Interaction of bilirubin and biliverdin with reactive nitrogen species

Harparkash Kaur[a,1], Martin N. Hughes[b], Colin J. Green[a], Patrick Naughton[a], Roberta Foresti[a], Roberto Motterlini[a,*]

[a] *Vascular Biology Unit, Department of Surgical Research, Northwick Park Institute for Medical Research, Watford Road, Y Block, Harrow, Middlesex HA1 3UJ, UK*
[b] *Department of Chemistry, King's College London, London WC2R 2LS, UK*

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Abstract Bilirubin (BR) and biliverdin (BV), two metabolites produced during haem degradation by haem oxygenase, possess strong antioxidant activities toward peroxyl radical, hydroxyl radical and hydrogen peroxide. Considering the importance attributed to nitric oxide (NO) and its congeners in the control of physiological and pathophysiological processes, we examined the interaction of BR and BV with NO and NO-related species in vitro. Exposure of BR and BV to agents that release NO or nitroxyl resulted in a concentration- and time-dependent loss of BR and BV, as assessed by high performance liquid chromatography. Peroxynitrite, a strong oxidant derived from the reaction of NO with superoxide anion, also showed high reactivity toward BR and BV. The extent of BR and BV consumption largely depended on the NO species being analysed and on the half-lives of the pharmacological compounds considered. Of major importance, BR and BV decomposition occurred also in the presence of pure NO under anaerobic conditions, confirming the ability of bile pigments to scavenge the gaseous free radical. Increasing concentrations of thiols prevented BR consumption by nitroxyl, indicating that bile pigments and thiol groups can compete and/or synergise the cellular defence against NO-related species. In view of the high inducibility of haem oxygenase-1 by NO-releasing agents in different cell types, the present findings highlight novel anti-nitrosative characteristics of BR and BV suggesting a potential function for bile pigments against the damaging effects of uncontrolled NO production.

Key words: Bilirubin; Nitric oxide; Nitroxyl; Nitrosonium cation; Antioxidant; Nitrosative stress; Anti-nitrosative capacity; HO-1

1. Introduction

The linear tetrapyrroles (LTPs) biliverdin (BV) and bilirubin (BR) are formed intracellularly during the degradation of haem. The protoporphyrin ring is first oxidised by haem oxygenase to produce carbon monoxide, Fe(II) and BV [1]; biliverdin reductase subsequently reduces BV to BR [2]. It is noteworthy that birds, amphibians and reptiles directly excrete BV whereas in mammals an energy-consuming reaction is employed to transform BV to BR, suggesting an evolutionary pressure in favour of the formation of BR [3]. Both bile pigments contain an extended system of conjugated double bonds and reactive hydrogen atoms that probably account for their potent antioxidant properties [3]. Indeed, the redox potential of BV/BR is of the same order of magnitude as that of NADP/NADPH, the redox system largely used for electron transfer within cellular compartments. It is not surprising then that oxidative stress characterises the majority of stimuli responsible for up-regulation of HO-1, the inducible isoform of haem oxygenase, which appears to protect tissues against stressful conditions [4].

It is also intriguing that nitric oxide (NO) and NO-related species (NORS[2]) can markedly increase the cellular expression of HO-1 [5–8]. NO is a versatile signaling molecule that plays a major role in the control of cardiovascular and neuronal functions. The nature of the biological activity exerted by NO will depend upon its concentration and whether or not it is converted to some other reactive species in the cellular milieu [9]. Thus, excessive production of NO leads to the generation of reactive nitrogen species, such as peroxynitrite ($ONOO^-$) formed in the presence of NO and superoxide radical, which may cause tissue damage in a variety of disease states [10]. Moreover, the nitrosonium cation (NO^+) and nitroxyl (HNO), the respective one-electron oxidation and one-electron reduction products of NO, can react with proteins, DNA and lipids, thereby altering their function [11]. Preferential target sites for reactive nitrogen species include thiol groups, metal centres and amines [9].

The biological significance of HO-1 induction in response to NO and NORS remains obscure; however, the possibility that the HO-1 system may act as an endogenous modulator of NO generation and function has been previously highlighted [8].

*Corresponding author. Fax: (44)-208-8693270.
E-mail address: r.motterlini@ic.ac.uk (R. Motterlini).

[1] Present address: Department of Infectious and Tropical Diseases, Gates Malaria Programme, London School of Hygiene and Tropical Medicine, University of London, London WC1B 3DP, UK.

Abbreviations: BV, biliverdin IXα; BR, bilirubin IXα; HO-1, haem oxygenase-1; NO, nitric oxide; NORS, nitric oxide-related species; $ONOO^-$, peroxynitrite; NO^+, nitrosonium cation; HNO, nitroxyl; SNAP, *S*-nitroso-*N*-acetylpenicillamine; GSNO, *S*-nitrosoglutathione; SNP, sodium nitroprusside; NAC, *N*-acetylcysteine; Cys, cysteine; Hcy, homocysteine; DEA-NO, diethylamine NONOate; AS, sodium trioxodinitrate or Angeli's salt; SIN-1, 3-morpholinosydnomine; meso-BR, meso-bilirubin IXα; LTP, linear tetrapyrrole; HPLC, high pressure liquid chromatography

[2] For convenience, we use the term NORS to define collectively the different compounds used in this study to release or transfer NO and/or various related species.

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The fact that BR decreases the degree of oxidative injury caused by hydrogen peroxide [5,12,13], $ONOO^-$ [14,15] and peroxyl radicals [3] led us to hypothesise that the scavenging properties of BR could be extended to NO and its congeners [8]. Therefore, the present study was designed to investigate in vitro whether BR and BV directly interact with NO and NORS.

2. Materials and methods

2.1. Chemicals and reagents

S-Nitroso-*N*-acetylpenicillamine (SNAP), *S*-nitrosoglutathione (GSNO), sodium nitroprusside (SNP), *N*-acetylcysteine (NAC), cysteine (Cys), homocysteine (Hcy) and Iscove's modified Dulbecco's medium were purchased from Sigma (Poole, Dorset, UK). ^{15}N-labelled GSNO was prepared by a standard synthetic route using $Na^{15}NO_2$ (98% enrichment) [16]. Diethylamine NONOate (DEA-NO), sodium trioxodinitrate or Angeli's salt (AS) and 3-morpholino-sydnomine (SIN-1) were from Alexis (Bingham, Nottingham, UK). BV, BR and meso-bilirubin (meso-BR) were obtained from Porphyrin Products (Utah, USA). $ONOO^-$ was prepared by reacting an acidic solution of hydrogen peroxide with nitrous acid and by quenching the product with NaOH as previously described [17]. The $ONOO^-$ solution was passed over manganese dioxide to remove residual hydrogen peroxide. The concentration of $ONOO^-$ was determined spectrophotometrically ($\varepsilon_{302} = 1670\ M^{-1}\ cm^{-1}$) before each experiment. NO was prepared in the absence of oxygen by slowly adding a saturated solution of sodium nitrite to concentrated sulphuric acid in a reaction system that had previously been thoroughly deoxygenated using a nitrogen stream. The nitrogen flow was reduced as the NO was generated. The NO was passed through three Dreschler bottles (via sinters) containing 1 M sodium hydroxide solution (to remove any acid) and then through three Dreschler bottles containing distilled water. The NO was then passed into a sample tube (fitted with a suba seal and needles) containing buffer or distilled water at 2–8°C for 30 min. The solution was stored in a refrigerator until required. The concentration of NO in solution at 37°C was noted from standard solubility tables to be 1.6 mM. The half-lives of the NORS under our specific experimental conditions were measured to ensure that these values agreed with literature values for decomposition in standard buffers.

2.2. Experimental protocol

BR, BV and meso-BR were used in these experiments. Because these LTPs are sensitive to light, all preparations and reactions were carried out in dim light. Amber borosilicate vials, which fit directly onto the autosampler tray for high pressure liquid chromatography (HPLC) analyses, were also used to protect the samples from light exposure. Stock solutions of each NORS (10 mM) were generally prepared in dimethyl sulphoxide (DMSO), except for SNP (in H_2O), AS and DEA-NO (in 0.01 M NaOH). LTPs (BR, meso-BR or BV) were dissolved in DMSO and added to 0.5 ml Iscove's modified Dulbecco's medium supplemented with 10% foetal bovine serum at a final concentration of 5 μM. Solutions of NORS were then added and the reaction mixture was incubated at 37°C for various periods of time. $ONOO^-$ was added as a bolus to the LTP at the appropriate concentration. In some experiments, AS (100 μM) was reacted with BR in the presence of increasing concentrations (0–400 μM) of the thiol compounds NAC, Cys or Hcy. At the end of the incubation period, samples were cooled and immediately analysed by HPLC for measurements of LTP concentrations. For experiments carried out in anaerobic conditions, a stream of nitrogen was bubbled through the solution of LTP for 5 min, the mixture was then transferred to a vacuum tube through a rubber septum prior to the addition of the NO-saturated buffer. At the end of the incubation, samples were immediately transferred to amber borosilicate vials prior to HPLC analyses.

2.3. HPLC analysis

An HPLC-based method was used to measure the decrease in LTP concentration during reaction with the various NORS. All HPLC equipment and software were from Dionex (Camberley, Surrey, UK). Samples were separated on a Lichrosorb RP-8 10 μm column (250×4.6 mm, Jones Chromatography, Mid Glamorgan, UK) with gradient elution from 100% solvent A to 100% solvent B over 11 min at a flow rate of 1.4 ml/min. Solvent A consisted of 100 mM ammonium acetate (pH 5.5), 2-methoxyethanol and methanol (v/v; 45:5:50) and solvent B consisted of 2-methoxyethanol and methanol (v/v, 5:95). The UV-photodiode array detector (UV-PDA; Dionex model UVD-340S) was set at 450 nm. BR and meso-BR were determined at 450 nm and spectral extraction at 376 nm was employed to determine the amount of BV detected. The detection limit was determined to be 0.01 μM for BR, 0.01 μM for meso-BR and 0.02 μM for BV. Peak identity was confirmed by measuring the retention time, spiking the sample with commercially available standards and determination of absorbance spectra using the UV-PDA.

2.4. GS-MS analysis

Analysis of the gaseous products from the reaction of $GS^{15}NO$ with BR were measured by gas chromatography-mass spectrometry (GS-MS) techniques using a JEOL JMS AX505W instrument. Reactions were carried out in septum-capped vials. At the end of the reaction, head-space samples were withdrawn with a Hamilton gas-tight syringe and injected into the column. Peak areas for ions at *m/z* 31 (^{15}NO) and *m/z* 46 ($^{15}N_2O$) were measured at the appropriate retention times, as determined for appropriate standards.

2.5. Spectrophotometric studies

All BR spectra were recorded using a Helios α Unicam UV-Visual spectrophotometer (Unicam, Cambridge, UK). The absorbance readings between 300 and 750 nm were conducted at 37°C against a blank (phosphate-buffered saline (PBS), pH 7.4) containing an equal volume of vehicle (0.01 M NaOH). An initial spectrum of BR (5 μM final concentration in PBS) was obtained and compared to a second absorption spectrum recorded 5 min after reaction of BR with freshly prepared AS or DEA-NO (40 μM). Changes in spectra were also obtained after exposure of the bile pigment to decomposed AS or DEA-NO, which were prepared by pre-incubating the compounds at 37°C for 1 h.

2.6. Statistical analysis

Differences in data among the groups were analysed by using one-way ANOVA combined with the Bonferroni test. Values were expressed as a mean ± S.E.M. and differences between groups were considered to be significant at $P < 0.05$.

3. Results

The reactions of BR and BV with NORS were studied in a cell culture medium containing 10% serum. This was done for two important reasons: (1) to simulate the growing conditions in the cell culture in order to maintain a continuity between data obtained in vitro and potential results originating from experiments in cell culture or tissues; (2) the presence of serum mimics physiological conditions whereby BR and NO can easily interact with blood components such as albumin [18]. The compounds selected for the study were grouped as follows: (1) SNP, an NO^+ donor that is particularly active towards thiolate groups rather than nitrogen centres; (2) $ONOO^-$ and SIN-1 (a $ONOO^-$ source via the simultaneous release of NO and superoxide anion); (3) AS, which decomposes to give HNO ($pK_a \approx 11.4$) [19] and nitrite ion; (4) the NO releaser DEA-NO; and (5) *S*-nitrosothiols such as GSNO, a releaser of NO by homolysis but a reactive nitrosating agent, and SNAP, which releases NO rapidly but is a poor NO^+ donor.

3.1. BR and BV interact with NORS

We initially performed experiments in which 5 μM BR was incubated with 40 μM NORS. As shown in Fig. 1A, all NORS except SNP caused a decrease in BR concentration over time. Based on the half-lives of the NORS and the pre-

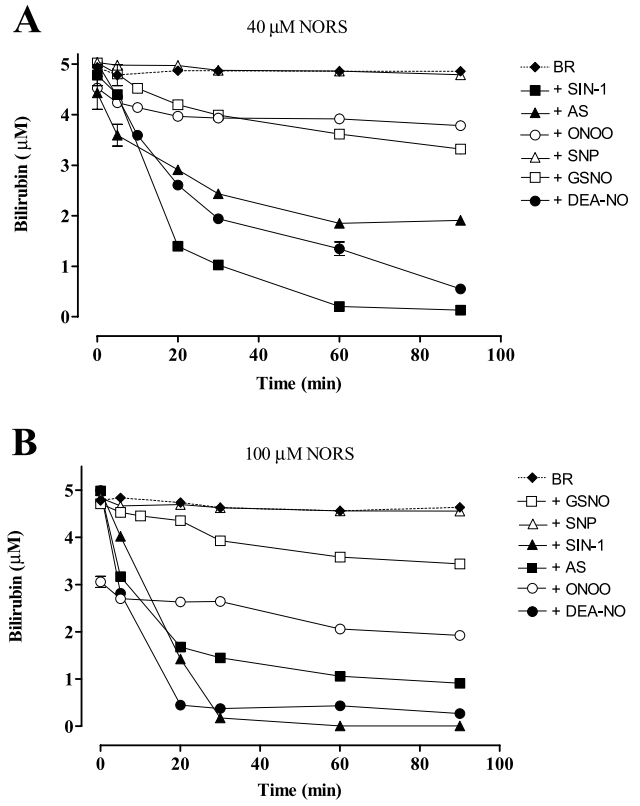

Fig. 1. Time-dependent disappearance of BR following interaction with NORS. A: BR, at the initial concentration of 5 μM, was incubated in Iscove's modified Dulbecco's medium with 40 μM of the following NORS: SNP, GSNO, $ONOO^-$, AS, SIN-1 and DEA-NO. At indicated time points, samples were analysed for BR levels using the HPLC method described in Section 2. B: BR was incubated with 100 μM NORS (as above) for various periods of time and BR disappearance was analysed by HPLC. Results represent the mean ± S.E.M. of three independent experiments.

liminary results obtained, we could identify three distinct modes of reactivity of NORS with BR. In the first case, typified by SIN-1 and DEA-NO, the reaction effectively went to completion by 90 min; in the second case, exemplified by $ONOO^-$ and AS, a complete disappearance of BR did not occur probably due to the fact that the extent of reaction was limited by the instability of these species ($t_{1/2}$ = seconds and $t_{1/2}$ = 2.3 min, for $ONOO^-$ and AS respectively). Finally, in the case of GSNO, only partial reaction with BR occurred.

Since BR was still detected at the end of the incubation period with some NORS, a further set of experiments was carried out at 100 μM NORS (Fig. 1B) to determine if the concentrations of NORS used was a factor limiting the extent of BR decomposition. Under these conditions, SNP was still unreactive towards BR. This lack of reactivity was also observed at concentrations of SNP up to 5 mM (data not shown). Likewise, the reactivity of 100 μM GSNO was unchanged compared to 40 μM. In general, as anticipated, the increase in concentrations of the other NORS led to an increase in the initial rate of disappearance of BR and the extent of reactivity. The kinetics of reaction of SIN-1 with BR were zero order in BR, with an excellent straight line plot of concentration against time, and successive half-lives of 15, 7.5 and 3.8 min at 37°C (zero order rate constant $k_0 = 2.73 \times 10^{-9}$ M s^{-1}). This showed that the rate-determining step in the reaction was the decomposition of SIN-1 to give NO and superoxide, followed by fast reaction of BR with one of these species or a species derived rapidly from them ($ONOO^-$) [20]. $ONOO^-$ added as a bolus also reacted rapidly with BR, but the reaction did not go to completion due to its rapid decomposition in the medium. It is significant that BR has been proposed to protect plasma proteins from $ONOO^-$-mediated oxidation [15] and that we have shown the cytoprotective effects of BR against apoptosis induced by $ONOO^-$ in cultured endothelial cells [14]. DEA-NO reacted more rapidly with BR than did SIN-1, but again the reaction was approximately zero order in BR, showing that the rate-determining step was the decomposition of DEA-NO. The reaction of BR with AS did not go to completion (80%), reflecting the rapid decomposition of AS as noted earlier. The dimerisation of HNO to give N_2O will be more favoured at higher AS concentration. The kinetics of loss of BR are not clearly zero order in BR.

The products of reaction of BR with GSNO were studied by MS-GC techniques using ^{15}N GSNO. This showed qualitatively the presence of nitrous oxide as a product although the peak area corresponding to *m/z* 31 was about twice as great as that expected for the formation of ^{15}NO by fragmentation of $^{15}N_2O$. The additional ^{15}NO probably results from homolytic fission of GSNO. The high reactivity of NO and HNO towards the bile pigment was also confirmed by measuring changes in the BR spectra after its reaction with DEA-NO or AS (40 μM) in PBS (pH 7.4) at 37°C. As shown in Fig. 2A,B, both DEA-NO and AS caused a rapid decrease in the maximal absorption peak of BR; since the changes in BR spectra were much less pronounced in the presence of decom-

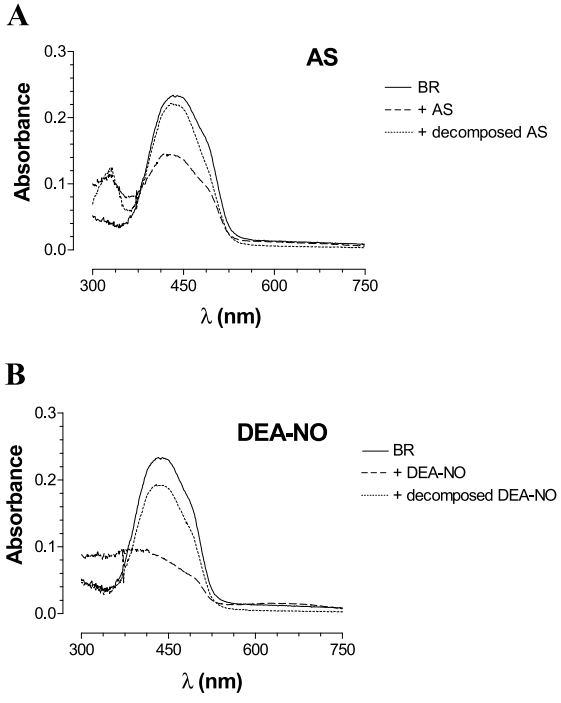

Fig. 2. Effect of AS and DEA-NO on BR absorption spectra. Absorption spectra of BR (5 μM) were recorded 5 min after incubation in PBS buffer (pH 7.4, 37°C) and after interaction with 40 μM AS (A) or DEA-NO (B). Changes in spectra were also obtained after reaction of BR with decomposed AS or DEA-NO, which were prepared by pre-incubating stock solution of compounds in PBS at 37°C for 1 h.

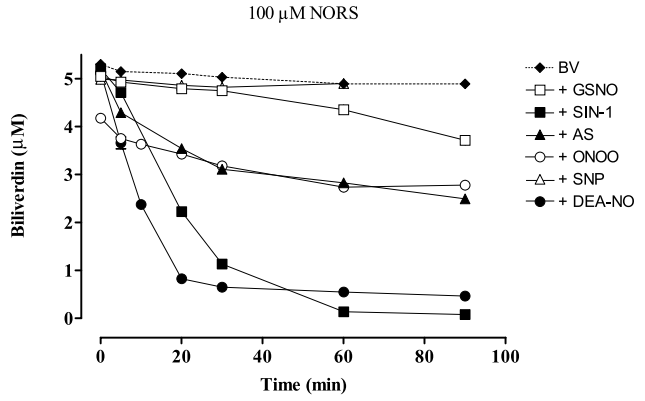

Fig. 3. Time-dependent disappearance of BV following interaction with NORS. BV, at the initial concentration of 5 μM, was incubated in Iscove's modified Dulbecco's medium with 100 μM of the following NORS: SNP, GSNO, $ONOO^-$, AS, SIN-1 and DEA-NO. At indicated time points, samples were analysed for BV determination using the HPLC method. Results represent the mean ± S.E.M. of three independent experiments.

posed DEA-NO and AS, these data suggest that a direct interaction with NO and HNO leads to BR degradation.

Having established that BR is susceptible to attack by various NORS, we wanted to investigate whether BV, the oxidation product and precursor of BR, would show reactivity towards these compounds. Incubation of 5 μM BV with 100 μM NORS resulted in degradation of the pigment over time (Fig. 3). As in the case of BR, SNP did not produce any detectable loss of BV while SIN-1 and DEA-NO essentially reacted with all BV available with zero order kinetics ($k_0 = 2.37 \times 10^{-9}$ M s^{-1} for SIN-1 and $k_0 = 4.46 \times 10^{-9}$ M s^{-1} for DEA-NO). Reaction of AS with BV occurred to a markedly lesser extent than the reaction of AS with BR. GSNO and $ONOO^-$ reacted similarly with both bile pigments.

Under aerobic conditions NO is oxidised to nitrite (NO_2^-) which might react with LTPs. However, we found that incubation of BR or BV with sodium nitrite (0–100 μM) did not produce any detectable loss in bile pigments (data not shown). Collectively, these data confirm the ability of NORS to interact with BR and BV and cause their decomposition in a time-dependent fashion.

3.2. BR, BV and meso-BR interact with NO in anaerobic conditions

In the above experiments, we used pharmacological agents that can liberate in physiological buffers NO (GSNO, DEA-NO and SIN-1) or HNO (AS), or transfer NO^+ to an appropriate receptor molecule (SNP and GSNO). In addition, $ONOO^-$ was used since it oxidises and nitrates biological targets [20]. As these experiments were conducted under aerobic conditions, the participation of oxygen in the reaction of NORS with bile pigments could not be excluded. Therefore, although the results obtained with the different NORS are significant in determining the potential susceptibility of BR and BV to the attack of NO and its congeners, it was necessary to ascertain whether NO per se would react chemically with the bile pigments. Fig. 4A illustrates that incubation of NO under anaerobic conditions resulted in a concentration-dependent disappearance of BR and BV. BV appeared to be more sensitive than BR as concentrations of NO in the range of 20–40 μM produced a substantial decrease of the LTP. In contrast, much higher NO concentrations were needed to degrade partially BR.

Meso-BR was also exposed to NO in an attempt to define if the site of attack of NO was the pyrrole rings or the vinyl side chains in BR. In meso-BR the vinyl side chains are saturated and, therefore, not susceptible to reaction. As the reactivity of NO to meso-BR was similar to its reactivity towards BR (Fig. 4A), it can be concluded that the vinyl group is not a target of attack by NO.

3.3. Reaction products of BR with $ONOO^-$

Oxidation of BR bound to albumin results in formation of BV [21]; therefore, the hypothesis that BV could also be produced upon exposure of BR to $ONOO^-$ was tested. Incubation of BR with increasing concentrations of $ONOO^-$ caused a disappearance of BR and formation of BV (Fig. 4B). However, while BR degradation was dependent on the concentration of $ONOO^-$, only small amounts of BV were formed and remained stable within the range of $ONOO^-$ concentrations used. Mostly, the reaction led to formation of colourless prod-

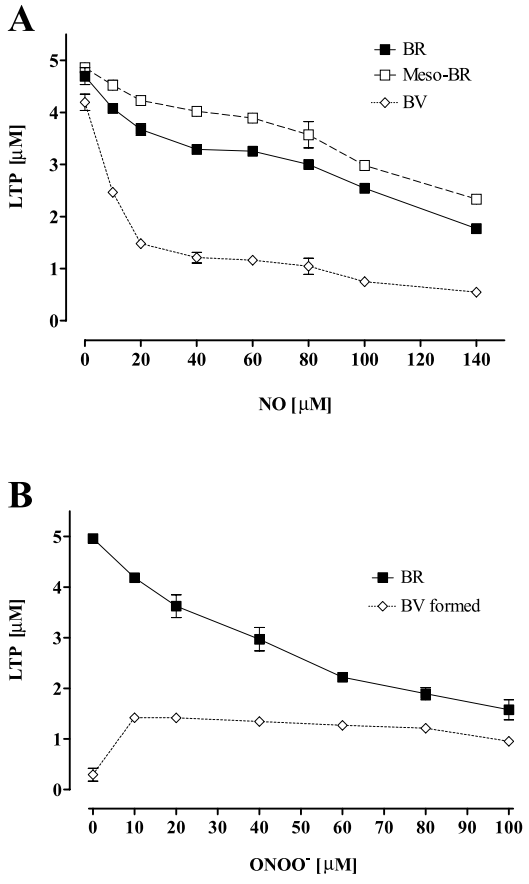

Fig. 4. Disappearance of LTPs following exposure to NO and $ONOO^-$. A: BR, BV and meso-BR, here indicated as LTP (5 μM), were incubated for 60 min with increasing concentrations (0–140 μM) of pure NO in anaerobic conditions. At the end of incubation, samples were analysed by the HPLC method for levels of LTP. Results represent the mean ± S.E.M. of three independent experiments. B: BR (5 μM) was incubated for 30 min with increasing concentrations (0–100 μM) of $ONOO^-$. At the end of the incubation, BR and the formation of BV were determined by the HPLC method. Results represent the mean ± S.E.M. of three independent experiments.

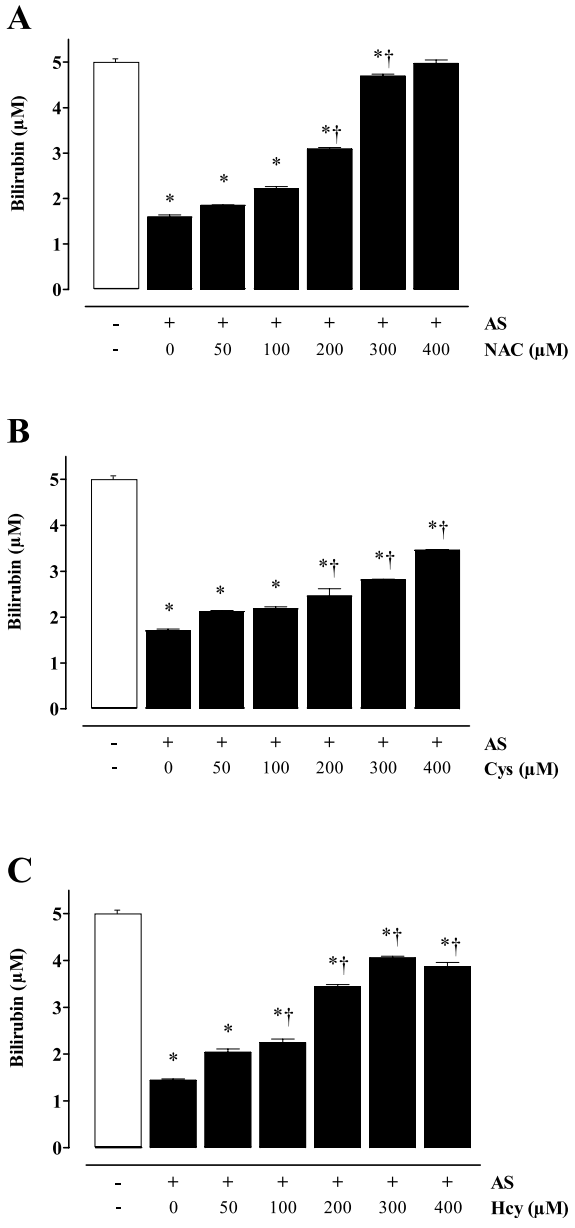

Fig. 5. Thiol compounds attenuate BR decomposition by HNO. BR (5 μM) was incubated with AS (100 μM), a HNO generator, for 60 min in the presence of increasing concentrations (0–400 μM) of the following thiols: NAC (A); Cys (B); and Hcy (C). At the end of incubation, samples were analysed by the HPLC method. Results represent the mean ± S.E.M. of three independent experiments, *$P < 0.005$ versus BR alone (open bar); †$P < 0.005$ versus BR+AS.

ucts, which have not been characterised. This is indicative of cleavage of the tetrapyrrole moiety of BR [22]. It has to be noted that some BV was formed following reaction of BR with all other NORS (data not shown).

3.4. Reaction of BR with AS in the presence of thiols

The reaction of BR with AS has been assumed to involve HNO, formed by self-decomposition of the trioxodinitrate group. This hypothesis was tested by studying the effect that thiols have on the reaction of BR with AS, since HNO reacts rapidly with sulphydryl groups [23]. The reaction of BR with AS was carried out in the presence of NAC, Cys and Hcy. This last compound, which has been shown to interact with NO to modulate vascular HO-1 expression [24], is potentially significant as an increased level of plasma Hcy is an independent risk factor associated with development of cardiovascular disease. Interestingly, thiols prevented BR degradation by AS in a concentration-dependent manner (Fig. 5). The most effective was NAC, which completely blocked decomposition of BR at 400 μM (Fig. 5A). However, some BR loss occurred even in the presence of the highest concentrations of Cys and Hcy used (Fig. 5B,C, respectively).

4. Discussion

The results described in this paper show clearly that BR and BV react with NO and its congeners (NORS) and that these reactions are accompanied by loss of BR and BV. These observations broaden our view on the potential scavenging properties of bile pigments. In fact, BR has already been recognised as an important physiological chain-breaking antioxidant against peroxyl, hydroxyl, hydroperoxyl and superoxide anion radicals [3,21,22]. Since increased amounts of NO and NORS have been suggested to impose a cellular threat termed 'nitrosative stress' (in analogy with the ability of oxidants and reactive oxygen species to cause oxidative stress), the data presented here highlight novel 'anti-nitrosative' characteristics of BR and BV.

The NORS utilised in this study release or transfer in physiological buffers not only NO but also redox forms of NO. We observed a direct correlation between the rate of the NO species released and the loss of BR over time inasmuch as DEA-NO ($t_{1/2} = 2$ min) and AS ($t_{1/2} = 2.3$ min) quickly consumed BR irrespective of the NO redox form liberated. GSNO was the slowest to react with BR, which is consistent with a mode of reaction involving release of NO from GSNO, as this *S*-nitrosothiol usually has a half-life for homolysis to NO of many hours. This is supported by the observation (using ^{15}N GSNO) that reaction of BR with GSNO gives nitrous oxide as a product. This suggests that NO oxidises BR with the formation of HNO and hence, by dimerisation, nitrous oxide. If GSNO acts like a nitrosating agent, then this reaction would also be impeded because NO^+ transfer to N nucleophiles is slow [9]. Conversely, the short-lived $ONOO^-$ ($t_{1/2} = < 1$ s) reacted so rapidly with BR that at time 0 min we could only detect 3 μM of the initial 5 μM BR employed. This is in agreement with findings of Minetti and colleagues, who reported that addition of increasing concentrations of $ONOO^-$ results in a rapid destruction of the bile pigment in vitro [15]. SIN-1 was also of particular interest as its fast decomposition releases NO and superoxide, which can combine to give $ONOO^-$; potentially, all three species could interact with BR and our results showed that the pigment was especially susceptible to attack by this specific NORS. It should also be noted that $ONOO^-$ decays to produce the highly toxic hydroxyl and nitrogen dioxide radicals, which have been shown to oxidise the LTP [22,25]. The failure of some of these reactions to go to completion was probably due to the faster rate of self-decomposition of NORS in comparison to the rate of reaction with BR. These preliminary data revealed that BR can effectively scavenge reactive nitrogen species derived from varied classes of NORS.

The lack of reactivity of SNP to BR and BV reflects the preferred targets of the NO^+ group in the nitroprusside anion

$[Fe(CN)_5(NO^+)]^{2-}$. This is well known to be selective towards sulphur groups such as RS^- [26], and is not reactive towards the O-centred and N-centred nucleophiles such as the one found in the LTP molecules.

The fact that BV also reacted with NORS was not unexpected, since BV shares the antioxidant properties of BR and is its direct metabolic precursor [3]. Accordingly, the pattern of BV loss in the presence of NORS was parallel to that of BR. Speculation on the type of reaction is not obvious but the possibility that some BV could be produced upon oxidation of BR by NORS was examined. This was, in fact, the case with $ONOO^-$ and all the other NORS used (not shown). Under these circumstances, it is plausible to suggest that competing consecutive reactions could follow whereby BV itself formed from BR would immediately react with NORS. However, if this event were to take place in vitro, it might have few repercussions in cells and tissues. Because of the presence of biliverdin reductase in these systems, BV could be promptly reduced to BR in a redox cycle specifically aimed at maintaining high concentrations of BR [27]. BV was only a negligible product formed following reaction of BR with $ONOO^-$, in agreement with a previously published report [15]. We observed, however, the formation of colourless products, which implies cleavage of the tetrapyrrole ring. Bonnet and Ioannu have identified colourless fragments arising from oxidative cleavage at the meso-bridges of BR [28]. They included pyrrole fragments, haematinic acid imide and dipyrroles, but the major products were in vitro propentdyopent adducts, which are readily soluble in water and difficult to isolate.

The fundamental finding presented in this study is that BR and BV react with NO under anaerobic conditions. Because of the lack of interference of oxygen and the use of pure NO, these data unequivocally confirm the ability of the bile pigments to scavenge NO and point to the potential function of BR and BV to counteract the cellular stress induced by excessive amounts of NO and its redox forms. We cannot explain at present the reasons why BV was more sensitive to attack by NO than BR.

To examine the relative importance of BR as a possible physiological scavenger of reactive nitrogen species, we determined BR disappearance caused by AS in the presence of thiol compounds. In fact, it has been shown that sulphydryl-containing compounds act as competitive 'traps' for HNO by reacting irreversibly with it [23]. Our results showed that thiols inhibited BR loss in a concentration-dependent manner, although complete prevention of BR consumption was only obtained with NAC. These findings reinforce the idea that HNO is the active species generated by AS that interacts with BR. Therefore, there is competition between BR and thiols for reaction with reactive nitrogen species and both antioxidants may act together to combat nitrosative stress in pathophysiological conditions. The recent findings showing that AS promotes induction of cardiac HO-1 with a consequent increase in haem oxygenase activity might reflect the need of cellular systems to increase endogenous BR production to restore the redox imbalance imposed by HNO [29].

Classically, BR has been regarded as a potentially cytotoxic and lipid-soluble waste product that needs to be eliminated from the body, until its antioxidant properties were reported by Stocker and colleagues [3]. More recently, the influence of BR on nitrergic neurotransmission processes mediated by NO has been reported [30]. In addition, experimental evidence highlighted the correlation between high levels of plasma BR and a reduced risk of developing coronary artery disease [31,32] and a prospective study considering subjects with benign hyperbilirubinaemia (Gilbert's syndrome) indicated a protective effect of BR on the prevention of ischaemic heart disease [33]. This is in line with our studies demonstrating that HO-1-derived BR ameliorates post-ischaemic cardiac functions of isolated rat hearts and protects cardiomyocytes against hypoxia–reoxygenation [34,35]. In addition, new published observations described a lower risk of cancer mortality associated with high concentrations of BR [36]. Because concomitant increased generation of reactive oxygen and nitrogen species appears to play a major role in the pathogenesis of vascular, inflammatory and other diseases, we suggest that the positive and beneficial actions of BR are ascribed not only to its powerful antioxidant activities, but also to the emerging anti-nitrosative capacities of the bile pigment [8].

Further studies on the potential scavenging role of BR against NO and its congeners are indicated; however, the data herein presented may explain why $ONOO^-$ and a variety of NO donors tested so far possess the ability to strongly induce HO-1 in different tissues [8,37]. It is also peculiar that haem and NO synergistically activate HO-1 in endothelial cells resulting in enhanced haem uptake and BR production [38]. The rationale for this ubiquitous response would be to increase the levels of intracellular and circulating BR to neutralise the damaging effects of uncontrolled NO production.

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