S-Nitrosylated S100A8: Novel Anti-Inflammatory Properties

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S-Nitrosylated S100A8: Novel Anti-Inflammatory Properties

Su Yin Lim,* Mark Raftery,† Hong Cai,* Kenneth Hsu,* Wei Xing Yan,* Hsiao-Ling Hseih,* Ralph N. Watts,§ Des Richardson,¶ Shane Thomas,§ Michael Perry,‡ and Carolyn L. Geczy³*

S100A8 and S100A9, highly expressed by neutrophils, activated macrophages, and microvascular endothelial cells, are secreted during inflammatory processes. Our earlier studies showed S100A8 to be an avid scavenger of oxidants, and, together with its dependence on IL-10 for expression in macrophages, we postulated that this protein has a protective role. S-nitrosylation is an important posttranslational modification that regulates NO transport, cell signaling, and homeostasis. Relatively few proteins are targets of S-nitrosylation. To date, no inflammation-associated proteins with NO-shuttling capacity have been identified. We used HPLC and mass spectrometry to show that S100A8 and S100A9 were readily S-nitrosylated by NO donors. S-nitrosylated S100A8 (S100A8-SNO) was the preferred nitrosylated product. No S-nitrosylation occurred when the single Cys residue in S100A8 was mutated to Ala. S100A8-SNO in human neutrophils treated with NO donors was confirmed by the biotin switch assay. The stable adduct transnitrosylated hemoglobin, indicating a role in NO transport. S100A8-SNO suppressed mast cell activation by compound 48/80; intravital microscopy was used to demonstrate suppression of leukocyte adhesion and extravasation triggered by compound 48/80 in the rat mesenteric microcirculation. Although S100A8 is induced in macrophages by LPS or IFN-γ, the combination, which activates inducible NO synthase, did not induce S100A8. Thus, the antimicrobial functions of NO generated under these circumstances would not be compromised by S100A8. Our results suggest that S100A8-SNO may regulate leukocyte-endothelial cell interactions in the microcirculation, and suppression of mast cell-mediated inflammation represents an additional anti-inflammatory property for S100A8. The Journal of Immunology, 2008, 181: 5627–5636.

Nitric oxide regulates vascular homeostasis, but excessive NO production and subsequent generation of reactive nitrogen species can cause nitrosative stress (1). This is particularly relevant in pathologies associated with inflammation, in which NO production is mediated by inducible NO synthase (iNOS), expressed by activated macrophages and other cell types (2). S-nitrosylation is the coupling of a NO moiety to a reactive cysteine thiol (3). S-nitrosylation of hemoglobin stabilizes NO generated by endothelial NO synthase, and regulates blood pressure by acting as an endogenous NO donor (4). S-nitrosothiols (SNOs) are increasingly implicated in regulating apoptosis, ki-

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are protected from endotoxin-induced septic shock (21). S100A8 and S100A9, expressed by microvessels in areas of neovascularization in human atheroma (15), are implicated in wound (22) and arterial repair (23). Their genes are regulated by TNF and IL-1β in microvascular endothelial cells (MEC) in vitro (24).

Corticosteroids induce S100A8 and S100A9 in human monocytes and, with LPS, superinduce S100A8 in murine macrophages and MEC (25). S100A8 gene induction is IL-10 dependent and involves the MAPK and JNK/p38 pathways (26); its regulation suggests that S100A8 may have a protective role and/or is involved in resolution of inflammation. In keeping with this, injection of S100A8/A9 into rats given LPS may scavenge excess NO, thereby reducing oxidative damage caused by acute inflammation (27). S100A8 is a potent scavenger of peroxide and hypochlorite generated by activated neutrophils and macrophages (28, 29). The chemotactic activity of murine (m)S100A8 is altered upon oxidation (28), and human (h)S100A8 has an anti-inflammatory, neutrophil-repelling property that is also oxidation sensitive (30).

PMA-activated neutrophils generate an oxidative burst that oxidizes exogenous S100A8 (28), which are effectively oxidized by low ratios of hypochlorite (HOCl) in vitro (15), to products containing sulfonamides (29). The capacity of Cys residues in particular proteins to exist in this, and the reduced state, makes them good candidates as regulators of oxidative/nitrosative stress. NO donors may induce protein sulfonation (31), although transnitrosation, involving reversible attachment of NO to target thiols, is a particular proteins to exist in this, and the reduced state, makes them good candidates as regulators of oxidative/nitrosative stress. NO donors may induce protein sulfonation (31), although transnitrosation, involving reversible attachment of NO to target thiols, is an alternate mechanism.

Generation of NO is key to antimicrobial defense. However, in the intestine, inhibition of NO synthesis exacerbates inflammation, leading to increased mast cell (MC) degranulation, neutrophil recruitment, and microvascular permeability. NO donors generally reduce acute inflammation (32). Under oxidative stress, NO may protect cells through S-nitrosylation of critical thiols, thereby preventing further oxidative damage (33). Importantly, immunotranslocation-related proteins that may modulate such responses have not been identified.

In this study, we demonstrate the conversion and characterization of S-nitrosylated S100A8 (S100A8-SNO). S100A8-SNO was generated in vitro and in human neutrophils with NO donors. S100A8-SNO suppressed MC degranulation in vitro and inflammation triggered by MC activation in the rat mesenteric microcirculation. Unlike iNOS, LPS and IFN did not induce S100A8 in macrophages, suggesting no role in antimicrobial defense. S100A8-SNO may modulate leukocyte-endothelial cell interactions in the microcirculation by regulating MC activation.

Materials and Methods

General

Chemicals were analytical grade (Sigma-Aldrich; Bio-Rad); solvents were HPLC grade (Malinckrodt). S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosogluthathione (GSNO), and diethylenetriamine NONOate (DEANO) were from Calbiochem; glutathione (GSH; reduced form) was from Sigma-Aldrich. Rabbit IgGs against S100A8 were raised and purified, as described (34). SDS-PAGE and Western blotting used 10% gels and a Tris/Tricine buffer system. Recombinant hS100A8 and mS100A8 and murine Cys41-Ala41 S100A8 mutants were produced using the pGEX expression system (34). Liquid chromatographic separations were performed using a nonmetallic LC26 HPLC system (Waters) and A214, A234, A254, A280 nm (monitored with a Waters 996 photodiode array or 490 UV/visible detector). C4 and C18 reverse-phase (RP)-HPLC columns (30 Å, 5 μm, 250 × 4.6 mm) were supplied by Vydra, Separations Group.

Preparation and characterization of nitrosylated S100 proteins

Stock solutions of NO donors (SNAP, GSNO, DEANO) were prepared in water, 10 mg/mL (5 nmoL) were incubated with donors (25 nM) in PBS (25 mM phosphate, 250 mM NaCl (pH 7.5)) for 15–120 min at 20°C (light-protected) or with NaNO2 (50 nM) in acetoni trile/0.1% trifluoro acetic acid (TFA) (pH 2.0) for 30 min at 25°C. Protein was isolated by C4 RP-HPLC (25–70% CH3CN/0.1% TFA at 1 ml/min over 30 min) by monitoring A214/335 nm. Nitrosylated peptides (5 nM) were similarly prepared and isolated by C18 RP-HPLC (5–75% CH3CN/0.1% TFA at 1 ml/min over 30 min). For characterization, C4 RP-HPLC-isolated nitrosylated proteins (~50 μg) were digested with endoprotease AspN (sequencing grade; Roche) in ammonium bicarbonate (50 mM (pH 8.0)), or protease K (Sigma-Aldrich) in sodium formate (50 mM (pH ~2.5)), at 37°C to substrate ratio of ~1:100 at 37°C for 1–6 h. The pH was lowered to ~2 with 1% TFA before isolating peptides by C18 RP-HPLC (5–75% CH3CN/0.1% TFA at 1 ml/min over 30 min). Major peaks at A214 nm were collected for electrospray ionization (ESI) mass spectrometry. Mass spectra were acquired using a triple quadrupole mass spectrometer equipped with an electrospray ionization source (TSQ7000; Finningan). Samples (~50 pmol) in water/acetoni trile (50:50, 1% acetic acid), flowing at 10 μl/min, were injected into the electrospray source via a fused silica capillary. Nitrogen was the nebulizer gas (15 °C). Sample droplets were ionized at a positive potential of 4.5 kV, and the capillary was maintained at 150°C. Spectra of proteins were acquired over m/z 600-1800, and peptides over m/z 200-1500. Mass spectra were acquired using a triple quadrupole mass spectrometer equipped with an electrospray ionization source (TSQ7000; Finningan). Samples (~50 pmol) in water/acetoni trile (50:50, 1% acetic acid), flowing at 10 μl/min, were injected into the electrospray source via a fused silica capillary. Nitrogen was the nebulizer gas (15 °C). Sample droplets were ionized at a positive potential of 4.5 kV, and the capillary was maintained at 150°C. Spectra of proteins were acquired over m/z 600-1800, and peptides over m/z 200-1500.

S-nitrosylation of S100A8 in neutrophils

Human neutrophils (2 × 109/mL), isolated from citrated blood by density gradient centrifugation with Ficol/Paque™ (Pharmacia Biotech), were incubated with 1 mM SNP, 1 mM SNAP, or in RPMI 1640-HEPES (Life Technologies, Invitrogen) at room temperature (RT) for 1–2 h, and harvested by centrifugation. Nitrite in supernatants was measured by Greiss reagent (35). Cell pellets were washed twice before lysis, and S-nitrosylated proteins were detected by the biotin switch assay using the S-nitrosylated Protein Detection Assay Kit (Cayman Chemical). For validation, S100A8 (75–500 μg) was treated for 1 h with 1 mM GSNO or untreated before assay; a minor modification included blocking in 20 mM methyl methanethiosulfonate (Sigma-Aldrich) for 20 min at 50°C instead of using S-nitrosylation buffer A. Acetone-precipitated proteins were resuspended in PBS (50 μl for S100A8, 100 μl for neutrophil samples), separated by SDS-PAGE before Western blotting. Biotinylated proteins were detected with streptavidin-HRP (1:1000 v/v; Bio-Rad) and S100A8 with anti-S100A8 IgG (15). To confirm S-nitrosylation, an ELISA was developed because the anti-S100A8 IgG did not work well for immuno-precipitation. Microtiter wells (Maxisorp; Nunc) coated overnight with anti-S100A8 IgG (0.25 μg/well) in 0.05 M sodium carbonate buffer (pH 9.6) were washed three times with Tween 20 (0.05%) in PBS, then blocked with 1% BSA in PBS for 2 h at RT. After washing, acetylated-precipitated samples and standard (biotinylated S100A8, 7.8–250 ng/ml; EZ-Link Sulfo-NHS-LC Biotin; Pierce) were added. Plates were incubated at RT for 2 h and washed, and streptavidin-HRP conjugate (1:3000 v/v) was added for 30 min and, after washing, incubated with substrate 3,3’,5’,5’-tetramethylbenzidine (Pierce) solution for 20 min at RT in the dark. The reaction was stopped with 2 N H2SO4, and absorbance was measured (Titertek Multispec MCC/540; Labsystems). Values are reported as S100A8-SNO/10 6 neutrophils.

S-nitrosylation of hemoglobin by S100A8-SNO

Oxygenated hemoglobin (OxyHb) was extracted from erythrocytes, as described (36); 250 μM S100A8-SNO and 25 μM OxyHb were mixed and scanned at A505–650 nm on a Beckman Coulter DU Series 600 spectrophotometer every 2 h until a steady state was reached. As a control for the...
S100A8-SNO/OxyHb reaction, S100A8 was used. The reaction was compared with GSN (50 μM), with GSH (50 μM) as its control.

**Effect of S100A8-SNO on chemotaxis**

Experiments were approved by the Animal Care and Ethics Committee of the University of New South Wales. Peritoneal neutrophils from BALB/c mice were obtained by lavage 16-h postinjection of 2 ml of thioglycolate solution. Chemotaxis was performed, as described (37), using 96-well chambers separated by a polycarbonate membrane (NeuroProbe; pore size, 5 μm). Cells in the lower chamber were measured by fluorescence at λex = 485 nm and λem = 530 nm using a CytoFluor multwell plate reader system (Applied Biosystems) and readings converted to cell numbers using a standard curve obtained with known numbers of neutrophils. C5a (Sigma-Aldrich; 10^{-8} M) was the positive control.

**Effects of S100A8-SNO on mast cell degranulation in vitro and mast cell-mediated inflammation in vivo**

Peritoneal cells of male Sprague-Dawley rats (6–8 wk; Biological Resource Centre, University of New South Wales), obtained by lavage and separated on a series of two 22% metrizamide (Sigma-Aldrich) gradients, were 99% MC by toluidine blue staining. MC (2×10^6/ml) were separated on a series of two 22% metrizamide (Sigma-Aldrich) gradients, 103×36.6 Da). C18 RP-HPLC chromatograms of peptic digests of S100A8 showed two peaks, with retention times of 17.6 min (peak 2) and 18.4 min (peak 1), respectively. The peak with retention time of 18.4 min (peak 1) was observed after SNAP treatment. A single peak with retention time of 18.4 min (peak 1) was isolated from digest of S100A8 treated with SNAP and GSNO. A single peak with retention time of 18.4 min (peak 1) was isolated from digest of S100A8 treated with SNAP and GSNO.

Induction of S100A8 and iNOS mRNA in murine macrophages

Murine RAW 264.7 macrophage-like cells were stimulated with LPS (50 ng/ml; Difco), IFN-γ (100 U/ml; Genzyme), IL-10 (10 ng/ml; Genzyme), or their combination for 4 or 24 h. RNA preparation and real time RT-PCR were performed, as described (25). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) or β-actin was endogenous control. PCR primer sequences for S100A8 and HPRT were as described (25). Primer sequences for iNOS were 5’-GGTTACTCGACCCCAACAAATACAAAGA-3’ and 5’-GGTG GACCGGTGACTGATGCAATCG-3’. β-actin, 5’-AGTGTGACGTTGACATC GC-3’ and 5’-GGCGACGACGTAATCCTCTTCT-3’.

**FIGURE 1.** S100A8 is S-nitrosylated by NO donors: identification by peptide mapping and mass spectrometry. C4 RP-HPLC chromatograms of S100A8 treated with SNAP and GSN O. A, A single peak with retention time of 18.4 min (peak 1) was observed after SNAP treatment. B, Two peaks, with retention times of 17.6 min (peak 2) and 18.4 min (peak 1), were isolated after GSN treatment. C, ESI mass spectrum of multiply charged ions of peak 1. D, The deconvoluted mass derived from charged ions of peak 1. E, The deconvoluted mass derived from charged ions of peak 2. F, The deconvoluted mass derived from charged ions of peak 2.
**Statistical analysis**

Data were analyzed using ANOVA, followed by Bonferroni’s correction for multiple comparisons and Student’s t test (unpaired) to compare differences between two groups. All values are reported as means ± SEM. Statistical significance was set at p < 0.05.

**Results**

**Structural characterization of S100A8 adducts**

Recombinant mS100A8 (has a single residue: Cys41) or Ala41-S100A8, treated with NO donors, was isolated by C4 RP-HPLC, and masses were determined. The HPLC retention times of reaction products were compared with untreated S100A8 (18.5 min); SNAP and GSNO generated high yields of a component eluting at 18.4 min, whereas the retention time of Ala41-S100A8 (18.25 min) treated with SNAP was unchanged.

The major reaction product (yield of >95% after ~40 min) of SNAP-treated S100A8 was the Cys41-S100A8-SNO derivative (Fig. 1A). GSNO generated two products over 2 h: S100A8-GSH and S100A8-SNO (Fig. 1B). The ESI mass spectrum of S-nitrosoylated S100A8 is shown in Fig. 1C; each peak corresponded to multiply protonated S100A8-SNO ions, which, when deconvoluted, gave a mass of 10,337 Da (Fig. 1D). The mass addition of +29 Da was identical with the theoretical mass for addition of NO, with the loss of a hydrogen atom. The location of the nitrosyl group was confirmed by peptide mapping and mass spectrometry. After digestion of S100A8 and S100A8-SNO with pepsin, the HPLC retention time of S100A812–44-SNO was increased by 0.8 min compared with the unmodified peptide, with a mass increase of +29 Da (Fig. 1E and F). An increase in A335 nm, characteristic of S-nitrosyl groups, was observed exclusively for S100A812–44-SNO. The mass of Ala41-S100A8 did not change after incubation with SNAP, and no modified peptides were obtained following peptic digestion (data not shown), confirming the site of NO addition to be the single Cys41 residue of S100A8.

S100A8-SNO products were isolated using the same conditions of treatment with other NO donors, albeit with varying yields. GSNO, NaNO2, and DEANO generated additional products (Table I). The S100A8-GSH mixed disulfide (HPLC retention time of 17.6 min; mass increase of +305 Da) was a major product of GSNO, together with S100A8-SNO (ratio ~4:1). An additional uncharacterized adduct (total mass addition, +46 Da; 29 Da accounted by the –SNO product, plus another component of 17 Da) formed with NaNO2 or DEANO. Ala41-S100A8 did not change after incubation with SNAP, and no modified peptides were obtained following peptic digestion (data not shown), confirming the site of NO addition to be the single Cys41 residue of S100A8.

S100A8-SNO products were isolated using the same conditions of treatment with other NO donors, albeit with varying yields. GSNO, NaNO2, and DEANO generated additional products (Table I). The S100A8-GSH mixed disulfide (HPLC retention time of 17.6 min; mass increase of +305 Da) was a major product of GSNO, together with S100A8-SNO (ratio ~4:1). An additional uncharacterized adduct (total mass addition, +46 Da; 29 Da accounted by the –SNO product, plus another component of 17 Da) formed with NaNO2 or DEANO. Ala41-S100A8 had an addition of 17 Da, indicating a Cys-independent modification; peptide mapping indicated a mass addition of m/z 18 (±0.1) within the N-terminal amino acid residues (GSSELE-KAL5NL) (data not shown). Tyr nitrosylation was not detected under any conditions.

Murine S100A8 and S100A8-SNO were readily separated by C4 RP-HPLC and had distinct elution times at 15.2 and 15.6 min, respectively (data not shown). S100A8-SNO was stable in

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** S100A8/A9 was S-nitrosylated in the presence and absence of calcium. A. The S100A8/A9 heterodimer, after treatment with SNAP and SNAP plus Ca²⁺, was separated by C4 RP-HPLC. The deconvoluted ESI mass spectra of SNAP-treated S100A8/A9 (B and C) and SNAP plus Ca²⁺-treated S100A8/A9 (D and E). Spectra show products with molecular masses corresponding to S100A8 (10,847 Da) and S100A8-SNO (10,876 Da) (B and D), and S100A9 (13,254 Da) and S100A9-SNO (13,284 Da) (C and E).

**Table I.** Masses of murine S100A8 and its Cys41-Ala41 mutant modified by treatment with NO donors

<table>
<thead>
<tr>
<th>NO Donors</th>
<th>mS100A8</th>
<th>Ala41-S100A8</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10,308 (0)</td>
<td>10,275 (0)</td>
</tr>
<tr>
<td>SNAP</td>
<td>10,337 (+29)</td>
<td>10,275 (0)</td>
</tr>
<tr>
<td>GSNO</td>
<td>10,337 and 10,356 (+29 and +305)</td>
<td>10,275 (0)</td>
</tr>
<tr>
<td>DEANO</td>
<td>10,336 and 10,354 (+29 and +46)</td>
<td>ND</td>
</tr>
<tr>
<td>NaNO2</td>
<td>10,292 (+17)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Murine S100A8 or the Ala41-S100A8 mutant was eluted from C4 RP-HPLC after treatment with NO donors, as described in Materials and Methods. Retention times (RT) and ESI masses are given; mass increases (Da) are shown in parentheses.*
PBS over 4.5 h, but GSH (5 nM) caused a time-dependent conversion to S100A8 within 2 h with ~40% conversion. Increasing GSH concentrations reduced the conversion time (data not shown).

To investigate whether the S100A8/A9 heterodimer was S-nitrosylated, an equimolar mixture of S100A8/A9 was treated with SNAP ± calcium and separated by C4 RP-HPLC; S100A8 eluted at 14.8 min and S100A9 at 16 min; SNAP and SNAP plus Ca²⁺ generated an additional component with retention time of 14.9 min (Fig. 2A). The deconvoluted ESI mass spectrum of SNAP-treated S100A8/A9 showed components with masses of 10,847 Da and 10,876 Da, corresponding to monomeric S100A8 and S100A8-SNO, respectively (Fig. 2B). The single peak eluting at 16 min contained two components with deconvoluted masses of 13,254 and 13,284 Da, identical with the theoretical masses of monomeric S100A9 and S100A9-SNO, respectively (Fig. 2C). Similar components were generated when the complex was treated in the presence of Ca²⁺ (Fig. 2, D and E). S100A8-SNO was the prevalent product, and its relative abundance was independent of calcium, unlike S100A9-SNO, which increased in the presence of calcium.

S-nitrosylation was tested in the presence of HOCl, which generates posttranslational modifications in S100A8 (29). HPLC elution profiles of HOCl-treated, and HOCl plus SNAP-treated S100A8 contained an additional peak (14.7 min) compared with the single peak (14.9 min) of SNAP-treated S100A8 (Fig. 3A). The ESI mass spectrum of HOCl-treated S100A8, when deconvoluted, gave masses of 10,847 Da (Fig. 3B) and 21,694 Da (Fig. 3C), corresponding to monomeric and disulfide-linked S100A8, respectively. The sulfinamide-containing form was a minor product. HOCl plus SNAP-treated S100A8 had a similar profile, but contained an additional product of 10,877 Da, corresponding to S100A8-SNO (Fig. 3D). S100A8-SNO was half as abundant as the dimer.

S100A8 in human neutrophils can be S-nitrosylated
Nitrite accumulation was used to confirm NO generation in SNAP- or GSNO-treated neutrophils; basal levels (0.3 ± 0.2 μM) increased to 5.4 ± 3.4 μM and 14.3 ± 9.5 μM, respectively. S100A8 was detected as a single band (10 kDa) in Western blots

S100A8-SNO nitrosylated hemoglobin. Reactions of OxyHb absorbance spectra with two NO donors and their controls were at RT; all samples were in PBS. A, 25 μM OxyHb reacted for 20 h with 250 μM S100A8-SNO; B, 25 μM OxyHb reacted for 20 h with 250 μM S100A8; C, 25 μM OxyHb reacted for 2 h with 50 μM GSNO; and D, 25 μM OxyHb reacted for 2 h with 50 μM GSH.
S100A8-SNO SUPPRESSES MAST CELL ACTIVATION AND INFLAMMATION

of neutrophil lysates (Fig. 4A). Endogenously biotinylated proteins, identified as weak streptavidin-reactive components (~12, ~15, and ~25 kDa), were present in nonbiotin-labeled samples (Fig. 4B, lanes 3 and 5). SNAP-treated neutrophils contained more S-nitrosylated proteins than untreated control. A ~10-kDa component, characteristic of S100A8 (Fig. 4B, lane 2; arrow), was particularly prominent, whereas the component at ~14 kDa, presumably S100A9, was much less abundant. Components migrating at 20, 40, and 60 kDa may represent complexes of S-nitrosylated S100A8 or other S-nitrosylated proteins. Basal levels of S100A8-SNO were detected by ELISA; these increased significantly in SNAP-treated neutrophils from 35.17 ± 5.7 ng to 62.0 ± 5.9 ng/10⁶ cells (p < 0.01), and in GSNO-treated samples to 74.5 ± 5.4 ng/10⁶ cells (p < 0.01; Fig. 4C). GSH-treated S100A8 under the same conditions was unreactive (data not shown).

S100A8-SNO can nitrosylate hemoglobin

The spectrum of OxyHb is defined by characteristic peaks at A₅₄₁, A₅₇₆ nm. Fig. 5A shows the reaction of S100A8-SNO with OxyHb after it reached steady state at 20 h. The spectrum represents the characteristic change from OxyHb to SNO-OxyHb (40), with decreased absorbance at A₅₄₁, A₅₇₆ nm and initiation of peaks at A₄₇₇, A₆₀₁ nm. In contrast to S100A8-SNO, the reaction between GSNO and OxyHb (Fig. 5C) reached steady state within 2 h, with a similar SNO-OxyHb spectrum. S100A8 (Fig. 5B) or GSH (Fig. 5D) did not alter the OxyHb spectrum.

S100A8-SNO is chemotactic, but suppresses mast cell degranulation in vitro and mast cell-mediated inflammation in the microcirculation

Murine S100A8 is chemotactic for phagocytes, and oxidation can modify this function (28). S100A8 induced maximal neutrophil migration at 10⁻¹¹ and 10⁻¹³ M, with potency somewhat less than that of 10⁻⁸ M C5a (Fig. 6A). S-nitrosylation shifted the optimal concentration from 10⁻¹³ to 10⁻¹² M. Similar numbers of neutrophils were recruited at the optimal concentrations of S100A8 and S100A8-SNO.

To confirm that NO donors inhibit MC activation (41), rat MC were treated with SNAP; β-hex release from activated MC was significantly reduced (Fig. 6B). S100A8-SNO had little direct effect, but significantly reduced the CMAP48/80-provoked response by 45% when tested at 10⁻⁵ M (p < 0.01); 10⁻⁷ M S100A8-SNO followed the same trend (41% reduction). In contrast, S100A8 did not significantly alter MC activation by CMAP48/80 (data not shown). Suppression of the CMAP48/80-provoked response was dose dependent; 10⁻⁵ M S100A8-SNO significantly reduced β-hex release from CMAP48/80-activated murine mast cells (p < 0.01), with less degranulation at lower dilutions (Fig. 6C).

Table II

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DV (µm)</th>
<th>V_wbc (µm/s)</th>
<th>V_m (µm/s)</th>
<th>V_wbc/V_m (µm²/mm)</th>
<th>Shear Rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 5)</td>
<td>37 ± 2</td>
<td>68 ± 3</td>
<td>3.1 ± 0.2</td>
<td>0.023 ± 0.001</td>
<td>410 ± 14</td>
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<tr>
<td>A8-SNO (n = 5)</td>
<td>38 ± 2</td>
<td>66 ± 4</td>
<td>3.0 ± 0.1</td>
<td>0.023 ± 0.001</td>
<td>388 ± 14</td>
</tr>
<tr>
<td>Control (n = 5)</td>
<td>39 ± 2</td>
<td>58.6 ± 11.5</td>
<td>3.7 ± 0.2</td>
<td>0.017 ± 0.004</td>
<td>485 ± 27</td>
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<tr>
<td>CMAP48/80 (n = 8)</td>
<td>36 ± 2</td>
<td>36.8 ± 7.5</td>
<td>3.4 ± 0.2</td>
<td>*0.012 ± 0.004</td>
<td>485 ± 25</td>
</tr>
<tr>
<td>Control (n = 5)</td>
<td>37 ± 2</td>
<td>62 ± 8</td>
<td>2.7 ± 0.2</td>
<td>0.023 ± 0.002</td>
<td>366 ± 24</td>
</tr>
<tr>
<td>CMAP48/80 + A8-SNO (n = 5)</td>
<td>36 ± 2</td>
<td><strong>55 ± 5.5</strong></td>
<td>2.6 ± 0.1</td>
<td>*0.019 ± 0.003</td>
<td>360 ± 19</td>
</tr>
</tbody>
</table>

* Mean venular diameter (DV), velocity of rolling leukocytes (V_wbc, µm/s), red cell velocity (V_m, µm/s), V_wbc/V_m ratio, and blood shear rate (s⁻¹) in the mesenteric circulation of rats. For each group, baseline measurements (controls) were made before treatment, and after 60-min superfusion with additives. *, p < 0.05 compared with their respective controls; **, p < 0.05, CMAP48/80 + A8-SNO compared with CMAP48/80.
activation of MC in the microcirculation by CMP48/80 causes rapid recruitment and extravasation of neutrophils (41), and quantification of responses by intravital microscopy provides an excellent in vivo model. Superfusion with CMP48/80 ± S100A8-SNO did not alter microvessel diameter, red cell velocity, or venular wall shear rate (Table II). Leukocyte velocity significantly decreased from 58.6 ± 11.5 to 36.8 ± 7.5 μm/s with CMP48/80 (p < 0.05); however, S100A8-SNO attenuated this (55 ± 6 μm/s; p < 0.01 compared with CMP48/80 alone).

Leukocyte flux increased 37 ± 10% above baseline with S100A8-SNO superfusion (Fig. 7A), but, in contrast to CMP48/80, had little direct effect on leukocyte adhesion (Fig. 7B) or extravasation (Fig. 7C). Following MC activation with CMP48/80, leukocyte adhesion was significantly reduced by 76 ± 7%, and extravasation by 63 ± 14%, in rats treated with S100A8-SNO. Leukocyte flux was not affected, and no effects on an established response were observed when inflammation was initiated with CMP48/80, followed by superfusion with S100A8-SNO for 10–30 min (data not shown). S100A8-SNO was tested at a single concentration because of the large amounts required for in vivo experiments. Notwithstanding, the significant reduction of the acute inflammatory response in the microcirculation with 2 × 10⁻⁸ M S100A8-SNO strongly supports its potential relevance in vivo.

S100A8 nitrosylation is unlikely to compromise macrophage-mediated antimicrobial activity

NO is important in antimicrobial defense, and LPS plus IFN-γ enhance iNOS expression. Because S100A8 is induced in macrophages by LPS (19), we considered that this synergy may potentiate its expression as well, thereby scavenging excess NO. LPS plus IFN-γ induced high levels of iNOS mRNA within 4 h (Fig. 8A). In contrast, no S100A8 mRNA was induced over 24 h with this combination. As reported previously, LPS induced S100A8 mRNA, a response that is IL-10 dependent and optimal 24–36 h poststimulation (26). IL-10 had little effect on iNOS mRNA (Fig. 8B). Amounts of nitrite and S100A8 in culture supernatants reflected mRNA levels (data not shown).

Discussion

Modulation of vascular tone, blood flow, and physiological effects, including inhibition of MC activation and leukocyte adhesion by NO, are well established; some functions can be reproduced by S-nitrosothiol (4). S-nitrosylation of cysteine is now considered a prototypic cellular signaling mechanism and has broad regulatory significance, analogous to protein phosphorylation (42). Although some S-nitrosylated proteins such as S-nitrosylation of hemoglobin have functions associated with NO shuttling, no protein generated by an inflammatory response with similar capacity has been described.

S100A8 is a major cytoplasmic protein of neutrophils and is induced in macrophages (19, 20), MEC (26), fibroblasts (43), and keratinocytes (44) by inflammatory mediators or oxidative stress. Its conserved Cys residue is particularly sensitive to oxidative modification (29); the single N-terminal Cys8 residue in human S100A9 is somewhat less susceptible (15, 28). Moreover, Cys8-S100A9 is not exclusively expressed, due to an alternate start site for translation at position 4 (45). Murine S100A9 does not have a conserved Cys residue, and, unlike S100A8, is not induced in macrophages (19, 20) or in keratinocytes by oxidative stress (44). For these reasons, this investigation focused on S100A8. Reaction
products were generated with NO donors, in mS100A8 and hS100A8, but not in the mutant Ala41-S100A8 (Table I). GSNO, the physiologically important cell storage form of NO (46), generated a mixture of nitrosothiol and GSH adducts; the mixed disulfide may have formed directly from GSNO, or due to degradation of S100A8-SNO. S100A8-SNO was generated in the presence of S100A9 even though calcium binding and heterodimer formation with S100A9 induce conformational changes (47). S-nitrosylation of both subunits was seen, but S100A8-SNO was predominant; S100A9 S-nitrosylation required calcium (Fig. 2). Similarly, S100A8-SNO was consistently the predominant product in SNAP-treated neutrophils (Fig. 4B).

S-nitrosylation may occur via an acid-base-catalyzed SNO/SH exchange, in which the target Cys residue is located next to basic and acidic amino acids (48). The glutamic acid and arginine residues surrounding the reactive cysteine (49, 50) are conserved in mS100A8 and hS100A8 and may facilitate SNO formation. Following HOCl oxidation, Cys84 initially forms a sulfenic acid that rapidly reacts with ε-amino groups of Lys34 or Lys35 in mS100A8, forming intramolecular sulfinamide bonds. These Lys residues, conserved in hS100A8, are in close structural proximity to Cys82 (51), and may enhance Cys-SNO formation through thiol deprotonation. As expected, concurrent treatment of hS100A8 with HOCl and NO generated a mixture of oxidation products, S100A8-SNO was formed, although relative abundance was half that of the homodimer.

Hydrophobic pockets within proteins may contribute in targetting NO to specific Cys residues, allowing additional selectivity (52). S100A8 is hydrophobic and interacts with unsaturated fatty acids (11). S100A8/A9 binds cytosolic p67 (53). Interestingly, translocation of cytosolic NADPH oxidase components, such as p47phox and p67phox to the plasma membrane, and subsequent superoxide generation are inhibited by SNOs (54). Cytosolic SNOs form in neutrophils treated with NO donors (55); S100A8-SNO was a major reaction product following treatment with SNAP or GSNO. Given this, and its association with NADPH oxidase components, S100A8-SNO could regulate NADPH oxidase assembly in these cells.

Denitrosylation occurs with reduced GSH, and protein glutathiolylation may be a feature of NO bioactivity (56). GSH removed NO from S100A8-SNO within 2 h to yield S100A8. No mixed disulfides were identified. In pure form, S100A8-SNO did not noticeably degrade at 37°C over 4.5 h and was stable at −70°C for up to 3 wk. NO is diffusible and short-lived, and some SNOs mediate its storage and transport. In general, transnitrosation (addition of NO +) is a slow reaction (57), with kinetics dependent on the properties of the donor as well as the surrounds. As reported (40), the reaction between GSNO and the NO scavenger, OxyHb, occurred within 2 h, whereas transfer of NO to OxyHb from S100A8-SNO took 20 h to reach steady state (Fig. 5). Taken together, the results indicate that S100A8-SNO may be a relatively stable NO adduct, depending on acceptor characteristics. The 10-fold increase in chemotaxis optima (Fig. 6A) could indicate reversion to the native form during the course of the assay, with transnitrosation of NO acceptor molecules on neutrophils.

NO plays important autocrine and paracrine roles in regulation of blood pressure and leukocyte adhesion. S-nitrosylation of endothelial cell proteins is implicated in altered vascular function (42). S100A8 and S100A9, induced in MEC by IL-1β and TNF in vitro (26), are observed in neovessels, but not larger vessels, in inflammatory lesions (15). The complex is deposited on the endothelium by transmigrating leukocytes; S100A9 binds endothelial cells (EC) via heparan sulfate proteoglycans (58) and to carboxylated N-glycans on activated EC (59). S100A8 has functions separate from those involving heterodimerization with S100A9 (60), and although we have not confirmed S100A8-SNO in EC, it may modulate microvascular function in inflamed vessels.

We demonstrated the potential for S100A8-SNO to regulate inflammatory responses in the microcirculation. NO suppresses mast cell degranulation, mediator release, cytokine expression, and adhesion, as mimicked by GSNO and SNAP (61), possibly via modification of acceptor proteins (62). S100A8-SNO significantly inhibited mast cell degranulation in vitro. Superfusion of the rat mesentery with CMAP8/80 activates perivascular mast cells, increasing P-selectin-dependent leukocyte rolling and CD18-dependent leukocyte adhesion via generation of histamine and platelet-activating factor, respectively (63). NO donors block adhesion-dependent alterations in vascular permeability, permeability changes induced by histamine, and modulate leukocyte flux and adhesion promoted by mast cell activation (41). In this study, we show, for the first time, that a protein generated by an inflammatory response, found systemically and within lesions, can be S-nitrosylated and may modulate mast cell-mediated leukocyte recruitment. S100A8-SNO had no apparent effect on vessel diameter, RBC velocity, or shear rate (Table II). However, when superfused together with CMAP8/80, S100A8-SNO attenuated the decreased leukocyte velocity caused by mast cell activation and significantly reduced numbers of adherent and extravasated leukocytes (Fig. 7, B and C). The effects of S100A8-SNO in the microcirculation were similar to those reported for NO donors (41). S100A8-SNO increased the flux of rolling leukocytes, suggesting effects on P- and/or L-selectin expression, although S100A8 has no effect on neutrophil adhesion or L-selectin expression in vitro (64). S100A8-SNO did not alter neutrophil adhesion in vitro (data not shown), but direct effects on the endothelium cannot be discounted. S100A8-SNO prevented mast cell activation and histamine release that could modulate P-selectin expression on the endothelium (63). Although our earlier studies indicated that hS100A8 had little chemotactic activity for monocytes due to sequence differences within the active hinge domain (50), this study suggests that because hS100A8 and mS100A8 were both readily S-nitrosylated at a conserved Cys residue, S100A8-SNO may have similar functions in the human microcirculation.

S100A8 is expressed in macrophages at inflammatory sites (15) and by stimulation with LPS or IFN-γ in vitro (20). Because IFN-γ in the presence of LPS induces high levels of iNOS in macrophages (65), we reasoned that concomitant induction of S100A8 with iNOS may compromise the antimicrobial functions as a consequence of S100A8 scavenging NO. However, S100A8 mRNA was not induced by LPS plus IFN-γ in murine macrophages over 24 h, even though iNOS levels were markedly elevated within 4 h (Fig. 8A). In contrast, LPS-induced S100A8 mRNA was elevated by IL-10, as reported previously (20). iNOS mRNA induced by LPS was not affected by IL-10 (Fig. 8B). Thus, S100A8 production would be suppressed in circumstances in which iNOS is required for optimal macrophage-mediated antimicrobial activity.

The results support our suggestion that oxidative modification of S100A8 may be important in resolution of inflammation. Because S100A8 is susceptible to both oxidation and nitrosylation, S100A8-SNO could represent a protective mechanism in the microcirculation, even when HOCl is generated. S100A8 was readily S-nitrosylated in vitro and in neutrophils, and formed a relatively stable adduct that transnitrosylated hemoglobin. S100A8-SNO reduced mast cell activation, and due to
their proximity to the vasculature, potential interactions between S100A8 and NO generated by EC may mediate alterations in EC-leukocyte interactions in the microcirculation.

Disclosures
The authors have no financial interest of conflict.

References


