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Albumin-Bound Bilirubin Interacts with Nitric Oxide by a Redox Mechanism

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ABSTRACT

Bilirubin, the final product of heme catabolism, plays a crucial role in the cellular defense against oxidative and nitrosative stress. This study investigated the interaction of albumin-bound bilirubin, the circulating form of the bile pigment, with nitric oxide (NO), a gaseous modulator involved in many physiological functions but able to induce cytotoxicity and cell death if produced in excess. A short-lived endogenous S-nitrosothiol such as *S*-nitroso-cysteine was used as NO donor. In PBS without chelators, bilirubin was bound to human serum albumin with an apparent affinity of 1.6 ± 0.2 μ*M* (*n* = 4). Furthermore, albumin (2–20 μ*M*) dose-dependently increased the half-life of BR (10 μ*M*) exposed to *S*-nitroso-cysteine (100 μ*M*) of 2.4 ± 0.4 times (*n* = 4). Albumin-bound bilirubin was almost completely oxidized by *S*-nitroso-cysteine–derived NO, and biliverdin was the major product formed; this reaction seemed to be rather specific for albumin-bound bilirubin because when free bilirubin was reacted with *S*-nitroso-cysteine the formation of biliverdin was significantly lower. Uric acid and reduced glutathione, two well-known plasma antioxidants, at physiological concentrations protected albumin-bound bilirubin from NO-mediated oxidation. Taken together, these data suggest that albumin-bound bilirubin maintains its ability to interact with NO also in the bloodstream counteracting extracellular nitrosative reactions. *Antioxid. Redox Signal.* 8, 487–494.

INTRODUCTION

Heme oxygenase (HO) is a microsomal enzyme which degrades the heme moieties of hemoproteins into ferrous iron, carbon monoxide, and biliverdin (BV). In mammals BV is then reduced into bilirubin (BR) by the cytosolic enzyme biliverdin reductase (BVR) (25). As a consequence of this two-step reaction, almost 300 mg of BR are produced per day in a healthy subject (45). Once formed within the cell, BR comes out and reaches the extravascular space and the bloodstream either by passive diffusion or by active transport. In the bloodstream, BR is bound primarily to serum albumin (HSA) which serves as a carrier and drives the bile pigment to the liver. Within the liver, BR dissociates from HSA and enters the hepatocytes where it is conjugated with glycuronic acid and excreted into the bile (21).

During the past 20 years much evidence arose in the literature demonstrating that BR is an endogenous molecule with both cytoprotective and cytotoxic effects (for an extensive and updated review on BR and its properties see Ref. 21). Peculiar is the dual role played by this bile pigment in the central nervous system. Neurons have copious amounts of BR produced by the constitutive isoform of HO (HO-2), and almost all BR is unconjugated because brain lacks BR conjugating enzymes (9, 40) . This finding, supported by the evidence that hydrogen peroxide toxicity is much higher in hippocampal neurons from HO-2-knockout mice and in cortical neurons from mice expressing the Swedish mutation of

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Alzheimer disease (in both the cases there is a marked reduction in neuronal BR level) and that the exogenous administration of BR improved neuronal survival (7, 47), prompted the investigators to hypothesize a key role for endogenous BR in the antioxidant defense of the brain. On the other hand if BR concentrations increase up to 300 μ*M*, as a result of pathological states such as newborn jaundice, hemolytic anemia, or sepsis, the bile pigment becomes toxic for brain, in particular for basal ganglia, hippocampus, and cranial nerve nuclei resulting in a pathologic condition called kernicterus (4, 21, 27).

Nitric oxide (NO) is an endogenous gaseous modulator involved in several physiologic functions such as neurotransmission, the control of immune response, inhibition of platelet aggregation, and the control of vascular tone (15, 29, 30). On the other hand, if produced in excess, NO has been shown to cause nitration of proteins as well as lipid peroxidation leading to cytotoxicity and cell death (46, 49). Recently some research groups, including ours, demonstrated that BR is an endogenous scavenger for NO and other reactive nitrogen species (RNS) (22, 26, 27). For this reason a new hypothesis was raised about the protective role of the bile pigment in pathologic situations in which nitrosative stress plays a major role such as atherosclerosis, neurodegeneration, and inflammation.

The rationale of our work was to study the interaction between BR and NO when the bile pigment was complexed with HSA. This experimental condition is representative of the pathophysiologic situation in which NO reacts with the BR–HSA complex within the bloodstream and the extravascular space. As a source of NO we used an endogenous *S*-nitrosothiol (RSNO) such as *S*-nitroso-cysteine (SNOC). *S*-nitrosothiols are a group of substances formed by the attachment of the NO group to the sulphydryl centers (*S*-nitrosylation) of proteins and nonprotein molecules (42). Notably, low molecular weight RSNO (i.e., SNOC and *S*-nitroso-glutathione (GSNO)), are the main nonprotein RSNO in cells and extracellular fluids (13, 19). The much greater half-life of RSNO with respect to free NO and their ability to release the gas in response to many stimuli, makes RSNO a useful tool to deliver NO in many experimental settings (17, 28, 35, 36).

MATERIALS AND METHODS

Chemicals

Bilirubin, reduced glutathione (GSH), essentially fatty acid free HSA, L-cysteine (L-cys), sodium nitrite, sulphanilamide (SA), *N*-naftyl-ethylene-diamine (NNED), HCl, sodium hydroxide, uric acid (UA), and PBS were from Sigma Chemicals (Sigma-Aldrich, St. Louis, MO, USA). BR was dissolved in sodium hydroxide (0.1 *M*) at a concentration of 10 m*M* and further diluted in double-distilled water. BR solutions were freshly prepared before each experiment and protected from light. The formation of the BR–HSA complex was allowed incubating the two substances for 10 min at 37°C in the dark (34). UA and GSH were dissolved in PBS at the stock concentration of 5 m*M* and 10 m*M*, respectively. SA and NNED were dissolved in HCl (0.5 *M*) at the working solution of 1% (w/v) and 0.02% (w/v), respectively.

Preparation of RSNO and stability of solutions

SNOC was prepared as described by Arnelle and Stamler (2). Briefly, a stock solution (100 m*M*) of L-cys was prepared by dissolving the compound in HCl (1 *M*). Nitrite (100 m*M*) was prepared in Millipore MilliQ water. Equal volumes of L-cys and nitrite were then mixed in an Eppendorf tube in order to obtain a brown solution of SNOC (50 m*M*). The effective concentrations of the stock solution of SNOC formed after the reaction between thiols and nitrite were assessed by the Saville assay (37). Previous studies have established that essentially no nitrite remains in the RSNO solutions using this synthetic method (41, 43). SNOC was further diluted in PBS to the desired concentration. Since RSNO are rather unstable in physiological solutions, SNOC was prepared freshly before the experiments, protected from light, and used within 10 min from the preparation.

Spectrophotometric determination of bilirubin consumption and biliverdin formation

BR consumption was assessed spectrophotometrically using a spectrophotometer [8453 UV-VIS-NIR, Hewlett-Packard (Agilent Technologies Inc., Palo Alto, CA, USA)] equipped with a water-bath, allowing the cuvette to be kept constantly at 37°C throughout the entire experiment. Spectra were performed on solutions (PBS, pH 7.4) containing BR or BR-HSA in the presence or absence of SNOC, as described below.

Bilirubin and BR-HSA consumption by SNOC was calculated monitoring, over time, the decrease of the BR specific peak at 457 nm until a plateau was reached. The oxidation of BR (1–10 μ*M*)–HSA (20 μ*M*) in BV in the presence of SNOC (100 μ*M*) was evaluated as the appearance, within the spectrum, of a peak/shoulder at 657 nm, a wavelength specific for BV. Finally, BR and BV concentrations were calculated as previously described by Minetti *et al.* (27).

Selected experiments to assess the protective role of HSA on the degradation of BR by SNOC (100 μ*M*) were carried out as follows:

Experiment A: Determination of BR half-life, at fixed concentration, as a function of HSA concentration. The BR (10 μ*M*) half-life was measured as function of HSA (2–20 μ*M*). The apparent affinity of the two molecules was obtained by nonlinear least-squares fitting of experimental data analyzed according to the model proposed by Lohman and Bujalowski (24) and Eftink (8) for tight-binding systems, assuming the existence of BR as a free (BR_f) and bound (complex BR-HSA) form.

Experiment B: Determination of BR half-life at different total (BR_t) concentrations but constant distribution between BR–HSA complex and BR_f form. Data were obtained from four experiments at total BR concentration (BR_t) of 1, 2.5, 5, and 10 μ*M*. In each case the amount of HSA to obtain a constant ratio between the bound form (i.e., BR–HSA complex) and total BR concentration was added on the basis of the affinity measured in experiment A.

Because stock solutions of SNOC and BR were prepared in strong acidic and alkaline environments, respectively, the pH of the reaction mixture was always measured before the be-

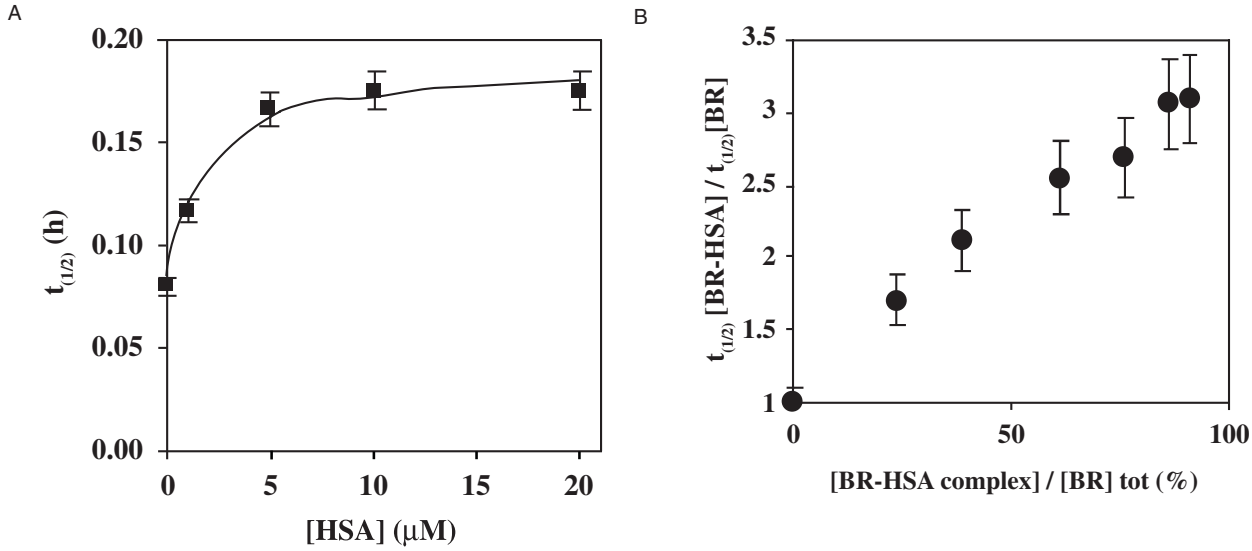

FIG. 1. (A) Effects of S-nitrosocysteine (SNOC) on bilirubin (BR) half-life ($t_{1/2}$) as a function of human serum albumin (HSA) concentrations. Bilirubin (10 μ*M*) was incubated with HSA (2–20 μ*M*) in the presence of SNOC (100 μ*M*) at 37°C as described under Materials and Methods. The continuous line was obtained by nonlinear least-squares fitting of experimental data. A binding parameter $K_d = 1.6 \pm 0.2$ μ*M* was calculated. (**B**) As in (**A**) with the ratio between the BR–HSA complex and BR_f half-life as a function of the percentage of the complex in respect to the BR_t concentration. In each case the amount of HSA to obtain a constant ratio between the bound form (i.e., BR–HSA complex) and total BR concentration was added on the basis of the affinity measured in experiment A. Data are expressed as mean ± SEM of four experiments per group.

ginning of the experiment. Only minor modifications with respect to pH 7.4 were observed. Because BR is light sensitive, all experiments were performed in subdued light.

Competition studies using uric acid and reduced glutathione

BR (10 μ*M*)–HSA (20 μ*M*) complex was incubated at 37°C and in the dark with SNOC (100 μ*M*) in the presence of UA (300 μ*M*) or GSH (20 μ*M*). BV formation was monitored over time until a plateau was reached and the concentrations calculated as described above. The pH of the reaction mixture containing UA or GSH *plus* BR–HSA and SNOC was confirmed to be in the neutral range before the beginning of the experiment.

Nitrite assay

The concentration of nitrite was determined as previously described by Griess (14). Briefly, BR (10 μ*M*)–HSA (20 μ*M*) were incubated with SNOC (100 μ*M*) in 1 ml of PBS at 37°C with continuous shaking. After 2, 10, 20, and 30 min of incubation, 300 μl of sample was mixed with equal volumes of 1% SA and 0.02% NNED and incubated for an additional 15 min at room temperature. Absorbance values were measured at 540 nm and nitrite concentrations were calculated by reference to a standard curve of nitrite in PBS.

Statistical analysis

All the data are shown as mean ± SEM of (*n*) independent experiments per group. Statistical analysis was performed using one-way ANOVA combined with Student two-tailed *t* test. Differences among groups were considered significant at $p < 0.05$. Half-life values ($t_{1/2}$) were calculated by linear and nonlinear regression analysis using a Prism 2.0 software (GraphPad Software, Inc. San Diego, CA, USA).

RESULTS

Due to the relative instability of BR in aqueous solutions and its rapid degradation in the presence of chelators such as EDTA and DTPA (26), all experiments were carried out in PBS without chelators in order to prolong BR stability in solution. We decided to use SNOC as a NO donor instead of GSNO because, in the absence of chelators, the former donates NO rapidly with a half-life of ~0.5–1 h, whereas the latter is stable, decomposing with a half-life of ~40 h (26, 48).

Figure 1A shows the variation in BR half-life as a function of HSA concentration (Experiment A). The apparent affinity of the two molecules was 1.6 ± 0.2 μ*M*, in good agreement with previously published data (34). Following the exposure to SNOC (100 μ*M*) the BR_f half-life was 0.08 ± 0.01 h, whereas the presence of HSA at saturating concentrations (2–20 μ*M*) was protective on the BR consumption, increasing the BR half-life 2.4 ± 0.4 times ($n = 4$). Figure 1B illustrates the *ratio* between the BR–HSA complex and BR_f half-life as a function of the percentage of the complex in respect to the BR_t concentration (Experiment B). Each point was calculated from the average of multiple measures at different BR concentrations. The figure emphasizes the increase of about

three times in the BR half-life in the presence of HSA. In order to see if the increase in BR half-life was due to a non-specific nitros(yl)ation of HSA, thus diverting NO from the interaction with BR, control experiments were performed studying the interaction between SNOC and HSA. In our experimental system, SNOC (100 μ*M*) was not able to induce any significant nitros(yl)ation of HSA as demonstrated by the Saville assay (data not shown).

In our previous work we demonstrated that the interaction between NO and BR_f primarily led to the rapid and irreversible degradation of BR_f, which is transformed into a *N*-nitroso derivative as demonstrated by a specific modification in the absorbance spectrum of BR_f at 316 nm (26). This *N*-nitrosative reaction seems to be rather specific for BR_f because when the bile pigment is bound to HSA, another kind of reaction occurs. As shown in Figure 2, when BR (1–10 μ*M*) is complexed with a saturating dose of HSA (20 μ*M*) and exposed to SNOC (100 μ*M*) the bile pigment was dose-dependently oxidized by NO, reaching a plateau at ~10 μ*M* and BV was the major product. This finding was not unexpected and confirms previous data showing that when BR is bound to albumin or in plasma, the bile pigment is oxidized in BV in response to strong oxidative conditions such as those elicited by peroxyl radicals or peroxynitrite (3, 27, 44). In contrast, if BR_f (1–10 μ*M*) reacted with SNOC (100 μ*M*), the amount of BV produced was significantly lower (Fig. 2).

The interaction between NO and BR–HSA was also evaluated over time by measuring nitrite formation as an index of NO degradation. SNOC (100 μ*M*) alone and in the presence of BR (10 μ*M*)–HSA (20 μ*M*) complex was incubated at 37°C and aliquots of the reaction mixture were taken at different time points and assayed for nitrite production. As shown in Figure 3, SNOC (100 μ*M*) was almost completely degraded into nitrite after 10 min of incubation reaching a plateau after 30 min, whereas when SNOC was incubated in the presence of the BR–HSA complex, the RSNO decomposed into nitrite with a higher rate than SNOC alone (initial rates: 22.1 ± 0.25 and 13.9 ± 1.5 μ*M* nitrite/min, respectively, $n = 8$, $p < 0.01$). In our experimental system HSA (20 μ*M*) *per se* increased the rate of oxidation of SNOC-derived NO into nitrite of less than 10%. Therefore, the data shown above and in Figure 3 have been corrected, subtracting from the effect of BR–HSA on nitrite formation the contribution of HSA alone.

To evaluate the physiological importance of BR–HSA as a plasma trapper of NO, we studied its interaction with the gas in the presence of other plasma antioxidants by measuring the rate of BR oxidation as the formation of BV. Analyzing the data shown in Figure 4, it emerged that when BR (10 μ*M*)–HSA (20 μ*M*) reacted with SNOC (100 μ*M*) in the presence of physiological concentrations of UA (300 μ*M*) and GSH (20 μ*M*) (1, 51), both the antioxidants significantly decreased the formation of BV (initial rates: SNOC, 0.21 ± 0.01; SNOC + UA, 0.07 ± 0.01; and SNOC + GSH 0.12 ± 0.02 μ*M* BV/min, $n = 4$, $p < 0.01$ *vs.* SNOC alone). Notably UA was not able to decrease the total amount of BV formed whereas GSH did (SNOC, 3.26 ± 0.11; SNOC + UA, 3.30 ± 0.12; and SNOC + GSH, 1.56 ± 0.11 μ*M* BV/90 min, $n = 4$, $p < 0.01$ SNOC + GSH *vs.* SNOC alone).

DISCUSSION

Until 20 years ago, BR was merely considered as a toxic by-product of heme metabolism. In 1987 Stocker *et al.* (45) raised the hypothesis that BR could serve as an endogenous antioxidant molecule and proposed that this property was due to the extended system of conjugate double bonds and a reactive hydrogen atom which the bile pigment can donate, transforming itself in a carbon centered radical (BR•) with resonance stabilization extending over the entire molecule. Following this first observation, many papers demonstrated that this bile pigment is involved in cytoprotection in many cell types including neurons, leukocytes, endothelial and smooth muscle cells (5, 7, 10, 11, 18, 31). Similarly, Clark and colleagues reported that 100 n*M* BR ameliorates postischemic myocardial dysfunction using an isolated heart model (6). Recently, Baranano *et al.* (3) suggested a novel mechanism for the cytoprotective action of BR based on an amplification cycle whereby the bile pigment, in the presence of albumin, is itself oxidized to BV by ROS (and probably RNS) and then recycled by BVR back to BR. The protective role of BVR against oxidative stress is emphasized by the evidence

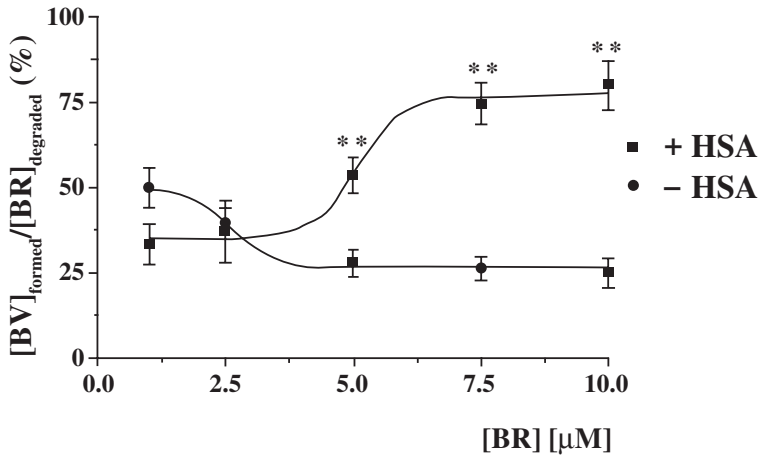

FIG. 2. Albumin (HSA)-bound bilirubin (BR) is dose-dependently oxidized into biliverdin (BV) in the presence of *S*-nitrosocysteine (SNOC). Bilirubin (1–10 μ*M*) was incubated with or without a saturating dose of HSA (20 μ*M*) and exposed to SNOC (100 μ*M*) as described in Materials and Methods. BR and BV concentrations have been calculated as described by Minetti *et al.* (27). Data are expressed as mean ± SEM of six experiments per group. $^{**}p < 0.01$ *vs.* samples without HSA.

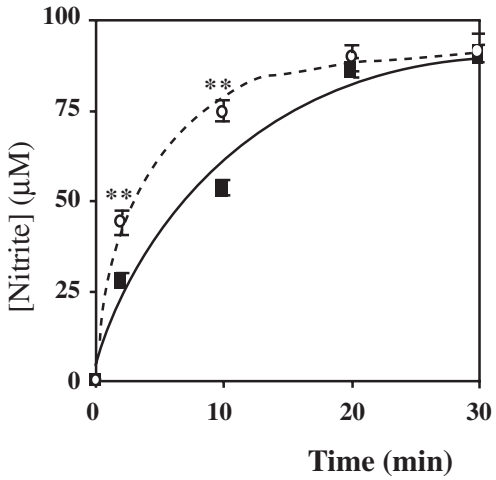

FIG. 3. Albumin (HSA)-bound bilirubin (BR) increases the rate of transformation of NO into nitrite. *S*-nitrosocysteine (SNOC, 100 μ*M*) either alone (*black squares*) or in the presence of BR (10 μ*M*)–HSA (20 μ*M*) (*white circles*) was incubated at 37°C in the dark; aliquots of the reaction mixture were taken at different time points and assayed for nitrite production as described in Materials and Methods. Data are expressed as mean ± SEM of eight experiments per group. $^{**}p < 0.01$ *versus* SNOC alone.

that HeLa cells or primary cerebral cortical neurons, depleted of BVR by using the RNA interfering technique, have a significant increase in intracellular ROS levels and undergo apoptosis (3). Collectively, these studies corroborated the notion that BR is an efficient scavenger of oxygen free radicals and suggested that the protective role of this pigment might be extended to other reactive species originated within the

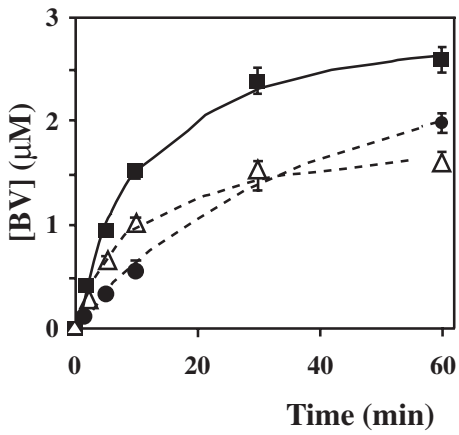

FIG. 4. Effects of uric acid (UA) and reduced glutathione (GSH) on biliverdin (BV) formation elicited by SNOC-derived NO. BR (10 μ*M*)–HSA (20 μ*M*) reacted with SNOC (100 μ*M*) (*black squares*) in the presence of UA (300 μ*M*) (*black circles*) or GSH (20 μ*M*) (*white triangles*). BV formation was monitored over time and the concentrations calculated as described in Materials and Methods. Data are expressed as mean ± SEM of four experiments per group.

cellular milieu. In a seminal paper Minetti *et al.* (27) have shown that BR is able to reduce the propagation of cell damage triggered by the pro-oxidant peroxynitrite, suggesting that the bile pigment could also prevent cellular injury elicited by RNS. In addition, Kaur *et al.* and our group demonstrated that BR, at physiological concentrations, also interacts with other RNS such as nitroxyl anion, nitrosonium, and with *S*-nitrosothiols (22, 26).

In the bloodstream BR, at concentrations ranging between 5 and 15 μ*M*, is almost completely unconjugated and bound primarily to HSA (27, 44); therefore, the vascular wall is continuously in contact with the BR–HSA complex. Considering that the plasma HSA concentration is ~500 μ*M*, it is possible to argue that, under physiological circumstances, plasma BR is completely saturated by HSA. Furthermore, even if the BR_f concentration increases up to 100 μ*M* (common levels found in patients affected by Gilbert's syndrome) (50) the bile pigment is still saturated by HSA.

It is well established that the binding of BR to HSA is physiologically useful because the protein stabilizes the bile pigment, prevents its transfer from blood to tissues, thus reducing the potential toxic effects. Moreover, previous studies clearly demonstrated that the complexing of BR with HSA does not reduce its antioxidant effect because albumin-bound BR maintains its efficacy to scavenge peroxyl radicals (7, 44). In line with these findings are the results shown in this paper; in fact BR–HSA was much more resistant than BR_f to the degrading effect of SNOC-derived NO and even increased the rate of transformation of this radical into nitrite (Figs. 1 and 3). In a recent paper Foresti *et al.* (12) have shown that the serum-containing medium from endothelial cells treated with hemin increases the rate of NO release from *S*-nitroso-*N*-acetylpenicillamine, and they conclude that this effect is mediated by a product of the HO activity . Our results enforce this evidence and propose BR as the HO product able to accelerate the NO release and decomposition from RSNO.

Furthermore, we have shown that BR–HSA interacts with SNOC-derived NO by a redox-based mechanism. In fact when BR–HSA reacted with SNOC, BV was the major product formed (~90% of the decomposed BR was converted in BV) (Fig. 2). This evidence is in contrast with the data showing that BR_f when interacts with SNOC undergoes *N*-nitrosative modification and its oxidation in BV is very low (Ref. 26 and Fig. 2). This different reactivity of BR_f versus BR–HSA can be explained considering that when the bile pigment is bound to the protein, the former exposes the reactive hydrogen atom at C-10 (27, 44), which can be oxidized by NO while protecting the dipyrrole rings from the *N*-nitrosation. We also observed an acceleration of nitrite formation from SNOC in the presence of BR–HSA. At the moment we can not rule out the possibility that such acceleration reflects a facilitated release of NO from SNOC rather than increased NO catabolism. Either way the ability of BR to interact with NO is quite important to explain some of the pathophysiologic mechanisms which are involved in the cytoprotective function of the bile pigment against RNS-related pathologies such as atherosclerosis, liver disease, and neurodegenerative disorders.

Recently, clinical studies demonstrated that low serum concentrations of BR correlate with a higher risk to develop atherosclerosis and undergo cardiovascular accidents such as

myocardial infarction (38). Moreover, Vitek *et al.* have shown that hyperbilirubinemic subjects with Gilbert's syndrome have a very low 3-year incidence of ischemic heart disease with respect to general population (50) and Ishizaka *et al.* demonstrated that high serum BR levels decrease the risk to develop carotid plaques (20). These clinical data become much more interesting considering that NO and RNS are involved in the initial phase of the atherosclerotic process by oxidizing both the circulating and vascular lipoproteins HDL and LDL, thus leading to the formation of the foam cells and atherosclerotic plaque (32, 33). Our data showing that BR–HSA inactivated NO provide a quite plausible explanation of a possible mechanism(s) by which BR–HSA can exert protective effects in the circulation.

Since BR–HSA is the predominant form of the bile pigment within the bloodstream, it was of interest to compare the NO trapping activity of BR–HSA with that of the water-soluble antioxidants UA and GSH. These two molecules have been chosen for this competition study because of their intrinsic ability to interact directly with NO and RNS (39, 51). Our results demonstrated that both UA and GSH reduced the rate of SNOC-induced oxidative conversion of BR into BV, indicating that they have a greater affinity for NO than BR–HSA, even if only GSH significantly reduced also the total concentration of BV formed. Based on these data it is possible to hypothesize a preferential role for BR–HSA in selected conditions in which the plasma concentrations of other antioxidants are reduced. In particular GSH plasma concentration can be significantly decreased in clinical syndromes such as liver disease and atherosclerosis (16, 23) and it is in the light of these findings that BR-HSA could exert its role as a circulating protective agent.

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ABBREVIATIONS

BR, bilirubin; BR_f, free bilirubin; BR_t, total bilirubin; BV, biliverdin; BVR, biliverdin reductase; GSH, reduced glutathione; GSNO, *S*-nitrosoglutathione; HO, heme oxygenase; HCl, hydrochloric acid; HSA, human serum albumin; L-cys, L-cysteine; NNED, *N*-naftyl-ethylene-diamine; NO, nitric oxide; PBS, phosphate buffered saline; RSNO, nitrosothiol(s); SA, sulphanilamide; SNOC, *S*-nitrosocysteine; UA, uric acid.

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