Supplementary Information

Glutathione and thioredoxin type 1 cooperatively denitrosate HepG2 cells-derived cytosolic *S*-nitrosoproteins

Detcho A. Stoyanovsky,[a*] *Melanie J. Scott,*[b] *and Timothy R. Billiar*[b]

Departments of Environmental and Occupational Health[1] and Surgery,[2] University of Pittsburgh, Pittsburgh, Pennsylvania 15213.

*Corresponding author: stoyanovskyd@upmc.edu

Reagents - All reagents used were purchased from Sigma Chemical Co. (St. Louis, MO). The solutions used in the experiments were prepared in deionized and Chelex-100-treated water or potassium phosphate buffer.

Analysis of S-nitrosothiols – GSNO and PSNOs were quantified following their Cu^+-catalyzed breakdown to RSH and nitric oxide with concomitant chemiluminescence measurements of the latter in the gas-phase using a Sievers Nitric Oxide analyzer (NOA™ 280i; Boulder, CO). 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), where 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 – 20 μL) containing either GSNO or PSNOs could be made without any significant loss of analytical sensitivity, as indicated by the release of NO after injection of a standard solution of GSNO upon completion of the reactions. Under these experimental conditions, $NaNO_2$ (up to 0.1 mM) did not interfere with the analysis of RSNOs. Calibration of the experimental peaks was performed by injection of standard solution of GSNO (0.1 – 50 μM).

S-nitrosation of cytosolic proteins from HepG2 cells - Cells were disrupted by three cycles of freezing and thawing. The resulting suspension was first centrifuged for 15 min at 14,000g and then, after discarding the pellet, for 90 min at 100,000g. From the resulting supernatant, LMW compounds and small proteins were removed via ultrafiltration through a 10 kDa cut-off filter (Vivaspin™ 500; Cole Palmer, Vernon Hills, IL). To minimize loss of PSNOs (< 5 %), the pores of the filters were pre-equilibrated with protein via centrifugation of 0.2 mL phosphate buffer containing 2 mg of cytochrome c/mL; after removal of the remaining protein, the filters were subjected to 2 washing cycles with 0.2 mL phosphate buffer. The filtrate from the 100,00 g supernatant 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 latter step was repeated three times. The final protein extract (~ 5 mg of protein/mL; MW $\geq$ 10 kDa) was treated with GSNO (0.3 mM) for 30 min at 20 °C; then, the excess of GSNO and GSH were 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 (C18 column; Alltima 4.6 x 250 mm, 5u; Alltech Associates, Inc.; Deerfield, IL; mobile phase, 50 mM phosphate buffer containing 10 % CH_3OH; Coulochem detector, E = + 900 mV). The PSNOs thus obtained could be kept at -70 °C for up to 1 month without any significant losses of SNO functions.