1 resulted in a mass increase of between 30 and 50 Da, corresponding to the oxidation of a cysteine to sulfinic and sulfonic acids. Cotreatment of cathepsin K with NOR-1 plus the sulfenic acid reagent dimedone resulted in a mass increase of approximately 141 Da, which is consistent with the formation of a dimedone adduct. This result demonstrates that the NOR-1-dependent formation of cathepsin K sulfinic and sulfonic acids occurs via a sulfenic acid. These results show that inhibition of cathepsin K activity and its autocatalytic maturation represent two potential mechanisms by which NO can exert its inhibitory effect on bone resorption. This work also shows that oxidative thiol modifications besides S-nitrosylation should be considered when the effects of NO and NO donors on critical thiol-containing proteins are investigated.

Cathepsin K is a lysosomal cysteine protease and a member of the papain superfamily which is highly expressed in osteoclasts, the cells responsible for bone resorption (*1*, *2*). Cathepsin K is regarded as a key protease in bone resorption because of its localization (*3*) and because it is one of a few proteases able to efficiently hydrolyze native collagen which makes up 90% of the protein in bone (*4*). The phenotype of the cathepsin K knockout mouse (*5*) includes increased bone mass, or osteopetrosis, a condition similar to that displayed by sufferers of pycnodysostosis, a human genetic disease which results in the production of inactive cathepsin K (*6*). The implied role of cathepsin K in bone resorption makes this enzyme a possible drug target to treat diseases such as osteoporosis where the rate of osteoclastic bone resorption outpaces the laying down of new bone by osteoblasts (*7*).

Nitric oxide (NO)[1] has been shown to modulate bone resorption in a number of studies (reviewed in refs *8* and *9*). Both osteoclasts (*10*) and osteoblasts produce NO (*11*), and the induction of NO release by cytokines, or the addition of NO donors, leads to a reduction in bone resorption by mature osteoclasts in vitro. Conversely, nitric oxide synthase inhibitors increase bone resorption both in vitro and in vivo. The mechanism by which NO inhibits bone resorption is not clear and likely involves multiple factors, affecting both osteoclast formation and function. A recent study showed that both a cell-permeable cGMP analogue and the NO donor SIN-1

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[1] Abbreviations: BSA, bovine serum albumin; CHO, Chinese hamster ovary; GSNO, *S*-nitrosoglutathione; HBSS, Hank's balanced salt solution; LC/MS, liquid chromatography/mass spectrometry; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; NO, nitric oxide; NOR-1, (*E*)-methyl-2-((*E*)-hydroxyimino)-5-nitro-6-methoxy-3-hexenamide; NOR-3, (*E*)-ethyl-2-((*E*)-hydroxyimino)-5-nitro-3-hexenamide; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside.

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cause osteoclasts to contract (*12*). The effect of SIN-1 was inhibited by the soluble guanylate cyclase inhibitor methylene blue, providing a link between NO, a rise in cGMP levels, and osteoclast detachment from bone. However, the inhibition of bone resorption by SIN-1 in a bone slice assay was unaffected by either methylene blue or a cell-permeable cGMP antagonist, indicating that another mechanism or mechanisms are also involved (*12*). In this work we show that the activity of cathepsin K and its autocatalytic processing to the mature catalytically active form are both inhibited by NO donors, thus providing another potential mechanism by which NO can inhibit bone resorption.

The activities of a number of cysteine-containing enzymes and proteins (*12*), notably hemoglobin (*14*), are modulated by *S*-nitrosothiols and NO donors. The present dogma for the mechanism of inhibition of critical thiol-containing enzymes by NO and NO donors is the formation of a protein *S*-nitrosocysteine (*13*, *15*−*17*). This species, which has been identified by spectroscopic and colorimetric quantitation techniques in a number of proteins, is reducible to the free thiol by DTT with the recovery of enzyme activity (*18*). This latter criterion has been used to postulate the presence of a *S*-nitrosothiol group in a number of recent studies on the effects of NO donors on proteins, sometimes in the absence of direct evidence (*19*−*21*). Here we show that the inhibition of cathepsin K with the *S*-nitrosothiols GSNO and SNAP leads instead to the formation of mixed disulfide species with the active site cysteine, whereas inhibition with the NO donor NOR-1 results in the oxidation of a single cysteine to a sulfenic acid, resulting ultimately in the formation of a mixture of sulfinic and sulfonic acids. Thus, the possibility of other cysteine modifications besides S-nitrosylation should be considered when the effects of NO donors on critical thiol-containing proteins are investigated.

MATERIALS AND METHODS

The NO donors GSNO, SNAP, NOR-1, and NOR-3 were from Biomol and SIN-1 and SNP were from Calbiochem. Z-Phe-Arg-AMC and Z-Phe-Arg-pNA were from Bachem. Other reagents were from Sigma. Solutions of NO donors were prepared fresh daily in DMSO, with the exception of SIN-1 which was made up in 50 mM MES and 2.5 mM EDTA, pH 5.5. Solutions were kept on ice until used. The concentrations of GSNO and SNAP were determined by absorption using $\epsilon_{330nm} = 767$ and 717 M^{-1} cm^{-1}, respectively (*22*). The concentrations of the remaining NO donors were determined by weight. The purity of GSNO was confirmed by reverse-phase HPLC (Nova-Pak C18, 3.9 × 150 mm, Waters) with elution at 1.5 mL/min using 0.1% acetic acid in water and detection at 240 nm. The retention times of GSH and GSNO were 1.24 and 2.95 min, respectively.

Cathepsin K Preparation. Recombinant human cathepsin K was expressed as the preproenzyme and was a gift from Axys Pharmaceuticals Inc. (*23*). Treatment of the concentrated harvest supernatant at pH 4.0 with pepsin followed by purification on SP-Sepharose yielded purified mature cathepsin K (*23*). Deglycosylated procathepsin K was isolated by treatment of the concentrated harvest supernatant (2.8 mg of protein) with 35 000 units of PNGase F (New England Biolabs) in 50 mM phosphate, pH 7.5, 2 mM DTT, and 2 mM EDTA at 37 °C for 7 h. The mixture was loaded at 1 mL/min on a HR5/5 Mono Q anion-exchange column (Pharmacia) in 20 mM Tris, pH 8.0, and 1 mM DTT and eluted with a linear gradient of 0−2 M NaCl and procathepsin K eluting around 1 M NaCl. Protein concentrations were determined by Coomassie blue binding (Bio-Rad) against standard cathepsin K, the concentration of which was determined by an active site titration with the inhibitor E-64.

Titrations of NO Donors against Purified Cathepsin K. Titrations with cathepsin K were conducted in either 50 mM MES, 2.5 mM EDTA, and 10% DMSO, pH 5.5, or 50 mM sodium phosphate, 2.5 mM EDTA, and 10% DMSO, pH 7.5. Cathepsin K stock was diluted to 1 mg/mL in pH 5.5 buffer containing 1 mM DTT. After 15 min the enzyme was diluted 3600-fold in pH 5.5 or 7.5 buffer, and 1.0 mL was added to 10 μL of NO donor or vehicle control in a deep well 96-well plate (Beckman). After the preincubation period, 225 μL of the mixture was added to 75 μL of 100 μM Z-Phe-Arg-pNA in either pH 5.5 or pH 7.5 buffer in a 96-well plate (Nunc, Nunclon), and the remaining activity was measured for 5 min by increase in absorbance at 405 nm (SpectraMax 190, Molecular Devices) resulting from the liberation of *p*-nitroanilide. Activities at each preincubation period were compared with the corresponding vehicle-treated control for calculation of the percentage inhibition.

Time Dependence of Inhibition of Cathepsin K by GSNO and NOR-1. Cathepsin K activity was determined using the fluorogenic substrate Z-Phe-Arg-AMC in 50 mM MES, 2.5 mM EDTA, and 10% DMSO, pH 5.5 at 22 °C. Fluorescence was measured using Perkin-Elmer LS-5 or Photon Technologies QuantaMaster fluorometers with excitation and emission wavelengths of 365 and 440 nm, respectively. Cathepsin K was diluted to 2.5 mg/mL in buffer containing 1 mM DTT for 15 min and then further diluted to 5 μg/mL (200 nM) and kept on ice until used. Vehicle or NO donor and substrate in DMSO (200-fold dilution, 25 μM final concentration) were premixed in 2 mL of buffer, and the reaction was initiated in the fluorometer by the addition of cathepsin K (1 nM final concentration). The progress curves were fitted to a single-exponential equation to obtain the observed first-order rate constant for inactivation (k_{obs}) using Kaleidagraph software. The K_m of the substrate Z-Phe-Arg-AMC under our assay conditions was approximately 25 μM. Higher concentrations showed substrate inhibition, likely due to the formation of substrate micelles (M. D. Percival, unpublished observations). For reactivation experiments cathepsin K was premixed with the NO donor or vehicle in the cuvette for 240 s prior to the addition of substrate (25 μM). The test thiol was added after approximately 60 s as a 100-fold concentrated stock in buffer. The progress curve was fitted to an equation (*24*) of the form $P = v_s t + (v_0 - v_s)(1 - e^{-kt})/k$, where P is the amount of product formed, v_s is the steady-state rate, and v_0 is the initial rate, to obtain the first-order rate constant for the recovery of activity (k).

Titrations of Whole Cell Cathepsin K Activity with NO Donors. Inhibitors were prepared as 200-fold concentrated stocks in DMSO. NO-depleted NOR-1 and NOR-3 were prepared by incubating each inhibitor for 5 days at pH 9 (50% DMSO), prior to neutralization before assaying. The intracellular cathepsin K activity in intact cells was determined by incubation of cathepsin K expressing CHO cells

with 5 μM (Z-Leu-Arg)$_2$-rhodamine-110 substrate in the presence of extracellular protease inhibitors (D. Claveau, unpublished material). Briefly, 24 h prior to the experiment, mock or human cathepsin K expressing stable CHO cell lines was trypsinized and dispensed in sterile 96-well cell culture plates (Nunc) at 50 000 cells per well. The next day, cells were washed twice in Ca^{2+}- and Mg^{2+}-containing PBS, and 200 μL of HBSS, pH 7.4, supplemented with 15 mM Hepes, 20 μg/mL cystatin (Calbiochem), and 100 μg/mL soybean trypsin inhibitor (Sigma) was added to each well. After a 15 min preincubation with the NO donors at room temperature, the reaction was initiated by the addition of 2 μL of a 500 μM stock solution of (Z-Leu-Arg)$_2$-rhodamine-110 in DMSO. Substrate hydrolysis was monitored over a period of 5 min at room temperature in a Cytofluor 4000 fluorescent plate reader (Perseptive Biosystems) with excitation and emission filters of 485 and 530 nm, respectively. For reactivation experiments, CHO cells expressing cathepsin K were treated with 10 μM E-64, 200 μM GSNO, 500 μM NOR-1, and vehicle for 15 min. At this time the cathepsin K activity of half of each inhibitor-treated cell was determined as above. The other half was washed twice with the above HBSS buffer and then incubated for 30 min at 37 °C in culture medium. The cells were then washed twice with the HBSS buffer, and the cathepsin K activity was determined as above.

Effect of GSNO on Maturation of Procathepsin K. Procathepsin K (16 μg) was desalted to remove DTT using a CentriSep spin column (Princeton Separations) in 50 mM MES, 0.5 M NaCl, and 2.5 mM EDTA, pH 5.5. The protein was then diluted 2-fold into 0.3 M citric acid/phosphate buffer, pH 4.0 or 5.0, containing either 100 μM GSNO or vehicle (1% v/v) and incubated for 120 min at 25 °C prior to quenching with SDS sample buffer. After being heated at 90 °C for 5 min the samples (0.5 μg) were run on a 12% Tris−Glycine gel (Novex) and silver stained (Novex).

Thiol Content of Native and Inactivated Cathepsin K. Cathepsin K (0.5 mg/mL) was freed of DTT by dialysis under anaerobic conditions for 6 h (50 mM MES, pH 5.5, 2.5 mM EDTA) and then treated with vehicle, 500 μM GSNO, or 500 μM NOR-1. After 30 min aliquots were removed and assayed for cathepsin K activity, which showed greater than 90% inhibition compared to vehicle. The cathepsin K samples were then dialyzed as above to remove excess NO donor and the thiol (*25*), and protein concentration (Bio-Rad) was determined.

LC/MS of NO Donor-Treated Cathepsin K. Cathepsin K was desalted to remove DTT by binding to a HS/F cation-exchange column (Perseptive Biosystems) in 20 mM MES/HEPES/acetate buffer, pH 6.0, followed by elution with a step gradient of 2 M NaCl. Samples of cathepsin K (0.1 mg/mL) were treated with NO donors and other reagents from 100-fold concentrated stocks in DMSO or buffer. Aliquots were removed to test for inhibition and the effect of the presence of DTT in the assay mixture on reactivation prior to freezing on dry ice until the sample was analyzed by LC/MS. Experiments to determine the effect of dimedone on NOR-1 and GSNO inhibition were conducted at pH 6.5 in 100 mM phosphate buffer. This slightly higher pH was used to ensure that dimedone (pK_a 5.5) was fully deprotonated. Electrospray LC/MS was carried out on a Finnigan TSQ7000triple quadrupole mass spectrometer (Finnigan MAT) using a homemade microspray source. The protein sample (10 μL of ~0.1 mg/mL) was injected onto an on-line protein trap (Michrom BioResources Inc.) coupled with a 1.0 mm i.d. × 50 mm Jupiter C4 column (Phenomenex), using a flow rate of 50 μL/min. The protein trap was washed with a 2 × 250 mL solution containing 90% H_2O, 10% CH_3CN, and 0.05% TFA for desalting. A linear gradient of 25% mobile phase A (CH_3CN, 0.05% TFA):75% mobile phase B (H_2O, 0.05% TFA) to 95% A:5% B in 20 min was used. The flow after the column was split using a Valco tee to ~1 μL/min to the microspray tip. The mass spectrometer was tuned and calibrated with myoglobin prior to protein sample analysis and was scanned from 800 to 1800 amu at a scan rate of 3 s in profile mode. The protein mass spectra were deconvoluted using the Finnigan BioWorks software.

Analysis of NO Donor-Treated Cathepsin K for S-Nitrosothiol Content (Greiss Reaction). Cathepsin K was desalted by cation-exchange chromatography as described above and an aliquot (0.4 mg/mL) was treated with 500 μM NOR-1 or 300 μM GSNO. After 10 min inhibition was complete, and the enzyme was desalted again by the same method and an aliquot removed to demonstrate that the enzyme was still fully inhibited. The sample (22.4 μL, 0.12 mg/mL) was then treated with 32 μL of $HgCl_2$/sulfanilamide solution (prepared from 1 volume of 1% $HgCl_2$ in water and 4 volumes of 3.4% sulfanilamide in 0.4 M HCl). After 5 min 25.6 μL of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 0.4 M HCl was added and the absorbance read after 5 min at 540 nm in a masked microcuvette. The increase in absorbance compared to a control treated as above but without $HgCl_2$ was less than 0.005 OD and corresponds to less than 0.1 mol of *S*-nitrosothiol/mol of cathepsin K using $\epsilon = 42\,000\ M^{-1}\ cm^{-1}$ (*26*, *27*).

S-Nitrosylation of Cathepsin K with Acidified Nitrite. Cathepsin K was S-nitrosylated according to a modification of the method of Stamler et al. (*28*). Cathepsin K in 2.1 M guanidine hydrochloride (3.5 mg/mL) was added to an equal volume of 1 M HCl, plus or minus 3 mM $NaNO_2$. After 30 min, analysis by UV−visible difference spectroscopy showed the appearance of absorption bands at 330 nm (0.74 OD) and 550 nm (0.015 OD).

Effect of Dimedone on GSNO and S-Nitrosylated BSA. The free thiol content of BSA (fraction V, Boehringer) was determined by titration with 2,2-dithiopyridine as 0.38 mol/mol of albumin and was S-nitrosylated as previously described (*28*). The product had absorption maxima at 335 and 540 nm with extinction coefficients (based on BSA concentration) of 1100 and 27 $M^{-1}\ cm^{-1}$, respectively. After dialysis against 100 mM sodium phosphate, pH 6.5, the material (1 mM) was treated with 1 mM DTT and the absorbance at 335 nm monitored with time. The OD_{330nm} of the mixture decreased in an apparently first-order manner with a $t_{1/2}$ of 10 min, consistent with the destruction of the *S*-nitrosothiol group (*29*). When S-nitrosylated BSA or GSNO was treated with 10 mM dimedone, no spectral changes were observed over 1 h. The above results were confirmed using the Greiss reaction (*30*) showing that DTT, but not dimedone, can destroy the *S*-nitrosyl group of BSA-SNO.

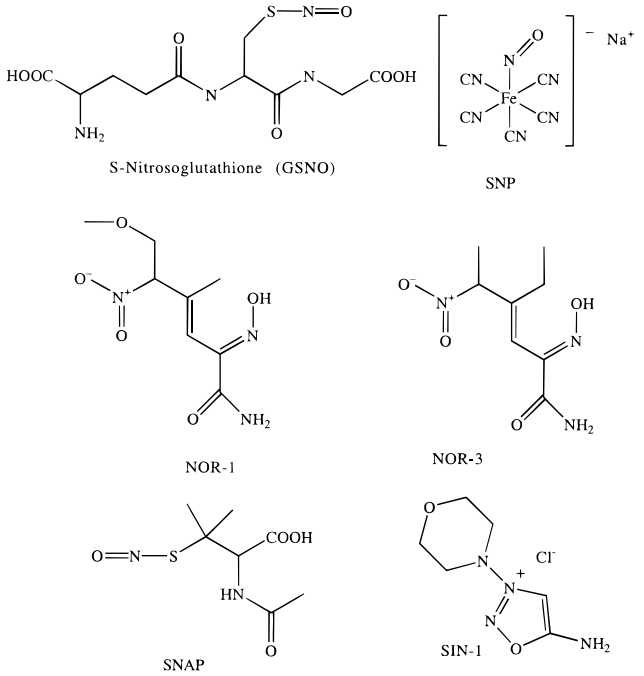


FIGURE 1: NO donors used in this study.

RESULTS AND DISCUSSION

The sensitivity of cathepsin K to inhibition by NO donors was tested with the *S*-nitrosothiols GSNO and SNAP and the non-thiol NO donors NOR-1, NOR-3, SIN-1, and SNP (Figure 1). Concentrated cathepsin K was pretreated with 1 mM DTT prior to the assay to ensure complete activation and then diluted such that the resulting concentration of DTT in the assay was 0.3 μM. The test compound and enzyme were then preincubated for periods of 1−80 min at pH 5.5 prior to the initiation of the reaction with the substrate Z-Phe-Arg-pNA. The NO donors were all concentration- (Figure 2A) and time-dependent (not shown) inhibitors of cathepsin K activity. After a preincubation period of 15 min, GSNO and NOR-1 were potent inhibitors, having IC_{50} values of approximately 5 μM (Table 1). SNAP and NOR-3 were weaker inhibitors (IC_{50} values of 72 and 40 μM, respectively). Less than 50% inhibition from SIN-1 and SNP was observed at 1 mM, the highest dose tested, although after an 80 min preincubation, both gave IC_{50} values of approximately 200−300 μM. Cathepsin K has a pH optimum of around 5.5 in vitro (*1*) and is about half as active at pH 7.5. Like other cathepsins (*31*, *32*), it is unstable at neutral pH in the absence of substrate and was rapidly ($t_{1/2} \approx 5$ min) inactivated under our preincubation conditions at pH 7.5. This compares with a half-life at pH 5.5 of approximately 45 min. However, to compare the effects of the NO donors against cathepsin K in a whole cell assay (which are described later and are conducted at neutral pH) with those against the purified enzyme, the titrations were repeated at pH 7.5.

Each NO donor was more potent against the purified enzyme, with the exception of the two *S*-nitrosothiols, when titrated at pH 7.5 (Figure 2B, Table 1), NOR-1 having an IC_{50} of 0.4 μM after a 15 min preincubation. The influence of pH on NO donor potency is most likely due to the increased rate of NO release at neutral pH. The decomposition of oximes such as NOR-1 and NOR-3 is pH dependent,

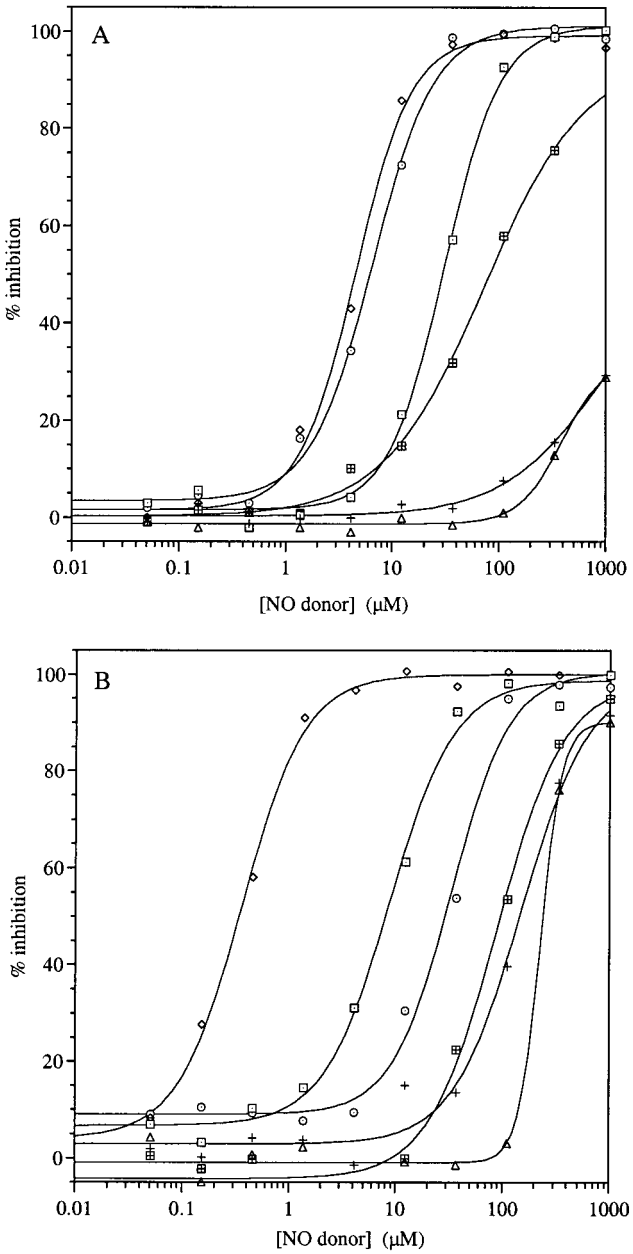


FIGURE 2: Titration of cathepsin K with NO donors at pH 5.5 and 7.5. Cathepsin K (11 nM) was preincubated in (A) 50 mM MES, 2.5 mM EDTA, pH 5.5, and 10% DMSO or (B) 50 mM sodium phosphate, 2.5 mM EDTA, pH 7.5, and 10% DMSO for 15 min with increasing concentrations of GSNO (⊙), SNAP (⊞), NOR-1 (◇), NOR-3 (⊡), SIN-1 (+), and SNP (△) prior to the addition of the chromogenic substrate Z-Phe-Arg-pNA (25 μM final concentration). Enzyme activity was followed at 405 nm for 5 min. Results are expressed as percentage inhibition compared to a vehicle-treated control.

these compounds being stable at pH <4, but releasing NO with $t_{1/2}$ values of approximately 1.7 and 30 min, respectively, at pH 7.4 (*27*, *33*). It has been established that NO will not react directly with thiols to form *S*-nitrosothiols under anaerobic conditions but must first be oxidized to species such as NO_2 and N_2O_3 (NO_x) (*34*−*36*). At pH 7.5 the inhibition of cathepsin K by NOR-1 was barely time dependent; only a 5-fold reduction in IC_{50} was observed between 1 and 15 min preincubation. This can be explained by the rapid oxidation of NO and other partially oxidized, potentially reactive species (NO_x) to nitrite. The concentration

Table 1: Effect of NO Donors on the Activities of Purified and Whole Cell Cathepsin K

	IC_{50} value (μM)[a]		
NO donor	purified cathepsin K, pH 5.5	purified cathepsin K, pH 7.5	whole (CHO) cell cathepsin K
GSNO	5	28	105
SNAP	72	105	107
NOR-1	6	0.4	13
NOR-3	40	10	20
SIN-1	>1000	90	53
SNP	>1000	270	336

[a] NO donor and cathepsin K or CHO cell expressing cathepsin K were preincubated for 15 min at the pH indicated prior to initiation of the reaction with substrate. Each value is the average of at least two determinations.

of reactive species released by NOR-1 would therefore quickly fall to zero under aerobic conditions at pH 7.5 (*34*). Both SIN-1 and SNP are also stable at pH 5 but decompose at neutral pH to release NO (*27*). SNP also requires light irradiation or a one-electron reduction to release NO. In our experiments light exposure was ambient and was not strictly controlled.

In contrast to the non-thiol NO donors, the *S*-nitrosothiols GSNO and SNAP are fairly stable at neutral pH, having half-lives of greater than 10 h (*29*). EDTA, which was present in our assay buffers, also reduces the rate of *S*-nitrosothiol decomposition by chelating trace amounts of Cu^{2+} which catalyze this process. The lack of correlation between the rate of NO release via homolysis from *S*-nitrosothiols and their biological effects has been explained by the observation that *S*-nitrosothiols act as NO^+ donors and undergo transnitrosylation reactions with thiols rather than release NO by homolysis (*13*, *29*).

The inhibitory activities of the above NO donors against cathepsin K activity in a whole cell were tested using CHO cells stably transfected with cathepsin K. The cells were pretreated with the NO donors for 15 min at pH 7.4 prior to the initiation of the reaction with the cell-permeable fluorogenic substrate $(Z\text{-Leu-Arg})_2$-rhodamine-110. As was the case with purified cathepsin K, NOR-1 was the most active inhibitor of whole cell cathepsin K activity (Table 1). SIN-1, which releases NO plus O_2^- (*27*) and inhibits bone resorption in an in vitro osteoclast assay (*12*), also has significant activity against cathepsin K in the whole cell assay. Titrations of whole cell cathepsin K activity with the non-NO releasing parent compounds, i.e., GSH, *N*-acetylpenicillamine, and NO-depleted NOR-1 and NOR-3, gave IC_{50} values for each compound of greater than 500 μM. Although an exact correlation between inhibition of whole cell and purified cathepsin K activities for each NO donor would not be expected due to differences in cellular permeability and whether the release of NO occurred in or outside the cell, the more potent inhibitors of purified cathepsin K were also more active in the whole cell. It should also be considered that the exact mechanism of inhibition of cellular cathepsin K by these NO donors may be different from that with the purified enzyme as NO could in principle be transferred to intracellular thiols or other protein or nonprotein carriers and hence to cathepsin K.

Cathepsin K is synthesized as a preproenzyme, and the proenzyme is believed to undergo autocatalytic activation

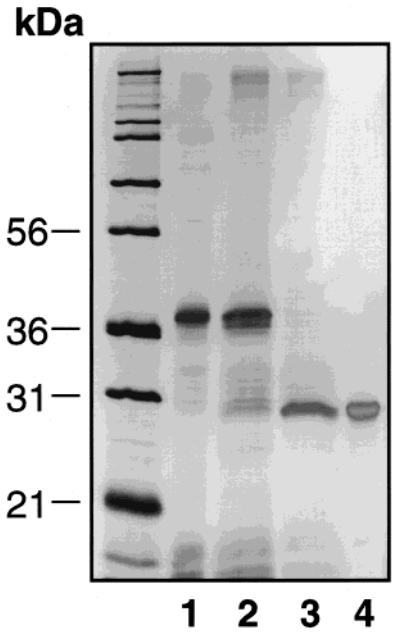


FIGURE 3: SDS−PAGE analysis showing inhibition by GSNO of the autocatalytic maturation of procathepsin K to cathepsin K. Procathepsin K was treated with vehicle or 100 μM GSNO in 0.2 M citric acid/phosphate buffer, pH 4.0, for 120 min at 25 °C and quenched with SDS sample buffer prior to analysis (0.5 μg) on a 12% Tris−glycine gel. The lanes represent (1) procathepsin K standard, (2) procathepsin K plus 100 μM GSNO, incubated at pH 4.0, (3) procathepsin K plus vehicle, incubated at pH 4.0, and (4) cathepsin K standard.

to the mature, catalytically active form in the acidic environment of the lysosome (*37*). Since NO donors can inhibit the activity of the mature form of cathepsin K, we performed experiments to determine whether the activity of the proenzyme is also similarly affected. When procathepsin K was incubated at pH 4.0 with 100 μM GSNO for 2 h, an almost complete inhibition of the autocatalytic processing compared to vehicle-treated control was observed (Figure 3). This effect was also blocked at pH 5.0 by 100 μM GSNO, but in this case the autocatalytic processing of the vehicle-treated control amounted to only approximately 20% (data not shown).

The above results show that either NO donors or *S*-nitroso species can act as inhibitors of cathepsin K, as well as its autocatalytic maturation, and indicate that NO and *S*-nitrosothiols may be able to directly modulate cathepsin K mediated bone resorption in vivo.

In light of the instability of cathepsin K at neutral pH the remainder of the experiments in this study were conducted at slightly acidic pH, conditions under which cathepsin K has maximal stability. The mechanism of the inhibition of cathepsin K by GSNO and NOR-1 was investigated in more detail in assays at pH 5.5 in which the inhibitor was premixed with the fluorogenic substrate Z-Phe-Arg-AMC prior to the initiation of the reaction with cathepsin K. As noted above, the inhibition of cathepsin K by GSNO is time and concentration dependent (Figure 4). Replotting the observed pseudo-first-order rate constants for the onset of inhibition versus inhibitor concentration gives a straight line passing near the origin (Figure 4 inset), consistent with a single-step reaction in which the reverse rate (obtained from the *y*-intercept) is close to zero. The slope of the line gives the apparent second-order rate constant for the onset of inhibition as 136 M^{-1} s^{-1}. This value was somewhat variable as a value

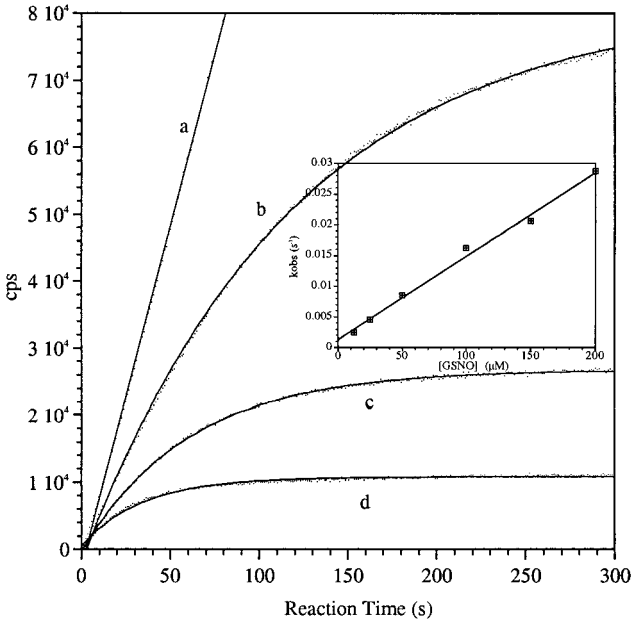


FIGURE 4: Time dependence of the inhibition of cathepsin K with GSNO. The *S*-nitrosothiol GSNO at 0 μM (a), 50 μM (b), 100 μM (c), and 200 μM (d) was premixed with the fluorogenic substrate Z-Phe-Arg-AMC (25 μM) in 50 mM MES, 2.5 mM EDTA, pH 5.5, and 10% DMSO and the time course of the reaction measured after the addition of cathepsin K (1 nM) using excitation and emission wavelengths of 365 and 440 nm, respectively. The solid lines represent the fitting of the data (in the presence of GSNO) to a single-exponential equation, from which the observed first-order inactivation rate constant (k_{obs}) was obtained. Inset: Plot of the values of the observed first-order inactivation rate constant (k_{obs}) versus concentration of GSNO. The line represents a linear fit to the data.

of 880 M^{-1} s^{-1} was obtained when the experiment was repeated a second time. The reversibility of this inhibition in the presence of thiols was determined in experiments in which cathepsin K was treated with 25 μM GSNO for 240 s prior to the addition of substrate followed by the addition of the test thiol. Cathepsin K was initially completely inhibited as determined by the absence of activity after the addition of substrate, but after the addition of 1 mM DTT the activity recovered in a first-order manner with a rate constant of 0.006 s^{-1}. After 500 s, 80% of the vehicle-treated control activity was recovered. Reactivation was also attempted with 5 mM GSH. In this case, reactivation was slower and only reached approximately 15% that of the control rate after 500 s.

The effect of NOR-1 on cathepsin K is also time dependent in experiments in which the reaction is initiated by the addition of cathepsin K. The rate at which cathepsin K activity declines will be a complex function of the rates of NO release from NOR-1, its oxidation to the reactive species (NO_x) (*34*, *35*), the rate of hydrolysis of NO_x to inactive species, and the rate at which the protein modification occurs. Under the conditions used here the half-time for inactivation of cathepsin K activity by 1500 μM NOR-1 at pH 5.5 was 38 s, giving an apparent second-order inactivation rate constant (k_{obs}/[I]) of 12 M^{-1} s^{-1}. In contrast to inhibition by GSNO, only approximately 20% of the enzyme activity was recoverable within 300 s upon treatment of NOR-1 inhibited enzyme with 5 mM DTT. The reversibility of GSNO and NOR-1 inhibition of cathepsin K activity was further examined in titrations conducted in 96-well plate format. Cathepsin K was preincubated at pH 5.5 (15 min) or pH 7.5 (2 min) with 0−1 mM GSNO or NOR-1 and the remaining activity measured after the addition of substrate. Following the completion of the measurement of activity (5 min), 5 mM DTT was added to each well and the plate incubated a further 15 min prior to the addition of fresh substrate for redetermination of enzyme activity. At both pH 5.5 and pH 7.5 the IC_{50} values for GSNO were increased approximately 100-fold following the addition of DTT, whereas less than 3-fold increases in IC_{50} values were observed for cathepsin K treated with NOR-1.

The reversibility of inhibition of cathepsin K expressed in CHO cells by GSNO and NOR-1 was also examined. Cells were treated for 15 min with 10 μM E-64, 200 μM GSNO, 500 μM NOR-1, and vehicle, and the remaining activity was determined by the addition of substrate. In parallel, another set of cells were treated with the same inhibitors, but after 15 min the cells were washed twice and incubated for 30 min prior to two more washes and the addition of substrate for the determination of cathepsin K activity. No reactivation of cathepsin K activity was observed for the irreversible cysteine protease inhibitor E-64 or for NOR-1. In contrast, complete activity was recovered for GSNO-treated cells. This difference in the ability of DTT to reactivate the inhibited enzyme suggests that the treatment of cathepsin K with the two types of NO donors results in different protein modifications.

To prepare sufficient protein to determine the identity of the modifications caused by GSNO and NOR-1, cathepsin K was subjected to ion-exchange chromatography under anaerobic conditions to remove stabilizing DTT. The protein was treated with an excess of GSNO or NOR-1 and rapidly rechromatographed to remove the excess NO donor. The inhibited protein was then analyzed for *S*-nitrosothiol content by displacement of NO with Hg^{2+}, followed by quantification of nitrite via formation of a diazo dye according to the method of Saville (*26*). For both GSNO and NOR-1 inhibited cathepsin K, less than 10% Hg^{2+}-displaceable NO was detected, showing that a stable *S*-nitrosothiol does not result from either NO donor treatment. A more sensitive fluorescence detection method also gave negative results (*38*).

The absence of formation of a cathepsin K *S*-nitrosothiol in the presence of GSNO or NOR-1 was surprising since treatment of the cysteine protease caspase-3 with SNAP did result in S-nitrosylation (*39*). Papain and calpain, two other cysteine proteases, were also reported to be inhibited by NO donors, the effects of which were attributed to S-nitrosylation (*20*, *40*, *41*). However, we could readily demonstrate S-nitrosylation of cathepsin K under denaturing conditions using acidified nitrite as evidenced by the appearance of new absorption bands at 330 and 550 nm which are characteristic of the *S*-nitrosyl group (*28*). It appears therefore that the tertiary structure of native cathepsin K causes the destabilization of the cysteine NO group, resulting instead in a different modification.

Evidence for thiol modification by the NO donors was obtained in experiments in which cathepsin K was inhibited by GSNO and NOR-1 and then dialyzed to remove the excess NO donor prior to analysis for remaining thiol content by DTNB. The results showed that GSNO and NOR-1 inhibited cathepsin K contained 52% and 41%, respectively, free thiol content of the vehicle-treated enzyme. As cathepsin

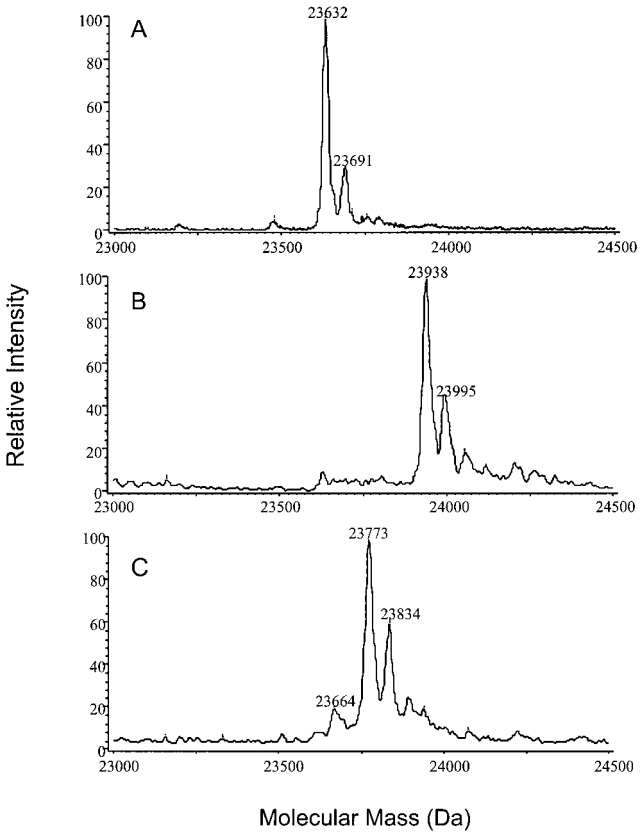


FIGURE 5: Identification of GSNO-dependent cathepsin K protein modifications by electrospray LC/MS. Cathepsin K (0.1 mg/mL) was treated with vehicle (A), 500 μM GSNO (B), or 500 μM NOR-1 plus 10 mM dimedone (C) prior to analysis (10 μL) by electrospray LC/MS as described in Materials and Methods. The MS spectra represent data from the maximal height of the LC peak and were deconvoluted using the Finnigan BioWorks software.

K contains two free thiols (*42*, *43*), the results indicate that inhibition by both NO donors results in the modification of a single thiol.

Electrospray LC/MS was used to investigate the identity of the protein modification resulting from treatment of cathepsin K with the NO donors GSNO and NOR-1. LC/MS of cathepsin K gave two peaks with masses of 23 630 $\pm$ 2 Da (major) and 23 690 $\pm$ 2 Da (Figure 5A). The masses of the peaks are the same as that predicted from the published amino acid sequence, with six of eight cysteine residues as disulfides (*42*, *43*), a Ser^{149} to Ala mutation (*42*), and N-terminal sequences of Arg^{114}APD...(23 630 Da) and Gly^{113}RAP...(23 687 Da) (*37*).

Cathepsin K treated with 500 μM GSNO at pH 5.5 showed a LC/MS spectrum similar to that of untreated enzyme except that both major peaks were shifted to approximately 306 Da higher mass (Figure 5B). The mass increase expected for the formation of a glutathione adduct is 305.3 Da, and therefore the results obtained are consistent with this species. The formation of a mixed disulfide in GSNO-treated cathepsin K is consistent with the almost complete recovery of enzymatic activity upon treatment with DTT. LC/MS of GSNO-treated cathepsin K which was then incubated with 5 mM DTT for 15 min showed the removal of GSH and the complete re-formation of unmodified cathepsin K (not shown). The formation of the cathepsin K−glutathione disulfide in the presence of GSNO was dependent on the NO moiety as treatment of cathepsin K with 0.3 or 2 mM GSH under the same conditions as above did not lead to any change in the LC/MS spectrum or inhibition of cathepsin K activity (data not shown).

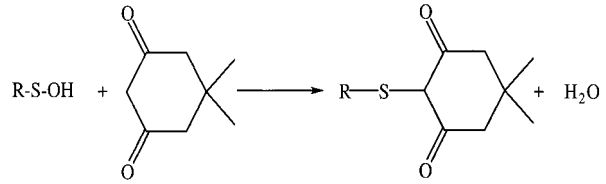
Further experiments were conducted to determine the site of the modification caused by GSNO. Cathepsin K is irreversibly inactivated by the epoxide-containing inhibitor E-64, and X-ray crystallography shows that this results in the covalent linking of the active site cysteine (Cys^{25} in the mature form with the N-terminal sequence Ala^{115}PDS) via opening of the inhibitor epoxide moiety (*43*). Treatment of cathepsin K, or cathepsin K in the presence of 2 mM DTT, with 20 μM E-64 (mass 357.4 Da) resulted in the formation of an adduct having a mass increase of approximately 360 Da (data not shown). When cathepsin K−glutathione disulfide (prepared from cathepsin K plus GSNO) was treated with 20 μM E−64, the mass of the resulting species was unchanged, showing that the glutathione disulfide blocked the effect of E-64 and providing good evidence that the active site Cys^{25} is the site of modification by GSNO. Conversely, cotreatment of the cathepsin K−glutathione disulfide with 2 mM DTT plus 20 μM E-64 resulted in a mass increase of approximately 359 Da over native protein, showing that removal of the glutathione disulfide allows the modification of Cys^{25} by E-64 to occur.

The LC/MS spectrum of cathepsin K treated with 500 μM SNAP was measured to determine whether the mixed disulfide formed with GSNO can also occur with other *S*-nitroso-containing compounds. In this case, after 15 min only 50% inhibition of cathepsin K activity was observed, and the LC/MS spectrum gave an almost equal mixture of species with approximate masses of 23 634 and 23 822 Da (data not shown). The lower mass species is that of unmodified cathepsin K, and the difference of approximately 188 Da is consistent with the addition of *N*-acetylpenicillanine (mass = 191 Da), showing that the formation of cathepsin K mixed disulfides is possible with other *S*-nitroso-containing species.

Analysis of NOR-1 inhibited cathepsin K for the presence of *S*-nitrosothiol by the Greiss reaction also gave a negative result, but in contrast to the effect of GSNO, this inhibition was only partially (10−20%) reversible. To determine the identity of this modification, cathepsin K was treated with 500 μM NOR-1. After 10 min, inhibition was complete, and the samples were frozen on dry ice until analysis by LC/MS (1−2 h). The resulting LC/MS spectrum showed two broad peaks corresponding to the addition of approximately 30−50 Da to both species present in native cathepsin K. Analyzing the MS data from the beginning and end of the LC peak showed sharper peaks at 23 664 and 23 678 Da, respectively, which correspond to mass increases of approximately 32 and 46 Da (data not shown). A mass increase of approximately 46 Da could be ascribed to cysteic acid formation ($-SO_3H$, +48 Da), whereas a mass increase of approximately 30 Da is likely due to sulfinic acid ($-SO_2H$, +32 Da) formation since S-nitrosylation (+30 Da) was ruled out by the results of the quantitation using the Greiss reaction.

To determine whether the cathepsin K sulfinic and sulfonic acids were formed via a sulfenic acid species (−S−OH), cathepsin K was coincubated with 500 μM NOR-1 plus 10 mM dimedone. Dimedone has been previously shown to react specifically with sulfenic acids to form a stable thioether

product (*44*).



The resulting LC/MS spectrum shows two major peaks that correspond to the addition of approximately 141 Da to each corresponding peak of native cathepsin K (Figure 5C). The increase in mass observed is within experimental error of that which would be expected (+138 Da) from the formation of a dimedone adduct and is clear evidence that NOR-1 treatment results in the oxidation of a single cysteine to sulfenic acid. The control, in which cathepsin K was incubated with 10 mM dimedone alone, showed no inhibition of activity and no change in LC/MS spectrum from that of native cathepsin K (data not shown). The enzymatic activity of the cathepsin K sample treated with NOR-1 alone could be reactivated approximately 20% in the presence of 2.5 mM DTT; however, less than 5% reactivation was obtained with the sample cotreated with NOR-1 plus dimedone, results also consistent with the formation of a stable dimedone thioether adduct in the latter case.

Thiols also react with sulfenic acids to give the mixed disulfide according to the reaction (*44*):

$$R-S-OH + R'-SH \rightarrow R-S-S-R' + H_2O$$

When cathepsin K was coincubated with 500 μM NOR-1 plus 2 mM GSH, the majority of cathepsin K was converted to the glutathione disulfide, thereby providing more evidence for the formation of a sulfenic acid.

The above experiments provide good evidence that the cathepsin K sulfinic and sulfonic acids produced in the presence of NOR-1 are formed via the corresponding sulfenic acid. Protein sulfenic acids are generally unstable and are readily oxidized irreversibly by molecular oxygen but can also be reduced back to the sulfhydryl by thiols and other reducing agents (*44, 45*). Since protein sulfenic acids can be prepared by the action of H_2O_2 (*44, 46, 47*), the possibility was considered that the cathepsin K sulfenic acid and glutathione disulfide observed here were due to the actions of peroxides generated in situ. When titrations of cathepsin K with NOR-1 and GSNO were repeated in the presence of 10 μg/mL catalase and superoxide dismutase, both alone and combined, less than a 5-fold increase in IC_{50} with both inhibitors was observed. These results show that it is unlikely that the effects of these inhibitors are due to oxidation events mediated by either H_2O_2 or O_2^-. These experiments also reduce the probability that the active species is peroxynitrite, formed by the reaction of NO and O_2^-. Peroxynitrite rapidly oxidizes protein and nonprotein thiols and can cause the S-nitrosylation of the active site thiol of glyceraldehyde-3-phosphate dehydrogenase (*48, 49*).

The possibility was also considered that the observed dimedone adduct produced in the presence of NOR-1 was formed via the nucleophilic attack of dimedone on the sulfur atom of cathepsin K −S−NO rather than cathepsin K −S−OH. However, under the conditions of our experiments this appears unlikely since 10 mM dimedone at pH 6.5 did not displace NO from either BSA-SNO or GSNO (see Materials and Methods).

The cathepsin K sulfenic acid formed in the presence of NOR-1 could be produced directly by reaction with NO (or NO_x) or via an initial *S*-nitroso species. A mechanism involving the direct reaction of NO under anaerobic conditions with the single thiol of BSA was proposed to account for the formation of a sulfenic acid species (*50*). In contrast, some evidence for an *S*-nitrosocysteine intermediate was obtained in the formation of a sulfenic acid of glutathione reductase in the presence of GSNO (*51*). Stamler and Hausladen (*16*) have proposed a reaction in which the ionized form of an *S*-nitrosothiol ($RS^+{=}NO^-$) is hydrolyzed to the sulfenic acid. Thus, literature precedents exist for the NO-dependent formation of a protein sulfenic acid with or without an *S*-nitrosothiol intermediate.

The most likely mechanism for the formation of the cathepsin K−glutathione disulfide is the direct nucleophilic attack of the cathepsin K thiolate on the sulfur of GSNO. This reaction, which will also produce HNO, has been proposed to account for the products obtained in the reaction of GSH with GSNO (*52*) and for the intramolecular reaction of *S*-nitrosothiols containing an adjacent thiol to form a disulfide (*28*).

$$Prot-SH + G-S-N{=}O \rightarrow Prot-S-S-G + HNO$$

The thiol modifications observed here with cathepsin K and the NO donors GSNO and NOR-1 are relatively novel, especially when compared to the number of reports which ascribe the effects of NO on proteins to S-nitrosylation (*13*). A glutathione adduct and sulfenic and sulfinic acids were detected in glutathione reductase treated with GSNO and $GS_2Fe(NO)_2$ (*51*). However, in this case the modifications were detected by X-ray crystallography, after a prolonged exposure to the NO donors under aerobic conditions, and it was considered that these modifications were due instead to the concomitant oxidative stress (*16*). In this work, the time between treatment with the NO donor and analysis was minimized and was likely on the same scale as studies in which protein S-nitrosylation has been identified. Moreover, EDTA was included in buffers to avoid the effects of contaminating metal ions, and SOD and catalase did not significantly affect the inhibition by GSNO or NOR-1. Thus, the observed glutathione adduct and sulfenic acid are not likely due to oxidative stress and may reflect a physiologically relevant modification in response to *S*-nitrosothiols and NO.

A recent report of work using a NO electrode with intact osteoclasts (*10*) showed that these cells can produce local concentrations of approximately 1 μM NO in response to stimulation of constitutive NO synthase with NADPH. The NO donor NOR-1 gave an IC_{50} of 13 μM against whole CHO cell cathepsin K activity (Table 1). Recalling that this IC_{50} reflects the concentration of the parent NO donor and that the concentration of NO itself will be considerably lower indicates that cathepsin K is inhibited in vivo by physiologically relevant concentrations of NO.

In vivo, the GSNO inhibition of cathepsin K may represent a reversible switch that could be turned on and off depending on the glutathione redox state of the cell as glutathione disulfides are reducible to the free thiol by glutaredoxin (*54*).

Under certain circumstances a cathepsin K sulfenic acid could also represent a reversibly inhibited form via conversion to the glutathione disulfide by glutathione (*44*) or direct reduction by the thioredoxin system (*55*).

The S-nitrosylation of cathepsin K was readily detectable under denaturing conditions, indicating that the native protein structure stabilizes the sulfenic acid and glutathione disulfide relative to the *S*-nitrosothiol. The same features that stabilize sulfenic acids, namely, an absence of vicinal thiols, the presence of a H-bond acceptor or the ionization of −SOH, and an apolar environment or solvent inaccessibility, also stabilize protein *S*-nitrosothiols (*16*). Interestingly, both papain, which shares the same catalytic triad (*53*) as cathepsin K, and glutathione reductase have been shown to form stable sulfenic acids (*56, 57*). Now, cathepsin K and glutathione reductase are two proteins in which sulfenic acid modifications in response to NO have been identified. Perhaps stabilization of the ionized form of an initially formed protein *S*-nitrosothiol ($R-S^{+}=N-O^{-}$) facilitates its hydrolysis to the sulfenic acid in these cases. In the case of cathepsin K the *S*-nitrosothiol may be stabilized by His^{59}, which forms a salt bridge with the active site thiolate anion.

The paucity of other examples in the literature of the formation of glutathione adducts and sulfenic acids due to interactions with *S*-nitrosothiols and non-thiol NO donors may in part be due to the difficulty in their detection and their lability. Both modifications are reversible with DTT, which is also the case with the more frequently described *S*-nitrosothiol. Thus, reactivation by DTT should not be taken as evidence alone for the formation of this species. The recently described use of NBD-Cl as a spectral probe to identify sulfenic acids (*46, 47*) and the use of other spectroscopically silent reagents such as dimedone in conjunction with mass spectroscopy techniques as described here should facilitate the identification of these species.

While this paper was in the final stages of preparation, a report appeared on the inhibition of glyceraldehyde-3-phosphate dehydrogenase by NO donors (*58*). When GAPDH was treated with BF_4NO, an NO^{+} donor, results consistent with protein S-nitrosylation were obtained. Treatment instead with GSNO resulted in the formation of a glutathione mixed disulfide. The authors also considered that this reaction was due to the direct attack of the highly reactive active site thiolate on the sulfur of GSNO.

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