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Cellular uptake mechanisms and responses to NO transferred from mono- and poly-S-nitrosated human serum albumin

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Abstract

Endogenous *S*-nitrosated human serum albumin (E-Mono-SNO-HSA) is a large molecular weight nitric oxide (NO) carrier in human plasma, which has shown many beneficial effects in different animal models. To construct more efficient SNO-HSA preparations, SNO-HSA with many conjugated SNO groups has been prepared using chemical modification (CM-Poly-SNO-HSA). We have compared the properties of such a preparation to those of E-Mono-SNO-HSA. Cellular uptake of NO from E-Mono-SNO-HSA partly takes place via low molecular weight thiol, and it results in cytoprotective effects by induction of heme oxygenase-1. By contrast, transfer of NO from CM-Poly-SNO-HSA into the cells is faster and more pronounced. The influx mainly takes place by cell-surface protein disulfide isomerase. The considerable NO inflow results in apoptotic cell death by ROS induction and caspase-3 activation. Thus, increasing the number of SNO groups on HSA does not simply intensify the cellular responses to the product but can also result in very different effects.

Keywords: *nitric oxide, S-nitrosation, cell-surface protein disulfide isomerase, cytoprotection, apoptosis*

Abbreviations: *HSA, human serum albumin; SNO-HSA, S-nitrosated HSA; E-Mono-SNO-HSA, endogenous mono-SNO-HSA (Cys-34-SNO, < 1 mol SNO/mol HSA); CM-Poly-SNO-HSA, chemically modified poly-SNO-HSA (>1 mol SNO/mol HSA); cs-PDI, cell-surface protein disulfide isomerase.*

Introduction

The sulfhydryl moiety can interact with nitric oxide (NO) and thereby form *S*-nitrosothiols [1-3]. *S*-nitrosothiols may function as NO reservoirs, and preserve the antioxidant and other activities of NO [4,5]. For example, it has been reported that endogenous mono *S*-nitrosated human serum albumin (E-Mono-SNO-HSA, Fig. 1) may serve *in vivo* as a circulating reservoir for NO produced by the endothelial cells [6]. The reservoir function was also reported to be operative when application of E-Mono-SNO-HSA to animals suffering from ischemia-reperfusion injury minimized the extent of tissue damage associated with reperfusion [7-9]. Therefore, E-Mono-SNO-HSA is under investigation as a therapeutic agent in humans.

However, E-Mono-SNO-HSA could have some drawbacks— the most important of which is that the number of NO molecules bound to HSA (<1 SNO group) is limited— because HSA has only one free cysteine residue (Cys-34) available for conjugation. Therefore, some research groups have attempted to synthesize Poly-SNO-bovine serum albumin

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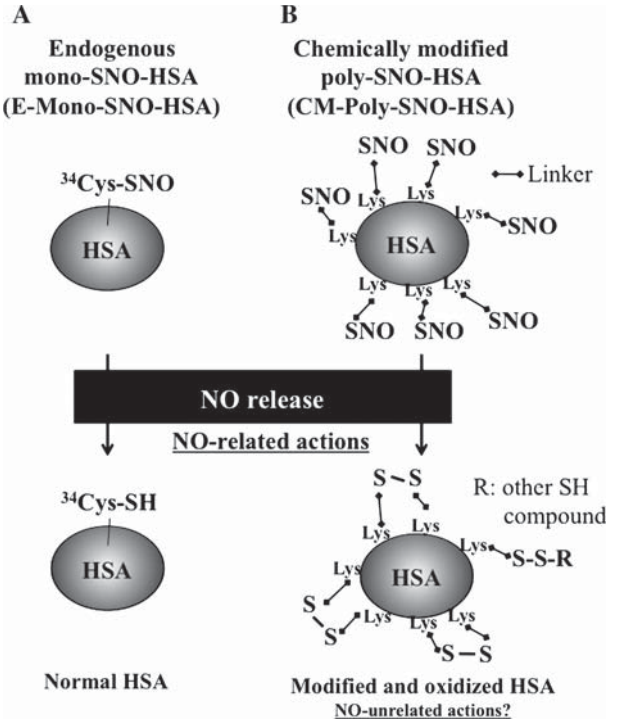

Figure 1. Scheme showing simplified structures of E-Mono-SNO-HSA and CM-Poly-SNO-HSA before and after NO release. E-Mono-SNO-HSA possesses one SNO group at position Cys-34, and after NO release it turns into normal HSA. By contrast, CM-Poly-SNO-HSA has approximately 7 SNO groups linked to lysine residues by chemical spacers such as 2-imminothiolane. After NO release (NO-related action), the spacers still possess the sulfur atom from the SNO groups and therefore can oxidize to different disulfides. Because the latter albumin form could have biological effects, the CM-Poly-SNO-HSA species may exert both NO-related and NO-unrelated activities.

(Poly-SNO-BSA) [10,11] in which several *S*-nitrosothiols were formed in BSA after reduction of the intramolecular disulfide linkages. However, Poly-SNO-BSA derivatives synthesized in this manner easily aggregated because of the formation of intermolecular disulfide linkages during synthesis. Other research groups (including our group) have attempted to produce chemically modified CM-Poly-SNO-HSA (or CM-Poly-SNO-BSA) using a chemical spacer such as 2-imminothiolane or *N*-Succinimidyl *S*-acetylthioacetate [12-14]. These chemical spacers were successful in attaching many NO groups via an *S*-nitrosothiol linkage to HSA (Fig. 1). It is possible that such CM-Poly-SNO-HSA preparations have the potential for anti-tumor and cytoprotective activity through NO release.

Recently, the important issue about the characterization of CM-Poly-SNO-HSA was raised by Tsikas in a letter published by Nitric Oxide [15]. For example, CM-Poly-SNO-HSA may possess both NO-related and NO-unrelated actions, because CM-Poly-SNO-HSA turns into chemically modified and oxidized forms of HSA after NO release (Fig. 1). Thus, the biological equivalence between E-Mono-SNO-HSA and CM-Poly-SNO-HSA should be examined, and CM-Poly-SNO-HSA should be characterized further before clinical application of such SNO-HSAs.

In the present work, we synthesized E-Mono-SNO-HSA by incubating HSA with isopentyl nitrite and CM-Poly-SNO-HSA and using 2-imminothiolane as the spacer. The NO release from the two preparations and the effects thereof were studied both *in vitro* and in cell cultures, and they were found to be quantitatively as well as qualitatively different. We also found strong evidence for the existence of cell surface-protein disulfide isomerase (cs-PDI) and for its involvement in NO transfer to the cells.

Methods

Materials

20% recombinant HSA solutions were donated by the Nipro Pharmaceutical Research Center (Shiga, Japan). The albumin was defatted by treatment with charcoal as described by Chen [16], dialyzed against deionized water, freeze-dried, and then stored at -20°C until used. According to density analysis of Coomassie Brilliant Blue (CBB)-stained protein bands on 12.5% SDS-PAGE, the purity of albumin in the original solutions and the defatted samples was more than 97%. 4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) used as a NO detector was purchased from Daiichi Pure Chemicals. Traut's Reagent (2-imminothiolane) was purchased from Pierce Chemical Co. (Rockford, IL, USA). Isopentyl nitrite was purchased from Wako Pure Chemical Industries (Osaka, Japan). Sulfanilamide, naphthylethylenediamine-hydrochloride, $HgCl_2$ and $NaNO_2$ were obtained from Nakalai Tesque (Kyoto, Japan). *S*-Nitrosoglutathione (GS-NO), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and diethylenetriaminepentaacetic acid (DTPA) were obtained from Dojindo Laboratories (Kumamoto, Japan), and 1,4-dithiothreitol (DTT) was from Sigma-Aldrich (St. Louis, MO). Other chemicals were of the best grades commercially available, and all solutions were made in deionized water. Sephadex G-25 desalting column (φ 1.6 × 2.5 cm) was from Amersham Pharmacia Biotech (Tokyo, Japan).

Synthesis of E-Mono-SNO-HSA, CM-Poly-SNO-HSA and N-ethyl-maleimide- (NEM-)HSA

For preparing CM-Poly-SNO-HSA, linkers with terminal sulfhydryl groups were added to the HSA molecule by incubation of 0.15 mM recombinant HSA with 3 mM Traut's Reagent (2-imminothiolane) in 100 mM potassium phosphate buffer containing 0.5 mM DTPA (pH 7.8) for 1 h at room temperature. After incubation, the number of sulfhydryl groups

of the resultant modified HSA, 4-imino-butane-1-thiol-modified HSA, was determined from a standard curve prepared with cysteine using the DTNB assay. The modified HSA was then divided into two portions: one was concentrated, exchanged with saline, adjusted to 2 mM ,and designated as control HSA, and the other was *S*-nitrosated via 3 h incubation with 15 mM isopentyl nitrite at room temperature. The resulting CM-Poly-SNO-HSA was concentrated and exchanged with saline using a PelliconXL filtration device (Millipore Corporation, Billerica, MA, USA). The final concentration was adjusted to 2 mM CM-Poly-SNO-HSA. This sample and the designated control HSA were stored at -80°C until use.

E-Mono-SNO-HSA was prepared according to previous reports [9,17]. In brief, protein (300 μM) was incubated with DTT (molar ratio, protein/DTT = 1:10) for 5 min at 37°C. DTT was then quickly removed by Sephadex G-25 gel filtration and eluted with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mM DTPA. Samples of DTT-treated proteins (0.1 mM) were incubated with isopentyl nitrite (molar ratio, protein/isoamyl nitrite = 1:10) in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mM DTPA for 60 min at 37°C. E-Mono-SNO-HSA was purified by Sephadex G-25 gel filtration, eluted with pure water, and concentrated by ultrafiltration (cut-off size of 7,500 Da). The sample was stored at -80°C until use.

The number of *S*-nitroso moieties on CM-Poly-SNO-HSA and E-Mono-SNO-HSA were determined using HPLC coupled with a flow reactor system, as previously reported [18,19]. The number of moles of NO per mole of protein was estimated using $NaNO_2$ standard; the values obtained for CM-Poly-SNO-HSA and E-Mono-SNO-HSA were 6.6 ± 0.5 and 0.3 ± 0.02 mol NO/mol HSA, respectively [9,12,13]. We have previously demonstrated that the *S*-nitroso site on Mono-SNO-HSA is Cys-34. However, the *S*-nitroso sites of Poly-SNO-HSA have not been identified, but 2-iminothiolamine, which is used as spacer, can react with lysine residues on the surface of HSA. Therefore, we suppose that the *S*-nitroso sites of Poly-SNO-HSA are Lys-159, Lys-199, Lys-281, Lys-439, Lys-444, Lys-500 and Lys-525. We suggest that the chemical nomenclatures of Mono-SNO-HSA and Poly-SNO-HSA are *S*-nitroso HSA and *S*-nitroso iminothiolated HSA, respectively. Other characteristics of mono-SNO-HSA and poly-SNO-HSA are given in Table I.

To synthesize Cys-34-blocked HSA, which thus is not able to interact with NO, NEM-HSA was prepared by incubating HSA with NEM (molar ratio, HSA/NEM = 1:5) dissolved in phosphate-buffered saline (pH 7.4) for 60 min at 35°C in dark. Excess NEM was removed by ultrafiltration [20].

Cell culture

Murine colone 26 (C26) cells were maintained in culture in 75-cm^2 polystyrene flasks (Costar) with 15 mL of RPMI 1640 medium supplemented with 10% fetal bovine serum. Human hepatoma cells HepG2 were maintained in culture in 75-cm^2 polystyrene flasks with 15 mL of Dulbecco's modified Eagle's medium liquid (DMEM) containing 5 mL/500 mL of Pen-Strep and 10% of fetal bovine serum. The cells were kept in a humidified incubator (95% air, 5% CO_2).

DAF-FM DA method

Measurement of intracellular NO production in C26 cells, seeded onto 96-well plates, was done by monitoring changes in the fluorescence of DAF-FM [20]. Cells were incubated with 5 μM DAF-FM DA for 30 min at 37°C, and then washed twice with 10 mM phosphate-buffered saline, pH 7.4 (PBS). Treated cells were reacted with various concentrations of *S*-nitrosothiols in PBS for 1 h at 37°C. During this reaction, the fluorescence was monitored every 5 min with excitation at 385 nm and monitored at 535 nm using a monochromator (TECAN SPECTRA FLUOR). After the reaction, we also observed the fluorescence using fluorescence microscopy Biozero (Keyence, Osaka, Japan). In some experiments, 1 mM of bacitracin (protein disulfide isomerase inhibitor) dissolved in PBS was added after 30 min of DAF-FM DA exposure.

Determination of CM-Poly-SNO-HSA stability

The amount of CM-Poly-SNO-HSA in C26 cell culture medium was quantified by HPLC coupled with a flow-reactor system, as previously reported [9,18]. Samples were collected every 10 min after adding CM-Poly-SNO-HSA to the cells. The HPLC column was a gel filtration column for *S*-nitrosated proteins ($\phi 8 \times 300$ mm), Diol-120, YMC, Kyoto, Japan. Briefly,

Table I. Chemical characterization of mono-SNO-HSA and poly-SNO-HSA.

	mol SH/ mol HSA	mol SNO/ mol HSA	SNO site/sites	Half lifiss ($T_{1/2}$) of SNO In PBS at 25°C (day)	Decomposition product
EL-Mono-SNO-HSA	0.78 ± 0.13	0.3 ± 0.02	Cys-34	23 ± 2	HSA
CM-Poly-SNO-HSA	$8.61 = 0.51$	6.6 ± 0.5	Cys-34 and 4-imino-butanc-1-tliiol	21 ± 3	4-imino-butanc-l-thiol-modificd HSA

the eluate from the HPLC column was mixed with a $HgCl_2$ solution to decompose SNO compounds to yield NO_2^- [9]. The NO_2^- generated was then detected after reaction with Griess reagent in the flow–reactor system.

Immunofluorescence

Measurement of cs-PDI expression in C26 cells, seeded onto 8-well chamber slides, was done using fluorescence microscopy Biozero (Keyence, Osaka, Japan). The cells were washed twice with PBS, and then fixed in 4% paraformaldehyde for 30 min. The cells were either reacted directly with antibodies (non-permeabilized) or permeabilized with 0.1% Tween 20 before reaction with antibodies. Rabbit polyclonal antibody against Calnexin (cell signaling) and mouse monoclonal antibody against cs-PDI (Alexis Biochemicals) were used at 1:50 and 1:100 dilutions, respectively. Anti-rabbit IgG antibody conjugated with Alexa Fluor 594 and anti-mouse IgG antibody conjugated with Alexa Fluor 488 were used at 1:100 dilutions.

LDH assay

Cell death was evaluated by measuring lactate dehydrogenase (LDH) release from damaged cells. The LDH release was measured by assaying enzyme activity [21]. The LDH assay (Wako Pure Chemical, Osaka, Japan) was performed according to the instructions provided by the manufacturer. In brief, 5×10^4 C26 cells were incubated with 50 μM SNOs of each SNO-HSA in PBS for 12 h at 37°C. Then ten times diluted samples of the culture medium were added to the enzymatic reaction buffer containing lithium lactate, NAD, diaphorase, and nitro blue tetrazolium (NBT) dye and allowed to proceed for 30 min at 25°C. The absorption values at 560 nm were determined. The amount of released LDH (%), which represent cytotoxicity, was calculated according to the formula:

$$\frac{\text{LDH activity (sample) - LDH activity (negative)}}{\text{LDH activity (positive) - LDH activity (negative)}} \times 100$$

where 'LDH activity (negative)' represents absorbance at 560 nm of control cells incubated with HSA alone, and 'LDH activity (positive)' represents the activity of total input of LDH which was measured as described above after lysis of cells with 0.1% Triton X-100.

FACS analysis

C26 cells (1×10^6 cells/mL) were treated with CM-poly-SNO-HSA (50 μM of SNO) for 6 h in dark and then washed with PBS three times. The number of apoptotic cells was determined with an annexin V-fluorescein isothiocyanate (FITC) binding assay kit from BD Biosciences (Tokyo, Japan). The fluorescence of annexin V-FITC was measured via a FACSCalibur flow cytometer (BD Biosciences) [9]. For blocking cs-PDI, cells were incubated with 1 mM bacitracin for 30 min at 37°C before the SNO-HSA treatment.

Western blot analysis

HepG2 cells (1×10^6 cells/mL) were treated with 10 μM or 50 μM SNOs of SNO-HSAs for 18 h in dark. For preparation of whole HepG2 cell lysates, SNO-HSAs-treated cells were harvested in whole cell lysis buffer [10 mM Tris-HCl (pH 7.9), 250 mM NaCl, 30 mM sodium PPi, 50 mM sodium fluoride, 0.5% Triton X-100, 10% glycerol, 1 × proteinase inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 μM Na_3VO_4, 5 μM $ZnCl_2$,and 2 mM indole acetic acid] for 30 min on ice. Lysates were then collected by centrifugation at 15,000 × g for 30 min. Equal amounts (30 μg) of protein from the supernatant were loaded onto 12.5% SDS-PAGE gels and blotted onto polyvinylidine difluoride membranes (Millipore). Polyclonal antibody against rat heme oxygenase-1 (HO-1; StressGen) and horseradish conjugated secondary antibody against β-actin (Jackson ImmunoResearch) were used at 1:2,000 and 1:5,000 dilutions, respectively. The ECL Western blotting system (Amersham Life Sciences) was used for detection. Bands were visualized and quantified using a lumino-analyzer (LAS-1000, Fuji photo Film) [22].

Determination of S-transnitrosation efficiency from SNO-HSAs to cysteine

To measure *S*-transnitrosation to cysteine, 50 μM SNO-HSAs were reacted with 50 μM cysteine in PBS containing 0.5 mM DTPA, at 37°C for 10 min in dark. The amounts of the *S*-nitroso moiety of SNO-HSAs and SNO-cysteine were quantified by HPLC coupled with a flow-reactor system, as previously reported [18,19]. The HPLC column was a gel filtration column for SNO-HSAs (ϕ 8 × 300 mm), Diol-120. Briefly, the eluate from the HPLC column was mixed with a $HgCl_2$ solution to decompose SNO compounds to yield NO_2^- (via NO^+). The NO_2^- generated was then detected after reaction with Griess reagent in the flow-reactor system. The amount of the endogeous NO_2^- was quantified by this HPLC system without a $HgCl_2$ solution.

Statistical analysis

The statistical significance of collected data was evaluated using the ANOVA analysis followed by Newman–Keuls method for more than 2 means. Differences between the groups were evaluated by

the Student's t test. $P < 0.05$ was regarded as statistically significant.

Results

NO traffic properties of SNO-HSAs into C26 cells

Transfer of NO from *S*-nitrosothiols into C26 cells were evaluated by the DAF-FM DA fluorescence assay. The principle of the assay is that upon cellular uptake, ester groups in DAF-FM DA are cleaved by esterases, resulting in the formation of DAF-FM that can react with NO to give fluorescence. Immediately after adding the *S*-nitrosothiols to the cells (0 min, n = 4), the fluorescence intensities were 810 ± 23 (*S*-nitrosoglutathione), 982 ± 34 (E-Mono-SNO-HSA), and 1102 ± 44 (CM-Poly-SNO-HSA);

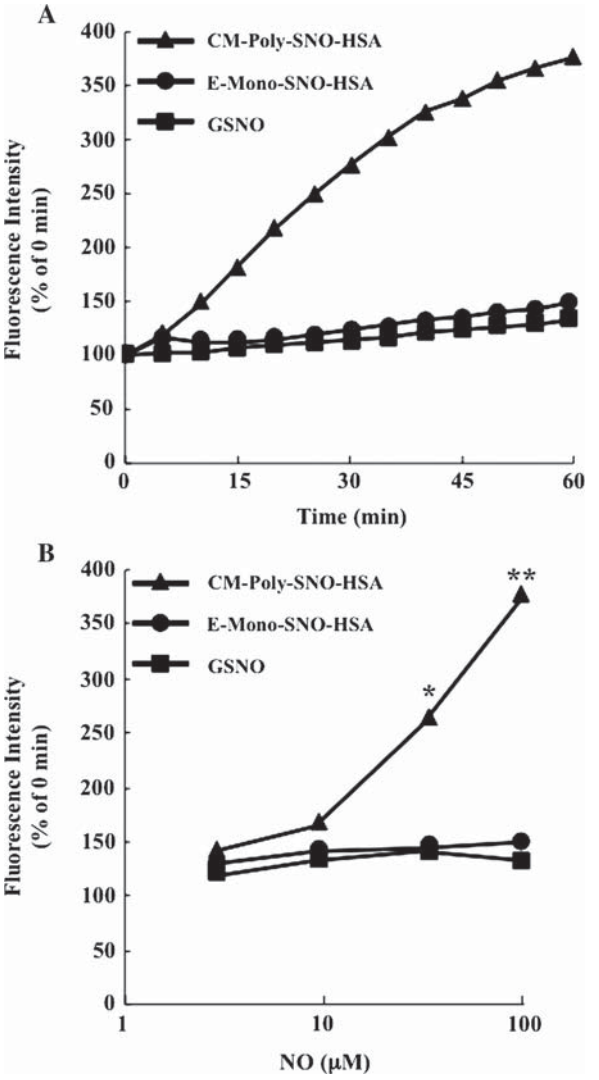

Figure 2. Transfer of NO from E-Mono-SNO-HSA and CM-Poly-SNO-HSA into C26 cells. The intracellular NO concentration was determined using DAF-FM fluorescence. (A) 100 μM SNO of E-Mono-SNO-HSA, CM-Poly-SNO-HSA or *S*-nitrosoglutathione was incubated with DAF-FM DA-treated cells at 37°C for 60 min. Fluorescence was measured at 5 min intervals. The time course curves were obtained from an average of five repeated independent experiments. (B) DAF-FM DA-treated cells were incubated with various concentrations of E-Mono-SNO-HSA, CM-Poly-SNO-HSA or *S*-nitrosoglutathione (SNO: 5-100 μM) at 37°C for 60 min. Fluorescence was measured at 60 min. Data are expressed as means ± SEM (n = 4). (A and B) During incubation with the SNO-HSAs, NEM-modified HSA was added to the media containing CM-Poly-SNO-HSA to give the same protein concentration as that used in the case of E-Mono-SNO-HSA (300 μM). *, $p < 0.05$, **, $p < 0.01$, compared with cells which had not been treated with E-Mono-SNO-HSA.

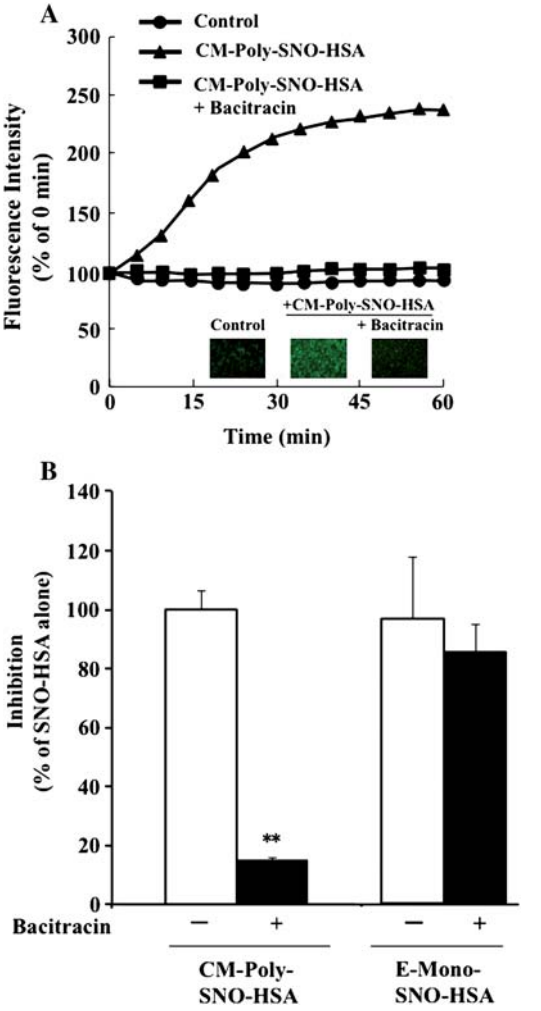

Figure 3. Effect of bacitracin on the NO traffic activity of CM-Poly-SNO-HSA and E-Mono-SNO-HSA into C26 cells. 50 μM SNO of CM-Poly-SNO-HSA or E-Mono-SNO-HSA was incubated with DAF-FM DA-treated cells at 37°C for 60 min. To investigate the effect of cs-PDI on the NO traffic activity of the SNO-HSAs, some of the DAF-FM DA-treated cells had reacted with 1 mM bacitracin at 37°C for 30 min before incubation with the SNO-HSAs. (A) DAF-FM DA-treated cells were reacted with control HSA (●) or 50 μM SNO of CM-Poly-SNO-HSA with bacitracin (■) or 50 μM SNO of CM-Poly-SNO-HSA without bacitracin (▲) in PBS for 1 h at 37°C. The fluorescence time course was measured at 5 min intervals. The fluorescence stainings in the inset to (A) show the DAF-FM fluorescence 60 min after treatment with HSA (control) or CM-Poly-SNO-HSA with or without bacitracin. The time course curves were obtained from an average of five repeated independent experiments. (B) The bacitracin inhibition of the NO traffic activity of SNO-HSAs was quantified using DAF-FM fluorescence intensity after 60 min of incubation. Data are expressed as means ± SEM (n = 4). **, $p < 0.01$, compared with cells which had not been treated with bacitracin.

these values represent 100% fluorescence intensity in Fig. 2A. Because the fluorescence intensity of cells, which had not been exposed to an *S*-nitrosothiol, was only 512 ± 22, it is apparent that treatment of the cells with E-Mono-SNO-HSA or *S*-nitrosoglutathione resulted in a fast uptake of NO, the amount of which did not increase much during incubation for 60 min (Fig. 2A). Incubation with CM-Poly-SNO-HSA also resulted in a fast uptake of NO, but in this case the amount of intracellular NO increased with time for 60 min, and reached a level that is about three times higher than that obtained for E-Mono-SNO-HSA or *S*-nitrosoglutathione. Principally the same trend was observed when studying cellular NO uptake from different SNO-HSA concentrations, i.e. a clear dose-dependent NO transfer from CM-Poly-SNO-HSA but an almost dose-independent uptake from E-Mono-SNO-HSA (Fig. 2B). The difference in albumin concentration between the two types of SNO-HSAs was compensated for by adding NEM-treated HSA, an albumin form that does not interfere with NO-transfer. This was also done in the following experiments.

Effect of cell-surface protein disulfide isomerase (cs-PDI) on NO traffic from SNO-HSAs into C26 cells

To clarify whether cs-PDI is important for the transfer of NO from extracellular SNO-HSAs into C26 cells, we studied the effect of adding bacitracin, which is a PDI inhibitor. From Fig. 3A, it can be seen that pre-incubation with bacitracin almost completely inhibits the transfer of NO from extracellular CM-Poly-SNO-HSA. The inset to Fig. 3A shows clear DAF-FM fluorescence in the C26 cells after 60 min incubation with CM-Poly-SNO-HSA but only a trace, when bacitracin was added. This finding is given in a quantitative manner in Fig. 3B, which shows an inhibition of NO transfer of 90%, approximately. By contrast to CM-Poly-SNO-HSA, the effect of bacitracin on NO transfer from E-Mono-SNO-HSA was only about 10% (Fig. 3B). We studied the NO transfer from CM-Poly-SNO-HSA further and found that pre-incubation of the cells with neutralizing anti-PDI mAb (RL90) and PAO, CXXC motifs inhibitor diminished NO traffic (Supplementary Figure 1). Thus, it is most likely that PDI is situated on the cell surface, and that it is directly involved in NO transfer from CM-Poly-SNO-HSA to C26 cells. To test for any effect of chemically modified HSA (CM-HSA) on the DAF-FM DA assay, we removed the NO groups from CM-poly-SNO-HSA by UV exposure. Afterwards, we added the resulting CM-HSA preparation to the DAF-FM DA-treated cells. The results obtained with CM-HSA are very similar to those obtained with control HSA, i.e. no detectable uptake of NO (Supplementary Figure 1). To investigate the stability of CM-poly-SNO-HSA in the medium, we measured its time-dependent concentration in cell supernatants (Supplementary Figure 2). The results show that bacitracin can completely inhibit simple degradation of CM-poly-SNO-HSA, indicating that its decrease in concentration mainly is caused by a PDI-dependent action, and that degradation of CM-poly-SNO-HSA by other factors within 60 min can be ignored.

To clarify more whether the involvement of PDI and the release of NO are really taking place on the cell surface and not in the endosomal compartment, we did additional experiments using two more PDI inhibitors. Thus, we measured NO uptake from

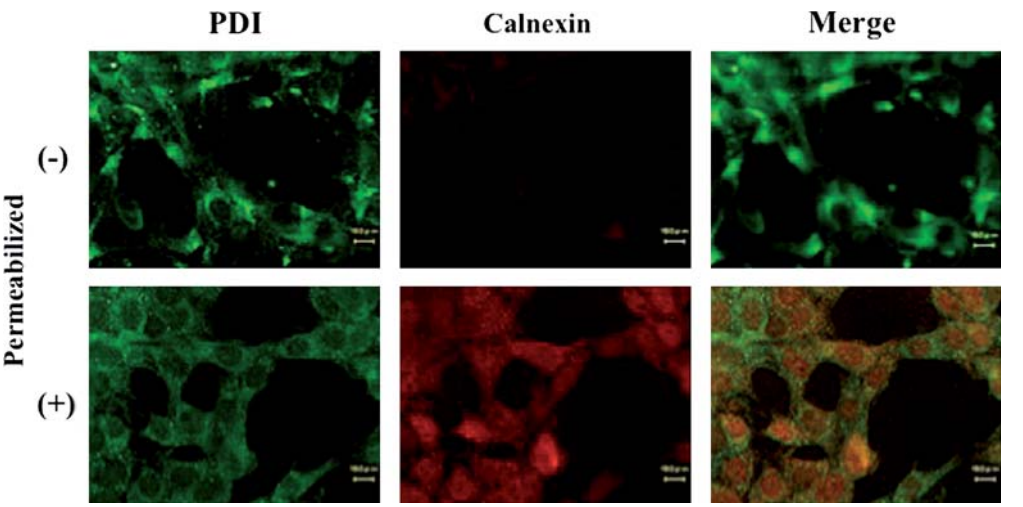

Figure 4. cs-PDI expression in C26 cells. The locations of cs-PDI in C26 cells were detected using fluorescence microscopy. cs-PDI is found on the cell surface, because mouse monoclonal antibody against cs-PDI reacts with intact cells (- permeabilisation with Tween 20). However, the antibody is also able to detect cs-PDI within the cells (+ permeabilisation). The intracellular location is the endoplasmic reticulum, because the antibody co-localizes with rabbit polyclonal antibody against calnexin, which is a marker for that organel. A control showed that calnexin is not associated with the cell surface (- permeabilisation). Green and red signals indicates cs-PDI and calnexin expression, respectively.

CM-poly-SNO-HSA in the presence of phenylarsine oxide (PAO) or neutralizing RL90. It is well-known that (i) bacitracin acts as a general PDI competitive inhibitor via redox thiols, (ii) PAO forms coordination bonds via As (III) with dithiols such as those from CXXC thioredoxin motifs, including PDI, and (iii) RL90 is directed against an epitope containing active site thiols of PDI on the cell surface [23]. Therefore, RL90 is a more specific inhibitor of PDI on cell surfaces. Supplementary Figure 1B shows that uptake of NO from CM-poly-SNO-HSA is strongly inhibited by PAO or RL90 pretreatment, supporting the proposal that PDI on the cell surface plays an important role for NO uptake from CM-poly-SNO-HSA.

Next, we investigated in a more direct way whether PDI can be found on the C26 cell surface. It is known that PDI acts as a chaperone molecule in the endoplasmic reticulum, where it catalyses protein thiol exchange reactions, but the expression and function of PDI on the C26 cell surface is still unclear. Treatment of the cells with fluorescent RL90 showed that the enzyme is indeed on the cell surface (Fig. 4). Additionally, if the cells were made permeable by treatment with the detergent Tween 20, then the antibody was also found intracellularly. The location could be identified as the endoplasmic reticulum, because the antibody co-localized with a fluorescent antibody raised against calnexin.

E-Mono-SNO-HSA and CM-Poly-SNO-HSA treatment result in different cellular responses

Next, we investigated whether the SNO-HSAs can promote cytotoxity (caused by apoptosis) in the C26 cells and cytoprotection (caused by HO-1 induction) in the HepG2 cells. In our previous studies, we found that CM-poly-SNO-HSA possesses cell death activities (cytotoxic activity) via apoptosis [12,13]. To evaluate the mechanism of apoptosis by CM-poly-SNO-HSA, we measured ROS induction, the mitochondrial membrane potential, the activation of caspase-3, and DNA fragmentation. These data suggested that the main initiator of apoptosis caused by CM-poly-SNO-HSA is ROS induction, and that ROS then promotes apoptosis via a change of mitochondrial membrane potential, an activation of caspase-3 and DNA fragmentation. In the present study, for investigating cytotoxity, we measured cellular LDH release (Fig. 5A). As seen from the figure, incubation with E-Mono-SNO-HSA resulted in a decrease in enzyme release, as compared to incubation with HSA (control). This finding proposes E-Mono-SNO-HSA-induced protection of the cells. Surprisingly, treatment of the cells with the same SNO concentration (50 μM) but in the form of CM-Poly-SNO-HSA caused apoptosis of the cells, because the release of LDH increased very much in this case. As a control, we made the degradation product of CM-Poly-SNO-HSA (cf. Fig. 1) by exposing it to UV-light and tested the effect of that compound; it had no effect on LDH release (Supplementary Figure 3). We also studied the effect of CM-Poly-SNO-HSA on C26 cells by determining the appearance of annexin V-positive cells using flow cytometry. This type of investigation (Fig. 5B) also showed that CM-Poly-SNO-HSA induces apoptotic cell death. The importance of cs-PDI for this process is revealed by the fact that bacitracin significantly inhibits apoptosis (Fig. 5B).

Cytoprotection was also studied by quantifying the amount of intracellular HO-1 (Fig. 6). It is apparent from the figure that E-Mono-SNO-HSA increases HO-1 expression in a dose-dependent manner. In

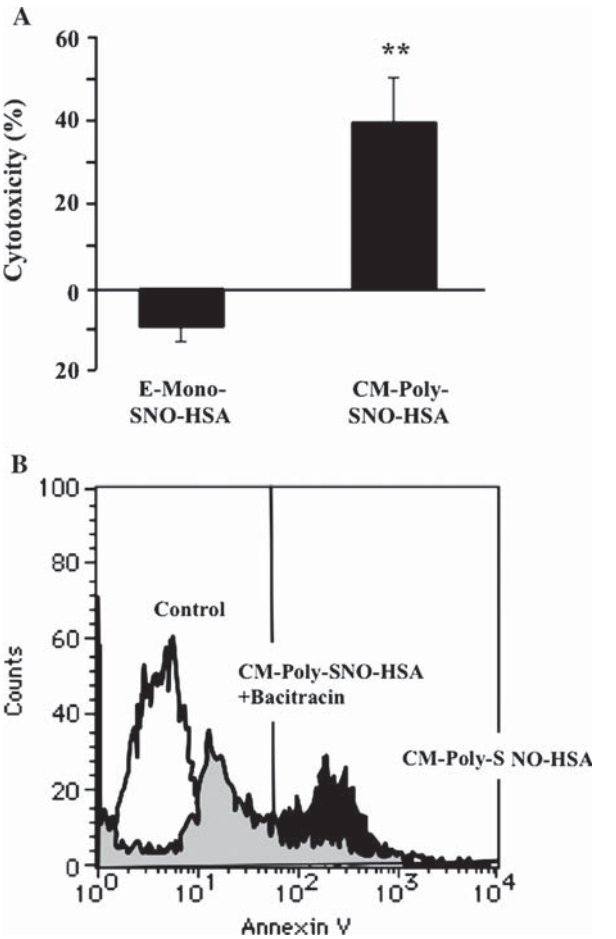

Figure 5. Effect of SNO-HSAs on LDH release from and apoptosis of C26 cells. (A) E-Mono-SNO-HSA (50 μM SNO), CM-Poly-SNO-HSA (50 μM SNO) or HSA (control) was adjusted to a final concentration of 150 μM HSA. C26 cells were incubated with such samples at 37°C for 12 h using 96-well plates. The amount of released LDH (%) was calculated according to the formula given in Experimental procedures. Data are expressed as means ± SEM (n = 5). **, $p < 0.01$, compared with E-Mono-SNO-HSA. (B) C26 cells were treated with 7.6 μM HSA (control) or CM-Poly-SNO-HSA (7.6 μM HSA, 50 μM SNO) with or without pretreatment with bacitracin. After 6 h of incubation, the number of apoptotic cells was determined with an annexin V-FITC binding assay kit from BD Biosciences (Tokyo, Japan). The fluorescence of annexin V-FITC was measured via a FACSCalibur flow cytometer. A representative of 3 repeated experiments is shown.

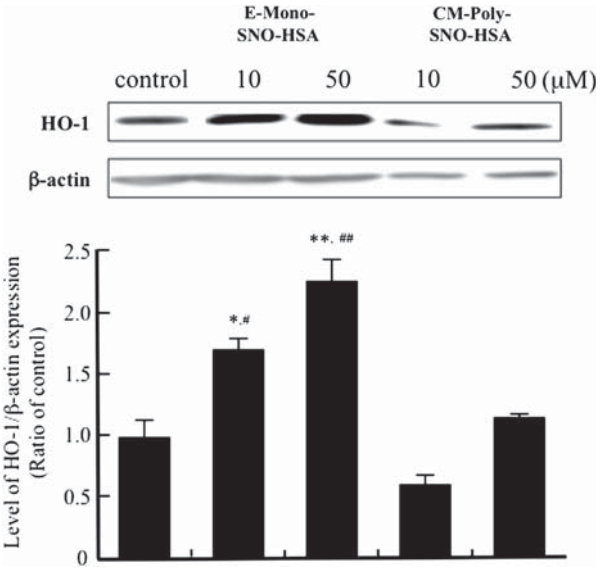

Figure 6. Western blot of HO-1 in HepG2 cells after E-Mono-SNO-HSA or CM-Poly-SNO-HSA treatment. HepG2 cells were incubated with 30 or 150 μM HSA (control), E-Mono-SNO-HSA or CM-Poly-SNO-HSA (10 μM or 50 μM of SNO) for 18 h in a CO_2 incubator. After incubation, whole HepG2 cell lysates were centrifuged, and equal amounts (30 μg) of protein from the supernatant were loaded onto 12.5% SDS-PAGE. Polyclonal antibody against HO-1 and horseradish conjugated secondary antibody against β-actin were used at 1:2,000 and 1:5,000 dilutions, respectively. The ECL Western blotting system was used for detection. Bands were visualized and quantified using a lumino-analyzer. The density of the bands for HO-1 and β-actin was quantitatively analyzed using the NIH Image J Software. Data are expressed as means ± SEM (n = 4). *, $p < 0.05$, **, $p < 0.01$, compared with control. $^{\#}$, $p < 0.05$, $^{\#\#}$, $p < 0.01$, compared with CM-Poly-SNO-HSA.

these experiments β-actin was used as an internal control. The increased expression of HO-1 is also evident when related to the expression of that protein. By contrast, treatment of the cells with CM-Poly-SNO-HSA resulted in decreased or unchanged HO-1 expression.

S-transnitrosation from E-Mono-SNO-HSA and CM-Poly-SNO-HSA to cysteine

According to Scharfstein and coworkers [24], NO exchange between plasma protein thiol-bound NO and available low molecular weight thiol pools (*S*-transnitrosation) occurs *in vivo*. To examine the relevance of free low molecular weight thiols for the biological functions of SNO-HSAs, we used free cysteine as a model. Incubation of E-Mono-SNO-HSA with an equimolar amount of cysteine (50 μM) for 10 min *in vitro* resulted in the formation of 30 μM of Cys-SNO (Fig. 7). By contrast, even though CM-Poly-SNO-HSA possesses its SNO groups on the surface of the molecule, a similar incubation with that SNO-form only resulted in the formation of approximately 10 μM. Wang et al. [25] have reported kinetics studies of transnitrosation between *S*-nitroso albumin and cysteine; and they determined the reaction

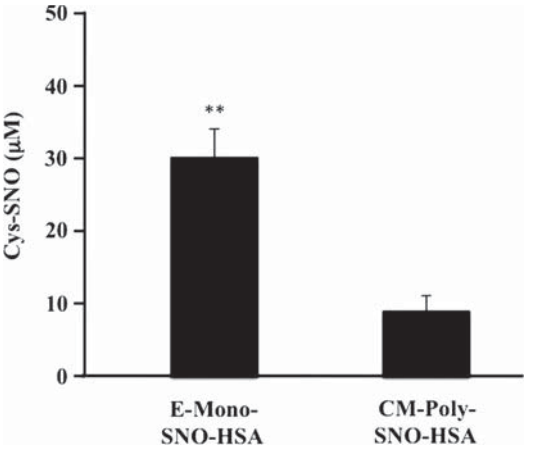

Figure 7. *S*-Transnitrosation from E-Mono-SNO-HSA and CM-Poly-SNO-HSA to cysteine. 50 μM SNO of E-Mono-SNO-HSA or CM-Poly-SNO-HSA was incubated with 50 μM cysteine (SNO: cysteine = 1:1) in PBS (pH 7.4) containing 0.5 mM DTPA at 37 °C for 10 min. The amounts of SNO-cysteine formed were determined by HPLC coupled with a flow-reactor system. The NO_2^- generated was then detected after reaction with Griess reagent in the flow-reactor system. Data are expressed as means ± SEM (n = 5). **, $p < 0.01$, compared with CM-Poly-SNO-HSA.

constant (k_2) to be 6.12×10^{-2} M^{-1} sec^{-1}. Here, we calculated 4.67×10^{-2} M^{-1} sec^{-1} as the value of k_2 for transfer from E-Mono-SNO-HSA to cysteine suggesting that our data are comparable to other reports on such reactions.

Discussion

E-Mono-SNO-HSA has been shown to be cytoprotective against free radical mediated damage and microvascular injury associated with ischemia-reperfusion or hemorrhagic shock as well as acute lung injury in a murine model of sickle cell disease. However, E-Mono-SNO-HSA has the potential drawback that it carries less than one NO molecule per protein molecule. Furthermore, the SNO group is located in 'a crevice' on the surface of the protein, and such a location could impede *S*-transnitrosation. Therefore, to prepare more efficient SNO derivatives, some research groups have synthesized CM-Poly-SNO-HSA, in which several NO moieties have been attached to surface residues of HSA, such as lysine residues, using a chemical spacer. In the present work we prepared CM-Poly-SNO-HSA by using 2-iminothiolane as a spacer, and we were able to bind 6.6 mol NO per mol of HSA.

Results obtained with C26 cells showed that cellular uptake of NO from CM-Poly-SNO-HSA is faster and quantitatively superior to NO uptake from E-Mono-SNO-HSA (Fig. 2). A possible explanation for these differences is that the NO of CM-Poly-SNO-HSA is more accessible than the SNO group of E-Mono-SNO-HSA. Pretreatment of the cells with the PDI inhibitors bacitracin, PAO or RL90 strongly

inhibited cellular NO uptake from CM-Poly-SNO-HSA and less so from E-Mono-SNO-HSA. These findings propose that PDI is located in the cell membrane, and that it is involved in NO transfer. The existence of cs-PDI was also shown in a more direct manner, namely by using fluorescent antibodies against it (Fig. 4). In a previous study, Zai et al. [26] have shown that cs-PDI catalyzes transnitrosation and regulates intracellular transfer of nitric oxide from SNO-4B beads. Additionally, in accordance with our findings, other groups have proposed that *S*-nitrosothiols interact with cs-PDI during nitric oxide delivery [27–29].

We studied the effect of the SNO-HSAs on cell growth by quantifying the expression of intracellular HO-1 and their effect on apoptosis by determining the outflow of LDH and the appearance of annexin V-positive cells (Figs. 5 and 6). The results showed that the relatively slow transfer of NO from E-Mono-SNO-HSA resulted in a clear cytoprotective effect, whereas a fast inflow of higher quantities of NO from CM-Poly-SNO-HSA resulted in pronounced apoptotic cell death. In accordance with the latter finding, we have recently shown that CM-Poly-SNO-HSA possesses anti-tumor activity against murine tumor LY-cells via ROS induction and caspase-3 activation [13]. The biological effect of CM-Poly-SNO-HSA could be partly due to, or influenced by, oxidized or otherwise modified HSA after NO release (see the illustrative overview in Fig. 1). However, the apoptosis induced by CM-Poly-SNO-HSA was completely inhibited by bacitracin, indicating that there is no effect of NO-unrelated action on this apoptosis induced by CM-Poly-SNO-HSA. Furthermore,

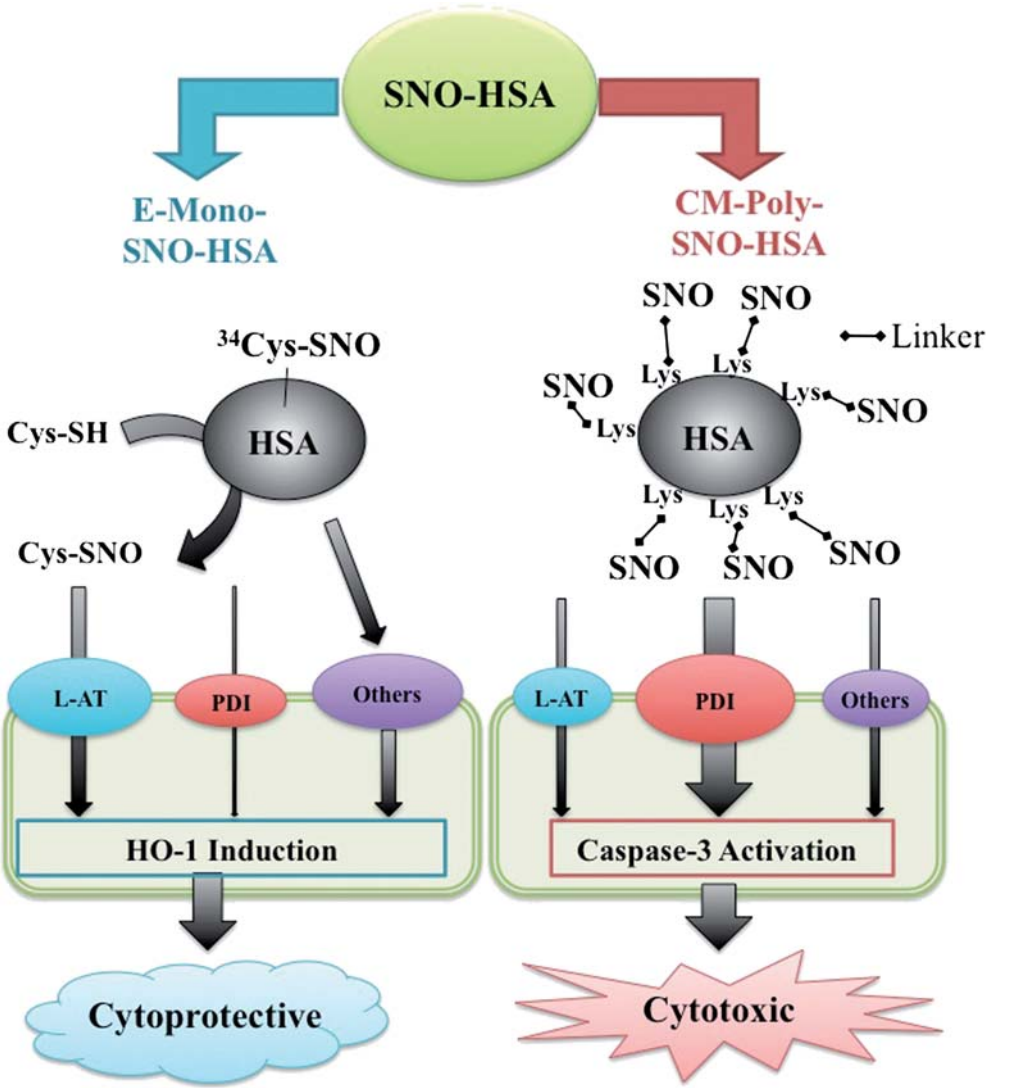

Figure 8. Differences in the mechanisms and consequences of NO traffic from E-Mono-SNO-HSA and CM-Poly-SNO-HSA to cells. NO transfer from the SNO group of Cys-34 on E-Mono-SNO-HSA to the cell is partly mediated by the L-amino acid transporter (L-AT) via *S*-transnitrosation to free low molecular weight thiol. By contrast, NO transfer from CM-Poly-SNO-HSA is mainly mediated by cs-PDI without *S*-transnitrosation to free low molecular weight thiol. The relatively slow transfer of NO from E-Mono-SNO-HSA avoids the presence of high intracellular NO concentrations and leads to cytoprotective activity through HO-1 induction. On the other hand, the NO influx from CM-Poly-SNO-HSA is very fast and pronounced and leads to cell death caused by apoptosis.

CM-Poly-SNO-HSA ,which had been exposed to UV-light, had no effect on LDH release.

The findings of the present work are summarized in Fig. 8. NO of E-Mono-SNO-HSA was easily *S*-transnitrosated to free cysteine (Fig. 7), suggesting that NO traffic into the cell from this SNO-species is partially mediated by the L-amino acid transporter (L-AT) [30]. By contrast, not much of the NO enters the cell via cs-PDI (Fig. 3). Finally, the NO entering the cell from E-Mono-SNO-HSA results in cytoprotection via HO-1 induction. The situation with respect to CM-Poly-SNO-HSA is quite different. The great majority of the NO-moities enters the cells via cs-PDI without *S*-transnitrosation to free low-molecular weight thiol, and the pronounced and fast inflow of NO results in cytotoxicity via caspase-3 activation.

We should mention that the SNO-HSA species have been used at high concentrations (micromolar level) in our work as compared with endogenous SNO-HSA concentrations (low nanomolar level). This was done, because it is very difficult to measure intracellar NO concentrations using DAF-FM DA at endogenous SNO-HSA levels. However, our data should be useful for the characterization and clinical applications of these SNO-HSAs.

CM-Poly-SNO-HSA can be used as a cytoprotective agent, if it is conjugated with polyethylene glycol [14], because this covalent modification adjusts the NO release. However, a clinical use of CM-Poly-SNO-HSA as an apoptosis inducer could be of greater help to the patient and have a broader application.

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Supplementary material available online

Supplementary Figure 1.
Supplementary Figure 2.
Supplementary Figure 3.