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Antioxidant Capacity of Reduced and Hydrogen-Peroxide-Oxidized Human Serum Albumin and Its Modulation by Hydroxychloroquine and Tigecycline

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ABSTRACT

BACKGROUND. Oxidative stress has long been implicated in the development of pathological conditions, and direct evidence of in vivo oxidative stress exists in oxidative stress-related diseases. Proteins are highly susceptible to oxidative stress, but studies of interactions among pharmacological agents, biologically active substances, and oxidatively modified proteins have been limited. The antioxidant capacity of HSA plays multiple key roles as a sensor and regulator of signal transduction and of drug efficacy in response to H_2O_2.

OBJECTIVES. The objectives of the study were to compare the antioxidant capacity of the reduced and H_2O_2-treated HSA and evaluate the dependence of hydroxychloroquine and tigecycline on the physiologically important function of HSA from its initial redox state.

METHODS. Human serum albumin (HSA) fraction V, a free fatty acid, was obtained from MP Biomedicals, LLC and then reduced with dithiothreitol (DTT), 10 mM, or oxidized by 10 mM hydroperoxide. The antioxidant capacity of HSA was studied in denaturing conditions using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity and measured by the UV-vis absorption spectrum maximum at 517 nm, and the native antioxidant capacity (AC) by converting the reaction of the cationic radical 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and the absorbance intensity at 734 nm. SPSS v.22.0 was used for statistical analysis of the data, and results are presented as mean ± standard deviation. Differences are considered significant when $p<0.05$.

RESULTS. H_2O_2-induced oxidation of HSA leads to a decrease in the AC of the protein, and after 3 min, it reaches only 43-46% of that observed for reduced HSA. Tigecycline (TGC, 2×10^{-4} M) increased the antioxidant potential of HSA, scavenging ABTS in native conditions, by 55.3% after 15 min of incubation, and that of the HCQ (2×10^{-4} M)-HSA complex by 43%. H_2O_2 treated HSA lost the AC more than 50% at all times of the experiments, and after incubation with HCQ and TGC, the AC reached 49% and 69.6% for reduced HSA. There were no significant differences between the AC of the reduced and oxidized forms of HSA under denaturing conditions (DPPH assay); only after 45 min did the changes become significant following the addition of free radicals.

CONCLUSIONS. The present findings provide insights into the relationship between the oxidative status of HSA, characteristic of oxidative-based diseases, and its functional deterioration in blood. H_2O_2-treated HSA showed decreased AC under native conditions in the ABTS assay. At the same time, HCQ and TG can restore AC in oxidatively modified HSA, and these changes do not appear when measuring AC under HSA denaturation conditions. This study may help elucidate the binding mechanism of drugs to serum proteins, providing additional rationale for their use as anti-inflammatory and antimalarial agents.

KEYWORDS. Antioxidant capacity; Human serum albumin; Hydrogen peroxide; Hydroxychloroquine; Tigecycline.

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BACKGROUND

Parasitic diseases are associated with an exponential rise in the number of recrudescent cases and a lack of vaccines and toxicity issues associated with chemotherapies, emphasizing drug resistance as a threat to global healthcare and the need for research to develop alternative therapeutic strategies. [1-3] Pharmacokinetics of pharmacological agents in infectious diseases, and parasitic injury particularly, complying with deterioration of protein synthesis and degradation, posttranslational modification, and increased formation of altered proteins, which leads to changes in the binding of small molecules/drug substances and becomes one of the most important and complex problems in the development of rational therapy for such diseases. [3,4] The key role in regulating distribution, excretion, therapeutic efficacy, and drug toxicity in the human body is played by the most abundant plasma protein, HSA. The protein-binding level of

ligands/pharmacological agents is very important for therapeutic purposes; thus, alterations in protein conformation can change the volume of distribution, clearance, and elimination of a drug, and, as a result, modulate therapeutic effect. This problem is exacerbated by the development of oxidative stress and pronounced reactive oxygen species generation, which induce modifications of target proteins, oxidative damage to proteins, and, in particular, impair the cellular functions of HSA. [5,6] The rate of HSA elimination is closely related to the hydrophobicity and net charge of the molecule, and alterations at Arg410 lead to a short half-life and a structure similar to that of oxidized HSA after oxidation. The results of in vitro experiments also lend support to this hypothesis because they show that HSA protects human low-density lipoproteins against copper-mediated oxidation and blood against hemodialysis by free radicals. [7,8] H_2O_2, a non-radical ROS, is less reactive with small, diffusible biomolecules and, as such, has a relatively long biological lifespan (cellular half-life of ~1 ms), [9,10] and is often used as the archetypal oxidant in redox cell biology. Hydrogen peroxide produced by Streptococci caused the oxidation of hemoglobin and heme degradation, conferring oxidative insult on the host cell induced by malarial parasites, whereas bacterial species that produce <1 μM H_2O_2 neither oxidized hemoglobin nor degraded heme. ROS are also involved in pathological changes in host tissue, such as damage to the vascular endothelial lining during a malaria infection (cerebral malaria) [11] and modulation of the expression of redox-responsive transcriptional factors.[12] An important link exists between the bloodstream pool and redox-state shifts in HSA during increased vascular permeability in acute inflammation and disease severity. [2-4,12]

The changes in the degree of binding to oxidized HSA suggested could influence the rate of metabolite clearance and the delivery of metabolites to cells and tissues. [3,5,8] Based on the conventional concept, the cellular and tissue uptake is proportional to the unbound fraction of drugs and their metabolites. In various pathological, extreme, and critical clinical conditions characterized by oxidative stress, the level of oxidized albumin ("damaged albumin") can increase to 70%, impairing its function. [4,13-15] The distribution of active drug within the body is proportional to the free concentration of unbound drug in circulating plasma. It was shown early that COVID-19-induced oxidative stress inflicts structural damage to HSA and accumulates H_2O_2 in plasma, and is coupled with mortality outcome in critically ill patients. [13] This provided evidence that "effective HSA "(the non-modified HSA) is more associated with disease severity and liver dysfunction and has greater prognostic power in patients with decompensated cirrhosis than "total albumin" measured with conventional methods. [16] Antibiotics have traditionally been used to treat patients with infectious and rheumatologic diseases of proven infectious etiology. Several antibiotics have shown promising antimalarial effects and have been useful in malarial chemotherapy, particularly when combined with standard antimalarial drugs. [17] Tigecycline (TGC), a tetracycline-class antibiotic, is the first clinically available drug in a new class of glycylcycline antibiotics (containing a tert-butyl-glycylamido side chain on the aromatic ring) and is widely used to treat infections caused by antimicrobial-resistant organisms. In vitro studies indicate that TGC exhibits significant antioxidant properties and can act as a radical scavenger [18], and that, even after degradation under oxidative conditions, it can reduce mitochondrial potential, increase oxidized thiol levels, and increase ROS content in melanocytes. [19] Moreover, it indicates prominent anti-parasitic action of TGC in vitro and in vivo when combined with chloroquine. It supports further evaluation of TGC as a potential combination candidate for treating drug-resistant malaria. [20] Another anti-parasitic, anti-virus (SARS-CoV-2) drug [21-23] with anti-inflammatory properties in rheumatoid arthritis and systemic lupus erythematosus [24] is a quinoline derivative that scavenges free radicals and, in turn, can help improve HSA antioxidant activity as a modulator. [25,26] The HSA binding mechanism with HCQ involves multiple residues and consists of hydrogen-bonding and van der Waals interactions.

We hypothesized that the H_2O_2-induced oxidative state of HSA could alter the protein's antioxidant capacity and that the effects of pharmacological agents on HSA's physiologically important function might depend on its initial redox state.

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METHODS

In vitro HSA reduction with redox agent 1,4-dithiotreitol, full regeneration of native albumin, reduced HSA, and determination of total HSA concentration were reproduced as described below. [27] Reduced HSA were prepared from HSA, fraction V paste, free fatty acid, pure >98% (MP Biomedicals, LLC, venous blood from which this product is manufactured was used after confirmation to currently approved FDA test in accordance with 21 CFR 640 and separate instituonal ethics approval was not required) and dissolved in buffer containing 146 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium phosphate (PBS), pH 7.4 with the addition of 11 mM NaN3 was used in all experiments prepared by this procedure contains 0.84±0.05 mol SH/mol HSA.

Preparation of HSA undergoing oxidative stress by a pro-oxidant molecule, hydrogen peroxide

Then, 2 µl of H_2O_2 (final concentration in albumin solution was 10 mM) was added to 1 ml of HSA (100 µM). After 1 h of incubation, HSA was dialyzed once (cellulose dialysis tubing, MWCO 14000) against PBS (4 h at 4° C on an automatic stirrer) to remove H_2O_2. [27]

Determination of antioxidant capacity of HSA in native conditions

The 7 mM 2,20-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS•+, Sigma-Aldrich, USA) working reagent (green) was created as a cationic radical ($5x10^{-3}$ M) in H2O and 140 mM potassium persulfate buffer (pH 7.4), stored in the dark at room temperature (22±2° C) for 16 h, and diluted to make its absorbance equal to one. To study the antioxidant activity, the ABTS solution was diluted with phosphate buffer (pH 7.4) to obtain an absorbance of 0.700±0.030 at 734 nm. [28] The ABTS was mixed with the tested sample (HSA, $5x10^{-5}$ M) without or with ligands in a 1:1 volume (v:v) ratio, and absorbance was measured at 734±2 nm. The % inhibition was evaluated as: Antioxidant activity = [(Acontrol−Asample)/Acontrol]x100%, where: A0, A1 – the absorbance of DPPH or ABTS in the absence and presence of the samples, respectively. Next, calibration curves for ascorbic acid (AA) at concentrations ranging from $2.2x10^{-6}$ to $4.2x10^{-5}$ M (DPPH assay) and from $5.3x10^{-6}$ to $5.3x10^{-5}$ M (ABTS assay) were determined. The % inhibition values were recalculated from absorbance values to ascorbic acid equivalent antioxidant capacity (AAEAC). The TGC stock solution, 8.5×10^{-5} M, was prepared fresh in 0.15 M NaCl and 0.1 M phosphate buffer, pH 7.4, at 22° C for incubations in the dark; sample tubes were wrapped in aluminum foil. HCQ was dissolved in the same buffer as HSA to achieve a final concentration of $5.0×10^{-6}$ M. The concentration of the drug in samples was measured spectrophotometrically at the absorbance maxima of 342 nm for HCQ and 555 nm for TGC.

Determination of antioxidant capacity of HSA in denaturing conditions

2,2-Diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich), dissolved in an ethanolic solution ($1x10^{-4}$ M), was mixed with the samples at a 1:1 (v/v) molar ratio. The concentration of added ligands was 2:1 to HSA ($2x10^{-4}$ M). The maximum absorption of DPPH measured at 517±2 nm (e= $1.09x10^4$ $M^{-1}cm^{-1}$) was recorded after 3, 10, 15, 30 and 45 min. [29] A blank probe was prepared by mixing 4 mL of a 0.1 mmol/L ethanolic DPPH solution with 200 µL of deionized distilled water. After 30 min of incubation in the dark at 25 °C, the absorbance was read at 517 nm against a prepared blank.

Statistical analysis

All experiments have been carried out Tat least three times in triplicate. The data obtained were analyzed using a one-way analysis of variance (ANOVA) in the Statistical Package for the Social Sciences (SPSS) version 22.0 (Armonk, NY) and Microsoft Excel. Data were presented as mean ± standard deviation (SD) and analyzed using Student's t-test; mean values with $p<0.05$ were considered significant.

RESULTS

Influence of H_2O_2-induced oxidation on the total antioxidant capacity of human serum albumin in native conditions

The reaction of ABTS radicals with HSA primarily involves interactions with specific surface residues rather than deep binding pockets, notably the free thiol group at Cys-34 (semi-open crevice in subdomain IA), suggesting that protein-ABTS interactions indicate

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that tyrosine residues (such as Tyr-84, which, with Asp [38] and His [39] located in the surroundings of Cys-34) can form covalent adducts with the ABTS radical, resulting in a colored, non-radical product.[30-32] Thus, H_2O_2-induced oxidation of HSA with conformational alteration near Cys-34 [33,34] leads to a significant decrease in the protein's antioxidant capacity, and after 3 min from the initiation of the radical reaction, it reaches only 43-46% of that observed for the reduced form of HSA (**TAB.1**).

TABLE 1. Total antioxidant capacity in native (non-denaturing) condition

Ascorbic acid equivalent antioxidant capacity (AAEAC), mM AA								
Sample/time	Pharmacological agents		HSA reduced 1x10^{-4} M			HSA oxidized 1x10^{-4} M		
n	6	7	11	12	10	9	8	10
3 min	1.4±0.4	0.9±0.7	5.1±0.6	5.2±0.7	4.5±0.5	2.2±0.7xx	2.2±0.7	3.2±0.5
5 min	2.1±0.7	2.1±0.9	10.4±1.1	15.9±1.0**	12.4±1.0	4.8±0.9xxx	10.9±0.8**	11.2±1.1***
15 min	3.5±0.9	7.5±0.9	15.9±0.9	22.8±1.9**	24.7±1.8***	7.9±0.9xxx	11.8±0.6**	13.4±0.8**
30 min	4.6±0.9	6.6±0.9	21.6±1.4	26.4±1.8*	27.2±2.3*	9.6±1.4xxx	13.1±1.5*	15.7±1.2**#
45 min	4.2±0.6	2.2±1.3	26.3±2.2	29.9±2.3	30.3±2.1	12.8±1.8xxx	14.3±1.9	16.9±2.1*

Explanations: n – sample sizes; * - comparison with HSA; # - with HSA + HCQ; x- between HSA red and oxidized form. The average plasma concentration of HCQ is 2.5-6.0x10^{-7} Mol/L for HCQ sulfate (155 mg HCQ) at a dosage of 200 mg/day, and up to 5x10^{-6} Mol/L in whole blood for autoimmune diseases under treatment with 400 mg/day 32. The significance of the difference in comparison: *- with HSA; # - with HCQ+HCQ; x - between HSA reduced and HSA oxidized; one symbol - p<0.05; two - p<0.01; three - p<0.001.

HCQ and TG also scavenge the cationic ABTS radicals, and TG shows higher antioxidant activity than HCQ. Both pharmacological agents, HCQ and TG, can form complexes with HAS [35,36] and increase HSA's antioxidant potential through a synergistic interaction for at least 30 min after the radical reaction is initiated. The maximum rate of increase in antioxidant capacity was observed within the first 10 min for HSAred-HCQ, whereas for HSAred-TG, it occurred within the first 15 min. After 15 min of scavenging, the cationic radical HCQ-HSA complex antioxidant capacity exceeds that of HSA alone by 43% and that of TG-HSA by 55.3%. In the case of H_2O_2-induced oxidation of HSA, a pronounced decrease in its ability to scavenge free radicals occurs in native conditions, more than 50% at all times of the experiment; however, the synergistic effects with pharmacological agents persist and, after 15 min, reach 49% for HCQ-HSA and 69.6% for TG-HSA. A synergistic drug–HSA interaction effect (as determined by the ABTS assay) was observed in studies of the binding of naproxen, ketoprofen, quinine, furosemide, and losartan to HSA. In contrast, an additive interaction effect was observed for diclofenac, which is known to induce antioxidant activity. [27,36] Based on the presented data, it can be assumed that, despite their low antioxidant activity, HCQ and TG may have been considered effective stimulators of HSA (mercaptoalbumin) antioxidant potential.

Influence of H_2O_2-induced oxidation on the total antioxidant capacity of human serum albumin in denaturing conditions (DPPH assay)

The data obtained show that HSA, HCQ, and TG exhibit antioxidant activity against the DPPH radical (**TAB.2**). However, no significant differences were observed in the antioxidant capacity between the reduced and oxidized forms of HSA; the changes became significant only after 45 min of free radical addition. The antioxidant capacity of the HSA red-TG complex was significantly higher than that of reduced HSA at all time points investigated, and HCQ treatment decreased antioxidant capacity after 30 min of reaction initiation for both HSA forms. HCQ dissolved in ethanol showed higher antioxidant activity than in phosphate buffer, while TGC showed higher antioxidant activity in the phosphate-buffered solution.

DISCUSSION

Progressively increasing imbalance between the excessive generation of pro-oxidants and insufficient antioxidant defense mechanisms leads to oxidative modification of albumin, formation of "damaged albumin" with alteration of protein structure and destruction of function, which correlates with the strengthening of infectious diseases, sepsis, renal and liver failure, diabetes mellitus, coronary artery disease, invasive surgery, and aging.[1-5,27] Oxidative stress can act as a concomitant factor or as a mediator of drug adverse effects and influence drug-protein adduct formation. H_2O_2-induced oxidation reduces HSA's propensity to form fibrils, suggesting that oxidation-induced side-chain changes shift the balance between critical intra- and inter-protein interactions, leading to different kinetics, intermediate species, and final aggregate morphologies. Spectroscopic measurements reveal increased compactness of the oxidized protein and reduced solvent accessibility in the HSA domain II environment. HSA antioxidant activity is associated with the presence of a free thiol group (-SH) at the Cys-34 residue and, to a lesser extent, at methionine (Met) residues. The Cys-34 amino acid residue is in domain IA on the HSA surface, close to Asp-38, His-39, and Tyr-84, and its accessibility to ligands is limited because its side chain is positioned at the bottom of a crevice.[35,36] As Rogóż et al.[37] showed, various drugs, such as losartan, furosemide, ketoprofen, and naproxen, can modulate the antioxidant potential of HSA. The conformational changes of HSA in reduced form, HSA (free fatty acid), and, to a lesser extent, H_2O_2-HSA, appear to result in a more open protein molecule with greater exposure of hydrophobic regions.[32] However, H_2O_2-induced oxidation selectively modified Cys34 and some Met residues (Met123, Met298, Met446, and Met548), thereby primarily inducing conformational changes within domain II, leading to increased protein compaction and reduced solvent accessibility, without significantly altering protein secondary structure. The DPPH scavenging antioxidant activity examined in the presence of ethanol indicates that proteins, including HSA, lose their native secondary and tertiary structures. In this condition, as shown in **TABLE 2**, the differences between the antioxidant capacities of the reduced and oxidized forms of HSA established in denatured-condition assays were no longer significant (**TAB.1** and **TAB.2**). However, the results showed that the reduced and oxidized forms of HSA in the presence of HSQ and TGC in the environment can retain their antioxidant activities despite conformational changes. Suggested that DPPH radicals interact predominantly in the IIA subdomain of HSA, while HCQ tended to bind to site I of HSA. HCQ and TG, at concentrations of about $2x10^{-4}$ M, scavenge the cationic ABTS radicals. TG shows higher antioxidant activity than HCQ (**TAB.2**).

TABLE 2. Total antioxidant capacity in denaturing conditions

Ascorbic acid equivalent antioxidant capacity (AAEAC), mM AA								
Sample/time	**Pharmacological agents**	**HSA reduced $1x10^{-4}$ M**			**HSA oxidized $1x10^{-4}$ M**			
-	HCQ	TG	-	+HCQ	+TG	-	+HCQ	+TG
15 min	2.1±0.3	2.5±0.6	6.1±1.1	5.2±0.7	9.9±0.8*	4.9±0.9	4.8±0.3	9.2±0.9*
30 min	5.9±0.6	7.3±0.9**	10.9±0.8	7.6±1.4*	16.7±1.4#	10.0±1.4	8.1±1.1	15.7±1.4##
45 min	6.4±0.8	5.2±1.4	12.1±1.4	13.9±1.9	17.9±1.1	9.8±1.2	10.9±1.2	16.3±2.1

Explanations: n – sample sizes; * - comparison with HSA; # - with HSA + HCQ; x- between HSA red and oxidized form. The average plasma concentration of HCQ is $2.5\text{-}6.0\times10^{-7}$ mol/L for HCQ sulfate (155 mg HCQ) at a dosage of 200 mg/day, and up to 5.1×10^{-6} mol/L in whole blood in autoimmune diseases treated with 400 mg/day 32. The significance of differences in comparison: *- with HSA; # - with HCQ+HCQ; x - between HSA reduced and HSA oxidized; one symbol - $p<0.05$; two - $p<0.01$; three - $p<0.001$.

The binding and reaction of ABTS radicals with HSA primarily involve interactions with specific surface residues rather than deep binding pockets, notably the free thiol group at Cys-34. Additionally, studies on protein-ABTS interactions indicate that tyrosine residues (such as Tyr-84) can form covalent adducts

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with the ABTS radical, resulting in a colored, non-radical product. [39] Early studies showed that two DPPH molecules can bind to a single HSA molecule via high- and low-affinity binding sites, with DPPH bound mainly in the IIA subdomain. They suggested that the presence of antioxidants in the HSA-DPPH reaction may confer a protective effect on the protein. [25,26,36,37] The binding of the ligand to HSA did not impair its ability to scavenge free radicals. Both drugs in the HSA complex also induced a synergistic effect. [37] The antiradical activity of the seven tetracyclines (tetracycline, chlortetracycline, oxytetracycline, doxycycline, methacycline, demeclocycline, minocycline) was confirmed by the DPPH assay and electron spin resonance. It was found that the tetracyclines exhibited high DPPH antiradical activity ranging from 26% to 96% at a concentration of 2.5 mmol/L. [38,39] Our findings suggest direct scavenging activity of the examined tetracyclines towards free radicals, and may be relevant to therapeutic strategy. DPPH radical-scavenging activity is widely used to measure antioxidant capacity. It offers a putative index of antioxidant activity, but it is limited in its ability to study differences in antioxidant capacity among conformational states of proteins, with HSA as an example.

CONCLUSIONS

Oxidative damage is linked to parasitic/inflammatory and autoimmune diseases, chronic liver and renal injury, clinically critical stages and states after hemodialysis, several aging-related diseases, and among the chemical pathways determining protein structural/conformational alterations, loss of its cellular function. Consistently, the amount of oxidized HSA has been found to correlate with systemic oxidative stress and to play an active role in the disease's pathophysiology. Therefore, the data obtained represent an important step forward in understanding the ROS-induced posttranslational modifications of HSA and the effects of pharmacological agents on modified forms of HSA, and lay the groundwork for future, larger-scale investigations into the clinical and prognostic significance of these alterations in a key, abundant blood protein.

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