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Bilirubin and *S*-nitrosothiols interaction: evidence for a possible role of bilirubin as a scavenger of nitric oxide

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Abstract

Bilirubin (BR), the final product of heme catabolism, plays a crucial role in the defense against reactive oxygen species in various cell types. In this study, we addressed the hypothesis that BR can act as a physiological scavenger of nitric oxide (NO), a gaseous mediator involved in many cellular functions and able to trigger the formation of reactive nitrogen species with pro-oxidant activity. We found that *S*-nitrosocysteine (SNOC) and *S*-nitrosoglutathione (GSNO), which have a half-life of 0.52 ± 0.07 hr and 38 ± 5 hr and release NO at a constant rate of 1.42 ± 0.2 hr^{-1} and 0.018 ± 0.002 hr^{-1}, respectively, were able to decrease BR half-life in a concentration-dependent manner under physiological conditions. This effect appears to be dependent on NO formation as L-cysteine and GSH did not affect BR consumption and nitrite was four to five times less efficient than SNOC in reducing BR half-life. Oxyhemoglobin, a well-known scavenger of NO, protected BR from SNOC-mediated degradation. In addition, the reaction between SNOC/GSNO and BR modified the absorption spectrum of the bile pigment showing a gradual increase in the absorbance at 316 nm. This change in the BR spectrum indicates that the bile pigment could be a target for *N*-nitrosation reactions, since it resembles the modifications occurred when other molecules such as di-peptides and uric acid are nitrosated. Taken together, these data suggest that BR should not be considered only as an endogenous antioxidant but also as a molecule with the potential ability to counteract intracellular nitrosative stress reactions.
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Keywords: Bilirubin; Nitric oxide; *S*-Nitrosothiols; Reactive nitrogen species; Nitrosation; Nitrosative stress

1. Introduction

BR is the final product of heme catabolism. In mammals, approximately 80–90% of the total BR formed originates from the degradation of heme moieties of hemoglobin whereas 10–20% derives from the catabolism of other hemoproteins [1]. Two enzymes play a key role in the formation of BR: the microsomal HO and the cytosolic BVR. HO degrades heme to carbon monoxide, iron, and biliverdin (BV), while BVR converts BV to BR [1]. BR is then bound to ligandins that transport it from inside the cell to the blood stream [2,3]. As a consequence of these enzymatic reactions, at least 300 mg of BR are produced per day in normal adult humans [4]. In the blood stream, BR is bound primarily to serum albumin at concentrations ranging between 5 and 15 μM [5]; therefore, the vascular wall is continuously in contact with the albumin–bilirubin complex. Interestingly, at concentrations between 5 and 25 μM, BR has been shown to exert a concentration-dependent cytoprotective effect against hydrogen peroxide-mediated damage in aortic endothelial cells [6]. The bile pigment may become toxic for many tissues if the concentration exceeds 300 μM resulting in pathological states such as jaundice and kernicterus [5]. In contrast, the concentration of BR inside the cells is much lower than in blood ranging between 20 and 50 nM [7]. Despite these very low concentrations of BR, the continuous production

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Abbreviations: BR, bilirubin; BV, biliverdin; BVR, biliverdin reductase; DTPA, diethylenetriaminepentaacetic acid; GSNO, *S*-nitrosoglutathione; HCl, hydrochloric acid; HO, heme oxygenase; L-cys, L-cystein; NNED, *N*-1-naphtylethylenediamine; NO, nitric oxide; oxyHb, human ferrous oxyhemoglobin; RNS, reactive nitrogen species; RSNO, *S*-nitrosothiol(s); SA, sulphanilamide; SEM, standard error of the mean; SNOC, *S*-nitrosocysteine.

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of this linear tetrapyrrole offers an important line of defense against the oxidant insults in particular in vascular and brain tissues. Snyder and coworkers showed that BR, in the nanomolar range, is able to protect neurons from hydrogen peroxide-mediated injury [8]. Similarly, Clark *et al.* reported that 100 nM BR ameliorates post-ischemic myocardial dysfunction using an isolated heart model [9]. These and other studies [4,8,10] corroborate the notion that BR is an efficient scavenger of oxygen free radicals and suggest that the protective role of this pigment might be extended to other reactive species originated within the cellular milieu.

NO is a labile gas, formed by a group of constitutive (nNOS and eNOS) and inducible (iNOS) NO synthase enzymes (NOS). It plays a pivotal role in several physiological and pathophysiological processes within the cardiovascular, nervous, and immune system [11–13]. From a chemical point of view, NO is a free radical as it contains an unpaired electron in the outer orbital which enables the molecule to exist in different redox-activated forms. NO and its redox-activated forms, nitrosonium (NO^+) and nitroxyl anion (NO^-), can react with molecular oxygen, superoxide anion and other reactive molecules to generate RNS, among them peroxynitrite and nitrogen dioxide radical [14]. The excessive production of these NO derivatives has been shown to cause nitration of proteins as well as lipid peroxidation leading to cytotoxicity and cell death [15,16]. RSNO are a group of endogenous substances formed by the attachment of the NO groups to sulphydryl centers (*S*-nitrosylation); low molecular weight RSNO, such as SNOC and GSNO, are more abundant in cells whereas high molecular weight RSNO (i.e. *S*-nitrosoalbumin) are present in plasma [17]. Several studies reported that RSNO are not only an endogenous reservoir for NO (thus avoiding its reaction with proteins or metals) but also exert physiologic actions being involved in smooth muscle cell relaxation, inhibition of platelet aggregation [18,19], immunosuppression and neurotransmission [20,21].

Recent studies have shown that BR is able to protect plasma proteins and endothelial cells from the pro-oxidant effects mediated by peroxynitrite [5,22]. The aim of our study was to examine whether BR can interact directly with NO. In particular, we investigated whether SNOC and GSNO were capable of consuming BR *in vitro*.

2. Materials and methods

2.1. Chemicals

BR, L-cys, GSH, sodium nitrite, SA, NNED, EDTA, DTPA, HCl, and PBS were from Sigma Chemicals (Sigma-Aldrich). BR was dissolved in sodium hydroxide (0.1 M) at a concentration of 10 mM and further diluted in double-distilled water. BR solutions were freshly prepared before each experiment and protected from light. SA and NNED were dissolved in HCl (0.5 M) at the working solution of 1% (w/v) and 0.02% (w/v), respectively. Purified oxyHb was a kind gift of Dr. Elisabetta Clementi (Institute of Biochemistry and Clinical Biochemistry, Catholic University).

2.2. Preparation of RSNO and stability of solutions

RSNO were prepared as described by Arnelle and Stamler [23]. Briefly, stock solutions (100 mM) of L-cys and GSH were prepared by dissolving the compounds in HCl (1 M). Nitrite (100 mM) was prepared in Millipore MilliQ water. Equal volumes of L-cys/GSH and nitrite were then mixed in an Eppendorf tube in order to obtain a brown solution of RSNO (50 mM). The effective concentrations of stock solutions of SNOC and GSNO formed after the reaction between thiols and nitrite were assessed by the Saville assay [24]. Previous studies have established that essentially no nitrite remains in the RSNO solutions using this synthetic method [25,26]. RSNO were further diluted in PBS to the desired concentration.

The stability of SNOC and GSNO in PBS was evaluated spectrophotometrically knowing the characteristic absorption peak of RSNO at 332–334 nm [23] and calculating the appropriate extinction coefficient in PBS. For this purpose, a standard curve was obtained by plotting the absorbance values at 334 nm vs. the respective RSNO concentrations (0.05–2.5 mM) in PBS. A linear fitting was obtained and the calculated extinction coefficients (SNOC: $870 \pm 11\ M^{-1}\,cm^{-1}$, $N = 4$; GSNO: $970 \pm 37\ M^{-1}\,cm^{-1}$, $N = 5$) were in good agreement with previously reported values [27–29]. Since RSNO are rather unstable in physiological solutions, the substances were prepared freshly before the experiments, protected from light and used within 10 min from the preparation.

2.3. Spectrophotometric determination of bilirubin consumption

BR consumption was assessed spectrophotometrically using a Hewlett-Packard 8453 UV–VIS–NIR spectrophotometer equipped with a water-bath, allowing the cuvette to be kept constantly at 37° throughout the entire experiment. Spectra were performed on solutions (PBS, pH 7.4) containing BR (10 μM), BR plus SNOC (1–100 μM), BR plus GSNO (10–100 μM), or BR plus nitrite (1–100 μM). Because stock solutions of SNOC/GSNO and BR were prepared in strong acidic and alkaline environments, respectively, the pH of the reaction mixture was always measured before the beginning of the experiment. Only minor modifications with respect to pH 7.4 were observed. BR concentration over time was calculated by the difference in absorbance between 464 and 530 nm using an extinction coefficient of $40\ mM^{-1}\,cm^{-1}$ [9]. Because BR is light sensitive, all experiments were performed in subdued light.

2.4. Nitrite assay

The concentration of nitrite was determined as previously described by Griess [30]. Briefly, SNOC solutions (10–100 μM) in the absence or presence of BR (10 μM) in 1 mL of PBS (pH 7.4) were incubated at 37° with continuous shaking. After 5, 15, 30, and 60 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.

2.5. Competition studies using hemoglobin

Since oxyHb avidly binds NO, it was of interest to examine the scavenging capacities of BR towards RSNO in the presence of oxyHb. BR (10 μM) was incubated at 37° with SNOC (100 μM) in the presence or absence of oxyHb (2–25 μM; final volume 1 mL in PBS, pH 7.4). The amounts of BR consumed and oxyHb levels were then monitored over time. The concentration of oxyHb was calculated spectrophotometrically using an extinction coefficient of 14.6 mM^{-1} cm^{-1} at 577 nm [31]. The percentage of oxyHb was calculated as described elsewhere [31]. As reported above, the pH of the reaction mixture containing oxyHb, BR, and SNOC was confirmed to be in the neutral range before the beginning of the experiment.

2.6. 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's two-tailed t test. Differences among groups were considered significant at $P < 0.05$. Chemical decomposition constants (k) and half-life values ($t_{1/2}$) were calculated by linear and nonlinear regression analysis using a Prism 2.0 software (GraphPad Software).

3. Results

BR is a polar molecule, highly soluble in basic environments and light sensitive; these characteristics explain why this bile pigment is unstable in aqueous solutions. In our experiments, BR (10 μM) showed an acceptable stability in PBS (pH 7.4) with a half-life of 0.645 ± 0.026 hr (N = 6). However, the presence of 1 mM EDTA or 100 μM DTPA in the PBS solution significantly decreased BR half-life to 0.456 ± 0.068 hr (N = 4, $P < 0.05$ vs. PBS alone) and 0.235±0.003 hr (N = 3, $P < 0.01$ vs. PBS alone), respectively. In view of these preliminary observations, we decided to perform all the experiments in PBS in the absence of chelators, in order to prolong BR stability in solution.

In our model, SNOC decomposed with a rate constant of 1.42 ± 0.2 hr^{-1} and a half-life of 0.52 ± 0.07 hr (N = 4), while GSNO showed a very high stability, decomposing with a rate constant of 0.018 ± 0.002 hr^{-1} and a half-life of 38 ± 5 hr (N = 3). These values are in good agreement with results obtained by others in similar experimental conditions [29].

We initially examined the disappearance of BR upon reaction with SNOC and GSNO. We found that RSNO accelerated BR consumption in a concentration-dependent

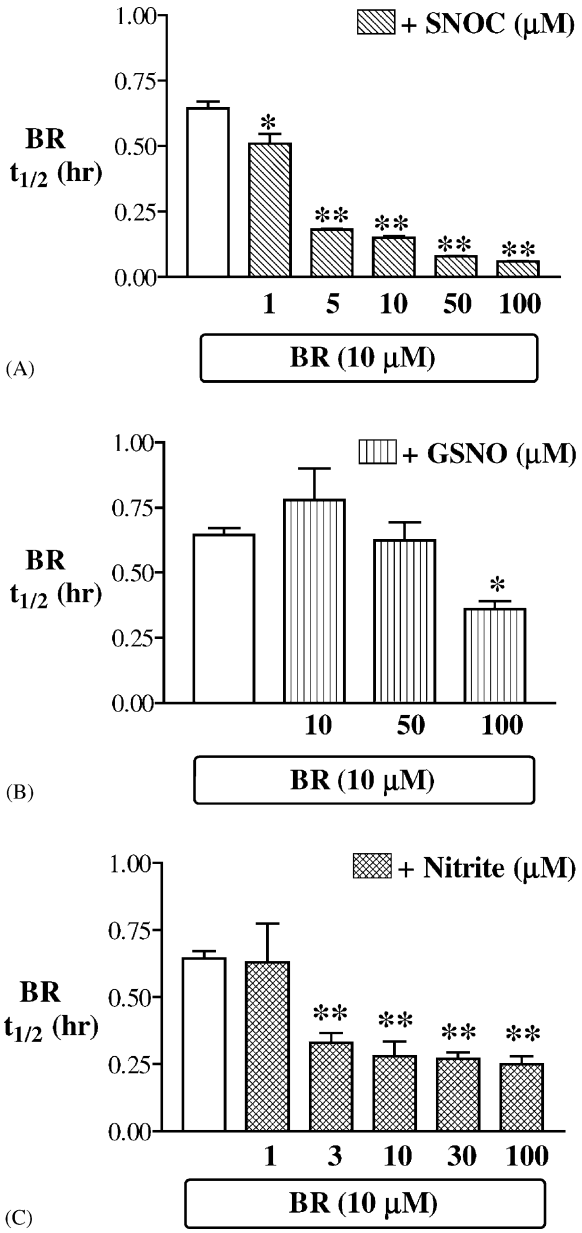

Fig. 1. (A) Effects of SNOC, (B) GSNO, and (C) nitrite on BR half-life. Different concentrations of SNOC, GSNO, and nitrite were added to BR (10 μM) in PBS (pH 7.4, 37°) and the change in the maximal absorbance peak characteristic of BR was monitored over time. The concentrations of BR consumed were calculated as described in Section 2. The half-lives ($t_{1/2}$) were then calculated using nonlinear fitting equations (Prism 2.0). Values are expressed as mean ± SEM of three to seven different experiments per group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* BR alone.

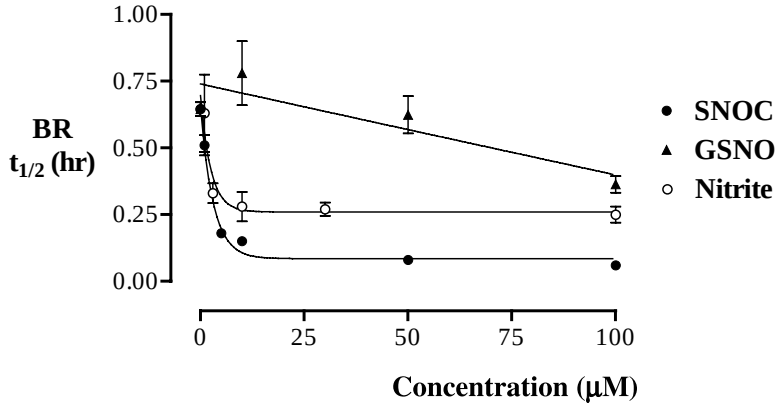

Fig. 2. SNOC, GSNO, and nitrite decrease BR half-life with different potency and efficacy. Different concentrations of SNOC, GSNO and nitrite (as in Fig. 1) were added to BR (10 μM) in PBS (pH 7.4, 37 °C) and the change in the maximal absorbance peak characteristic of BR was monitored over time. The concentrations of BR consumed were calculated as described in Materials and Methods. The half-lives ($t_{1/2}$) were then plotted *versus* the respective RSNO and nitrite concentrations and an hyperbolic fitting was obtained. The values related to potency and efficacy were calculated using nonlinear fitting equations (Prism 2.0). Values are expressed as mean ± SEM of three to seven different experiments per group.

manner. The results showed that SNOC (1–100 μM) and GSNO (10–100 μM) significantly consumed BR (10 μM) as demonstrated by the reduction in bile pigment half-life (Fig. 1A and B). To exclude the possibility that the decrease in BR half-life was due to the diluted HCl solution in which RSNO were dissolved, control experiments were performed by adding increasing concentrations of HCl to BR. At the lower concentrations tested (0.01–0.1 mM corresponding to the concentration of HCl present in 1–10 μM RSNO), HCl showed only a negligible effect in reducing BR half-life (1% of reduction). At higher concentrations (0.5–1 mM corresponding to the concentration of HCl present in 50–100 μM RSNO), HCl reduced BR half-life of less than 10%. Data in Figs. 1 and 2 were modified subtracting to SNOC and GSNO effects the contribution of HCl.

The interaction between SNOC/GSNO and BR exhibited different concentration dependence. By plotting RSNO half-lives vs. the respective concentrations (Fig. 2), a hyperbolic fitting was obtained for both SNOC and GSNO. In order to achieve the asymptotic value for GSNO (necessary for calculating its maximal efficiency) concentrations higher than 100 μM were tested on BR half-life (not shown). On this basis, we calculated the potency and efficiency of both SNOC and GSNO in consuming BR. Analyzing the data shown in Fig. 2, it emerges that SNOC is almost 30 times more potent than GSNO (EC_{50} 2.17 ± 0.03 μM and 60 ± 20 μM, respectively, $P < 0.05$) and about six times more efficient (maximal effect 0.04 ± 0.005 hr and 0.23 ± 0.1 hr, respectively, $P < 0.01$). Because NO is quantitatively converted into nitrite in oxygenated aqueous solutions, we considered the possibility that RSNO-induced BR consumption was dependent on nitrite. As shown in Fig. 1C, nitrite (1–100 μM) significantly reduced the half-life of BR, but it was at least four to five times less efficient than SNOC (0.04 ± 0.005 hr and 0.25 ± 0.03 hr, respectively, $P < 0.01$) and as efficient as GSNO (Fig. 2).

Under appropriate experimental conditions, RSNO decompose to the corresponding (di)sulfide and NO [32]. Therefore, we tested whether the consumption of BR was due to a thiolation or nitrosation reaction. We observed that L-cys and GSH, even at 100 μM, did not affect BR consumption (data not shown).

OxyHb is a well-known scavenger of NO, and its affinity for this gas is five to six orders of magnitude greater than that for oxygen. Under aerobic conditions, the reaction between oxyHb and NO results in the conversion of oxyHb to methemoglobin [33–35]. Our experiments confirmed that NO released from SNOC is able to convert oxyHb to methemoglobin as revealed by characteristic changes in the absorption spectrum (data not shown). When BR (10 μM) was reacted with SNOC (100 μM) in the presence of oxyHb (2–25 μM), we observed that high concentrations of oxyHb scavenged NO so efficiently to prevent completely the reaction between NO and BR; however, when the amount of oxyHb decreased below a critical value (∼6%), NO started to consume BR (Fig. 3). The ''protective'' effect of oxyHb on NO-mediated BR consumption was concentration dependent as demonstrated by the linear increase in the lag phase of BR consumption (Fig. 3, inset).

The interaction between NO and BR was also evaluated over time by measuring nitrite formation as an index of NO production/degradation. SNOC (10–100 μM) alone or in the presence of BR (10 μM) was incubated at 37° and aliquots of the reaction mixture were taken at different time points and assayed for nitrite production. As shown in Fig. 4, 10–100 μM SNOC was almost completely degraded into nitrite after 10 min of incubation reaching a plateau after 30–60 min. However, when SNOC was incubated in the presence of 10 μM BR, the amount of free nitrite markedly decreased.

Previous reports revealed that nitrosation of non-thiols-containing dipeptides or uric acid by RNS produces a chromophore with an absorption spectrum ranging from

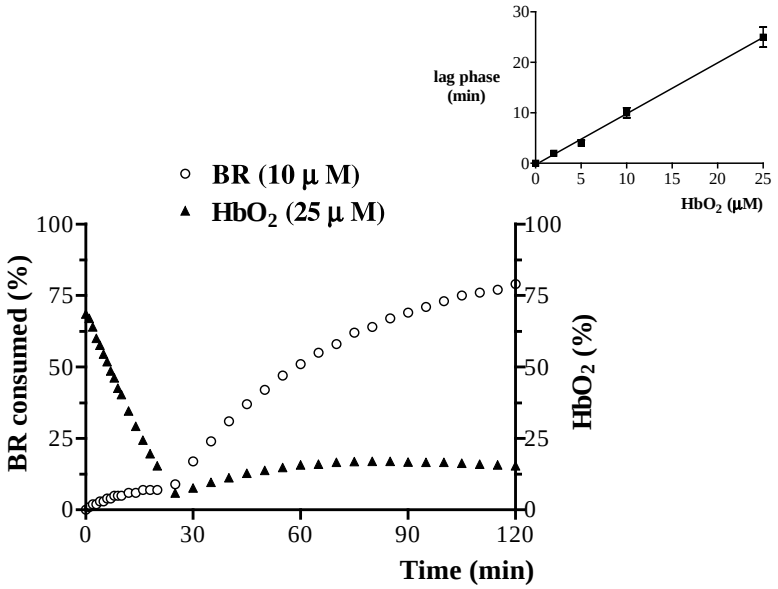

Fig. 3. Scavenging effects of oxyHb on BR consumption by SNOC. BR (10 μM) was incubated at 37 °C with SNOC (100 μM) with or without oxyHb (2–25 μM) in 1 mL PBS (pH 7.4) and the amounts of BR consumed and oxyHb were monitored over time. The plot is a representative experiment performed using BR (10 μM), oxyHb (25 μM) and SNOC (100 μM). The *inset* shows the linear increase of the lag phase of BR (10 μM) consumption *versus* the oxyHb concentration (2–25 μM). Data are expressed as mean ± SEM of 4 different experiments.

316 to 335 nm and a maximum at 316 nm [36,37]. Therefore, we assessed the possibility that a similar chromophore could be formed following the reaction of RSNO with BR. As shown in Fig. 5, BR alone does not absorb at 316 nm, but in the presence of SNOC and GSNO the spectrum changed and a peak at 316 nm appeared gradually increasing over time. Interestingly, BV, the major oxidation product of BR, was not formed during the reaction between RSNO and BR. In fact, we did not observe any increase at 378 and 670 nm, which represent the maximal absorption peaks for BV [5], following the treatment of BR with RSNO (Fig. 5A and B). The formation of BV was negligible when the concentration of RSNO was increased to 100 μM (data not shown).

4. Discussion

In the last few years, a new role of BR as an endogenously produced antioxidant has emerged and several reports have shown that BR contributes to prevent cell damage mediated by reactive oxygen species. In 1987, Stocker *et al.* [4] showed that BR is able to scavenge peroxyl radicals and they argued that the antioxidant mechanism was based on the extended system of conjugate double bonds and a reactive hydrogen atom which BR can donate, transforming itself in a carbon-centered radical (BR•) with resonance stabilization extending over the entire molecule. More recently Doré *et al.* reported that (i) hydrogen peroxide toxicity is much higher in hippocampal neurons from

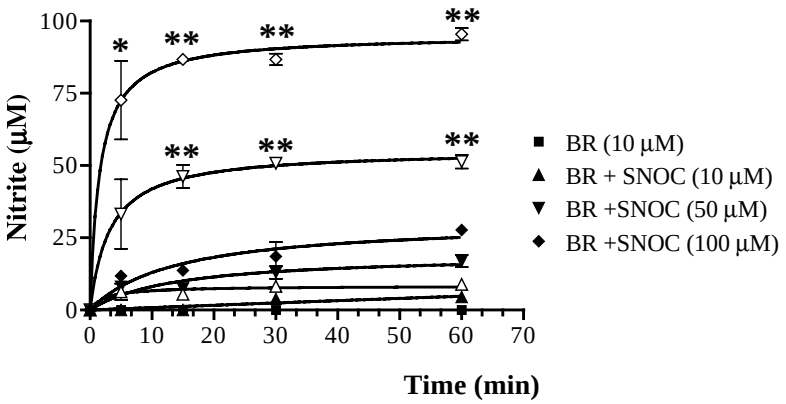

Fig. 4. Nitrite formation following reaction SNOC with BR. SNOC at the concentrations of 10 (△), 50 (▽), and 100 (◇) μM, either alone (open symbols) or in the presence of BR (10 μM; filled symbols) was incubated at 37°; aliquots of the reaction mixture taken at different time points and assayed for nitrite production as described in Section 2. Data are expressed as mean ± SEM of four to six different experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. SNOC alone.

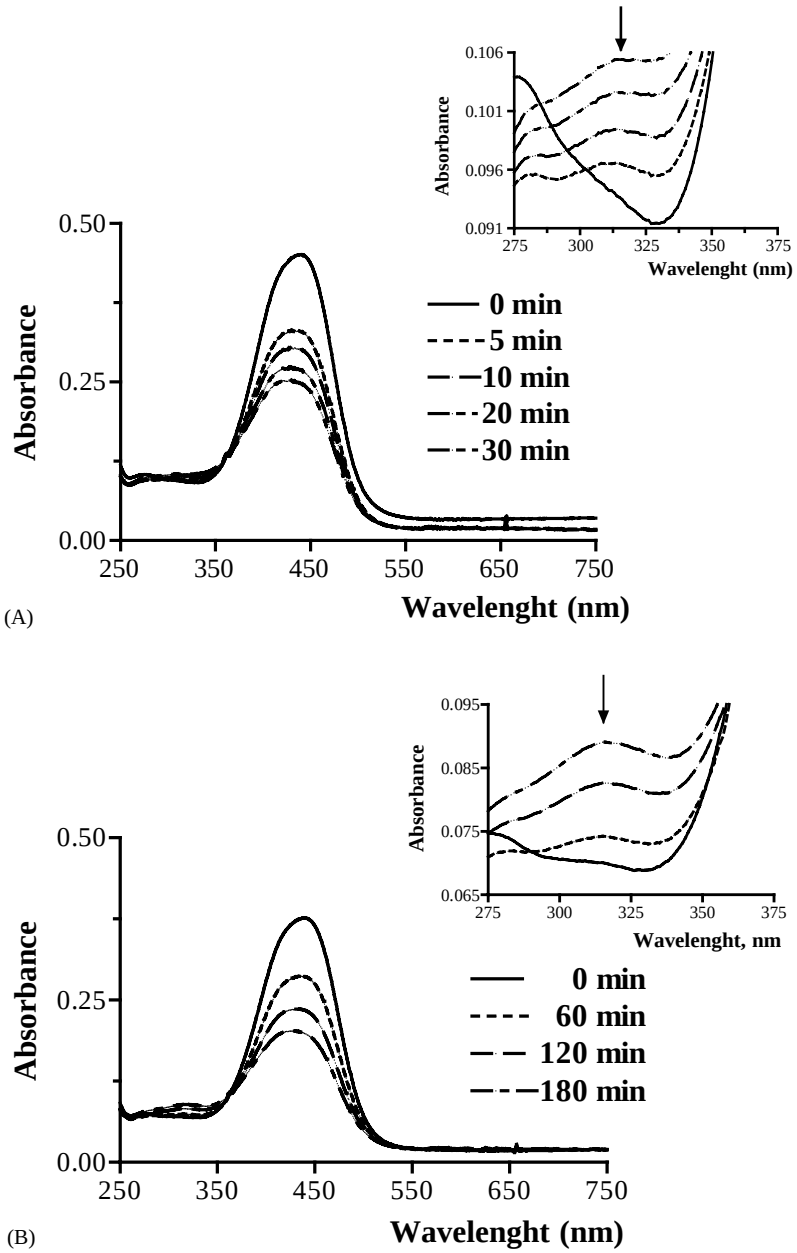

Fig. 5. Changes in the BR spectrum following treatment with (A) SNOC and (B) GSNO. SNOC or GSNO (10 μM) were added to BR (10 μM) in PBS (pH 7.4, 37°) and the absorbance spectra were recorded every 5 min. The insets show a detail of the spectra between 275 and 375 nm. The arrows indicate the peak at 316 nm.

HO-2-knockout mice (which have decreased BR production in neurons) compared to wild-type mice and that (ii) the exogenous administration of BR improved neuronal survival [8]. In line with these findings, it was found that cortical neurons cultured from mice expressing the Swedish mutation of Alzheimer's disease had defects in BR production with subsequent increase of hydrogen peroxide toxicity [38]. Studies on leukocytes, endothelial, smooth muscle and cardiac cells have highlighted the importance of BR in the defense of vascular tissue against the damage caused by reactive oxygen species and hypoxia-reoxygenation [6,10,22,39,40]. In addition, exogenous BR administered to isolated hearts at concentrations as low as 100 nM significantly reduced postischemic myocardial dysfunction [9]. With respect to the nervous tissue, it is interesting to note that the brain lacks BR conjugating enzymes, allowing the bile pigment to accumulate from neuronal HO-2 activity [41,42]. This finding becomes more intriguing in light of the evidence that neurons have relatively low concentrations of GSH [43,44], a tripeptide involved in detoxification of ROS and very abundant in almost all mammalian tissues; this suggests a possible role of BR as an alternative endogenous antioxidant molecule in neurons [45]. Recent studies have investigated the mechanism by which BR exerts its neuroprotective role. It was found that the antioxidant action of BR reflects an amplification cycle

whereby the bile pigment is itself oxidized to BV by hydrogen peroxide and recycled by BVR back to BR. The authors concluded that this redox cycle could explain how low concentrations of BR can protect against the cytotoxicity caused by 10,000-fold higher concentrations of the oxidant [7].

A previous study by Minetti *et al.* [5] has 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. Indeed, our group has recently demonstrated that BR interacts with other RNS such as nitroxyl anion [46]. Based on these observations, we decided to examine whether BR can interact specifically with RSNO, a group of substances formed by *S*-nitrosylation of sulphydryl groups. RSNO play a crucial role in modulating the physiological actions of NO and may prolong the bioactivity of NO. We noticed that BR had a significantly lower half-life in PBS supplemented with the chelators EDTA and DTPA. This is in agreement with findings obtained by Lee and Gartner [47] who reported that L-ascorbic acid and EDTA are both able to degrade BR. We cannot exclude that DTPA shares a similar effect in partially degrading the bile pigment. To prolong BR stability in our experiments, we decided to solubilize the bile pigment in PBS in the absence of chelators, with the knowledge that this would affect RSNO decomposition. In fact, the mechanisms of NO release from RSNO are complex and depend on physical and chemical factors such as light and presence of trace metals in the solution [32].

Our data showed that both SNOC and GSNO significantly reduced BR half-life and this effect appeared to be dependent on the direct interaction between BR and NO. In fact, L-cys and GSH alone did not decrease BR half-life whereas HCl *per se* has only a negligible effect. Particularly interesting is the different ability of SNOC and GSNO in reducing BR half-life. This difference reflects at least in part the different stability of RSNO in aqueous buffered solutions. The lower reactivity of GSNO raises the question why a such effective NO donor *in vivo* is apparently less effective *in vitro*. One intriguing hypothesis is that *in vivo*, GSNO is substrate for the enzyme γ-glutamil transpeptidase which releases intermediate *S*-nitroso compounds responsible for the rapid NO release [48]. Concerning the role of nitrite in BR consumption, this effect is also potentially relevant because *in vivo* there is a certain amount of cyclical conversion of nitrite in NO and these interchanges could affect the total amount of NO produced by tissues [20]. On this basis, the scavenger ability of BR for nitrite could reduce its conversion in NO thus avoiding potential toxic effects.

The effective role of NO in consuming BR was also evaluated in competition studies using oxyHb and BR as scavengers of NO. Our results showed that BR is able to scavenge NO even if its affinity for the gas is lower than that of oxyHb. Based on our data it is possible to hypothesize a preferential role for BR as scavenger for NO in particular in selected cells such as brain cells which have great amounts of the bile pigment and a very low amount of heme-containing proteins [41,49]. In the bloodstream, the potential role of BR as a scavenger of NO is overcome by oxyHb, the concentration of which is much higher than that of BR. Many studies showed that excessive production of NO in the brain, as a consequence of nitric oxide synthase induction in activated glia, participates in neurodegeneration [50,51] and it is in the light of these findings that BR could exert its role as neuroprotective agent. In addition, our results could have strong relevance in the context of liver physiology. Indeed, all the unconjugated BR generated in the body is transported to the liver in order to be detoxified and excreted; the liver *per se* further generates great amounts of BR through HO activity. For these reasons, the concentration of BR in and around the space of Disse should be much higher than that observed in the extravascular space of other organs. Our current and previous data [46] suggest that BR in the liver could exert potent scavenging activities towards NO and its redox forms and imply that the signaling and modulatory actions of NO in this organ have to be reconsidered in light of these findings. If BR diminishes the effects of NO in the liver, it is tempting to speculate that HO-derived CO could substitute NO in the control of liver functions. Consistent with this hypothesis, studies have shown that HO-derived CO, rather than NO, plays a key role in maintaining liver homeostasis by regulating sinusoidal tone and bile formation [52,53].

In the experiments described above, the interaction between NO and BR was evaluated by monitoring the consumption of BR. We also conducted another series of experiments whereby the formation of nitrite as an index of NO production/degradation was measured over time following reaction of BR with SNOC. The finding that BR was very effective in reducing the fast conversion of SNOC to nitrite strongly confirms that a fraction of total NO released from SNOC was scavenged by BR. This is indicative of binding of NO to BR and sustains a possible physiological participation of BR in nitrosative reactions.

Interestingly, some authors have shown that RNS such as nitrosonium and peroxynitrite can transfer NO to non-cysteine residues present in many model di-peptides as well as uric acid [36,37]. They found that the nitrosation reaction produces a chromophore with a spectrum ranging between 316 and 332 nm and maximal absorbance at 316 nm. We tried to reproduce this experiment in our system using SNOC and BR. We used a low concentration of SNOC and GSNO in order to minimize their interference with the absorbance at 332 nm and, therefore, with the absorption of the newly formed chromophore. After reaction with RSNO, the absorbance spectrum of BR showed a time-dependent increase at 316 nm strongly suggesting the formation of a *N*-nitroso product. This evidence *vis-à-vis* with the lack of formation of BV, the major product of BR

oxidation, is consistent with our hypothesis that NO primarily interacts with BR modifying its structure in the sense of nitrosation rather than oxidation.

The full characterization of nitrosated BR is now required and its possible physiologic role needs to be clarified. However, the consistent evidence showing that the HO-1/bilirubin pathway is very sensitive to activation by nitrosative reactions involving NO and its redox-activated forms [54,55] suggests a role for bile pigments as important intracellular metabolites with anti-nitrosative capacities.

Acknowledgments

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