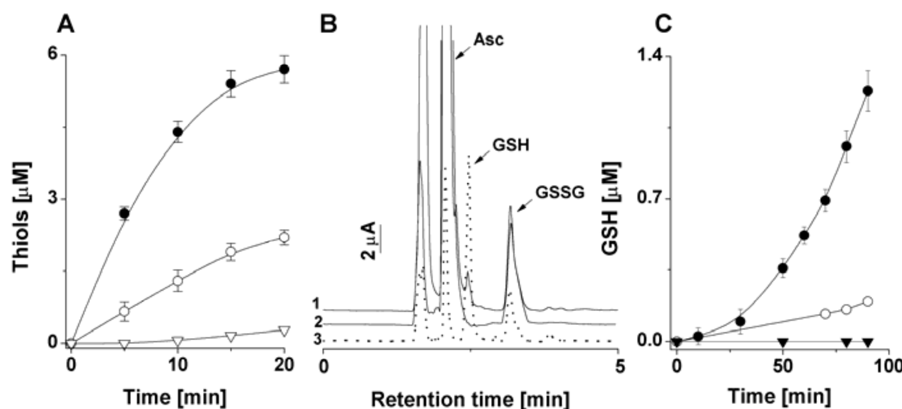

Figure 4. Reduction of disulfides by Asc and Cu^{n+}. Reactions were carried out at 25 °C in Chelex 100-treated 0.1 M phosphate buffer (pH 7.4). *A*- Spectrophotometric assessment of thiols was carried out with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by following increases in the absorbance at 412 nm. Triangles, DTNB (0.2 mM) and Asc (0.1 mM); open circles, DTNB, Asc and $CuSO_4$ (5 µM); Closed circles, DTNB, Asc, $CuSO_4$ and cystine (0.1 mM). *B*- HPLC analysis of the reduction of oxidized glutathione (GSSG) by Asc and $CuSO_4$ to glutathione (GSH). Asc (1 mM), GSSG (1 mM), and $CuSO_4$ (5 µM) were incubated for 1 min (chromatogram 2) and 60 min (chromatogram 1); chromatogram 3, standard solution of Asc (20 µM), GSH (10 µM; GSH) and GSSG (0.2 mM). *C*, Closed circles- Asc, GSSG and $CuSO_4$ (incubation time, 1 hour); triangles- Asc, GSSG and $CuSO_4$ incubated for 1 hour under helium. Additional identification of the HPLC peaks reflecting the elution of GSH was performed by their deletion with DTNB (0.2 mM; 5 min incubation at 25 °C; open circles).

Reagents: All reagents used were purchased from Sigma Chemical Co. (St. Louis, MO). DMPO/SG adduct (**12**) was synthesized and characterized by MS and ^{1}H-NMR as described in ref. (*18*). The solutions used in the experiments were prepared in deionized and Chelex-100-treated water or potassium phosphate buffer.

Photolysis of S-nitrosothiols: Visible (white) light provided by a Sylvania lamp type DWY equipped with a spherical reflector ("Sun Gun"; 625 W) was used for irradiation of *S*-nitrosothiols. The light source was positioned from 30 to 120 cm from the front of the EPR cavity or the thermostated at 25 °C glass tubes containing the corresponding samples. In selected experiments (Figures 2*C* and 3), the light was filtered through a 515 nm cut-off filter (colored glass filter OG515; Melles Griot BV; Aalsbergen, Nederland). Light intensity was measured with a Datalogging Light Meter (Model 850008; SPER Scientific, LTD; Scottsdale, AZ).

Western Blots: Protein-DMPO adducts were assessed by Western blot analysis as reported in ref. (*19, 22*). Briefly, proteins were resolved by SDS-PAGE (10%) and then transferred onto a nitrocellulose membrane. The membrane was consecutively treated with a blocking buffer (5% BSA/casein, 1:1), anti-DMPO nitrone adduct polyclonal antiserum (rabbit IgG; Cayman; Ann Arbor, MI; dilution, 1:1,000), and alkaline phosphatase-conjugated goat anti-rabbit IgG as a secondary antibody (Pierce; Rockford, IL; dilution, 1:5,000). Thereafter, the membrane was exposed to Lumi-Phos™ WB Chemiluminescent substrate (Pierce) and visualized by chemiluminescence on an autoradiography film.

Analysis of PSNOs: GSNO and PSNOs were quantified following their Cu^+-catalyzed breakdown to •NO with concomitant chemiluminescence measurements of the latter in the gas-phase using a Sievers Nitric Oxide analyzer (NOA™ 280i; Boulder, CO; (*3*)). The purge vessel of the NO Analyzer was filled with 5 mL of 0.1 M phosphate buffer (pH 7.4; 20 °C; gas carrier, He). In the reaction vessel, a steady-state concentration of Cu^+ was maintained by a large excess of ascorbic acid over $CuCl_2$ (50 mM vs. 0.2 mM). Thus, multiple injections of aliquots (5 µL) containing PSNOs could be made without any significant loss of analytical sensitivity. Under

these experimental conditions, $NaNO_2$ (up to 0.1 mM) did not interfere with the analysis of PSNOs. Calibration of the experimental peaks was performed by injection of standard solutions of GSNO (0.1 – 50 μM).

Protein S-nitrosation: For isolation of proteins, HepG2 cells were disrupted by three cycles of freezing and thawing. The resulting homogenate was centrifuged for 90 min at 100,000g, and low molecular mass compounds were removed from the supernatant via ultrafiltration through a 10 kDa cut-off filter (Vivaspin™ 500; Cole Palmer, Vernon Hills, IL). The filtrate was discarded while the protein fraction (~ 0.01 mL) was diluted with 0.1 M phosphate buffer (0.2 mL; pH 7.4) containing EDTA (0.2 mM) and subjected to a second ultrafiltration. The final protein extracts (5 mg of protein/mL; MW $\geq$ 10 kDa) was treated with GSNO (0.3 mM) for 30 min at 20 ^{o}C; then, the excess of GSNO was removed via ultrafiltration (10 kDa Vivaspin cut-off filter), which included 4 washing cycles with 0.1 M phosphate buffer containing 0.2 mM EDTA (4 x 0.2 mL). In the final protein fraction, the content of GSNO was less than 0.1 μM, as assessed by reverse phase HPLC-EC (*3*). The PSNOs thus obtained could be kept at -70 ^{o}C for up to 1 month without any significant losses of SNO functions.

S-nitrosation of $(HS)_3TrxnS_2^{(32,35)}$ was performed with GSNO (0.3 mM) at 20 ^{o}C for 30 min in 0.1 M phosphate buffer containing 0.2 mM EDTA. Thereafter, 14 was separated from GSH and the excess of GSNO via ultrafiltration (3 kDa Vivaspin cut-off filter), which included 4 washing cycles with 0.2 mL of the reaction buffer.

Measurement of NO_2^-: Aliquots from the reaction system were directly mixed with equal volumes of 1% sulfanilamide in 0.4 N HCl and 0.1% *N*-(1-naphtyl)ethylenediamine in 0.4 N HCl to determine nitrite anion (HNO_2). After an incubation of 10 min at room temperature, the absorption was measured at 540 nm. Quantitation of nitrite anion was achieved via comparison of the experimental results with those obtained with a standard solution of $NaNO_2$.

Determination of proteins and thiols: Protein concentration in samples was determined using the Bio-Rad Protein Assay Kit with bovine serum albumin as standard. Protein thiols ($(HS)_3TrxnS_2^{(32,35)}$) were determined colorimetrically at 412 nm following the reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to 2-nitro-5-mercapto-benzoic acid (NMB; $\varepsilon_{412\ nm}$ = 13,500 $M^{-1}cm^{-1}$).

HPLC analysis of Asc, GSSG and GSH. HPLC separations were achieved with 50 mM phosphate buffer (pH 3.0, adjusted with acetic acid) containing 2.5% methanol at a flow rate of 0.5 mL per min on a 3.2 x 150 mm C18 ESA column (3 μ; ESA Inc. Chelmsford, MA.). Electrochemical detection of the analytes was carried out with an ESA Coulochem II detector at a holding potential of +950 mV.