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Original Research Communication

Quantification of Oxidative/Nitrosative Modification of CYS_{34} in Human Serum Albumin Using a Fluorescence-Based SDS-PAGE Assay

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ABSTRACT

The SH group represented by cysteine in proteins is fundamental to the redox regulation of protein structure and function. Albumin is the most abundant serum protein whose redox modification modulates its physiologic function, as well as serves as a biomarker of oxidative stress. Measurement of selective Cys modification (*S*-oxidation/nitrosation, electrophilic substitution) on specific proteins, however, is problematic within complex biological mixtures such as plasma. We have utilized a maleimide fluorogenic SH reagent, ThioGlo™-1, to develop a fluorescence-based quantitative assay of SH modification of human serum albumin (hSA) using SDS–PAGE. Fully reduced native albumin containing one free SH (Cys_{34}) per molecule was utilized as a model protein to characterize the kinetics of ThioGlo™-1 reaction using a solution-based spectrofluorometric assay. Optimum labeling of hSA Cys_{34} was achieved within 10 min at 60°C using a threefold molar excess of ThioGlo™-1 relative to hSA and required SDS. Comparison of the solution spectrofluorometric assay to fluorescent image analysis of hSA bands localized by SDS-PAGE revealed that SH groups in hSA could be quantified after gel electrophoresis. The solution- and gel-based methods were in excellent concordance in their ability to quantify SH modification of hSA following exposure to phenoxyl radicals and nitric oxide. The application of ThioGlo™-1 staining and SDS-PAGE quantified the degree of hSA modification in complex human plasma exposed to oxidative or nitrosative stress and revealed that hSA is more sensitive to S modification than other SH-containing plasma proteins. *Antioxid. Redox Signal.* 4: 855–865.

INTRODUCTION

ONE OF THE MOST IMPORTANT FUNCTIONAL GROUPS in various proteins is the free sulfhydryl (SH) group contained in the amino acid cysteine. Many enzymes contain specific cysteine residues within their active sites where the bimodal proton-donating and proton-withdrawing properties of free SH or thiolate anion, respectively, play essential roles in catalysis (22, 30). In addition, protein SH groups can serve to bind and coordinate various metal ligands as in the case of metallothionein (12). More recently, important thiol-dependent redox-sensing mechanisms have been implicated in the signal transduction properties of fos, jun (1), and Oxy R (37) transcription factors in mammals and bacteria. Because of their reactive chemical nature, thiols are frequent sites of modification by electrophilic xenobiotics (2), as well as oxidative or nitrosative stress (3, 5).

The studies above confirm the need to develop quantitative methods that permit the precise experimental determination of thiol modification on specific proteins in complex mixtures *in vivo* and *in vitro*. The utilization of Ellman's reagent [5,5′-dithio-2-nitrobenzoic acid (DTNB)] (7) or dithiodipyridine (24) are often applied to quantify thiols in biological samples by absorption measurements. The relatively low sensitivity and other factors, however, limit their usefulness in analyzing small amounts of material, and it is difficult to obtain any information concerning thiol status of individual components in complex mixtures. Recent advances

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in fluorescence technology have led to the creation of numerous SH-reactive chemicals tagged with a variety of fluorophores with improved sensitivity. Several methods have been used to identify thiol-dependent modification of specific proteins following polyacrylamide gel electrophoresis (PAGE) (4, 9, 19). These technical approaches, however, are limited by several factors, including their lack of quantification, as well as reliance on the use of radioactivity and their specificity for only particular types of SH modification, such as *S*-thiolation with glutathione (GSH). Kim *et al.* have described a novel approach to resolve hydrogen peroxide (H_2O_2)-sensitive cysteines on PAGE-resolved proteins based on differential pH sensitivity and biotin-conjugated iodoacetamide labeling (15). Strict quantification, however, was not applied, and this method appears specific for labeling those cysteine residues that display a relatively low pK_a. Labeling proteins with thiol reagents such as bimanes (16) or maleimide coumarins (35) is complicated by the low aqueous solubility, high intrinsic fluorescence of unreacted reagent, as well as rapid hydrolysis of the protein thiol–probe adduct (18, 34).

Albumin is the major protein found in plasma and represents an important target for oxidation during systemic oxidative stress. Human serum albumin (hSA) contains 34 cysteines linked to form 17 disulfude bonds and an additional solitary free SH group contained at the Cys_{34} position. It has been suggested that albumin constitutes a major extracellular antioxidant (10, 26). As such, its oxidation state may serve as a useful biomarker for monitoring oxidative stress in human populations. Oxidative modification of Cys_{34} can alter the affinity of this protein for Cu^{2+} and other ligands, such as phenolsulfophthalein and free fatty acids (13, 28). Cys_{34}-nitrosoadduct of hSA represents a major carrier of releasable nitric oxide (NO) in human plasma (27). The aim of the present work was, therefore, to develop a quantitative method for determination of protein thiols in hSA following resolution by sodium dodecyl sulfate (SDS)–PAGE using the fluorescent maleimide-based reagent, ThioGlo™-1, which shows enhanced fluorescence upon derivatization to free thiols (18).

MATERIALS AND METHODS

Materials

Dithiothreitol (DTT), DTNB, etoposide, deferoxamine, H_2O_2, acetaminophen, *S*-nitrosoglutathione (GS-NO), and myeloperoxidase (from human leukocytes; EC 1.11.1.7) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The fluorescent maleimide-based reagent, ThioGlo™-1 [*N*-(2-carbomethoxy-9-methoxy-3-oxo-3*H*-naphthol[2,1-b]pyran-10-yl)maleimide], was from Covalent Associates, Inc. (Woburn, MA, U.S.A.). The NO-donor compound, (Z)-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate (NOC-15), was purchased from Alexis Biochemicals (San Diego, CA, U.S.A.). Normal human plasma was the kind gift from Dr. James Roberts at Magee Women's Hospital and collected with IRB approval as part of a large ongoing multicenter program project on preeclampsia.

Preparation of reduced hSA

hSA (fatty acid-free) was obtained from Sigma Chemical Co. Preliminary studies indicated that this batch of hSA contained a significant amount of oxidized Cys_{34} (60%). It was therefore necessary to prepare fully reduced hSA prior to experiments. hSA was dissolved in 50 m*M* phosphate buffer, pH 7.4 (15 mg/ml), and treated with DTT (2 m*M*, final concentration) for 1 h at room temperature essentially as described by Katchalski *et al.* (14). Reduced hSA was then extensively dialyzed against 50 m*M* phosphate buffer, pH 7.4, for 24 h at room temperature. Contents of the dialysis bag were collected, and the thiol content was measured by reacting with dithionitrobenzene and recording absorbance at 412 nm (extinction coefficient, 13,600 M^{-1} cm^{-1}). Protein content was measured by a Bradford assay using bovine serum albumin as a standard.

Exposure of hSA and human plasma to oxidative and nitrosative conditions

Stock solutions of purified reduced hSA were prepared in 50 m*M* phosphate buffer, pH 7.4, to a final concentration of 10 mg/ml. Human plasma samples were similarly diluted in 50 m*M* phosphate buffer, pH 7.4, with 30 μ*M* SDS to contain a final protein concentration of 8 mg/ml. hSA or plasma protein samples were subjected to oxidation by addition of myeloperoxidase/H_2O_2 alone (1 unit/ml myeloperoxidase, 100 μ*M* H_2O_2, final concentration) or in combination with substrates capable of stimulating enzyme-dependent phenoxyl radical-generating peroxidase activity such as acetaminophen, phenol, or etoposide (500 μ*M*, final concentration). Deferoxamine (100 μ*M*) was also added to chelate any adventitious iron. Samples were then incubated at 37°C for 20 min. Protein samples were subjected to nitrosative conditions by addition of the NO donors NOC-15 (0.5 or 2 m*M*, final concentration) or 0.5 m*M* GS-NO and incubation for 1 h at 37°C at room atmosphere. Following incubations, aliquots of these samples were taken for reaction with ThioGlo™-1 and SH group determination by standard fluorescence spectroscopy or SDS-PAGE and fluorescent image analysis.

Labeling of free SH groups with ThioGlo™-1

Purified reduced hSA was utilized in the initial experiments designed to characterize and validate our quantitative assay. hSA stock solutions or plasma samples were diluted in 50 m*M* phosphate buffer, pH 7.4, containing 30 m*M* SDS to yield protein concentrations ranging from 0.05 to 0.75 mg/ml. ThioGlo™-1 (10 μ*M*, final concentration unless indicated) was added to 2.5 ml of this protein solution (typically containing <3 μ*M* protein SH groups unless indicated), and the mixture was incubated at 60°C for 40 min followed by 20 min of cooling at room temperature. Following reaction of protein solutions with ThioGlo™-1, 2.5-ml samples were subjected to standard solution spectrofluorometry. Fluorescent content of the sample was determined using a Shimadzu spectrofluorometer RF-5301PC using excitation and emission wavelengths 388 and 500 nm, respectively. Data were analyzed using RF-5301PC Personal Fluorescence Software

(Shimadzu). For analysis of the specific protein thiols in plasma, samples were pretreated with 10 μ*M* ThioGlo™-1 prior to the addition of SDS in order to derivatize any GSH or other low-molecular-weight thiols present in plasma. The protein thiol component was then measured as the difference in the fluorescent responses obtained before and after addition of SDS.

Fluorescence determination following SDS-PAGE

The electrophoretic patterns of ThioGlo™-1-labeled proteins were determined using slight modifications of the SDS-PAGE method described by Laemmli (17). Samples of ThioGlo™-1-labeled proteins (20 μl) prepared as described above were combined with 5 μl of 5× loading buffer and placed onto 1.5-mm-thick 10% acrylamide gels. Electrophoresis was carried out under standard conditions with the exception that loading buffer did not contain bromophenol blue, which was found to have fluorescent properties similar to ThioGlo™-SH adducts. In addition, the samples were applied to the gel without additional heating in order to preserve the stability of the ThioGlo™-SH products. Fluorescence of ThioGlo™-1 derivatized to protein thiols was then visualized with a Bio-Rad Fluor-S MultiImager equipped with a scanning ultraviolet (UV)-light source (290–365 nm) and built-in CCD camera with the emission filter at 530 nm (broad range). Image capture and subsequent densitometric analysis of the intensity of the fluorescent bands were performed using Bio-Rad Multi-Analyst Software according to the manufacturer's directions.

RESULTS

Kinetics and characteristics of ThioGlo™-1/hSA reaction

We and others have previously reported on the use of the maleimide-based ThioGlo™ reagents to quantify both low-molecular-weight (primarily GSH) and protein thiols in model systems and cellular homogenates (8, 11, 18, 34). The ability to discriminate between these two separate pools depends on the initial titration of low-molecular-weight thiols in the absence of SDS followed by a second titration in the presence of SDS. SDS is required to fully denature tertiary protein structure and thus permit reaction between the SH groups on proteins and the maleimide-based ThiolGlo™-1 reagent. Our goal here was to extend this methodology to enable us to quantify the amount of free SH groups on specific proteins resolved from complex mixtures by SDS-PAGE. Such methodology would thus permit the evaluation of the degree of S modification on specific proteins contained in heterogeneous samples such as human plasma.

We first chose to characterize the kinetics of the reaction of ThioGlo™-1 with purified hSA, which under fully reduced physiological conditions contains a single SH group (Cys_{34}) and represents the major constituent of the complex protein mixture of human plasma. We first determined the reaction kinetics of ThioGlo™-1 with purified hSA. Figure 1A shows that, at room temperature in the absence of SDS, the combination of ThioGlo™-1 and hSA produced a very slow and minimal increase in fluorescence intensity over the time period measured. In contrast, the addition of SDS to this reaction mixture (Fig. 1B) resulted in a dramatic instantaneous increase in fluorescence intensity that then increased at a rate more rapid than that observed in control. Figure 1C represents an identical incubation as 1B except in the absence of any added protein and demonstrates that SDS has essentially a minimal effect on the fluorescence of unreacted ThioGlo™-1 and cannot itself account for any time-dependent increases in fluorescence intensity. Therefore, it appears that SDS greatly facilitates the reaction of ThioGlo™-1 with the single free SH group of Cys_{34} in hSA.

It was noted, however, that even with the addition of SDS a plateau in hSA/ThioGlo™-1 fluorescence was not achieved over the time period observed in these samples. For this reason, we examined whether increases in temperature would

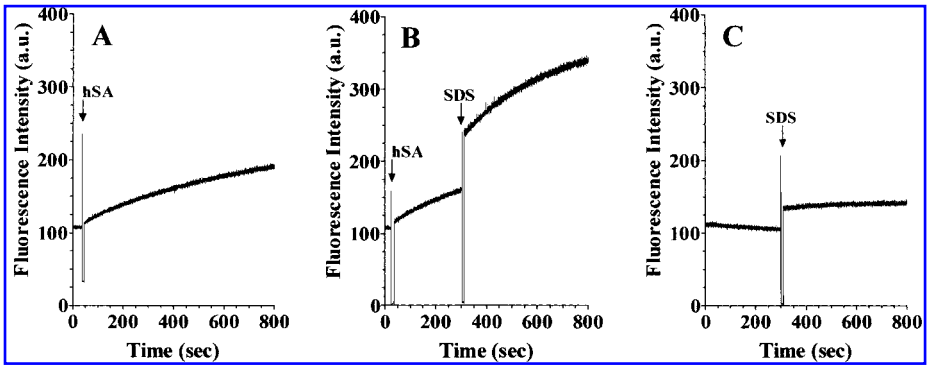

FIG. 1. Time-dependent titration of protein sulfhydryls on hSA by ThioGlo™-1 in the presence and absence of SDS. hSA (1.1 μ*M*) was placed in 2.5 ml of 50 m*M* phosphate buffer, pH 7.4, containing 10 μ*M* ThioGlo™-1. Following the addition of hSA, the fluorescence was recorded continuously in a spectrofluorometer at room temperature with constant mixing at 500 nm emission wavelength and 388 nm excitation wavelength. **A** shows the reaction of hSA thiols in the absence of SDS. **B** shows the rapidly enhanced accessibility of hSA thiols to ThioGlo™-1 upon the addition of SDS (30 m*M*, final concentration). **C** shows the nonspecific fluorescence response of ThioGlo™-1 alone in the absence of added hSA.

hasten the reaction. Figure 2 compares the rate of ThioGlo™-1 reaction with the available thiols on fully reduced hSA when incubations were performed at two temperatures (25°C and 60°C). As observed in Fig. 1, there was a slow progressive increase in ThioGlo™-1 labeling that continued over the first 60 min of the incubation at 25°C and appeared to plateau between 75 and 125 min. In contrast, ThioGlo™-1 reaction proceeded very rapidly at 60°C such that saturation was essentially achieved by the earliest time point tested (5 min). In this experiment, the maximal degree of labeling at both temperatures corresponded to >80% of the total SH groups theoretically available for labeling. The failure to achieve 100% efficiency of labeling may result from incomplete reduction of initial hSA stock by DTT or low spontaneous level of SH modification/oxidation occurring during the aerobic incubation conditions. The rapid kinetics of ThioGlo™-1 reaction with SH groups on hSA led us to adopt the 60°C incubation temperature for further experiments.

The optimum concentration of ThioGlo™-1 was then determined by incubating a single protein concentration over a range of ThioGlo™-1 concentrations in order to ascertain the optimum protein-to-ThioGlo™-1 ratios to achieve maximal labeling of SH groups. Figure 3 shows the results of incubating hSA corresponding to 3 μ*M* free cysteines with various concentrations of ThioGlo™-1 (1–50 μ*M*) for 20 min at 60°C. Prior to reading the resultant fluorescence, it was necessary to dilute the various samples to correspond to a uniform ThioGlo™-1 concentration during each reading (in this case, 1 μ*M*) in order to minimize any contribution of low level of fluorescence from unreacted ThioGlo™-1. Intensity of the increased fluorescence above background (that measured in the absence of added protein) was then normalized back according to the specific dilution factor for each sample.

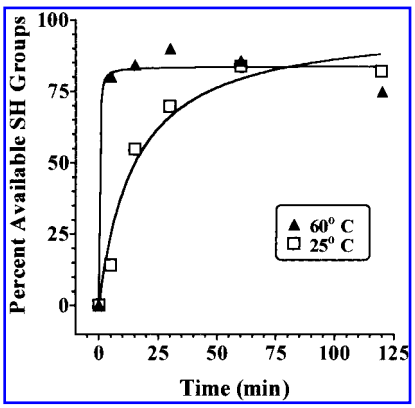

FIG. 2. Comparison of ThioGlo™-1 labeling of hSA protein thiols at 25°C and 60°C. Incubations of hSA and ThioGlo™-1 in the presence of 30 m*M* SDS were set up as described in Fig. 1 and incubated at either 25°C or 60°C. Aliquots were taken at the various times and fluorescence recorded as described in Fig. 1. Percentage of available thiols was calculated based on the fact that fully reduced hSA contains only a single free reduced cysteine (Cys_{34}) theoretically available to ThioGlo™-1 and comparison with a standard curve of ThioGlo™-1 response constructed with various concentrations of GSH.

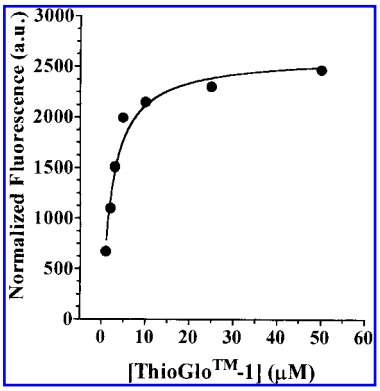

FIG. 3. Saturable titration of hSA thiols with ThioGlo™-1. Reduced hSA containing 1 mol of free SH/mol of protein (3 μ*M*, final concentraton) was incubated at 60°C in 2.5 ml of 50 m*M* phosphate buffer, pH 7.4, 30 m*M* SDS with a range of concentrations of ThioGlo™-1 (1–50 μ*M*). After incubation, samples were equilibrated to room temperature for 20 min and then diluted in phosphate buffer to yield a final ThioGlo™-1 concentration of 1 μ*M*. This was done to avoid the contribution of different levels of unreacted ThioGlo™-1 to the fluorescence reading of the undiluted reaction mixture. Fluorescence reading was then back normalized by the dilution factor in order to yield the protein-thiol-specific ThioGlo™-1 fluorescence.

ThioGlo™-1 fluorescence reflecting reaction with SH groups steadily increased over 1–5 μ*M* ThioGlo™-1 and leveled off at ~10 μ*M*, after which the normalized fluorescence remained constant. Therefore, it appears that under these reaction conditions the addition of an approximately threefold molar excess of ThioGlo™-1 was sufficient to achieve maximal labeling of the available reduced SH groups on hSA. Subsequent experiments were then conducted to ensure that ThioGlo-to-SH ratios were in the range of 3–10.

SH modification of purified hSA by SDS-PAGE

Once the incubation conditions for labeling were determined as described above, the ability of ThioGlo™-1-labeled hSA to be resolved and detected following SDS-PAGE was assessed. hSA (0.05–1.0 mg/ml, 0.7–15 μ*M*) was first labeled with ThioGlo™-1 in 50 m*M* phosphate buffer, pH 7.4, containing 30 m*M* SDS and 30 μ*M* ThioGlo™-1 for 1 h at 60°C. Aliquots containing 1–20 μg were then subjected to SDS-PAGE. Figure 4B shows the fluorescent protein bands corresponding to hSA detected after exposure to a scanning UV light source using the Bio-Rad Fluor-S MultiImager. A fluorescent band could be detected with as little as 1 μg of hSA loaded per well and increased over the range of protein amounts applied to the gel. To determine if the intensity of this fluorescence was linear over the range of hSA amounts used, the fluorescence intensity of each hSA band was quantified using the Bio-Rad Multi-Analyst Software. Figure 4A shows a plot of fluorescent signal amplitude versus the amount of hSA thiols applied to each well. A strict linear response ($r^2 = 0.9925$) was achieved over this 20-fold range of protein amounts and indicates that this assay can indeed be applied for quantitative purposes using protein amounts

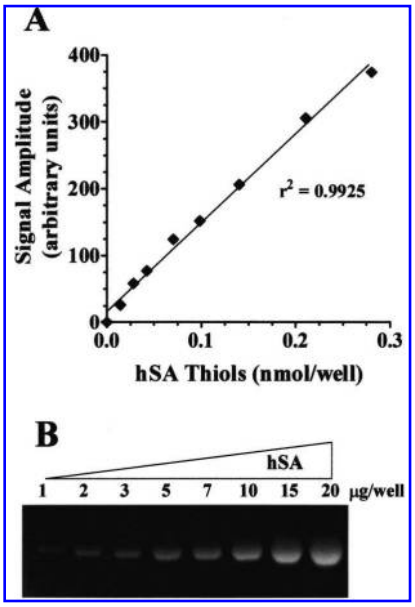

FIG. 4. Linearity of ThioGlo™-1 reaction with varying protein concentrations. Various concentrations of hSA (0.05–1.0 mg/ml) were incubated with 30 µ*M* ThioGlo™-1 in 50 m*M* phosphate buffer, pH 7.4, containing 30 m*M* SDS for 1 h at 60°C. Twenty-microliter aliquots of these mixtures corresponding to varying amounts of protein (1–20 µg) were then mixed 4:1 with 5× loading buffer and subjected to SDS-PAGE as described in Materials and Methods. **B** shows the typical fluorescent labeling pattern after visualization of gel under UV light. Quantification was performed using Bio-Rad Multi-Analyst Software after image acquisition with a Bio-Rad Fluor-S MultiImager equipped with UV light source (290–365 nm) and a CCD camera with 530 nm broad range emission filter. **A** shows the linear relationship between the intensity of the signal and amount of protein contained in the reaction mixture over this range of hSA concentrations.

within this assay range. The goodness-of-fit of this response observed over these amounts of fully reduced native hSA containing a single thiol per protein molecule was further exploited to construct a standard curve that would enable the quantification of ThioGlo™-1-reactive SH groups under a variety of experimental conditions.

We next sought to determine if this method could be applied to measure the degree of SH group modification following exposure of hSA to oxidative and nitrosative conditions. Phenoxyl radicals are well known for their potency to produce selective SH oxidation. A cell-free model system containing myeloperoxidase and H_2O_2 was used to generate phenoxyl radicals derived from the following phenolic substrates: phenol, acetaminophen, and etoposide. hSA was first exposed to these myeloperoxidase/H_2O_2-derived phenoxyl radicals and then subjected to analysis of reduced SH groups with ThioGlo™-1 using both conventional solution fluorospectroscopy and our SDS-PAGE fluorescent image analysis. Figure 5A shows that using the conventional spectrofluorometric assay, ThioGlo™-1 could be used to detect a loss of free SH groups following exposure of hSA to various phenoxyl radicals. Radicals derived from phenol produced the most significant modification of free SH groups with ~70% reduction in ThioGlo™-1 reactivity. More modest, but significant, reductions were observed after exposure to phenoxyl radicals derived from acetaminophen and etoposide (49% and 19%, respectively). A small, but statistically significant, reduction could also be observed after hSA was exposed to myeloperoxidase/H_2O_2 alone. Similarly, decrements in the fluorescence intensity of hSA bands detected after SDS-PAGE appeared to follow the same pattern as those seen with the solution-based spectrofluorometic assay (Fig. 5B). Marked decrease in fluorescence intensity in the hSA band could be observed after exposure to phenol/myeloperoxidase/H_2O_2 with smaller decrements observed when aceta-

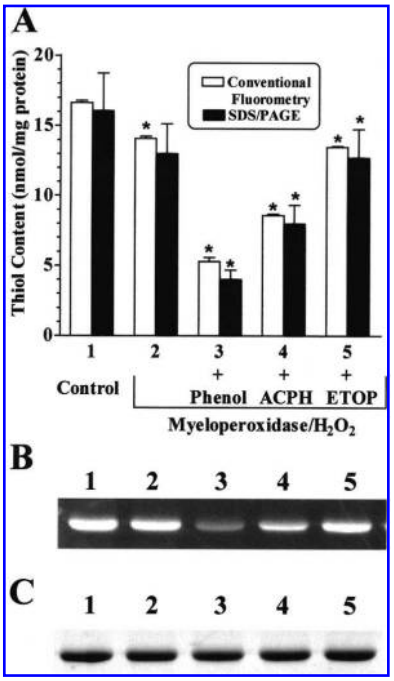

FIG. 5. Comparison of conventional fluorescence spectroscopy with SDS-PAGE with fluorescent image analysis to measure SH group content of hSA following exposure to myeloperoxidase/H_2O_2-derived phenoxyl radicals. hSA (10 mg/ml) was exposed to myeloperoxidase/H_2O_2 alone (1 unit/ml myeloperoxidase, 100 µ*M* H_2O_2) or myeloperoxidase/H_2O_2 in the presence of either 500 µ*M* phenol, acetominophen (ACPH), or etoposide (ETOP). Incubations were performed in 50 m*M* phosphate buffer, pH 7.4, in the presence of 100 µ*M* deferoxamine to chelate any adventitious iron at 37°C for 20 min. Samples were then taken and diluted in phosphate buffer, and the amount of ThioGlo™-1 reactive thiols was determined by both conventional fluorescence spectroscopy and SDS-PAGE as described. **B** and **C** show a typical gel containing proteins stained with ThioGlo™-1 and Coomasie Blue, respectively. **A** compares the number of ThioGlo™-1-titratable thiols on hSA obtained by the two methods after exposure to myeloperoxidase-generated phenoxyl radicals. Data represent means ± SEM from four experiments. Asterisks denote statistically significant differences from untreated control ($p < 0.05$) by one-way ANOVA with repeated measures and Dunnett's multiple comparison test.

minophen and etoposide were used as substrates for phenoxyl radicals. Staining of the gels with Coomasie Blue indicated that these differences were not due to differences in protein loading (Fig. 5C). When the fluorescence intensity of the individual hSA bands was quantified and compared with that obtained by conventional fluorescence spectroscopy, essentially identical measures of thiol content were obtained within each experimental condition (Fig. 5A).

The ability of our methods to measure SH modification following exposure to NO was similarly assessed after exposure of hSA to various NO donors. Figure 6 shows the thiol content of hSA after exposure to two concentrations of NOC-15 (500 μ*M* and 2 μ*M*), as well as 500 μ*M* GS-NO. It is clear that exposure of hSA to these nitrosative conditions results in significant reduction in free SH groups available for reaction with ThioGlo™-1. NO-mediated reductions in fluorescence intensity of the hSA band were clearly observed after SDS-PAGE analysis of the purified protein (Fig. 6A and B). When compared on a quantitative basis, essentially identical reductions in thiol content in hSA were observed when measured by either conventional fluorescence spectroscopy or fluorescence image analysis of samples subjected to SDS-PAGE.

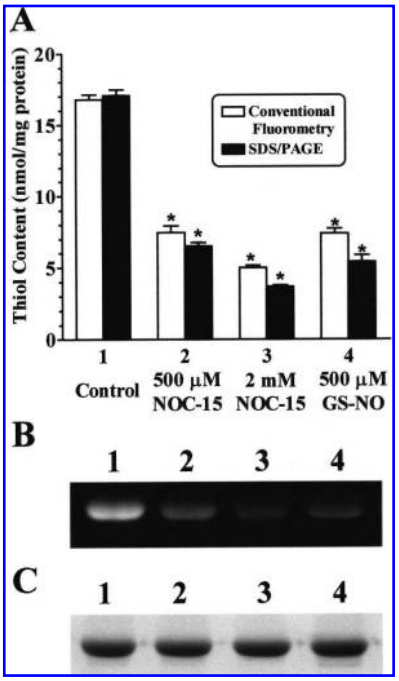

FIG. 6. Effect of NO donors on SH modification of purified hSA. hSA (10 mg/ml) dissolved in phosphate buffer was exposed to 0.5 or 2 m*M* PAPANONOate or 0.5 m*M* GS-NO for 1 h at 37°C. Following incubation, free SH groups on albumin were labeled with ThioGlo™-1. **A** compares the free SH groups determined by conventional solution fluorescence spectroscopy with quantification of fluorescence profiles after SDS-PAGE. Data represent means ± SEM of four experiments. **B** shows the ThioGlo™-1-stained hSA observed after SDS-PAGE from a typical experiment. **C** shows the same gel after staining with Coomassie Blue to demonstrate equal protein loading between lanes. Asterisks denote statistically significant differences from untreated control ($p < 0.05$) by one-way ANOVA with repeated measures and Dunnett's multiple comparison test.

SH modification of human plasma proteins by SDS-PAGE

Although it appears that this method worked well for evaluation of purified defined proteins, it was important to determine the application of this method to analysis for more complex samples that contained a mixture of diverse proteins. For this we utilized human plasma because it contains a plethora of proteins that possess potential SH groups for reaction with ThioGlo™-1, as well as serum albumin that had been used in our initial studies. Figure 7 shows the fluorescent spectroscopic determination and SDS-PAGE fluorescent image analysis of protein thiol content in proteins contained in normal human plasma and plasma exposed to phenoxyl radicals generated as described above. The Coomasie Blue-stained gel shown in Fig. 7C clearly reveals that multiple proteins could indeed be resolved by SDS-PAGE and that hSA (approximate MW, 67,000) represents the major protein species detected in these samples. Moreover, exposure of human plasma to the oxidizing conditions provided by the phenoxyl radical-generating system had no effect on the overall protein profile of these samples. Fluorescent image analysis for proteins stained with ThioGlo™-1 showed that several proteins could also be detected as possessing SH–ThioGlo™ adducts with hSA being the most prominent (Fig. 7B). It was also clear that SH groups in hSA contained in plasma were modified in a manner similar to that seen in purified hSA following exposure to various phenoxyl radicals. However, the ability of this system to modify SH groups on other proteins was not uniform. A significant, but unidentified SH-containing protein, which we termed Protein X (approximate MW, 200 kDa) was consistently observed in all plasma samples following fluorescent image analysis of the SDS-PAGE gel. In contrast to hSA, the SH groups contained in Protein X were essentially unchanged following exposure to these oxidizing conditions.

When plasma samples were exposed to NOC-15 and GS-NO, similar to conditions used for purified hSA, we again observed significant modification of SH groups. Using conventional fluorescence spectroscopy, exposure to NO resulted in an ~50% reduction in total SH groups available for reaction with ThioGlo™ (Fig. 8A). Similarly, NO produced dramatic reduction in the fluorescence intensity of the hSA bands after SDS-PAGE (Fig. 8B), but no change in the overall protein profile (Fig. 8C). As shown in Fig. 8A, the reduction in hSA was quantitatively greater (~85%) than that observed for the total thiol pool measured by conventional fluorescent spectroscopy. The ThioGlo™-labeled Protein X was again detected in these experiments and was essentially unchanged after exposure to NO donors, indicating that not all SH group-containing proteins in plasma are equally sensitive to nitrosative stress.

DISCUSSION

We report here for the first time on the use of ThioGlo™-1 as a probe that allows the quantitative measurement of SH modification in hSA resolved by SDS-PAGE following exposure to oxidative and nitrosative stress. ThioGlo™-1, a maleimide derivative of a naphthopyranone fluorophore, was

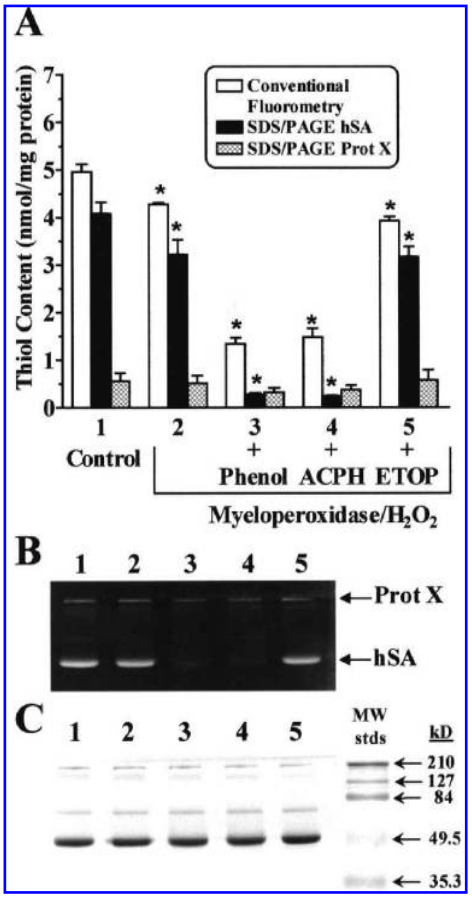

FIG. 7. Phenoxyl radical-dependent modification of protein SH groups in human plasma. Normal human plasma was diluted in 50 m*M* phosphate buffer, pH 7.4, containing 100 μ*M* deferoxamine to a final protein concentration of 8 mg/ml. Plasma samples were then exposed to 1 unit/ml myeloperoxidase and 100 μ*M* H_2O_2 alone (**2**) or with addition of 500 μ*M* phenol (**3**), 500 μ*M* acetaminophen (ACPH; **4**), or 500 μ*M* etoposide (ETOP; **5**). Control samples (**1**) were incubated in phosphate buffer containing only deferoxamine. Samples were incubated for 20 min at 37°C. SH group content was then analyzed by both solution-based fluorescence spectroscopy and SDS-PAGE using ThioGlo™-1 as described. **B** represents a typical gel of plasma proteins labeled with ThioGlo™-1 and visualized under UV light. hSA was located based on its comigration with purified hSA and location relative to a series of molecular weight standards. **C** shows the same gel stained with Coomassie Blue. **A** compares the amount of total thiols measured by solution-based fluorescence spectroscopy (open columns) with those specifically contained in hSA (black columns) and another unidentified ThioGlo™-1-labeled protein termed Protein X (~220 kDa) (hatched columns) after fluorescent image analysis of the SDS-PAGE gel. Total thiol content in the solution-based assay was calculated by comparing the fluorescence response to a series of GSH standards of known concentrations. Thiol content of hSA band was determined by quantitative analysis of the fluorescent image and compared with the responses observed with a range of hSA standards run under identical conditions (black columns). Data represent means ± SEM from four experiments. Asterisks denote statistically significant difference from untreated control ($p < 0.05$) by one-way ANOVA with repeated measures and Dunnett's multiple comparison test.

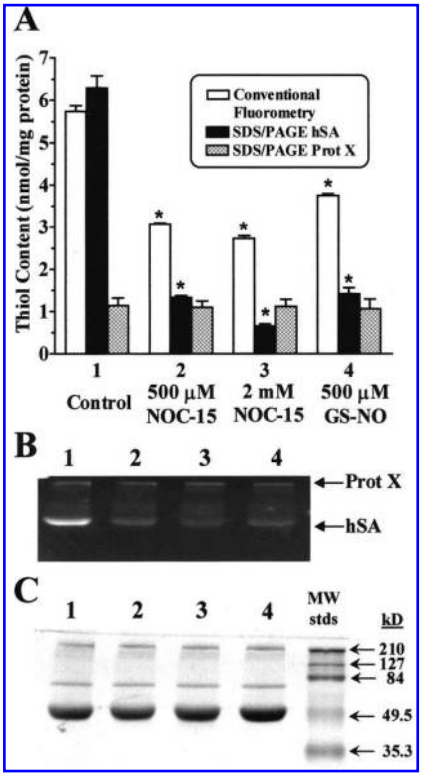

FIG. 8. Albumin SH modification in human plasma after exposure to NO donors. Normal human plasma was added to 50 m*M* phosphate buffer, pH 7.4 (8 mg of protein/ml), and incubated with various NO donors including NOC-15 (500 μ*M* or 2 m*M*) or GS-NO (500 μ*M*) for 1 h at 37°C. **A** shows the overall SH group content of the samples as measured by ThioGlo™-1 fluorescence determined by conventional fluorescence spectroscopy after addition of 30 m*M* SDS and 10 μ*M* ThioGlo™-1 (open columns). SDS-PAGE was then carried out on these samples after reaction with ThioGlo™-1 to visualize the pattern of SH groups in specific proteins contained in human plasma. **B** shows a representative gel stained with ThioGlo™-1 and visualized for fluorescence. **C** shows the same gel stained with Coomassie Blue. Black columns and hatched columns (A) show the amount of SH groups contained in hSA and Protein X, respectively, determined as described in Fig. 7. Data represent means ± SEM from four experiments. Asterisks denote statistically significant difference from untreated control ($p < 0.05$) by one-way ANOVA with repeated measures and Dunnett's multiple comparison test.

chosen because it has several advantages over other available SH probes (18). These include very low intrinsic fluorescence of the reagent before reaction with SH groups, but a significant, 50-fold increase in quantum yield after reaction. The hydrolysis rate of the SH–ThioGlo™ adduct is substantially lower than with other reagents, and the hydrolysis product is itself nonreactive and essentially nonfluorescent. Furthermore, its excellent water solubility permits its use over a wide range of concentrations and minimizes exposure of proteins to potentially denaturing organic solvents.

Several characteristics of the ThioGlo™–hSA interaction bear noting. First, it appears that the addition of SDS is required for the rapid and efficient interaction of ThioGlo™-1

with Cys_{34} on hSA. This suggests that Cys_{34} in hSA is located on a region in the native protein with somewhat restricted access to the relatively bulky ThioGlo™-1 molecule. This does not preclude, however, the ability of Cys_{34} to participate in redox reactions *in vivo,* because these may arise by reactants of much smaller size (reactive oxygen/nitrogen species) and could be assisted by specific protein–protein interactions. Indeed, in these studies, various species of phenoxyl radicals and NO produced substantial modification of hSA in the absence of SDS. It is possible that other proteins have SH groups more readily accessible to ThioGlo™-1 in the absence of SDS. In contrast, other proteins may have more limited accessibility to SH groups even after SDS denaturation. These particular issues would need to be investigated using purified proteins of interest whenever possible before applying this approach to analyze their modification in complex biological mixtures. For example, we were able to observe the ThioGlo™-1 reaction with only 1 Cys equivalent/molecule of metallothionein out of the 21 theoretically available (data not shown). This relative inefficiency could arise from difficulty in removing bound metal ions that may competitively inhibit ThioGlo™-1 interaction with reactive SH groups. Alternatively, as metallothionein SH groups occur as contiguous (Cys-Cys) or vicinal (Cys-X-Cys) SH pairs, the reaction between ThioGlo™-1 and one cysteine may limit the accessibility of neighboring cysteines to the fluorescent probe. Significant self-quenching may also be achieved when multiple fluorescent probe molecules exist in close proximity to each other, such as within the thiolate clusters of metallothionein.

We also observed that the reaction between ThioGlo™-1 and SH groups was temperature-dependent. The velocity of the reaction was considerably higher when performed at 60°C than at room temperature, and 60°C was therefore chosen as the standard assay condition. We did examine the use of 95°C as usually applied in SDS-PAGE analysis of proteins in preliminary experiments and observed variable results with frequent reductions in the extent of labeling. Thus, elevated temperatures may favor autooxidation of the protein SH groups during incubation and render them unreactive to ThioGlo™-1 or alternatively favor the breakdown of the SH–ThioGlo™-1 adduct with subsequent loss of fluorescence. In this regard, we also did not use the typical SDS-PAGE step of boiling the samples prior to loading. The absence of this step, however, did not appear to adversely affect the gel migration of purified hSA or of the multiple proteins found in human plasma. At 60°C, a plateau in the response curve was achieved within 5–10 min, a time easily incorporated into the assay scheme. Moreover, the plateau appeared quantitatively the same as that achieved with longer times at 25°C, indicating that autooxidation and degradation were minimal under these conditions. We found that equimolar concentrations of reduced albumin and reduced GSH gave essentially equivalent fluorescence intensities in the solution-based fluorometry assay. As there is negligible autooxidation of GSH during the nearly instantaneous reaction with ThioGlo™-1, we similarly surmise that minimal albumin oxidation occurred during this time despite the addition of SDS, prolonged incubation (up to 2 h at room temperature), or elevated temperature (60°C).

We cannot definitively rule out the possibility that some thiols on specific proteins may be oxidized during the analytical procedure and produce detection failures or underestimations of SH content. As with any assay measuring oxidation of biomolecules, sample collection and storage should be performed under conditions that minimize autooxidation (reduced temperature, rapid analysis, etc.). We avoided the addition of antioxidants or reducing agents for their obvious potential to reverse oxidations of interest or interfere with ThioGlo™–SH reaction. The most critical time to control for such oxidation is during the sample labeling with ThioGlo™-1, where conditions after this time are less relevant because all reduced SH groups have already been consumed during the labeling reaction. It is possible that certain proteins in human plasma might be extremely sensitive to thiol loss during incubation and, hence, not detected here. It is more likely, however, that the failure to detect many other SH-containing proteins reflects the overwhelming abundance of albumin relative to other proteins in plasma. hSA represents ~70% of the total protein content in plasma and therefore is present in massive abundance compared with any other single protein in plasma. The gel results were in excellent agreement with those of solution-based assay where the sum of albumin intensity and Protein X intensity equals the approximate response observed with total plasma protein by solution fluorometry. Faintly fluorescent bands corresponding to other proteins can indeed be observed, but are not of sufficient intensity to quantify. It should also be noted that the SH content in albumin as measured in the plasma samples (~4 nmol/mg of protein) is about half of what is theoretically available based on our experiments with purified hSA and its abundance in serum. This observation likely reflects the extent of SH modification that exists on hSA *in vivo.*

Quantitative measurements made in SDS-PAGE gels were compared with those made using solution-based fluorospectroscopy and revealed that measurements of thiol status made in the fluorescence-based image analysis could be directly compared with those obtained by conventional solution fluorospectroscopy. Standard curves constructed with known concentrations of fully reduced GSH and hSA could be applied to solution- and gel-based assays, respectively, for exact determination of thiol number. Similarly, O'Keefe has shown that fluorescence response of five different proteins labeled with monobromobimane was dependent on the number of cysteine residues present for each protein and independent of protein species (23). Most importantly, the degree of SH modification of hSA following exposure to myeloperoxidase-derived phenoxyl radicals or NO donors was essentially identical when measured by either conventional fluorospectroscopy or SDS-PAGE. Therefore, we are confident that this assay can be used to accurately quantify the degree of SH modification in hSA following oxidative and nitrosative stress.

It is important to point out that although this assay provides a sensitive and quantitative measurement of SH loss, it does not by itself define or describe the molecular nature of the SH-derived product formed. Therefore, it will be paramount to use this assay in conjunction with other biochemical assays to define the molecular species produced. For example, titration of protein thiols can be combined with the direct measurement of NO liberated from nitrosothiols. Using this approach, we have shown that exposure of HL-60 cells to NO results in substantial loss of metallothionein thiols (~30%), but that only a minor fraction of those modified (~2–3%)

could actually be recovered as *S*-nitrosothiols (20). In addition, the degree of SH oxidation ranging from thiolation with formation of mixed disulfides to subsequent creation of sulfinic and/or sulfonic acids may be discriminated based on their reversibility by a potent reducing agent such as DTT.

Our experiments with human plasma reveal that this approach can be used to quantitatively examine SH modification of hSA present in complex mixtures. As hSA represents by far the most abundant protein in human plasma, it is not surprising that most of the ThioGlo™-1 labeling occurs on this protein after SDS-PAGE. It was noted, however, that the measurements of fluorescence intensity in plasma made by conventional fluorospectroscopy were always slightly higher than those made on the specific hSA band observed in gels. The contribution of GSH and other low-molecular-weight thiols was subtracted out by pretreatment of plasma samples with ThioGlo™-1 in the absence of SDS. Therefore, this additional fluorescence most likely represents the multiple additional SH-containing proteins present in plasma because this difference was not apparent in experiments using purified hSA alone. The nature and reactivity of these proteins can be assessed in future experiments. For example, we observed an as yet undefined protein of approximate molecular mass 220 kDa (Protein X) that contained sufficient ThioGlo™-1 reactivity for reliable quantification. In contrast to hSA, the SH group(s) on Protein X were relatively resistant to oxidation/nitrosation. It may be that the large excess of SH groups represented by hSA simply protected other smaller pools by mass action and may imply an antioxidant role for albumin by protecting critical SH groups on other less abundant serum proteins. It may also mean that not all SH groups are equivalent in their sensitivity or accessibility to attack by reactive oxygen/nitrogen species, and that their susceptibility to modification depends on protein tertiary structure and other physicochemical properties, such as pK_a. It should be pointed out that we used relatively mild oxidizing conditions in our studies, and the effect of more severe oxidants, such as diamide, on ThioGlo™-1 labeling of proteins remains to be shown.

The ease of redox modification of hSA Cys_{34} likely has several physiological implications. For example, the formation of mixed disulfides with low-molecular-weight thiols, such as cysteine and GSH, modulates the affinity of hSA for Cu^{2+} and other ligands, such as phenolsulfophthalein and free fatty acids (28). Such an observation may challenge the antioxidant role of albumin because modification of Cys_{34} may enhance Cu-dependent redox cycling. Similarly, nitrosylation of hSA Cys_{34} can influence binding of various ligands to hSA (13). The free thiolate ion of hSA, in conjunction with Cu^{1+}, may catalyze the liberation of NO for various *S*-nitrosothiols, including hSA itself (6). As the Cys_{34}-nitrosoadduct of albumin is thought to represent a major physiologic carrier of releasable NO in human plasma (27), the redox status of Cys_{34} in hSA may have great potential to regulate various NO-dependent activities, including modulation of vascular tone.

Several serum- or plasma-based biomarkers, including albumin, have been utilized to study oxidative stress in various clinical situations. Toyokuni *et al.* have shown that 4-hydroxy-2-nonenal-modified albumin is elevated during type 2 diabetes mellitus (29). Similarly, advanced glycation end product and acrolein adducts of albumin have been proposed as novel markers of oxidative stress (32, 33). These approaches, however, shed little light on the specific site of modification within the albumin protein molecule. In addition, they rely on immunological detection of specific protein adducts and, thus, may report on only specific insults. The free Cys_{34} is a likely target for both covalent modification and simple oxidation, and, therefore, its loss may more globally reflect oxidative challenges in plasma. Many studies measuring the content or depletion of traditional antioxidants like vitamins C and/or E yield conflicting results, and often neglect to consider the large contribution of albumin Cys_{34} to the overall antioxidant potential of human serum whose oxidation may spare other less abundant antioxidants (21, 25, 36). In addition, the ability of Cys_{34} to transport and release NO points to the site-selective redox-dependent dynamic regulation of vasomotor tone. We have previously observed elevated levels of *S*-nitrosylated albumin in the plasma of preeclamptic pregnancy compared with normal pregnant women (31). Thus, the application of this assay to specifically quantify Cys_{34} modification of albumin will have great utility in assessing oxidative stress in various disease states, as well as during therapeutic/prevention clinical trials.

The data provided here indicate that we have developed a fully quantitative assay using the fluorescent probe ThioGlo™-1 that is amenable to measuring the degree of SH modification of hSA resolved by SDS-PAGE. Such an assay can be applied to analyzing the redox state of SH groups contained in specific proteins after exposure of complex mixtures such as cells or biological fluids to various challenges. This approach will provide a specific means to assess Cys_{34}-selective modification of hSA during *in vitro* and *in vivo* investigations. Furthermore, its application to other protein targets will assist in the molecular analysis of the cellular events associated with exposure to environmental chemicals, oxidative/nitrosative stress, and redox-mediated signal transduction.

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ABBREVIATIONS

Cys, cysteine; DTNB, 5,5′-dithio-2-nitrobenzoic acid; DTT, dithiothreitol; GSH, reduced glutathione; GS-NO, *S*-nitrosoglutathione; H_2O_2, hydrogen peroxide; hSA, human serum albumin; NO, nitric oxide; NOC-15, (Z)-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SH, sulfhydryl; UV, ultraviolet.

REFERENCES

1. Abate C, Patel L, Rauscher FJ, and Curran T. Redox regulation of fos and jun DNA-binding activity *in vitro*. *Science* 249: 1157–1161, 1990.

2. Albano E, Rundgren M, Harvison PJ, Nelson SD, and Moldeus P. Mechanisms of *N*-acetyl-*p*-benzoquinone imine cytotoxicity. *Mol Pharmacol* 28: 306–311, 1985.
3. Beckman JS. Physiological and pathological chemistry of nitric oxide. In: *Nitric Oxide: Principles and Actions*, edited by Lancaster J Jr. San Diego, CA: Academic Press, 1996, pp. 25–26.
4. Chai Y-C, Ashraf SS, Rokutan K, Johnston RB, and Thomas JA. *S*-Thiolation of individual human neutrophil proteins including actin by stimulation of the respiratory burst: evidence against a role for glutathione disulfide. *Arch Biochem Biophys* 310: 273–281, 1994.
5. Dean RT, Fu S, Stocker R, and Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* 324: 1–18, 1997.
6. Dicks AP and Williams DL. Generation of nitric oxide from *S*-nitrosothiols using protein-bound Cu^{2+} sources. *Chem Biol* 3: 655–659, 1996.
7. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 82: 70–77, 1959.
8. Fabisiak JP, Tyurin VA, Tyurina YY, Sedlov A, Lazo JS, and Kagan VE. Nitric oxide dissociates lipid oxidation from apoptosis and phosphatidylserine externalization during oxidative stress. *Biochemistry* 39: 127–138, 2000.
9. Gupta S, Rogers LK, Taylor SK, and Smith CV. Inhibition of carbamyl phosphate synthetase-I and glutamine synthetase by hepatotoxic doses of acetaminophen in mice. *Toxicol Appl Pharmacol* 146: 317–327, 1997.
10. Halliwell B. Albumin—an important extracellular antioxidant? *Biochem Pharmacol* 37: 569–571, 1988.
11. Kagan VE, Yalowich JC, Borisenko GG, Tyurina YY, Tyurin VA, Thampatty P, and Fabisiak JP. Mechanism-based chemopreventive strategies against etoposide-induced acute myeloid leukemia: free radical/antioxidant approach. *Mol Pharmacol* 56: 494–506, 1999.
12. Kagi JHR and Schaffer A. The biochemistry of metallothionein. *Biochemistry* 27: 8509–8515, 1988.
13. Kashiba-Iwatsuki M, Miyamoto M, and Inoue M. Effect of nitric oxide on the ligand-binding activity of albumin. *Arch Biochem Biophys* 345: 237–242, 1997.
14. Katchalski E, Benjamin GS, and Gross V. The availability of the disulfide bonds of human and bovine serum albumin and of bovine γ-globulin to reduction by thioglycolic acid. *J Am Chem Soc* 79: 4096–4099, 1957.
15. Kim JR, Yoon HW, Kwon KS, Lee SR, and Rhee SG. Identification of proteins containing cysteine residues that are sensitive to oxidation by hydrogen peroxide at neutral pH. *Anal Biochem* 283: 214–221, 2000.
16. Kosower EM and Kosower NS. Bromobimane probes for thiols. *Methods Enzymol* 251: 133–148, 1995.
17. Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227: 680–685, 1970.
18. Langmuir ME, Yang J-R, Lecompte KA, and Durand RE. New thiol active fluorophores for intracellular thiols and glutathione measurement. In: *Fluorescence Microscopy and Fluorescent Probes*, edited by Slavik J. New York, NY: Plenum Press, 1996, pp. 229–234.
19. Liu G, Abramson JJ, Zable AC, and Pessah IN. Direct evidence for the existence and functional role of hyperreactive sulfhydryls on the ryanodine receptor–triadin complex selectively labeled by the coumarin malemide, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin. *Mol Pharmacol* 45: 189–200, 1994.
20. Liu S-X, Kawai K, Tyurin VA, Tyurina YY, Borisenko GG, Fabisiak JP, Quinn PJ, Pitt BR, and Kagan VE. Nitric oxide dependent pro-oxidant and pro-apoptotic effect of metallothioneins in HL-60 cells challenged with cupric nitrilotriacetate. *Biochem J* 354: 397–406, 2001.
21. Mercuri F, Quagliaro L, and Ceriello A. Oxidative stress evaluation in diabetes. *Diabetes Technol Ther* 2: 589–600, 2000.
22. Miller DK, Myerson J, and Becker JW. The interleukin-1 beta converting enzyme family of cysteine proteases. *J Cell Biochem* 64: 2–10, 1997.
23. O'Keefe DO. Quantitative electrophoretic analysis of proteins labeled with monobromobimane. *Anal Biochem* 222: 86–94, 1994.
24. Pedersen AO and Jacobsen J. Reactivity of the thiol group in human and bovine albumin at pH 3–9, as measured by exchange with 2,2′-dithiodipyridine. *Eur J Biochem* 106: 291–295, 1980.
25. Polidori MC, Cherubini A, Senin U, and Mecocci P. Peripheral non-enzymatic antioxidant changes with human aging: a selective status report. *Biogerontology* 2: 99–104, 2001.
26. Quinlan JG, Margarson S, Mumby S, Evans TW, and Gutteridge MA. Administration of albumin to patients with sepsis syndrome: a possible beneficial role of plasma thiol repletion. *Clin Sci* 95: 459–465, 1998.
27. Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, Singel D, Valeri CR, and Loscalzo J. Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso adduct of serum albumin. *Proc Natl Acad Sci U S A* 89: 7674–7677, 1992.
28. Takabayashi K, Imada T, Saito Y, and Inada Y. Coupling of fatty acid binding and sulfhydryl oxidation in bovine serum albumin. *Eur J Biochem* 136: 291–295, 1983.
29. Toyokuni S, Yamada S, Kashima M, Ihara Y, Yamada Y, Tanaka T, Hiai H, Seino Y, and Uchida K. Serum 4-hydroxy-2-nonenal-modified albumin is elevated in patients with type 2 diabetes mellitus. *Antioxid Redox Signal* 2: 681–685, 2000.
30. Turk B, Turk V, and Turk D. Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. *Biol Chem* 378: 141–150, 1997.
31. Tyurin VA, Liu S-X, Tyurina YY, Sussman NB, Hubel CA, Roberts JM, Taylor RN, and Kagan VE. Elevated levels of *S*-nitrosoalbumin in preeclampsia plasma. *Circ Res* 88: 1210–1215, 2001.
32. Uchida K, Kanematsu M, Sakai K, Matsuda T, Hattori N, Mizuno Y, Suzuki D, Miyata T, Noguchi N, Niki E, and Osawa T. Protein-bound acrolein: potential markers for oxidative stress. *Proc Natl Acad Sci U S A* 28: 4884–4887, 1998.
33. Witko-Sarsat V, Friedlander M, Capeillere-Blandin C, Nguyen-Khoa T, Nguyen AT, Zingraff J, Jungers P, and Descamps-Latscha B. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int* 49: 1304–1313, 1996.

34. Wright SK and Viola RE. Evaluation of methods for the quantitation of cysteines in proteins. *Anal Biochem* 265: 8–14, 1998.
35. Yamamoto K, Takamitsu S, and Kanaoka Y. Fluorescent thiol reagents. XII. Fluorescent tracer method for protein SH groups using *N*-(7-dimethylamino-4-methyl coumarinyl) maleimide. An application to the proteins separated by SDS-polyacrylamide gel electrophoresis. *Anal Biochem* 79: 83–94, 1977.
36. Zhang C, Williams MA, Sanchez SE, King IB, Ware-Jauregui S, Larrabure G, Bazul V, and Leisenring WM. Plasma concentrations of carotenoids, retinol, and tocopherols in preeclamptic and normotensive pregnant women. *Am J Epidemiol* 153: 572–580, 2001.
37. Zheng M, Aslund F, and Storz G. Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279: 1718–1721, 1998.

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2. J.D. Uys, Y. Manevich, L.C. DeVane, L. He, T.E. Garret, C.J. Pazoles, K.D. Tew, D.M. Townsend. 2010. Preclinical pharmacokinetic analysis of NOV-002, a glutathione disulfide mimetic. *Biomedicine & Pharmacotherapy* **64**:7, 493-498. [CrossRef]
3. William E. Funk, He Li, Anthony T. Iavarone, Evan R. Williams, Jacques Riby, Stephen M. Rappaport. 2010. Enrichment of cysteinyl adducts of human serum albumin. *Analytical Biochemistry* **400**:1, 61-68. [CrossRef]
4. Ulrika Törnvall. 2010. Pinpointing oxidative modifications in proteins—recent advances in analytical methods. *Analytical Methods* **2**:11, 1638. [CrossRef]
5. Özgür F#rat, Ferit Karg#n. 2010. Individual and Combined Effects of Heavy Metals on Serum Biochemistry of Nile Tilapia Oreochromis niloticus. *Archives of Environmental Contamination and Toxicology* **58**:1, 151-157. [CrossRef]
6. Peiwei Han, Xixi Zhou, Bo Huang, Xu Zhang, Chang Chen. 2008. On-gel fluorescent visualization and the site identification of S-nitrosylated proteins. *Analytical Biochemistry* **377**:2, 150-155. [CrossRef]
7. Z RASHEED, R ALI. 2006. Reactive oxygen species damaged human serum albumin in patients with type 1 diabetes mellitus: Biochemical and immunological studies. *Life Sciences* **79**:24, 2320-2328. [CrossRef]