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Sinapinic acid can replace ascorbate in the biotin switch assay

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ABSTRACT

Background: Protein *S*-nitrosation is an important post-translational modification altering protein function. Interaction of nitric oxide with thiols is an active area of research, and is one of the mechanisms by which NO exerts its biological effects. Biotin switch assay is the method, which has been developed to identify *S*-nitrosated proteins. The major concern with biotin switch assay includes reducing disulfide which may lead to false positives. We report a modification of the biotin switch assay where sinapinic acid is utilized instead of ascorbate to eliminate potential artifacts in the detection of *S*-nitrosated proteins.
Methods: The denitrosation ability of sinapinic acid was assessed by monitoring either the NO or NO_2^- released by chemiluminescent NO detection or by the griess assay, respectively. DTNB assay was used to compare disulfide reduction by ascorbate and sinapinic acid. Sinapinic acid and ascorbate were compared in the biotin switch detection of *S*-nitrosoproteins in RAW 264.7 cells ± *S*-nitrosocysteine (CysNO) exposure.
Results: We show that sinapinic acid has the ability to denitrosate *S*-nitrosothiols at pH 7.0 and denitrate plus denitrosate at pHs 8 and 8.5. Unlike ascorbate, sinapinic acid degrades *S*-nitrosothiols, but it does not reduce disulfide bridges.
Conclusions: Sinapinic acid denitrosate RSNO and does not reduce disulfides. Thus can readily replace ascorbate in detection of *S*-nitrosated proteins in biotin switch assay.
General significance: The work described is important in view of protein *S*-nitrosation. In this study we provide an important modification that eliminates artifacts in widely used technique for detecting the *S*-nitrosoproteome, the biotin switch assay.

1. Introduction

Protein *S*-nitrosation is an important post-translation modification that affects various proteins involved in number of cellular processes, mediated by nitric oxide (NO) through redox-dependent conversion of cysteine thiols of proteins to *S*-nitrosothiols [1–4]. Earlier studies have shown that modifying cysteine residues of proteins may result in altered protein function thus nitrosothiols play major roles in human health and disease [5].

A major contribution to the field of *S*-nitrosothiols is the biotin switch assay, introduced by Jaffrey et al. in 2001 [6,7]. In this assay, proteins are denatured by sodium dodecyl sulphate (SDS) in the presence of methyl methane thio-sulfonate (MMTS) to block free thiols. Thiols present in the interior of the protein are exposed when they are incubated with SDS at 50 °C. After acetone precipitation to remove excess of MMTS, 1 mM ascorbate and biotin-HPDP are added to maintain the Cu (I)-catalyzed reduction of the nitrosothiols and to label the reduced thiol with biotin. Proteins are separated by non-reducing SDS-PAGE followed by immunoblotting. Biotinylated proteins are detected using streptavidin-HRP or anti-biotin HRP and chemiluminescence detection system. This assay is first method to identify *S*-nitrosated proteins on a gel, which enables their subsequent isolation and identification by techniques like mass spectrophotometry.

Many researchers have modified the biotin switch assay either by altering the concentration of ascorbate or the incubation times for the various steps, or adding metals or metal chelators. Several reports indicate that the high levels of ascorbic acid utilized for denitrosation can also reduce disulfide bonds in proteins thus leading to "false" biotinylation of proteins that have no *S*-nitrosothiols [8,9]. A recent article by Gladwin and coworkers indicated that the extent of biotin-labelling depends upon factors such as buffer composition and choice of the metal-ion chelators. Other modifications of the biotin switch method include the addition of trace amounts copper to increase the sensitivity of the assay without compromising its specificity [10].

Sinapinic acid (3, 5-dimethoxy-4-hydroxy cinnamic acid) is widely used as matrices in matrix-assisted laser desorption/ionization (MALDI) mass spectrometry [11]. Previous studies with sinapinic

Abbreviations: CysNO, *S*-nitrosocysteine; NO, nitric Oxide; MMTS, methyl methane thio-sulfonate; HRP, horse radish peroxidase; MALDI, matrix-assisted laser desorption/ionization; NOA, nitric oxide analyzer; GSNO, *S*-nitrosoglutathione; NEM, N-ethylmaleimide

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acid have shown that it reacts with peroxynitrous acid at neutral pH to yield a red product, *O*-nitrososinapinic acid. The *O*-nitrososinapinic acid is a direct analog of *S*-nitrosothiols and was shown to be capable of transnitrosating thiols [12]. However, *O*-nitroso intermediates were unstable, which would lead to their dimerization via the formation of mono-lactone like products at pH 7.4 [13, 14].

The objectives of our study were to determine if sinapinic acid could: (1) denitrosate *S*-nitrosothiols; and (2) to test if sinapinic acid could replace ascorbic acid as the denitrosating agent in biotin switch assay.

2. Materials and methods

2.1. Materials

Glutathione (GSH), L-cysteine, sinapinic acid, ascorbic acid, N-ethylmaleimide, phenylmethylsulfonyl flouride (PMSF), Sodium dodecyl sulfate (SDS), biotin maleimide, Hepes, ammonium persulfate (APS), TEMED, phosphoric acid, Sodium nitrite, N-(1-naphthyl)-ethylenediamine dihydrochloride, Sulphanilamide, 5,5′-ditiobis-2-nitrobenzoate (DTNB), bovine serum albumin, lipopolysaccharide (LPS), N^G-monomethyl-L-arginine (L-NMMA) were purchased from Sigma-Aldrich. Acrylamide solution (30%), Immunopure Streptavidin-HRP and Chemiluminescent HRP-substrate were purchased from Bio-Rad and Pierce respectively. RAW 264.7 were obtained from American Type culture collection (ATCC).

2.2. Synthesis of S-nitrosothiol

GSNO and CysNO were synthesized as described previously [15,16]. Briefly, GSH (Sigma) was dissolved in ice-cold 0.5 M HCl. Equimolar sodium nitrite was added, and the reaction was carried out in the dark at 4 °C for 40 min. The pH of the reaction mixture was adjusted to 7.4. The concentration of GSNO was determined by UV-

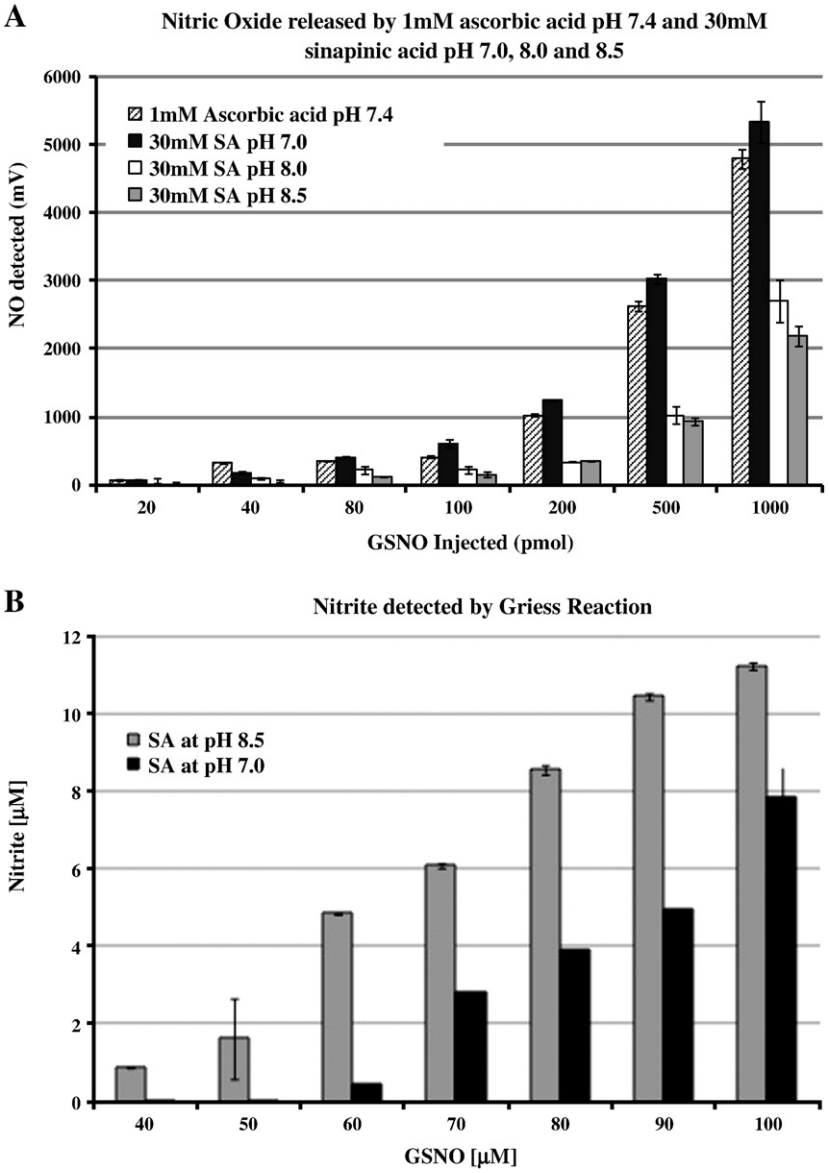

Fig. 1. (A) Comparison of denitrosation of GSNO by 1 mM ascorbic acid at pH 7.4 and 30 mM sinapinic acid at pH 7.0, 8.0 and 8.5 analyzed by ozone-based chemiluminescence nitric oxide analyzer. 20–1000 pmol of GSNO were injected into a glass vessel containing either 1 mM ascorbic acid or 30 mM sinapinic acid solution at varying pH. The error bars represent standard error ($n=3$). (B) Comparison of nitrite formed by reaction GSNO with 30 mM sinapinic acid at pH 8.5 and 7.0.

Vis spectrophotometer by measuring absorbance at 334 nm (extinction coefficient of 920 M^{-1} cm^{-1}). CysNO was synthesized as described by Greco et al. [16] and concentration was determined using extinction coefficient of 900 M^{-1} cm^{-1}. *S*-nitroso-BSA was synthesized by incubating BSA with GSNO at room temperature for 1 h [9], and purified on a G25 sephadex column. The yield of *S*-nitroso-BSA was calculated from the typical *S*-nitrosothiol absorbance spectra, using the reported molar absorption coefficients 3869 M^{-1} cm^{-1} [12,17].

2.3. Nitric oxide detection using NO analyzer (NOA)

Amount of NO formation was determined using Sievers® Nitric Oxide Analyzer (NOA 280i) based on gas-phase chemiluminescence reaction between NO and ozone. NO ($NaNO_2 + NaI$) standard was used to calibrate the NO-analyzer. GSNO (20–1000 pmol) were injected into purge vessel at room temperature containing either ascorbate (1 mM) at pH 7.4 or sinapinic acid (30 mM) at pH 7.0, 8.0 and 8.5.

2.4. Cell culture and treatment

RAW 264.7 cells were purchased from ATCC. Cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with streptomycin (200 μg/ml), penicillin (200 μg/ml) and 10% fetal bovine serum. Cells are incubated at 37 °C incubator at 5% CO_2 and 95% air. For the detection of *S*-nitrosothiols by biotin switch assay, experiments were carried out as previously described [18]. Briefly, cells were seeded in 100 mm dishes and grown to 70–80% confluence. Media was removed and washed thrice with PBS, followed by incubation of cells with CysNO for 1 h in Hank's balanced salt solution (HBSS) with 10 mM Hepes. Cells were washed thrice with PBS after treatment with CysNO, and 900ul of lysis buffer containing 250 mM Hepes pH7.7, 1 mM phenylmethylsulfonyl flouride and 50 mM NEM was added. After treatment, cells were scraped and sonicated (550 sonic Dismembrator from Fisher Scientific, power level 2 for 15 s) and centrifuged at 12000 *g* for 5 min. Supernatant was used for detection of *S*-nitrosothiol by biotin switch assay and sinapinic acid technique.

2.5. Detection of protein S-nitrosothiols by the biotin switch assay with ascorbic acid vs. sinapinic acid as the denitrosating agents

Biotin switch assay was performed as described by Jaffrey et al. [7,10] Briefly, SDS (2.5%) was added to cell lysate in NEM containing lysis buffer, followed by incubation at 50 °C for 1 h with frequent vortexing. Excess of blocking agent was removed by precipitating the proteins with 2 volumes of prechilled (−20 °C) acetone and incubated for 1 h at −20 °C, followed by centrifugation for 10 min at 8000 *g*, 4 °C. The protein pellets were then washed with acetone to remove the excess NEM.

Protein pellets were dissolved in PBS pH 7.4 with 1%SDS, followed by addition of 1 mM ascorbic acid and 1 mM biotin-maleimide for the normal biotin switch assay procedure, and incubated in dark at room temperature for 1 h. For the sinapinic acid modification, after removing excess of blocking agent as described above protein pellets were dissolved in PBS pH 7.0, 30 mM sinapinic acid and 1 mM biotin-maleimide were added and incubated at room temperature in dark for 1 h. After incubation, proteins were precipitated by adding prechilled acetone. Protein pellets were resuspended in PBS, mixed with sample buffer (without reducing agents) and separated on non-reducing SDS-PAGE gel followed by immunoblotting. The blots were incubated with streptavidin-HRP (1/100,000 dilution) antibody for 2 h and visualized by chemiluminescence substrate.

2.6. Griess assay

Determination of nitrite formed by reaction of GSNO with sinapinic acid at pH 8.5 and 7.0 was based on griess reaction [19]. Nitrite containing sample was mixed with equal volume of griess reagent (0.1% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride, and 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid) and incubated at room temperature for 5 min to form a purple azo dye, and absorption was determined at a wavelength of 546 nm [20].

2.7. DTNB assay

The Ellman's reagent (DTNB, 0.4 mM) was used to test if sinapinic acid (30 mM) reduces disulfide bridges. The thiol reagent DTNB bears

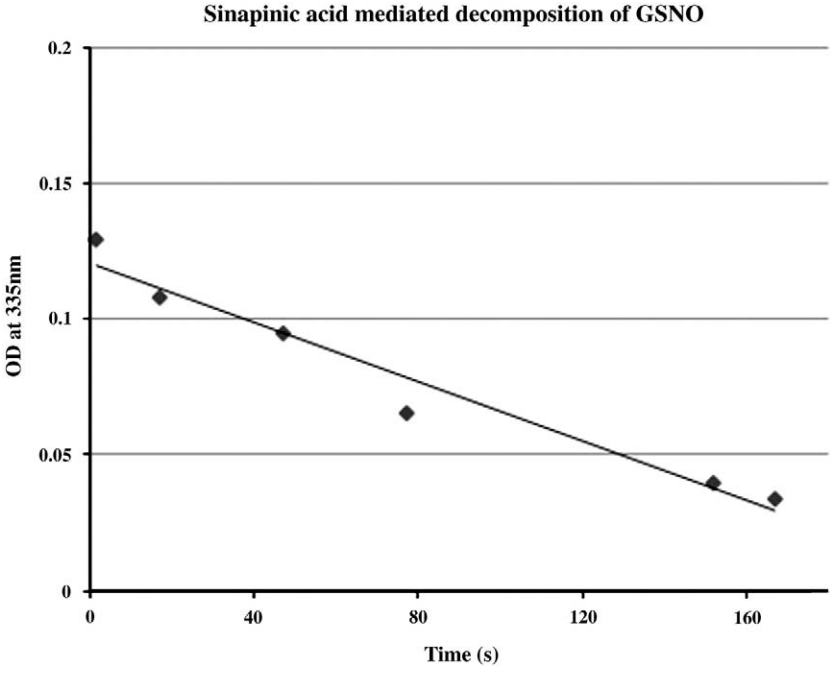

Fig. 2. UV-Vis spectra for decomposition of GSNO by sinapinic acid at pH 7.0.

an aryl-disulfide bond and once reduced possesses a strong absorbance at 410 nm wavelength [21]. 0.4 mM DTNB was added to 1 mM ascorbic acid pH 7.4 or 30 mM sinapinic acid pH 7.0 and formation of the thiol (TNB) was determined spectrophotometrically by measuring the absorbance at 410 nm. To determine free thiol generated by reaction of GSNO with sinapinic acid, 0.2 mM GSNO was added to 30 mM sinapinic acid at pH 7.0 and DTNB assay was performed.

2.8. Kinetic measurements

The reductive decomposition of S-nitrosoglutathione (GSNO) by sinapinic acid was monitored spectrophotometrically on Agilent 8453 UV-Vis spectrometer. The reaction was started by addition of 1 mM GSNO to 30 mM sinapinic acid at pH 7.0. Time dependent spectra were monitored immediately after addition of 1 mM GSNO. Loss of NO from GSNO was accompanied by decrease in absorption at 334 nm.

2.9. Detection of endogenous protein S-nitrosothiols from RAW 264.7 cells treated with and without NOS inhibitor by the biotin switch assay vs. sinapinic acid technique

In this study endogenous NO synthesis is utilized as basis for *S*-nitrosylation for detecting basal levels of protein S-nitrosothiols from RAW 264.7 cells using both biotin switch and sinapinic acid assay [22].The murine macrophage cell line RAW 264.7 was exposed to lipopolysaccharide (LPS) 1 μg/ml for 12 h to induce inducible nitric oxide synthase (iNOS). To detect basal protein *S*-nitrosothiols the cells were either stimulated with LPS or unstimulated in control

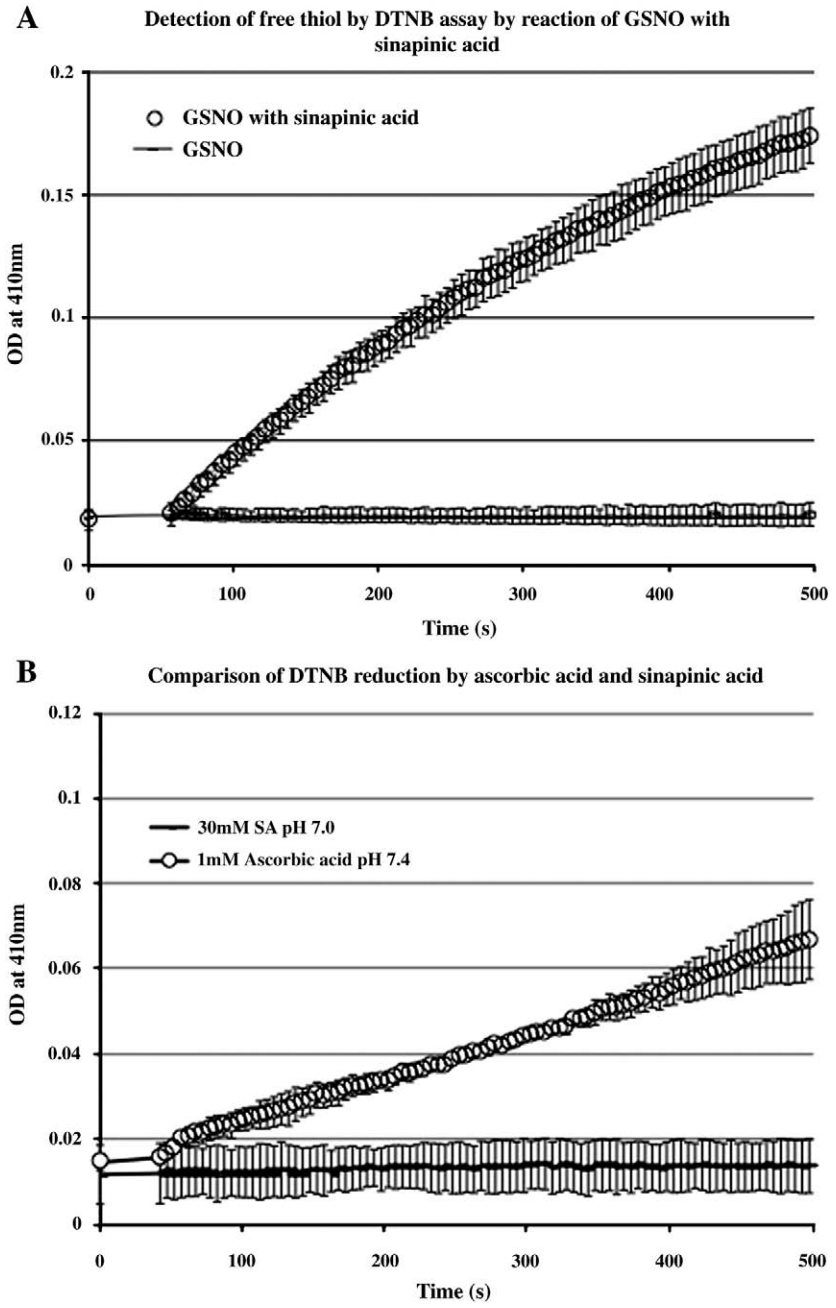

Fig. 3. (A) Detection of free thiol by DTNB assay by reaction of GSNO with sinapinic acid at pH 7.0. (B) Comparison of DTNB disulfide reduction by 30 mM sinapinic acid or 1 mM ascorbic acid at pH 7.0 and pH 7.4 respectively.

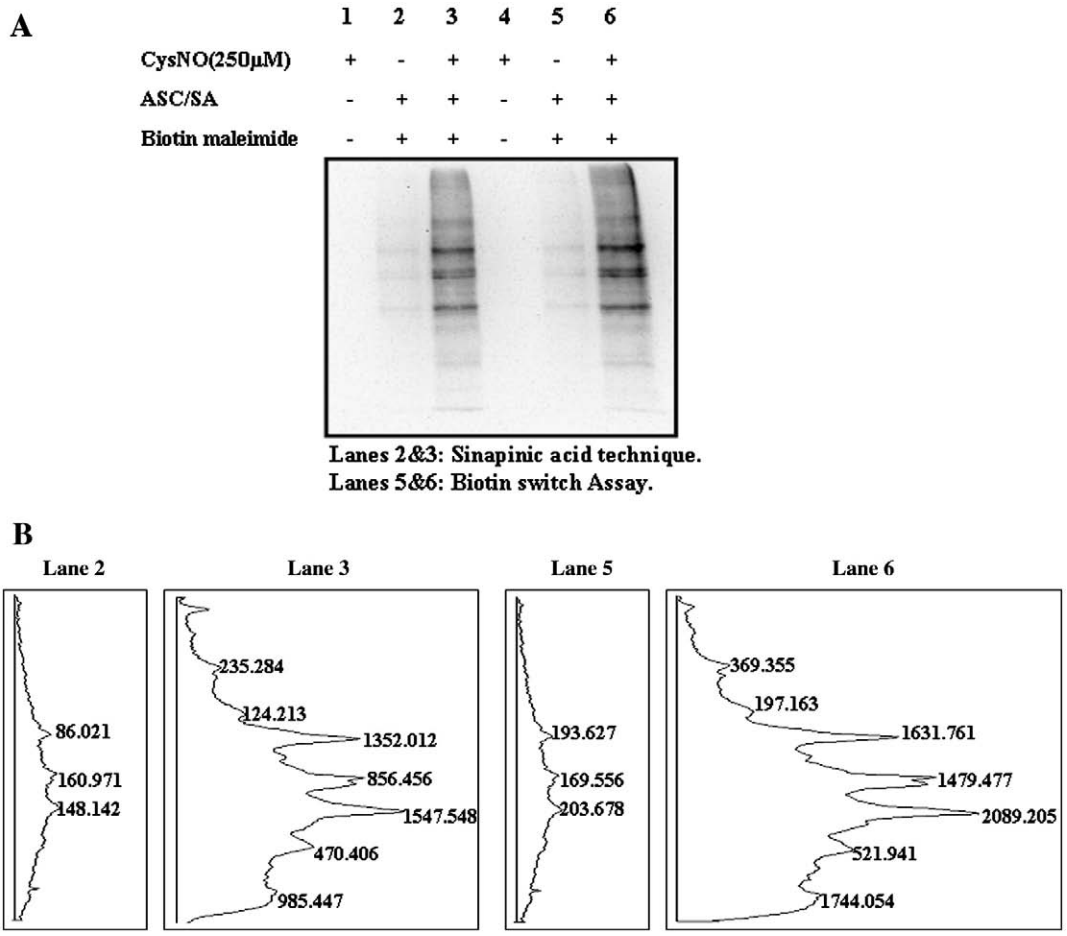

Fig. 4. (A) Detection of *S*-nitrosated proteins in murine macrophage- derived RAW 264.7 cell line using biotin switch assay with either sinapinic acid or ascorbic acid as the denitrosating agent. (B) Densitometry of the western blots in panel A with *Image-J* software.

samples followed by treating with or without NOS-specific inhibitor N^G-monomethyl-L-arginine (L-NMMA, 1 mM) [23,24]. After incubation for 12 h at 37 °C in 5% CO_2 cells were washed thrice with PBS and resuspended in lysis buffer containing 250 mM Hepes pH 7.7, 1 mM phenylmethylsulfonyl flouride and 50 mM NEM was added. After treatment, cells were scraped and sonicated (550 sonic Dismembrator from Fisher Scientific, power level 2 for 15 s) and centrifuged at 12000 *g* for 5 min. Supernatant was used for detection of *S*-nitrosothiol by biotin switch assay and sinapinic acid technique (as described in Section 2.6).

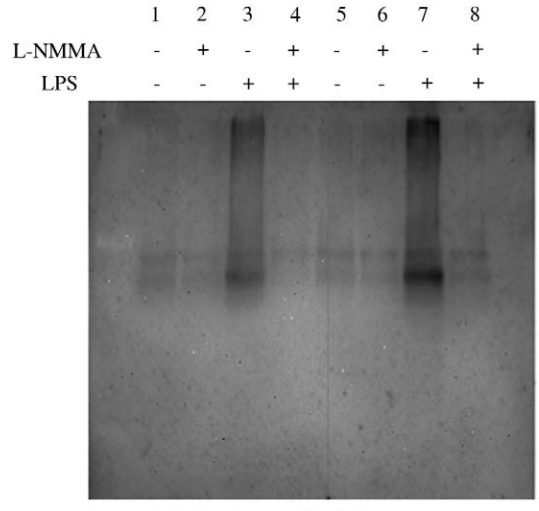

Fig. 5. Western blot of protein *S*-nitrosothiols in control and LPS-treated with and without L-NMMA in RAW 264.7 cells detected by both sinapinic acid assay and biotin switch assay.

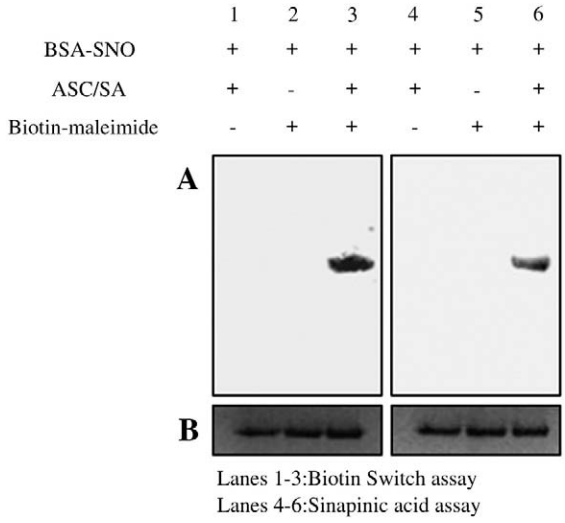

Fig. 6. (A) Western blot of S-nitroso-BSA detected by both sinapinic acid assay and biotin switch assay. (B) Coomassie staining of *S*-nitroso-BSA as loading control.

2.10. Detection of S-nitroso-BSA by biotin switch assay vs. sinapinic acid technique

For these experiments, *S*-nitrosated BSA was biotin labelled using either ascorbate or sinapinic acid as denitrosating agents, followed by SDS-PAGE and immunoblotting as described earlier [10]. The blots were incubated with streptavidin-HRP (1/100,000 dilution) antibody for 2 h and visualized by chemiluminescence substrate.

3. Results

We first sought to determine whether or not sinapinic acid had ability to denitrosate *S*-nitrosothiols. Denitrosation ability of sinapinic acid was studied at pH 7.0, 8.0 and 8.5 by injecting various concentrations of GSNO (20–1000 pmol) into glass vessel of a nitric oxide analyzer containing 30 mM sinapinic acid. Fig. 1A shows the amount of NO released by denitrosation of GSNO by sinapinic acid at varying pH compared to denitrosation of GSNO by 1 mM ascorbic acid at pH 7.4.

The data obtained suggest that the denitrosation ability of sinapinic acid at pH 7.0 is comparable to that of 1 mM ascorbic acid at pH 7.4. However, the denitrosation ability of sinapinic acid at pH 8.5 and 8.0 was less as compared to that observed at pH 7.0.

UV-Vis spectra were recorded for reduction of 1 mM GSNO by 30 mM sinapinic acid (Fig. 2). The spectra were characterized by time dependent decrease at 334 nm. The pseudo first order rate constant (k_{obs}) for the denitrosation of GSNO (1 mM) by sinapinic acid (30 mM) estimated form this data was 0.0079 ± 0.0005 s^{-1} corresponding to a half-life of 1.46 ± 0.009 min. The k_2 (extracted from k_{obs}) was 0.016 ± 0.001 M^{-1} s^{-1}.

The next question was does the denitrosation of RSNOs with sinapinic acid result in the generation of free thiols? To this end, we added DTNB to solution of GSNO ± sinapinic acid. Our data with DTNB assay show that free thiols are generated in the presence but not in the absence of sinapinic acid (Fig. 3A).

Next, we investigated the potential role of sinapinic acid in replacing ascorbic acid in biotin switch assay. RAW264.7 cells treated or untreated with CysNO (250 μM) and *S*-nitrosothiols were detected by both biotin switch assay where sinapinic acid replaced ascorbate for *S*-nitrosoprotein reduction (Fig. 4A). Densitometry of the western blots was calculated using *Image-J* software (Fig. 4B). 32pmol of SNO / μg of protein were detected in blot. It is evident from the western blot data that nearly identical *S*-nitrosoprotein band patterns were evident when either sinapinic acid or ascorbate was used to reduce the S-nitrosoprotein pool. However, the blot densities calculated for ascorbate were larger than sinapinic acid

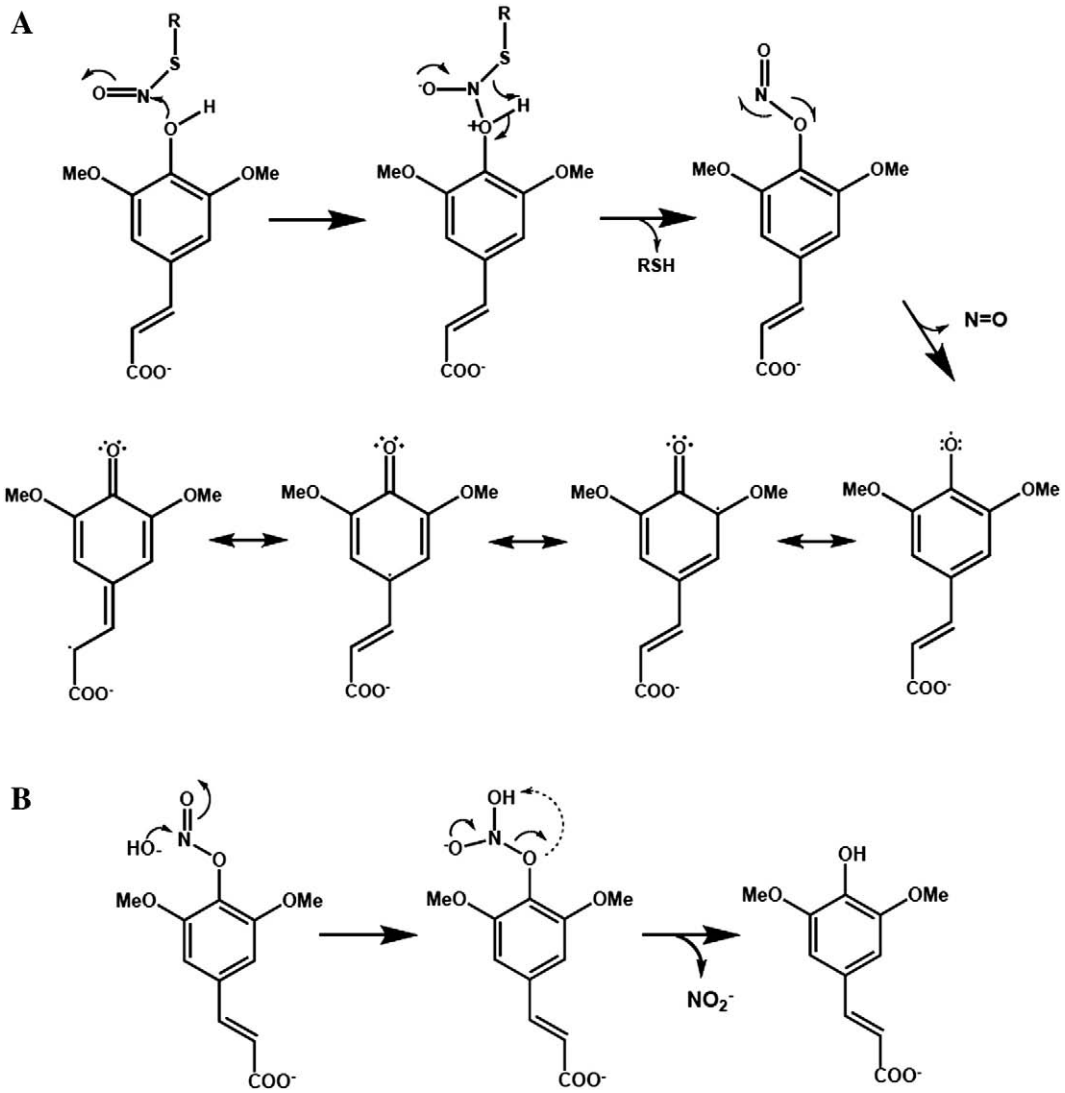

Fig. 7. (A) Proposed mechanism for denitrosation of *S*-nitrosothiols by sinapinic acid at pH 7.0. (B) Proposed mechanism for the formation of nitrite at pH 8.5 and 8.0 from *O*-nitroso sinapinic acid.

both in the absence (Fig. 4B lanes 2, 5) and presence (Fig. 4B lanes 3, 6) of CysNO.

Protein *S*-nitrosothiols were measured from RAW 264.7 cells as an index of NOS induction, using both sinapinic acid and biotin switch assay. LPS induced *S*-nitrosation (Fig. 5, Lanes 3 and 7) was inhibited in presence of L-NMMA (Fig. 5, Lanes 4 and 8). Both biotin switch assay and sinapinic acid technique are used for detecting *S*-nitroso-BSA (Fig. 6, Lanes 3 and 6).

4. Discussion

In an earlier study by Akhter et al. [12,13] we showed that sinapinic acid could get *O*-nitrosated with nitrite at acidic pH. Furthermore, *O*-nitrososinapinic acid could transnitrosate the free thiol of bovine serum albumin (BSA) forming *S*-nitroso-BSA at pH 7.0. Here we tested whether sinapinic acid could also be utilized to denitrosate *S*-nitrosothiols, with the goal of using it instead of ascorbate in the biotin switch assay.

Our data show that the reactions of sinapinic acid (30 mM, pH 7.0) or ascorbic acid (1 mM, pH 7.4) with GSNO resulted in the release of similar amounts of NO as detected by ozone-chemiluminescence (Fig. 1A). However, at pH 8.0 and 8.5 sinapinic acid yielded ~64% less (500 pmol GSNO) or ~56% less (1000 pmol GSNO) NO respectively than at pH 7.0 (Fig. 1A). We believe a plausible explanation for our observations is that at pH 7.0, sinapinic acid releases NO as shown in the proposed mechanism in Fig. 7A: when an *S*-nitrosothiol comes into contact with sinapinic acid at pH 7.0, the hydroxyl oxygen performs a nucleophilic attack on the nitrogen of the nitroso group. The oxygen of the nitroso group would abstract electrons from the double bond to help stabilize the nitrosothiol-sinapinic acid adduct. Upon interaction of the hydroxyl proton with the nitrosothiol sulphur, a protonated thiol as well as *O*-nitrososinapinic acid would be formed. This is similar to mechanism proposed for formation of *O*-nitrososinapinic acid by nitrite and peroxynitrite [12]. The intermediate *O*-nitrososinapinic acid is unstable and would decompose to yield nitric oxide and *O*-nitrososinapinic acid radical, which can be stabilized by resonance as proposed by Niwa et al. [13,14]. However, at pH 8.0 and 8.5 we believe the product of the reaction is nitrite, as proposed in mechanism shown in Fig. 7B: where, a hydroxide anion attacks the *O*-nitrososinapinic acid nitrogen forming *O*-nitrososinapinic acid which decomposes to yield nitrite. To test this, we compared the amount of nitrite formed by sinapinic acid at pH 7.0 and pH 8.5 via the griess reaction which has a detection limit for nitrite ~1–5 μM [19]. As a result we studied the degradation reaction at larger [GSNO] (40–100 μM). The data obtained (Fig. 1B) does show that sinapinic acid yields ~50% more nitrite at the higher pH thus supporting the proposed mechanism in (Fig. 7B).

Our data with DTNB assay indicate that free thiols are formed as result of denitrosation of GSNO by sinapinic acid at pH 7.0 (Fig. 3A). We also tested whether sinapinic acid, like ascorbate, could also reduce disulfide bridges. To this end, we employed the thiol reagent DTNB that bears an aryl-disulfide bond whose reduction can be monitored spectrophotometrically since the reduced product 2-nitro-5-thiobenzoate has a strong absorbance at 410 nm ($E_M = 13{,}6000$ L/mol cm). Our data indicate that at concentrations employed in the biotin switch assay procedure sinapinic acid does not reduced disulfide bridges and hence it is specific for the degradation of *S*-nitrosothiols. In contrast, ascorbic acid as previously shown [20] does result in the reduction of the DTNB disulfide (Fig. 3B).

The data obtained from western blots (Fig. 4) indicate that both the techniques (ascorbate or sinapinic acid) detect same set of *S*-nitrosated proteins. However, the blot densities calculated for ascorbate were larger than sinapinic acid both in the absence and presence of CysNO, likely due to the reduction of disulfides by ascorbate in biotin switch assay.

Our data from LPS activated RAW cells indicated that both the techniques detect basal levels of *S*-nitrosothiols (Fig. 5, lanes 1 and 5). The fact that the *S*-nitrosated proteins decreased significantly in the presence of and L-NMMA (Fig. 5, lanes 4 and 8), an inhibitor of all nitric oxide synthase (NOS) isoforms suggests that the LPS induced S-nitrosation (Fig. 5, lanes 3 and 7) is the result of NOS-dependent NO production.

In conclusion, we have shown that sinapinic acid has the ability to denitrosate *S*-nitrosothiols at pH 7.0 resulting in generating a free thiol and denitrate plus denitrosate at pHs 8 and 8.5. In addition, unlike ascorbate, sinapinic acid degrades S-nitrosothiols, but it does not reduce disulfide bridges. Furthermore, sinapinic acid produced nearly identical *S*-nitrosoprotein patterns/density, in comparison to ascorbate, in the biotin switch analysis of both CysNO treated or untreated RAW 264.7. These results clearly indicate that sinapinic acid can readily replace ascorbate in the analysis of the *S*-nitrosoproteome via currently available methods or those under development.

Acknowledgements

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