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ANTIOXIDANT PROPERTIES OF CONJUGATED BILIRUBIN AND BILIVERDIN: BIOLOGICALLY RELEVANT SCAVENGING OF HYPOCHLOROUS ACID

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Conjugated bilirubin at low micromolar concentrations strongly inhibits the luminol-enhanced chemiluminescence response of stimulated human polymorphonuclear leukocytes. In contrast, it does not inhibit either reduction of ferricytochrome c or lucigenin-mediated chemiluminescence of stimulated cells. Also, conjugated bilirubin and its metabolic precursor, biliverdin, do not inhibit the enzyme myeloperoxidase (MPO) since (i) the MPO-dependent oxidation of guaiacol is not affected by biliverdin and (ii) the spectral changes observed when conjugated bilirubin is oxidized by a MPO-H_2O_2-Cl^--system are very similar to those obtained with reagent HOCl. As judged from these spectroscopic studies, each molecule of conjugated bilirubin can scavenge one molecule of HOCl giving rise to an oxidation product that itself is capable of scavenging further molecules of HOCl. Importantly, at physiological pH, both bile pigments can efficiently protect the elastase-inhibitory capacity of α1-antiprotease against inactivation by reagent HOCl.

KEY WORDS: Bilirubin, biliverdin, superoxide radical, hydrogen peroxide, hypochlorite, α1-antiprotease.

ABBREVIATIONS: BV, biliverdin; CBR, conjugated bilirubin; EDTA, ethylenediaminetetraacetic acid; HBSS, Hanks' buffered salt solution; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; LM-CL, luminol-enhanced chemiluminescence; MPO, myeloperoxidase; PMA, phorbol myristate acetate ; PMNs, polymorphonuclear leukocytes

INTRODUCTION

Bile pigments are open-chain tetrapyrroles present in almost all organisms ranging from primitive life forms to mammals. They are formed continuously through oxidative cleavage of ferriprotoporphyrin IX (heme) whereby biliverdin (BV) is formed as the primary catabolic product.[1] While BV is not further metabolized in most organisms, mammals and some species of fish reduce it to bilirubin.[2] Due to its low solubility in aqueous solutions at physiological pH, a variety of bilirubin derivatives exist that enable the pigment to be transported from its site of production (mainly the spleen and liver) to the site of its excretion, i.e. bile.[2] We have shown previously that BV and the various physiologically relevant forms of bilirubin are very potent scavengers of peroxyl radicals[3–5] and have proposed that one function of bile pigments is to act as natural antioxidants.[3]

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Peroxyl radicals, generated during some biological events (e.g. lipid peroxidation)[6] or by the reaction of hydroperoxides with heme proteins)[7] are secondary oxidants derived from O_2^- and H_2O_2, the primary oxygen reduction products formed by biological systems.[8] Polymorphonuclear leukocytes (PMNs) generate and release O_2^- and H_2O_2 when undergoing the respiratory burst. In addition, these cells contain myeloperoxidase (MPO), an enzyzme that can be release from azurophilic granules upon cell activation and utilize H_2O_2 to oxidise chloride ions to the very potent oxidant hypochlorite, HOCl.[9] HOCl oxidatively degrades or inactivates biological compounds including nucleotides, heme proteins, porphyrins, albumin and α1-antiprotease.[10–13] Inactivation of α1-antiprotease, the main circulating antiprotease, may result in decreased tissue protection against proteolytic attack by elastase, another protease released by activated PMNs.[14] Ascorbate, urate and some hydroxyl radical scavengers efficiently react with HOCl[11] at rates sufficient to protect α1-antiprotease from inactivation by this oxidant.[15–17] Hence these antioxidants have been suggested to be important in tissues protection against uncontrolled proteolysis at sites of PMN activation.[15–17]

We have investigated the reactivities of bile pigments towards O_2^-, H_2O_2 and, in particular, HOCl. The results show that conjugated bilirubin (CBR) does not react readily with either O_2^- or H_2O_2 while both CBR and BV react with HOCl efficiently enough to protect α1-antiprotease from HOCl-mediated inactivation.

MATERIALS AND METHODS

Materials

The following compounds were obtained from Sigma Chemical Company: luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), lucigenin (*bis*-N-methylacridinium nitrate), phorbol myristate acetate (PMA), ferricytochrome c (from horse heart, type 3), superoxide dismutase (from bovine erythrocytes), α1-antiprotease (from human plasma), and elastase substrate (N-succinyl-ala-ala-ala-p-nitroanilide). Guaiacol, H_2O_2, taurine and elastase (from hog pancreas) were obtained from Fluka, Buchs, Switzerland, while bilirubin ditaurine (a model compound of CBR) and BV were purchased from Porphyrin Products, Logan, Utah. The two bile pigments were dissolved immediately before each experiment as described.[18] The commercial stock solution of NaOCl (sextemplex, Grogg Chemie, Berne, Switzerland) was about 3% and all other reagent of analytical grade. MPO ($A_{430}/A_{280} > 0.8$) from horse neutrophils was a gift from Dr. A. Jörg, University of Freiburg, Switzerland.

Methods

Human PMNs were prepared from freshly obtained blood[19] collected into heparin-containing vacutainers. PMNs were suspended at 10^5 cells/ml in Hank's balanced salt solution, pH 7.4 (HBSS) containing 5.6 mM glucose and buffered with 20 mM HEPES instead of bicarbonate. Luminol-enhanced cellular chemiluminescence (LM–CL) was measured in a modified Kontron Betamatic I Liquid scintillation spectrometer as described previously.[20] For some experiments lucigenin (final concentration 50 μM) was used as the chemilumigenic probe. CBR, or the corresponding amount of HBSS (control), was added to the dark-adapted reaction mixture imm-

ediately before phorbol myristate acetate (PMA, 10^{-7} M) was added to intitiate the respiratory burst of PMNs.

Production of superoxide anions by activated PMNs was measured as the reduction of ferricytochrome c (50 μM). Cells (2×10^6/ml HBSS) were preincubated in the dark at 37°C for 5 min in the absence (control) or presence of various amounts of CBR before PMA (1.6×10^{-6} M) was added to activate the PMNs. The amount of O_2^- produced was quantitated at 37°C in a Beckman DU 70 spectrophotometer using the dual wavelength mode (550–540 nm). Incubation of PMNs with CBR did not change the low rate of spontaneous cellular reduction of ferricytochrome c.

MPO activity was determined from the maximal rate of guaiacol oxidation at 37°C, as described by Klebanoff *et al.*[21]

HOCl for reagent-mediated oxidation of CBR or inactivation of α1-antiprotease (see below) was prepared freshly after adjusting the pH with diluted H_2SO_4 to 4.5 and 6.2, respectively. The concentration of HOCl was determined using an extinction coefficient of 100 $M^{-1}cm^{-1}$ at 235 nm.[22] HOCl-mediated oxidation of CBR (50 μM) was initiated at 37°C by either addition of aliquots (10 μM) of reagent HOCl or through enzymatic generation of HOCl.[23] EDTA (1 mM) was included in the reaction mixtures to partially prevent metal ion-catalyzed decomposition of CBR. Control experiments showed that EDTA at this concentration had no influence on the MPO-mediated oxidation of either guaiacol or CBR.

HOCl-mediated oxidative inactivation of α1-antiprotease and the resulting decrease in inhibition of elastase was assayed principally as described by Halliwell *et al.*[15] The minimal amount of α1-antiprotease for complete inhibition of elastase was determined prior to each set of experiments. Typically, HOCl (100 μM) was added to a reaction mixture (final volume 50 μl) containing the above determined amount of α1-antiprotease and the appropriate amount of phosphate buffered saline (pH 7.4) without (control) or with (sample) a defined amount of either CBR or BV. After 1 hr incubation at 25°C, 3 ml of the same buffer were added, followed by 50 μl of elastase (1 mg/ml). Elastase activity was measured at 25°C as an increase in OD at 410 nm after the addition of 100 μl of elastase substrate (12.5 mM).

RESULTS AND DISCUSSION

Physiologically relevant forms of bilirubin and BV are very powerful scavengers of peroxyl radicals.[3–5] However, little is known about their reactivities towards other reactive oxygen species produced by biological systems such as PMNs. These cells, upon exposure to PMA or other stimuli, produce large amounts of various reactive oxygen species some of which can be detected as LM-CL. Figure 1 shows that PMA-induced LM-CL of human PMNs was inhibited almost completely in the presence of 5 μM CBR. Inhibition was due to the bilirubin moiety of bilirubin-ditaurine, the model compound of CBR used, since addition of 10 μM taurine did not affect the amount of light emitted. CBR inhibited light emission in a concentration-dependent manner (data not shown). As LM-CL of human PMNs is dependent on MPO and HOCl,[24–25] the observed inhibitory activity of CBR may be explained by either direct scavenging of HOCl, or an inhibition of at least one of the cellular events preceeding formation of HOCl. These include: (i) processes leading to the activation of NADPH oxidase, (ii) production of O_2^- by activated NADPH oxidase, (iii) dismutation of O_2^- to give rise to H_2O_2, and (iv) MPO-mediated formation of HOCl

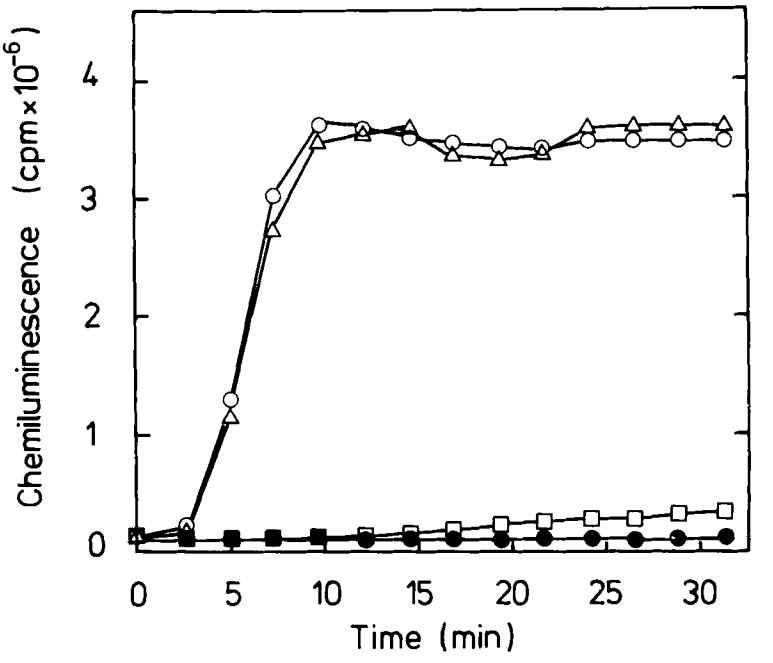

FIGURE 1 Inhibition of luminol-enhanced chemiluminescence of PMA-stimulated PMNs by CBR. At time zero PMA was added to the cells and LM-CL measured in the absence (○) or presence of either 5 μM CBR (□) or 10 μM taurine (△). (●) Mock stimulation with an appropriate amount of buffer.

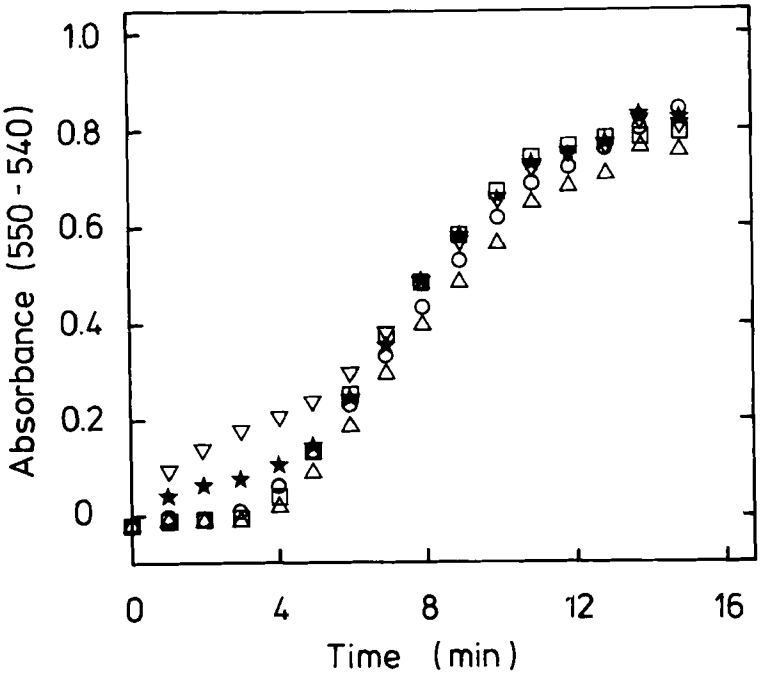

FIGURE 2 Effect of CBR on the reduction of cytochrome c by PMA-activated human PMNs. At time zero PMA was added to the cells in the absence (○) or presence of CBR at 5 μM (□), 20 μM (△), 50 μM (★), or 100 μM (▽).

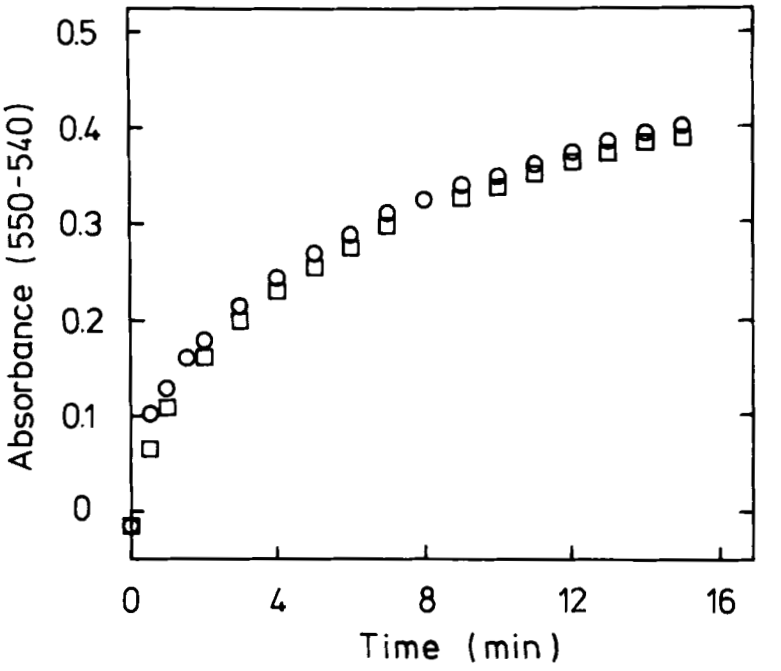

FIGURE 3 Reduction of cytochrome c by CBR. At time zero CBR (100 μM) was added to a solution of ferricytochrome c (50 μM in HBSS) in the absence (○) or presence (□) of superoxide dismutase (100 U/ml).

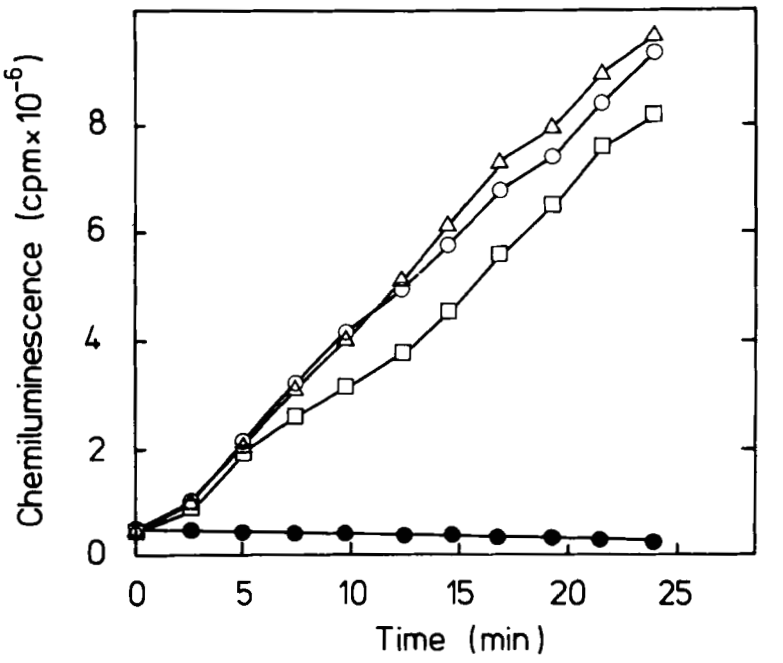

FIGURE 4 Effect of CBR on the lucigenin-enhanced chemiluminescence of PMA-activated PMNs. At time zero PMA was added to the cells in the absence (○) or presence of either 5 μM CBR (□) or 10 μM taurine (△). (●) Mock stimulation with an appropriate amount of buffer.

from H_2O_2 and chloride. As shown below, scavenging of HOCl is the only important process by which CBR inhibits LM-CL of stimulated PMNs.

PMA-induced activation of the respiratory burst in PMNs is dependent on protein kinase C,[26] and free bilirubin inhibits protein kinase C in a cell-free system[27] as well as PMA-induced production of O_2^- by PMNs isolated from human cord blood.[28] In contrast to these observation with free bilirubin, low micromolar concentrations of CBR did not significantly inhibit reduction of cytochrome c by PMA-stimulated PMNs (Figure 2). When CBR was present at higher concentrations we even observed a slight increase in the initial rates of cellular reduction of cytochrome c (Figure 2), most likely the result of direct, superoxide dismutase-insenstive reduction of cytochrome c by CBR (Figure 3).

We further examined the effect of low concentrations of CBR on lucigenin-enhanced cellular chemiluminescence as this type of detection system, like reduction of cytochrome c, is a direct indicator of O_2^- generation.[29] As expected, PMA-induced lucigenin-dependent chemiluminescence of PMNS was not significantly inhibited in the presence of 5 μM CBR (Figure 4). Taken together, these results clearly show that the inhibition of LM-CL in the presence of low concentrations of CBR is neither the result of an inhibition of the activation processes of NADPH oxidase nor due to direct scavenging of O_2^- or quenching of photoemissive excited species derived from luminol. In agreement with previous studies,[30] the results further indicate that CBR is unlikely to be an important biological target for O_2^- since the rate of its reaction with O_2^- must be significantly lower than $2.6 \times 10^5 M^{-1}s^{-1}$, i.e. the rate constant for the reaction of superoxide anion with ferricytochrome c.[31] We are not aware of any published rate constants for the reaction of O_2^- with lucigenin.

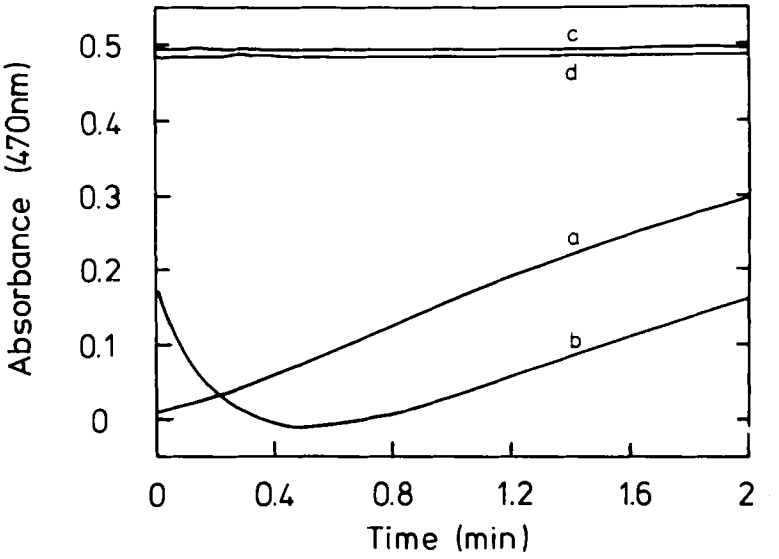

FIGURE 5 MPO-H_2O_2-mediated oxidation of guaiacol in a cell-free system. Guaiacol (6.7 mM) and MPO (73.5 mU/ml) were incubated in chloride-free phospate buffer (50 mM, pH 7.0) at 37°C for 5 min in the absence (trace a) or presence (trace b) of 10 μM CBR before guaiacol oxidation was initiated by the addition of H_2O_2 (133 μM). No change in absorbance at 470 nm was observed when H_2O_2 (133 μM) was added to a solution containing MPO (73.5 mU/ml) and CBR (10 μM) but no guaiacol (trace c); or CBR (10 μM) and guaiacol (6.7 mM) but no MPO (trace D).

Figure 5 shows the effects of CBR on the MPO-H_2O_2-mediated oxidation of guaiacol in a cell free system. In the absence of CBR guaiacol oxidation progressed smoothly as judged by a steady increase in OD at 470 nm.[21] In the presence of low micromolar concentrations of CBR, initially a rapid decrease in A_{470nm} was observed (Figure 5), the duration of which was directly proportional to the amounts of CBR (not shown). After this initial period guaiacol oxidation proceeded at rates very similar to those observed in the absence of bile pigment. The observed rapid decrease in A_{470nm} most likely reflected oxidation of CBR, as this pigment was the only component absorbing at 470 nm and present at the start of the reaction. All four components (i.e. MPO, H_2O_2, guaiacol and CBR) needed to be present in order to observe the initial decrease in A_{470nm} (Figure 5). In the absence of either guaiacol or MPO A_{470nm} remained constant, indicating that CBR does not react readily with H_2O_2 or compound I (the latter being formed in the reaction between MPO and H_2O_2), nor can act as a substrate for MPO. Free bilirubin, in contrast, is a substrate for horseradish peroxidase[32] and prostaglandin H synthase.[33] The observations make it likely that it is the tetraguaiacol product formed from guaiacol that oxidizes CBR. Indeed, addition of tetraguaiacol to a solution containing CBR resulted in a very rapid decrease in A_{470nm}, with kinetics very similar to those observed in Figure 5 (not shown). Taken together, the results strongly suggest that CBR does not inhibit MPO. In line with this, addition of up to 10 μMBV had no significant effect on the MPO-H_2O_2-mediated oxidation of guaiacol (not shown).

Further support of the idea that bile pigments do not inhibit MPO-mediated formation of HOCl comes from a comparison of the spectral changes observed during the oxidation of CBR by either reagent HOCl or a MPO–H_2O_2 – Cl^--system (Figure 6). Under both conditions the color of the reaction mixture changed from yellow to green, as indicated by the decrease in absorbance at 450 nm and an increase in absorbance at 375 and 665 nm, spectral features typical of BV-like compounds. Upon addition of an equimolar amount of reagent HOCl to CBR (Figure 6A) or during the initial phase of MPO–H_2O_2–Cl^--mediated oxidation of CBR (Figure 6B), two isosbestic points developed at 520 and 386 nm, respectively. Once the amount of HOCl added or produced by the enzymatic system exceeded the amount of CBR additional spectral changes were observed. This indicates that each molecule of CBR can scavenge one molecule of HOCl whereby a single oxidation product is formed that itself is capable of scavenging HOCl.

The above findings demonstrate that CBR does not inhibit the cellular activation of NADPH oxidase, nor does it efficiently scavenge O_2^- and H_2O_2, or inhibit MPO. Therefore, the inhibition of LM-CL of activated PMNs by CBR is most likely due to direct scavenging of HOCl. Indeed, both CBR and BV strongly prevented LM-CL in a cell free system containing MPO and H_2O_2 with 50% inhibition observed at 0.3 and 1.3 μM, respectively (not shown). Furthermore, the results in Table I indicate that the reaction between HOCl and bile pigments is fast enough to protect the biologically important target α-1-antiprotease from oxidative inactivation. Both bile pigments protected α1-antiprotease from HOCl-mediated inactivation in a concentration dependent manner. α1-Antiprotease was used at a concentration sufficient to inhibit elastase by 100%. The protective effects of BV and CBR were greatly increased when they were preincubated with HOCl for 5 min before α1-antiprotease was added (Table 1). Under these conditions, 25 μM of either CBR or BV offered almost complete protection against the effects of 100 μM HOCl, further supporting the notion that the

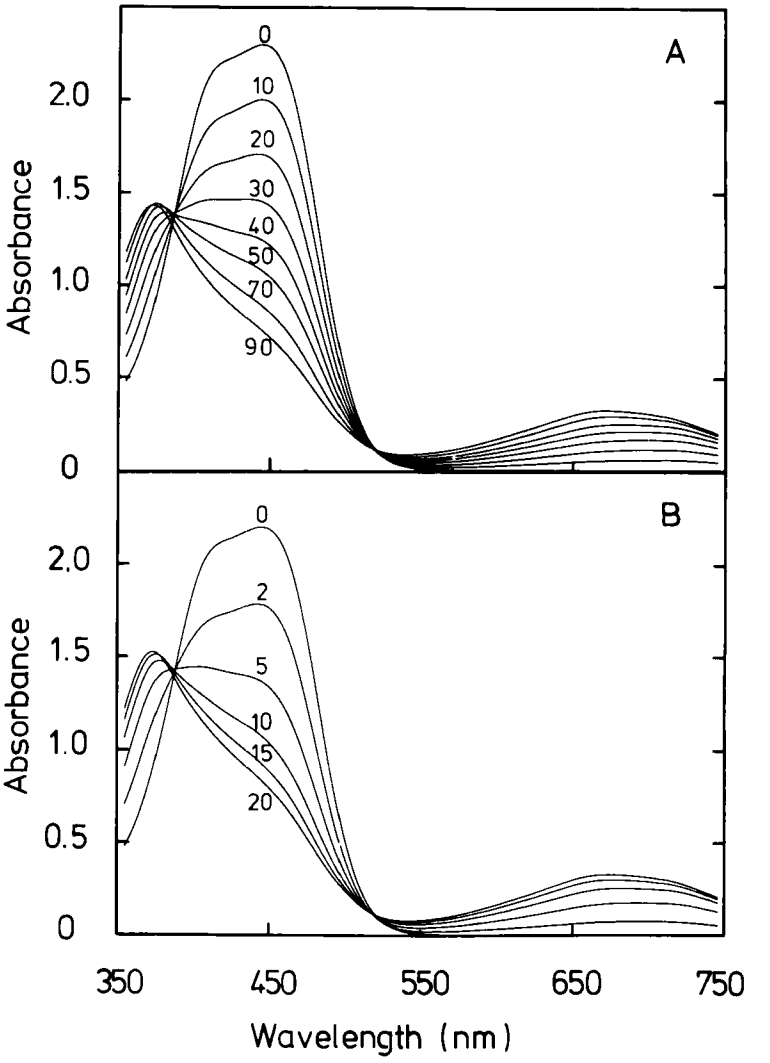

FIGURE 6 Spectral changes during the HOCl-mediated oxidation of CBR. Oxidation of CBR (50μM) was initiated in chloride (50 mM)-containing phosphate buffer (100 mM, pH 4.5) at 37°C by the addition of 10 μM aliquots of reagent HOCl (A), or by the simulataneous addition of MPO (73.5 mU/ml) and H_2O_2 (200 μM) (B). The numbers indicate the total amount of reagent HOCl added (A), or the time in minutes after initiation of enzymatic oxidation of CBR (B).

primary oxidation products formed when HOCl reacts with CBR or BV can also scavenge HOCl. Taurine did not offer any protection for α1-antiprotease (Table 1). This is in agreement with results published recently by others.[34] Besides, the amino group of taurine in bilirubin ditaurine, the model compound of CBR used in this study, is esterified with the propionyl side chains of bilirubin and hence not available to react with HOCl.[35]

TABLE I
Effect of bile pigments on the inactivation of α1-antiprotease by HOCl

Reagent added	Concentration* (μM)	Elastase activity† (% of maximal rate)	
		A	B
None	---	100	100
Biliverdin	25	74	3
	50	47	0
	75	41	0
	100	13	0
Bilirubin-ditaurate (CBR)	25	89	11
	50	58	11
	75	40	12
	100	27	6
Taurine	100	100	100
	200	100	100

*Concentrations stated refer to the final concentrations in the 50 μl preincubation mixture.
†Results are expressed as percentages of maximal elastase activity measured as the increase in absorbance at 410 nm (see Materials and Methods) and are typical for each set of experiments (n = 3). 100% is $\Delta A_{410} = 0.325$ units·min^{-1}. Column A; α1-antiprotease was added immediately after either BV, CBR, or taurine was added to HOCl (100 μM) in the preincubation mixture. Column B; BV, CBR, or taurine was preincubated with HOCl for 5 min at 25°C before α1-antiprotease was added. The presence of bile pigments had no effect on elastase activity or the ability of α1-antiprotease to inhibit elastase.

In summary, our results show that CBR does not react rapidly with O_2^- or H_2O_2 while both, CBR and BV do scavenge HOCl with high efficiency. The physiological relevance of these findings remain to be investigated. CBR occurs at high concentrations within the liver and bile whereas only submicrolar levels are present in the serum of healthy individuals.[36] In contrast to CBR, albumin-bound bilirubin, the major form of the pigment present within the circulation, does not react efficiently with HOCl.[37] In pathological sera, concentrations of bilirubin monoglucuronide and diglucuronide are increased by up to three orders of magnitude[38,39] and it can not be excluded that under some of these conditions (e.g. sepsis or trauma) scavenging of HOCl by CBR may take place *in vivo*.

Acknowledgements

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