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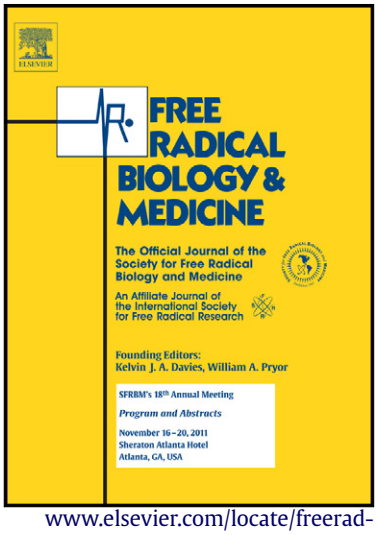

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A-C. Boon, C.L. Hawkins, J.S. Coombes, K-H. Wagner, A.C. Bulmer

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Bilirubin scavenges chloramines and inhibits myeloperoxidase induced protein/lipid oxidation in physiologically relevant hyperbilirubinaemic serum

Boon A-C.[a]*, Hawkins C.L.[b,c], Coombes, J.S.[d], Wagner K-H.[e], Bulmer A.C.[a]*

[a]Heart Foundation Research Centre, Griffith Health Institute, Griffith University, Gold Coast, Australia, QLD 4222.

[b]Heart Research Institute, Newtown, Sydney, Australia, NSW 2042.

[c]Sydney Medical School, University of Sydney, Australia, NSW 2006.

[d]School of Human Movement Studies, University of Queensland, St Lucia, Australia, QLD 4072.

[e]Department of Nutritional Science, University of Vienna, Austria, Vienna 1090.

*Corresponding Authors

Andrew C. Bulmer

Ph: +61 7 55528215

Email: a.bulmer@griffith.edu.au

Ai-Ching Boon

Ph:+61 7 55529184

Email: c.boon@griffith.edu.au

Abstract

Hypochlorous acid (HOCl), an oxidant produced by myeloperoxidase (MPO), induces protein and lipid oxidation, which is implicated in the pathogenesis of atherosclerosis. Individuals with mildly elevated bilirubin concentrations (i.e. Gilbert's syndrome; GS) are protected from atherosclerosis, cardiovascular disease (CVD) and related-mortality. We aimed to investigate

whether exogenous/endogenous unconjugated bilirubin (UCB), at physiological concentrations, can protect proteins/lipids from oxidation induced by reagent and enzymatically generated HOCl. Serum/plasma samples supplemented with exogenous UCB (≤250 μM) were assessed for their susceptibility to HOCl and MPO/H_2O_2/Cl^- oxidation, by measuring chloramine, protein carbonyl and malondialdehyde (MDA) formation. Serum/plasma samples from hyperbilirubinemic Gunn rats and humans with GS were also exposed to MPO/H_2O_2/Cl^- to; 1) validate *in vitro* data and 2) determine the relevance of endogenously elevated UCB in preventing protein and lipid oxidation. Exogenous UCB dose-dependently (P<0.05) inhibited HOCl and MPO/H_2O_2/Cl^- induced chloramine formation. Albumin-bound UCB efficiently and specifically (3.9-125 μM; P<0.05) scavenged taurine-, glycine- and *N*-α-acetyl-lysine- chloramines. These results were translated into Gunn rat and GS serum/plasma which showed significantly (P<0.01) reduced chloramine formation after MPO-induced oxidation. Protein carbonyl and MDA formation were also reduced after MPO oxidation in plasma supplemented with UCB (P<0.05; 25 μM and 50 μM, respectively). Significant inhibition of protein and lipid oxidation was demonstrated within the physiological range of UCB, providing a hypothetical link to protection from atherosclerosis in hyperbilirubinaemic individuals. These data demonstrate a novel and physiologically relevant mechanism whereby UCB could inhibit protein and lipid modification, by quenching chloramines, induced by MPO-induced HOCl.

KEYWORDS: Bile pigments, heme oxygenase, antioxidants, protein oxidation, myeloperoxidase, chloramines

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1. Introduction

Unconjugated bilirubin (UCB), a potentially toxic by-product of haem catabolism, also possesses powerful antioxidant properties. Epidemiological studies report that individuals with elevated UCB are protected from the development of intimal hyperplasia, atherosclerosis and cardiovascular disease (CVD) [1-7]. Furthermore, higher UCB concentrations (in the absence of haemolytic or liver disease) are associated with reduced incidence of CVD-related mortality [8-12]. The mechanisms responsible for CVD protection are not clear, however may be related to the potent antioxidant properties of UCB, given the potential role of oxidation in the development and progression of atherosclerosis [13]. Numerous *in vitro* reports demonstrate the radical scavenging activity of various bilirubin species, which protect proteins and lipids from oxidation. Current evidence indicates that UCB efficiently inhibits amino acid oxidation mediated by hydroxyl, hydroperoxyl and superoxide radicals generated by radiolysis [14] and irradiation [15]. Furthermore, UCB is a potent lipid chain-breaking antioxidant scavenging artificially generated peroxyl radicals (2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) [16] and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) [17] and inhibits copper (Cu^{2+}) induced lipid oxidation [18].

Whether, bilirubin and related compounds protect from oxidation mediated by biologically relevant myeloperoxidase (MPO) and hypochlorous acid (HOCl) [19], remains underexplored. This is an important phenomenon to investigate, because MPO is strongly linked to the development of atherosclerosis [20-23]. Evidence to support a role of MPO in the pathogenesis of atherosclerosis includes the deposition of MPO in atherosclerotic lesions [24,25], the extent of which is inversely related to plaque progression [26]. Myeloperoxidase generates HOCl, a potent oxidant, which reacts with most biological molecules and targets proteins for oxidation [27]. Reaction of HOCl with amine groups on proteins gives rise to

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chloramines, which decompose to form protein carbonyls [28,29] and can also induce the oxidation of low-density lipoprotein (LDL), which may be important in the development of atherosclerosis [30].

Some controversy exists regarding the role of bilirubin and related compounds in scavenging MPO-derived oxidants. Bilirubin ditaurate (BRT; a synthetic water soluble form of UCB) and biliverdin (product of oxidised UCB) inhibit MPO-mediated chemiluminescence [31] but fail to protect guaiacol from MPO oxidation [19]. However, both compounds prevent human polymorphonuclear leukocyte α1-antiprotease inactivation and tissue proteolytic attack by reagent HOCl [19]. Interestingly, preliminary *in vitro* data indicate that albumin-bound UCB does not react readily with HOCl [31]. This result is surprising when considering that persons with Gilbert's syndrome (GS) [32] who possess mildly elevated UCB concentrations (> 17.1 μM), demonstrate reduced protein carbonyl concentrations [33]. Given that the decomposition of intermediate chloramines is a pathway responsible for the formation of protein carbonyls, these observations support a potential role for UCB in the inhibition of MPO activity, and / or the scavenging of HOCl, or chloramines. Whether higher, physiologically relevant, UCB concentrations scavenge HOCl or prevent protein oxidation (e.g. chloramine/protein carbonyl formation) in plasma samples remains unpublished.

Therefore, the aim of this study was to investigate whether serum/plasma samples with exogenous UCB supplementation or endogenously elevated UCB in serum/plasma from unique models of benign hyperbilirubinemia (Gunn rat and human GS) protect from protein and lipid oxidation induced by reagent and enzymatically-generated HOCl. We hypothesised that bilirubin would protect from protein oxidation, by quenching HOCl-induced chloramine

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formation. These data may help to rationalise the association between bilirubin and protection from CVD.

2. Methods

2.1 Human subjects

Forty-two subjects (21 GS; 21 controls) were recruited and matched for gender, age, and body mass index (BMI). Equal numbers of female and male subjects existed in each group (11:11). A female control subject with normal UCB concentrations (10.3 μM) was used as a quality control for *in vitro* experimentation (ie. exogenous UCB supplementation for HOCl and MPO oxidation). Fasting human blood was collected in serum and ethylenediaminetetraacetic acid (EDTA) vacutainers. The recruitment of human subjects received ethical approval (MSC/02/10/HREC) from the Griffith University Human Research Ethics Committee prior to experimentation commencing.

2.2 Animals

Breeding pairs of heterozygote (genotyped) Gunn rats were imported from the Rat Research and Resource Center (Columbia, MO, USA) and kept within an animal housing facility at Griffith University (12-h light:dark cycle, constant temperature (22 °C) and humidity (60%)). Rats had continuous access to standard laboratory food pellets (Speciality Feeds, Glen Forrest, Australia) and fresh water. Female homozygous Gunn rat offspring were assumed to possess jaundice at birth, were ear-tagged, and housed together with female littermate (non-jaundiced) controls after weaning. Gunn rats (n=9) and littermate controls (n=5) at 12 months of age were anesthetized using intraperitoneal injection of pentobarbital sodium (concentration 60 mg/mL; 100 μL/100 g). A midline laparotomy was performed, and approximately 5 mL of whole blood was collected from the thoracic cavity using a syringe and was transferred into

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serum vacutainers and prepared for analysis (see section 2.3). All procedures were approved by the Griffith University Animal Ethics Research Committee prior to experimentation commencing (MSC/06/12).

2.3 Sample preparation

Whole blood was centrifuged (Thermo Scientific 5810R, Australia) at 2500 x *g* for 10 min (4 °C). Serum/plasma aliquots were prepared immediately and stored at −80 °C until analysis. Human serum samples were used for biochemistry, antioxidant activities and HOCl oxidation, whereas EDTA plasma samples were used for MPO oxidation, measurement of chloramine, protein carbonyl and malondialdehyde (MDA) concentrations. Serum samples obtained from animals were used to perform all analyses. All reagents used were of analytical reagent grade or better and obtained from Sigma-Aldrich (Australia) unless otherwise indicated.

2.4 Serum biochemistry

Serum samples from animals were diluted 1:1 with distilled water and analysed for the activity of liver enzymes (alanine aminotransferase, aspartate aminotransferase, γ-glutamyltransferase), glucose, uric acid and lipid parameters including total cholesterol, triglycerides and high-density lipoprotein using commercially available kits on a COBAS Integra 400 blood chemistry analyser (Roche Diagnostics, Australia). Cholesterol analyses were conducted using appropriate lipid standards (Calibrator for Automated Systems Lipids) and quality controls (Precinorm Control Clin Chem Multi 1 and 2; Roche Diagnostics, Australia). Total non-thiol antioxidant capacity including ferric reducing ability of plasma (FRAP) [34] was also assessed on an open channel of the COBAS Integra analyser. All analyses were conducted in duplicate.

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2.5 Determination of bilirubin concentrations

Serum UCB concentrations were quantified using high-performance liquid chromatography (HPLC) and a photodiode array detector (Waters, Australia) as previously described [35]. A slight variation to this method included the use of C18 reserve-phase HPLC guard and analytical column (4.6 mm x 150 mm, 3 µm; Phenomenex, Australia) that was perfused at 1 ml/min. Extracted samples were injected (100 µL) with a run time 15 min in duplicate. Unconjugated bilirubin (Frontier Scientific, USA; 0-100 µM) served as an external standard and was detected at 450 nm.

2.6 Induction of hypochlorous acid (HOCl) oxidation

Human quality control samples (serum) were thawed to room temperature and centrifuged at 2500 x g for 5 min and diluted (1:10 for reagent HOCl oxidation) with deionised water. Sodium phosphate buffer (0.1 M, pH 7.4) was prepared and treated with chelex resin prior to use, to minimise contamination with transition metal ions [36]. Buffer was stored at room temperature and used as a control in oxidation assays. Unconjugated bilirubin stock solutions were prepared fresh in 0.05 M sodium hydroxide (NaOH). Bilirubin solutions were protected from light using foil covered containers and used immediately. A final UCB concentration (1.56-25 µM; 5 µL) or NaOH as control (0.05 M; 5 µL) were added to diluted serum samples in new reaction tubes. Hydrochloric acid (HCl; 0.05 M; 5 µL; Ajax Finechem, Australia) was then added to the serum mixture to neutralise NaOH. Hypochlorous acid stock (1 mM) was prepared fresh in sodium phosphate buffer and 20 µL was added to the serum mixture (final HOCl concentration of 100 µM) and incubated in the dark at room temperature for 5 min. The serum mixture was transferred to 96-well plate which was then analysed for chloramine formation (see section 2.11).

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2.7 Reaction of unconjugated bilirubin with biologically relevant chloramine species

For *in vitro* experiments, which aimed to determine whether exogenous UCB could directly quench physiologically-relevant chloramines, UCB was associated with bovine serum albumin (BSA) to facilitate its aqueous solubility and to ensure that bilirubin was present in a biologically relevant form. Taurine- glycine- and *N*-α-acetyl-lysine chloramines (250 μM) were formed by the addition of HOCl to each amine in a 1:9 HOCl:amino acid molar ratio for 5 mins prior to the addition of BSA (250 μM) in the presence and absence of varying amounts of UCB (0 – 125 μM). UCB was added to BSA as a concentrated stock solution (10 mM) prepared in NaOH (0.05 M). Samples were incubated at 21 ℃ for 5 min prior to chloramine quantification (described below).

2.8 Induction of myeloperoxidase/hydrogen peroxide (MPO/H_2O_2) oxidation

Protein oxidation induced by MPO (PLANTA Natural Products, Austria) was conducted according to the method of Summers *et al.* [37]. Ten microliters of 2 μM MPO (final concentration of 100 nM) and phosphate buffer saline (PBS; 40 μL; Invitrogen, Australia) were added to human EDTA plasma (50 μL) in the presence of exogenous UCB (final concentration 0.78-25 μM; 25 μL) or NaOH as a control (0.05 M; 25 μL) containing HCl (0.05 M; 25 μL) to neutralise NaOH. Hydrogen peroxide (50 μL; final concentration 50 μM for chloramine analysis and 100 μM for protein carbonyl and MDA detection) was added to the mixture and incubated at 37ºC for 30 min for chloramine and four hours for protein carbonyl/MDA analysis respectively. Fifty microliters of human EDTA plasma and animal serum were added to similar MPO oxidation system with PBS (90 μL) for *ex vivo* experimentation (i.e. chloramine analysis). Catalase (100 μg/mL; 25-200 units) was added to quench any remaining MPO activity upon completion of the reaction. Sample oxidation was subsequently analysed by measuring chloramine, protein carbonyl and MDA concentrations.

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2.9 Measurement of protein concentrations

Human and animal serum protein concentrations were determined according to the bicinchoninic acid protein assay kit (Thermo Scientific, Australia). The samples were read at 540 nm using a 96-well plate reader (Thermo Scientific, Multiskan FC, Australia). The assay was performed in triplicate and results expressed in mg/mL of protein. The co-efficient of variation for this method was 3.4%.

2.10 Measurement of reduced thiol concentrations

Reduced thiol concentrations were quantified with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) reagent. Reduced thiols react with DTNB to generate 5-thio-2- nitrobenzoic acid (TNB), which was quantified in triplicate at 415 nm using a 96-well plate reader (Multiskan FC, Thermo Scientific, Australia) according to the method of Hawkins *et al.* [36]. Reduced glutathione (0–0.5 mM) served as an external standard. Thiol concentrations were expressed initially in μM and then converted to nmol/mg of protein. The co-efficient of variation for this method was 2.6%.

2.11 Measurement of chloramine concentrations

Chloramine is a stable product of protein oxidation and was quantified according to previously published protocols [38]. After HOCl and MPO oxidation, 150 μL of the developing reagent (2 mM 3,3′,5,5′-Tetramethylbenzidine in 400 mM acetate buffer, pH 5.4, containing 10% dimethylformamide [Merck, Australia] and 100 μM sodium iodide) was added to 96-well plate wells containing 50 μL of sample mixtures. Serum/plasma and BSA samples were incubated on a plate shaker at 50 rpm for 25 and 5 min, respectively. The plate was read at 620 nm using a 96-well plate reader. Samples were assayed 3-5 times within *ex*

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vivo and *in vitro* MPO and HOCl oxidation experiments. Results were expressed in nmols/mg of protein. The co-efficient of variation for this method was 5.6%.

2.12 Measurement of protein carbonyl concentrations

Detection of protein carbonyl with 2,4-dinitrophenylhydrazine was performed using enzyme-linked immunosorbent assay (ELISA; Sapphire Biosciences, Australia) with the above-mentioned 96-well plate reader. Each sample was assayed in triplicate, with experiments repeated five times and results expressed in nmols/mg of protein. The co-efficient of variation for this assay was 6.8%.

2.13 Measurement of plasma malondialdehyde concentrations

Malondialdehyde in human EDTA plasma was quantified in triplicate using HPLC via a modified method of Londero *et al.* [39], performed with a Hitachi Model F-1050 fluorescence spectrophotometer detector set at 532 nm excitation and 563 nm emission and a HPLC column (125 mm x 4mm, 5 μm; Merck, Austria). The extracted sample was injected (20 μL) with a retention time of 4 min with a flow rate of 1.3 mL/min. 1,1,3,3-Tetraethoxypropane (Sigma-Aldrich, Austria) ranging from 0-8.12 μM served as an external standard and the co-efficient of variation for this method was 8.8%.

2.14 Statistical analysis

SigmaPlot software (version 11.0) was used to analyse all data. Two-tailed, unpaired *t* tests (Student's *t* test or Mann–Whitney rank sum test) tested for significant differences in variables between GS individuals or Gunn rats and controls. One way analysis of variance (ANOVA) followed by Dunn's post-hoc analysis tested the dose effect of UCB on oxidized product formation. Group data are presented as a mean±standard deviation. Pearson

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correlation tested the relationship between UCB and oxidation products. The level of significance was set at $P<0.05$.

3. Results

3.1 Inhibition of chloramine formation by HOCl and MPO mediated oxidation

Hypochlorous acid reacts with amine groups on proteins to produce chloramines [28]. When HOCl was added to plasma, exogenous UCB inhibited chloramine formation in a dose-dependent manner. The lowest dose of UCB (versus vehicle treatment) that significantly inhibited chloramine formation was 15.6 μM ($P<0.05$; Figure 1A). Similar results were observed on addition of MPO/Cl^-/H_2O_2 system to plasma supplemented with exogenous UCB. Significant inhibition of chloramine formation was observed with an additional 12.5 μM UCB added to plasma (corrected for plasma dilution factor 1:4), representing the lowest effective concentration ($P<0.05$; Figure 1B). To determine whether UCB directly scavenged various model chloramines, albumin-bound UCB was added to biologically relevant chloramine species formed on taurine, glycine and *N*-α-acetyl-lysine. Addition of BSA (250 μM) in the absence of UCB to an equimolar concentration of each chloramine resulted in some quenching (170 – 210 μM) of the chloramines (Figure S1), which approximated the S-H content of BSA (data not shown). The remaining chloramines (~30-80 μM) were rapidly quenched by albumin bound UCB (0-125 μM) in a dose-dependent manner (Figure 2). The lowest dose to significantly scavenge chloramine was 3.9-7.8 μM ($P<0.05$), depending on the chloramine species tested. These data indicate that mildly increased *exogenous* UCB concentration (within the physiological range) can protect protein from MPO/HOCl mediated chloramine formation by directly quenching biologically relevant chloramine species.

To confirm whether *endogenously* elevated UCB would therefore protect from chloramine formation, serum/plasma samples from Gunn rats and humans with GS were also

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challenged, followed by chloramine assessment. Unconjugated bilirubin concentrations were significantly elevated in Gunn rats versus controls (82.3±11.6 vs. 2.55±0.16 μM; $P<0.01$; Figure 3A). Gunn rats have significantly improved antioxidant status (Ferric reducing ability of plasma, FRAP; $P<0.01$; Figure 4A) which was strongly and positively correlated with UCB concentrations (r=0.950; $P<0.01$; Figure S2A). Chloramine formation was inhibited by ~40% in hyperbilirubinemic Gunn rat serum, compared to control samples (0.20±0.02 vs. 0.34±0.07 nmols/mg protein; $P<0.01$; Figure 5A) when exposed to MPO/HOCl oxidation and was negatively correlated with UCB concentration (r=-0.840, $P<0.001$; Figure S3A). A sub-set of plasma samples from GS subjects with mildly elevated endogenous UCB concentrations were obtained from a previous study [33]. Similarly, UCB concentrations were significantly elevated in GS compared to controls (34.8±18.5 vs. 10.5±2.87 μM; $P<0.01$; Figure 3B), who tended to experience elevated FRAP concentrations ($P=0.095$; Figure 4C). Serum UCB concentration was significantly correlated with FRAP concentrations in the human cohort (r=0.521; $P<0.01$; Figure S2C). Reduced thiol concentrations were also significantly elevated in GS ($P=0.002$; Figure 4D) and were also positively correlated to UCB concentrations (r=0.403; $P<0.01$; (Figure S2D). Gilbert's syndrome plasma was also protected from MPO-mediated chloramine formation compared to controls (0.09±0.06 vs. 0.14±0.07 nmols/mg protein; $P<0.01$; Figure 5B) with chloramine formation being negatively correlated with UCB concentrations (r=-0.486, $P<0.01$; Figure S3B).

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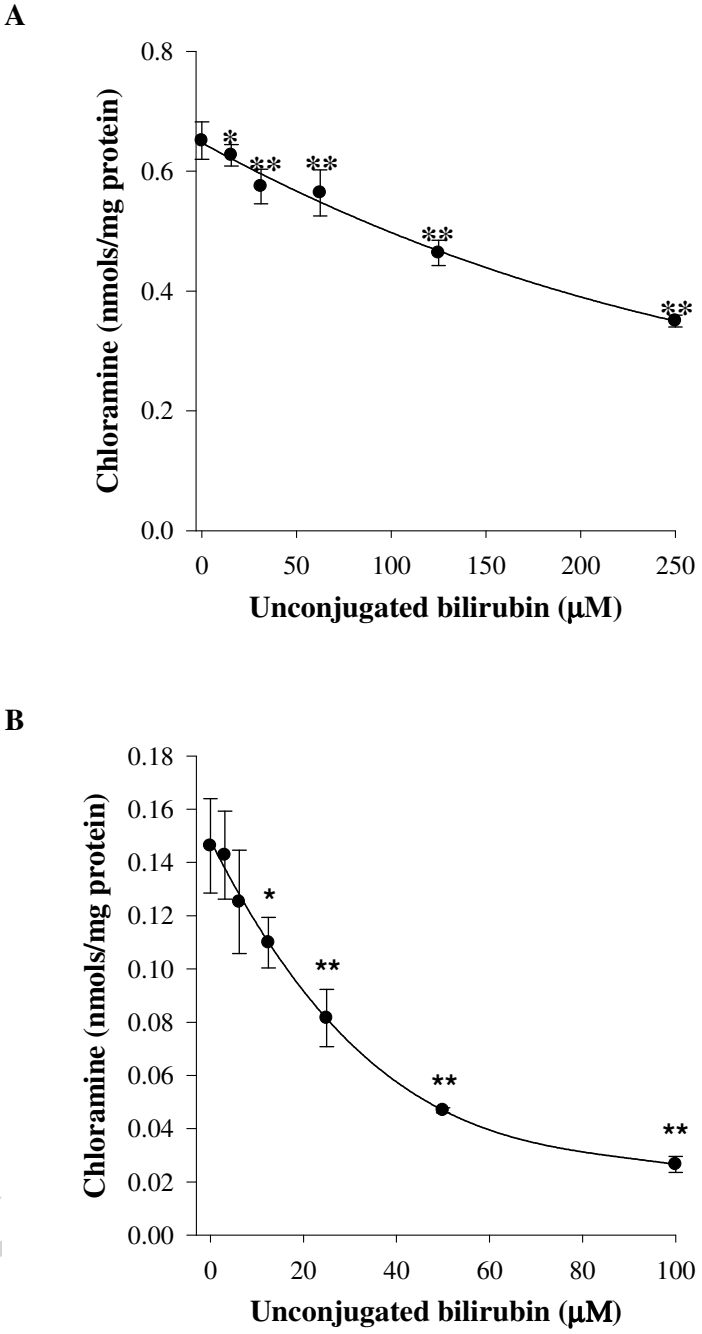

Figure 1. A) Exogenous UCB inhibits reagent HOCl (100 μM)-mediated chloramine formation (0, 15.6, 31.2, 62.5, 125, 250 μM; n=5). B) Exogenous UCB inhibits MPO/H_2O_2 (50 μM)-mediated chloramine formation in a concentration-dependent manner (0, 3.125, 6.25, 12.5, 25, 50 and 100 μM; n=4). **P*<0.05, ***P*<0.001 versus untreated samples (ANOVA; Dunn's post-hoc analysis).

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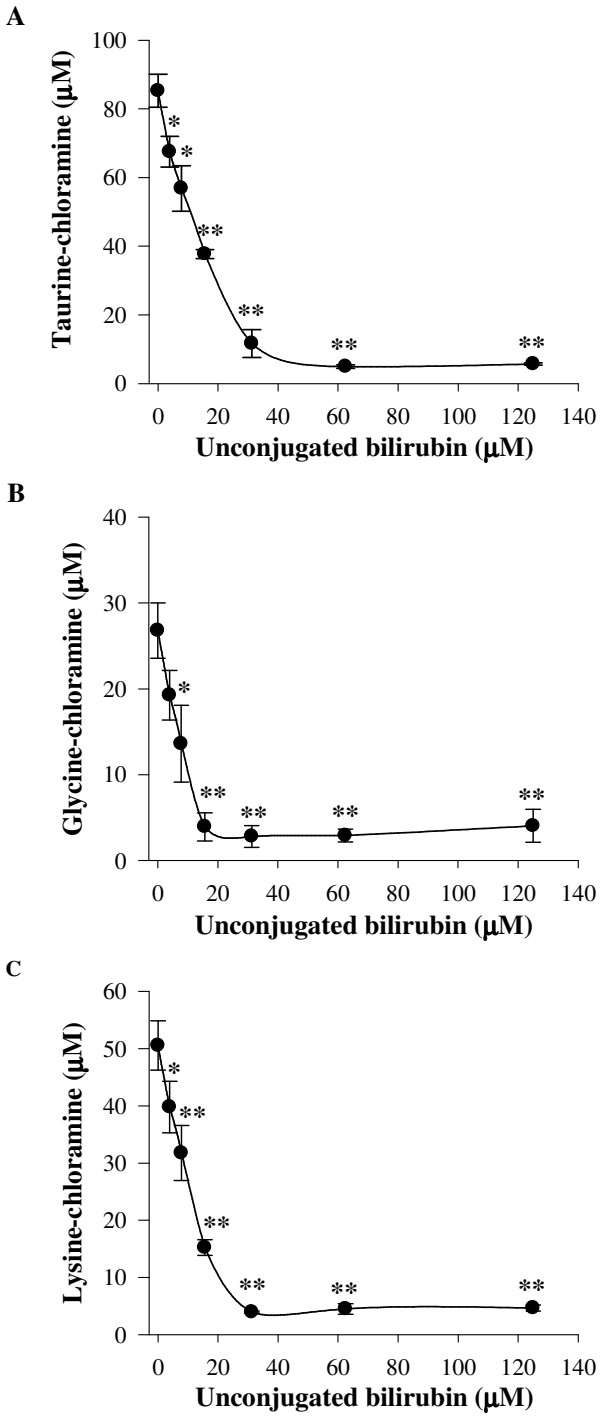

Figure 2. Exogenous UCB quenches A) taurine, B) glycine and C) N-α-acetyl lysine chloramines (250 μM) in a concentration-dependent manner (0, 3.9, 7.8, 15.6, 31.2, 62.5, 125 μM; n=3). ***P*<0.01 versus untreated samples (ANOVA; Dunn's post-hoc analysis).

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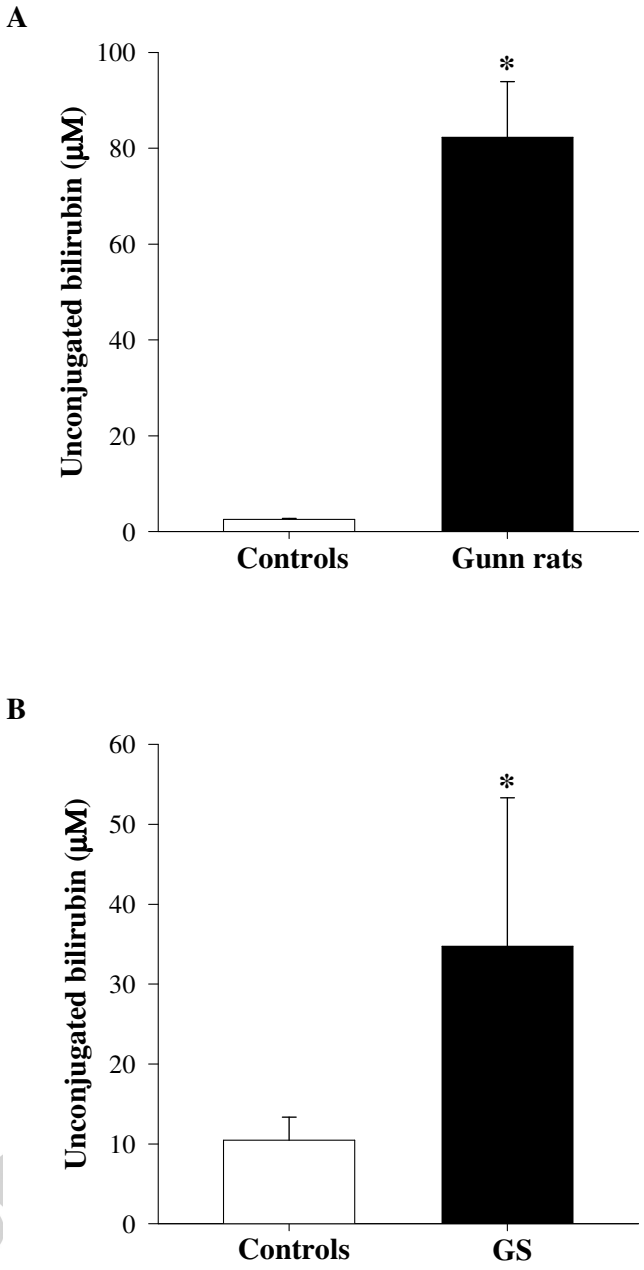

Figure 3. A) Unconjugated bilirubin concentrations in hyperbilirubinemic in Gunn rats (n=9) and littermate controls (n=5) and B) Gilbert's syndrome and controls (n=21 per group). **P*<0.01 between the groups.

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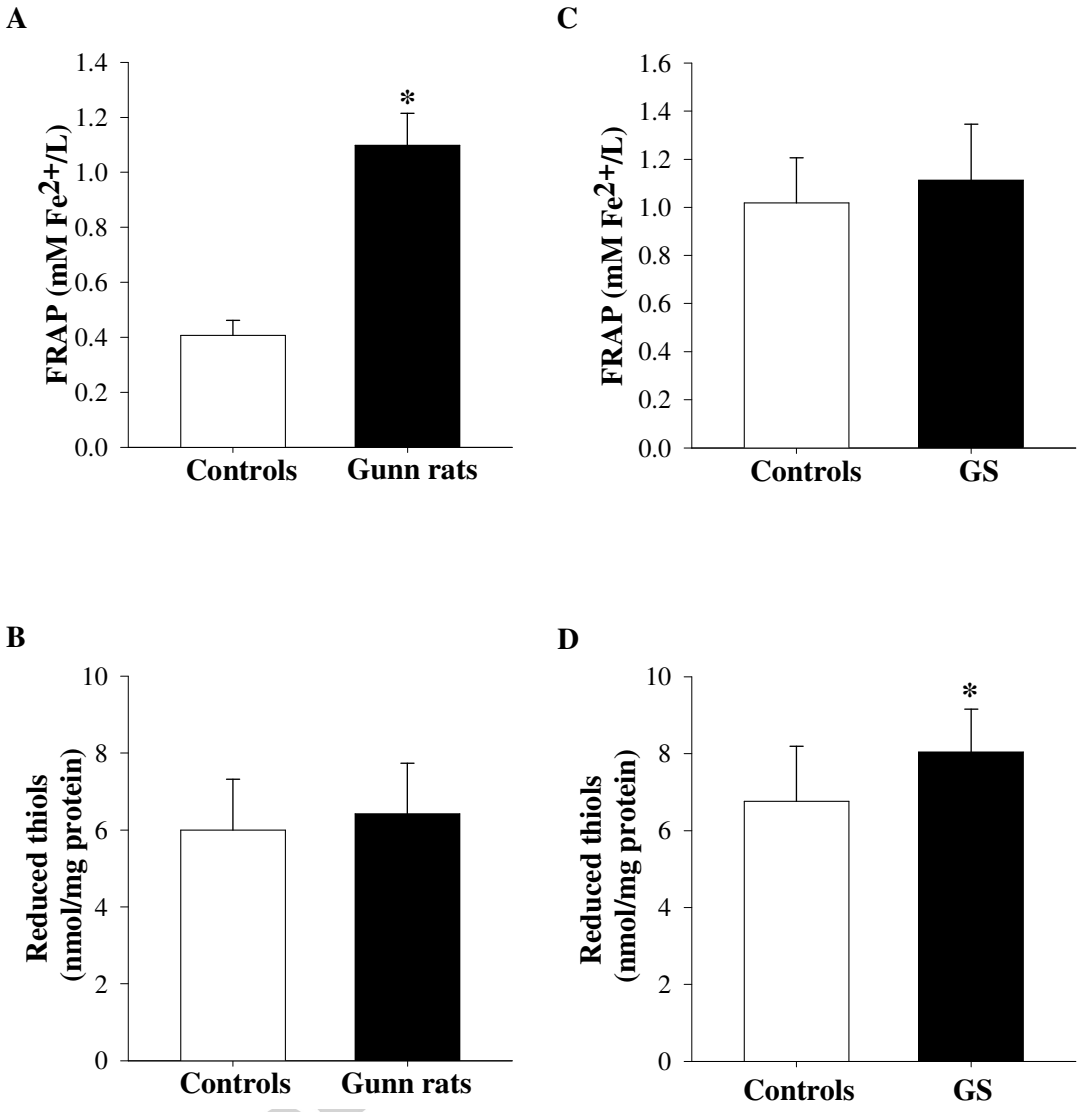

Figure 4. A) FRAP and B) reduced thiol concentrations in hyperbilirubinemic in Gunn rats (n=9) and littermate controls (n=5). C) FRAP and D) reduced thiol concentrations in Gilbert's syndrome and controls (n=21 per group). *P<0.05 between the groups.

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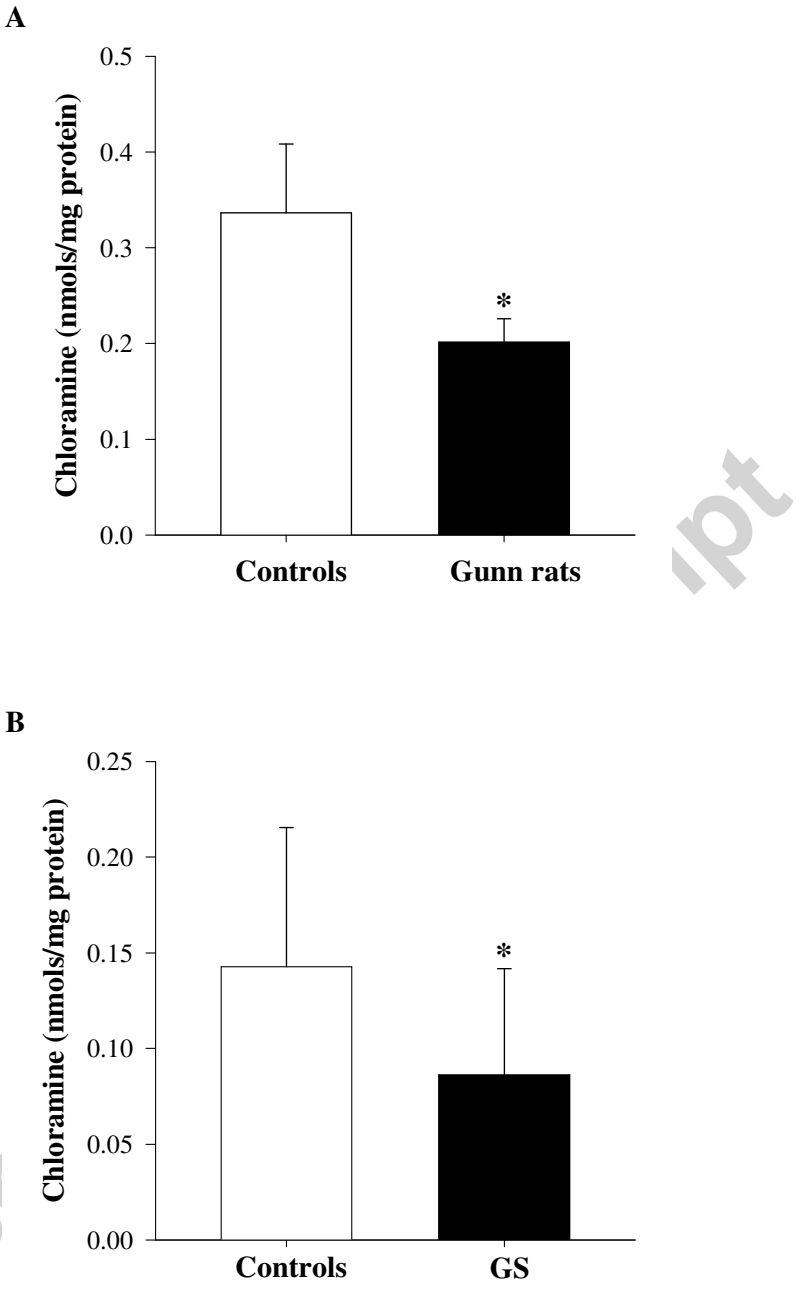

Figure 5. A) MPO-mediated chloramine formation in hyperbilirubinemic in Gunn rats (n=9) and littermate controls (n=5) and in B) Gilbert's syndrome and controls (n=21 per group). **P*<0.01 between the groups.

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3.2 Inhibition of protein carbonyl formation by MPO mediated oxidation

Chloramines decompose to yield protein carbonyls, a stable biomarker of protein oxidation and CVD risk [28,40-42]. Formation of protein carbonyl was inhibited in a concentration dependent manner with ≥25 μM UCB significantly inhibiting protein oxidation (P<0.05; Figure 6A).

3.3 Inhibition of malondialdehyde formation by MPO mediated oxidation

Oxidative damage to lipids, particularly LDL, is associated with the severity, and potentially development of CVD [43,44]. Despite the protective effects of UCB on MPO/HOCl-mediated protein oxidation (i.e chloramine and protein carbonyl formation), UCB may also inhibit MPO-mediated lipid oxidation (i.e. malondialdehyde; MDA). Myeloperoxidase-mediated MDA formation was significantly inhibited in the presence of UCB (50-100 μM; P<0.05), the inhibition of which occurred in a concentration-dependent manner (Figure 6B). Malondiadehyde and protein carbonyl formation were inhibited similarly by exogenous UCB supplementation, as indicated by a positive correlation between MDA and protein carbonyl formation mediated by MPO oxidation in the presence of various UCB concentrations (Figure 6C).

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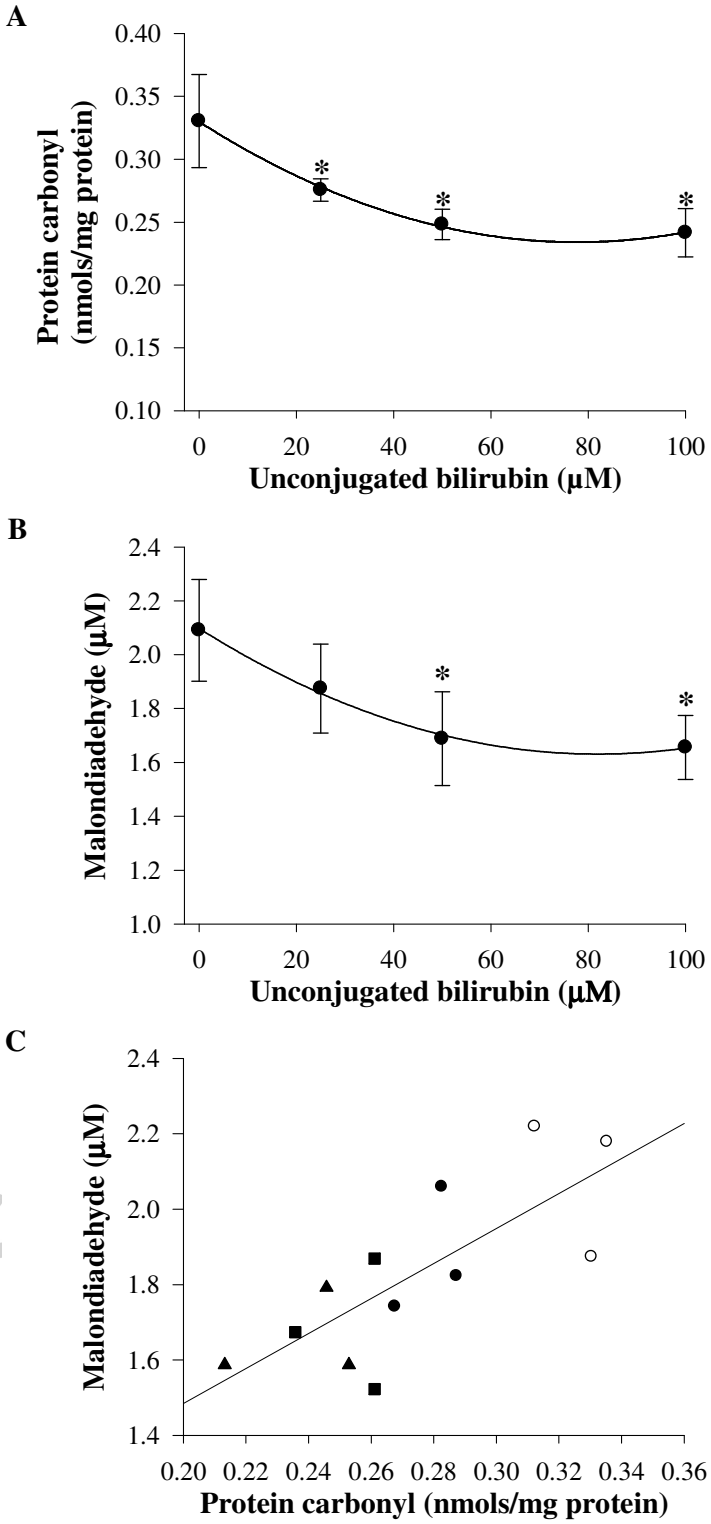

Figure 6. A) Exogenous UCB inhibits MPO/H_2O_2 (100 µM)-mediated protein carbonyl (n=5) and B) malondialdehyde (n=3) formation in a concentration-dependent manner (0, 25, 50 and 100 µM; ANOVA; Dunn's post-hoc analysis) **P*<0.05. C) Correlation between MPO/H_2O_2 (100 µM)-mediated protein carbonyl and malondialdehyde formation in plasma

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supplemented with exogenous UCB (0 μM, ○; 25 μM, ●; 50 μM, ■; 100 μM, ▲; r=0.760; P<0.01).

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4. Discussion

The present study demonstrates that UCB protects proteins and lipids from MPO induced oxidation, which represents an important biological source of oxidants implicated in disease pathogenesis [45-47]. Plasma supplemented with UCB reduced HOCl and MPO-induced chloramine formation. Importantly, albumin-bound UCB directly quenched taurine-, glycine- and *N*-α-acetyl-lysine- chloramines at low micromolar concentrations. Chloramine formation, induced by MPO, was also significantly inhibited by UCB in two physiologically relevant models of benign hyperbilirubinemia; in serum/plasma from humans with GS and Gunn rats. Unconjugated bilirubin further inhibited the formation of chloramine decomposition products including protein carbonyl and malondialdehyde, which was likely mediated by direct quenching of chloramine by UCB. Therefore, these data provide a biologically relevant mechanism to explain protection from protein and lipid oxidation and a hypothetical mechanism that may contribute to protection from atherosclerosis observed in individuals with mildly elevated UCB concentrations.

Individuals with benign hyperbilirubinemia often possess impaired uridine diphosphate-glucuronosyl transferase (UGT1A1) activity [48,49], underpinned by an increased TA repeat polymorphism in the UGT1A1 gene promoter, which contributes to elevated UCB levels observed in GS. Epidemiological reports indicate that hyperbilirubinemic individuals are protected from atherosclerosis and CVD [1-4,6,12]. Bilirubin is an endogenous antioxidant, which efficiently scavenges various oxidant species *in vitro* [14-16,18,50,51]. For example, one molecule of albumin-bound bilirubin efficiently inhibits the oxidation of albumin-bound fatty acids by scavenging two peroxyl radicals [16]. Unconjugated bilirubin protects against oxidative modification of BSA, lysozyme and melittin from radicals induced by irradiation of oxygen [15]. In a similar study, bilirubin also

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efficiently inhibited protein carbonyl formation mediated by radiolysis [14]. Nevertheless, testing whether UCB resists protein and lipid oxidation generated from biologically relevant systems, such as MPO-generated HOCl, in physiologically relevant models of hyperbilirubinemia is sparse and of great importance, if we are to understand the potential physiological importance of UCB in protecting from disease.

4.1 Reagent HOCl oxidation

Blood plasma is rich in proteins and amino acids including lysine, tyrosine and methionine, which are particularly susceptible to oxidative modification by HOCl [37,52]. Treatment of proteins with HOCl induces protein carbonyl formation via the hydrolysis of chloramines, which are considered biomarkers of protein oxidation [53,54]. Chloramines also decompose via pathways leading to protein side-chain modification, fragmentation and cross-linking [53]. However, the mechanisms by which UCB, at physiological concentrations, might inhibit the formation of these oxidized products in plasma are poorly described. In the present study, exogenous UCB (15.6- 250 μM; after correcting for serum dilution) efficiently inhibited reagent HOCl (100 μM) induced chloramine formation (measured at 5 min) in a concentration dependent manner, with 15.6 μM representing the lowest effective concentration. To determine whether bilirubin could directly quench chloramines we preformed chloramine on taurine, glycine and *N*-α-acetyl lysine. These biologically relevant chloramines react readily with protein thiols [37] and we show that albumin-bound UCB quenched chloramine formation at low micromolar concentrations (ie. 3.9-7.8 μM). These observations indicate that bilirubin co-operates with albumin to neutralise HOCl-mediated chloramine, which could otherwise decompose and further oxidize proteins and lipids. These data suggest that normal physiological UCB concentrations (5-10 μM) could contribute to quenching chloramine, and at bilirubin concentrations in GS (≥17.1 μM) may strongly inhibit

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chloramine formation. We show that 15.6 μM albumin-bound UCB quenched ~47, ~23, ~35 μM taurine, glycine and lysine chloramine species, suggesting that bilirubin quenches a ~1.5-3 molar excess of chloramine, when associated with albumin.

These observations are in general agreement with previous publications showing that BRT (≥25 μM), a synthetic water soluble bilirubin analogue, rapidly and efficiently scavenged HOCl (100 μM) in a concentration-dependent manner [19]. The authors concluded that higher concentrations of bilirubin (conjugated or unconjugated) within pathological conditions (eg. liver failure, GS, Dubin-Johnson, Rotor, Crigler-Najjar syndromes or conjugated bilirubin in bile) [55,56] may scavenge HOCl *in vivo*, however, did not test this specifically [31]. Interestingly, albumin-bound UCB (~15 μM) did not react efficiently with reagent HOCl (50 μM) *in vitro* [31]. These observations may also have been affected by the low concentration of bilirubin and HOCl tested in this *in vitro* system, the matrix within which it was tested (a BSA solution), which did not reflect the composition of human plasma or potentially that oxidation products of bilirubin contribute further to neutralising HOCl [19]. Despite these limitations, these data indicated that unconjugated bilirubin was unlikely to react sufficiently with HOCl to mediate protection from protein oxidation.

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4.2 MPO-generated HOCl oxidation

Stocker *et al*. showed that BRT (10 μM) did not inhibit the MPO-H_2O_2 mediated oxidation of guaiacol; however, showed that the tetraguaiacol product formed from guaiacol oxidised BRT [19]. The failure of BRT to inhibit guaiacol oxidation might be influenced by the relatively short oxidation period (ie. 5 min) used during testing. Plasma treated with MPO induces chloramine formation gradually after 30 mins of incubation [37]. In the present study however, the reaction of plasma with MPO for 30 mins resulted in chloramine formation, which was dose-dependently inhibited by exogenous UCB (12.5-100 μM). More importantly, Gunn rat serum and GS plasma were less susceptible to MPO-induced chloramine formation and suggested a dose response effect of UCB. Gunn rats possessed a greater antioxidant status (FRAP) versus controls, reflecting the contribution of bilirubin to antioxidant status in this assay. Strong, significant correlations between reduced thiols, glutathione and UCB concentrations were reported in humans within our previous study [33] and are in agreement with elevated circulating sulfhydryl groups reported in another GS cohort [57]. These data suggested dose-dependent protection from HOCl oxidation associated with UCB may also be influenced by improved antioxidant capacity (ie. reduced thiols), which may reduce chloramine formation by providing a kinetically more favourable target for reaction *in vivo* [27]. However, we have demonstrated that albumin-bound UCB efficiently reacts with preformed chloramine species, providing a novel mechanism to explain how UCB protects from MPO induced oxidation. Investigation of the rate constant for chloramine decomposition in the presence of bilirubin would provide important information as to whether bilirubin represents a kinetically favourable target for oxidation. However, methodological limitations including the need to associate bilirubin with BSA (which contains sulfhydryl groups that compete for oxidation) and/or sufficiently sensitive, specific

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and rapid quantitation of bilirubin currently preclude the accurate quantification of such a rate constant.

Plasma-derived chloramines decompose relatively slowly (over 4 hr) resulting in the formation of protein carbonyls [29,58]. Myeloperoxidase has previously been implicated as a source of protein carbonyls *in vivo* [59] and reduced protein carbonyl concentrations are associated with a decreased risk of CVD events [41,60,61]. Individuals with a greater UCB concentrations (≥ 40 μM), have reduced circulating protein carbonyls, which are negatively correlated with the UCB concentration *in vivo* [33]. Our present data shows that exogenous UCB supplementation (25-100 μM) effectively inhibited secondary protein carbonyl formation mediated by MPO-generated HOCl. Cumulatively, these data provide a mechanistic link to explain how endogenously elevated UCB inhibits protein carbonyl formation, via the direct quenching of intermediate chloramine formation.

Previous studies have reported that exposure of LDL to reagent HOCl or MPO-generated HOCl induces the modification of amino acid residues of apolipoprotein B-100 (apo B) associated with LDL, followed by lipid peroxidation [30,43,62-64]. Chloramines give rise to nitrogen centred radicals, in turn leading to the generation of secondary radicals [29,53]. These radicals derived from chloramine on apo B could initiate lipid peroxidation in LDL [30]. However, no data exists to link UCB and inhibition of lipid oxidation mediated by MPO-generated HOCl. We show that addition of exogenous UCB (≥50 μM) to plasma significantly inhibits MPO-generated MDA formation, a biomarker of lipid oxidation. Together these data suggest an important role of UCB in protecting protein and lipid oxidation by directly scavenging intermediate chloramines [30,65], which could potentially contribute to CVD protection (Figure 7). It should be noted however, such a statement is

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speculative and that animal models of CVD development (ie. ApoE$^{-/-}$ mice), with endogenously elevated and decreased bilirubin would need to be developed to provide a conclusive evidence for a role of bilirubin in atheroprotection.

5. Conclusion

This translational report shows that UCB, at physiologically relevant concentrations, protects from protein and lipid induced MPO-generated HOCl oxidation and suggests that this inhibition is accompanied by direct scavenging of chloramine by albumin-bound UCB. Inhibition of protein oxidation is reported upon exogenous UCB addition to human plasma, and within two independent models (rats and humans) of hyperbilirubinemia. These data provide a biologically relevant mechanism which could potentially contribute to reducing the incidence of CVD in GS. If this hypothesis is confirmed, strategies aimed at modifying haem catabolism and bilirubin metabolism, leading to mildly elevated circulating bilirubin, may provide a new target for therapy in humans at risk of atherosclerosis [66].

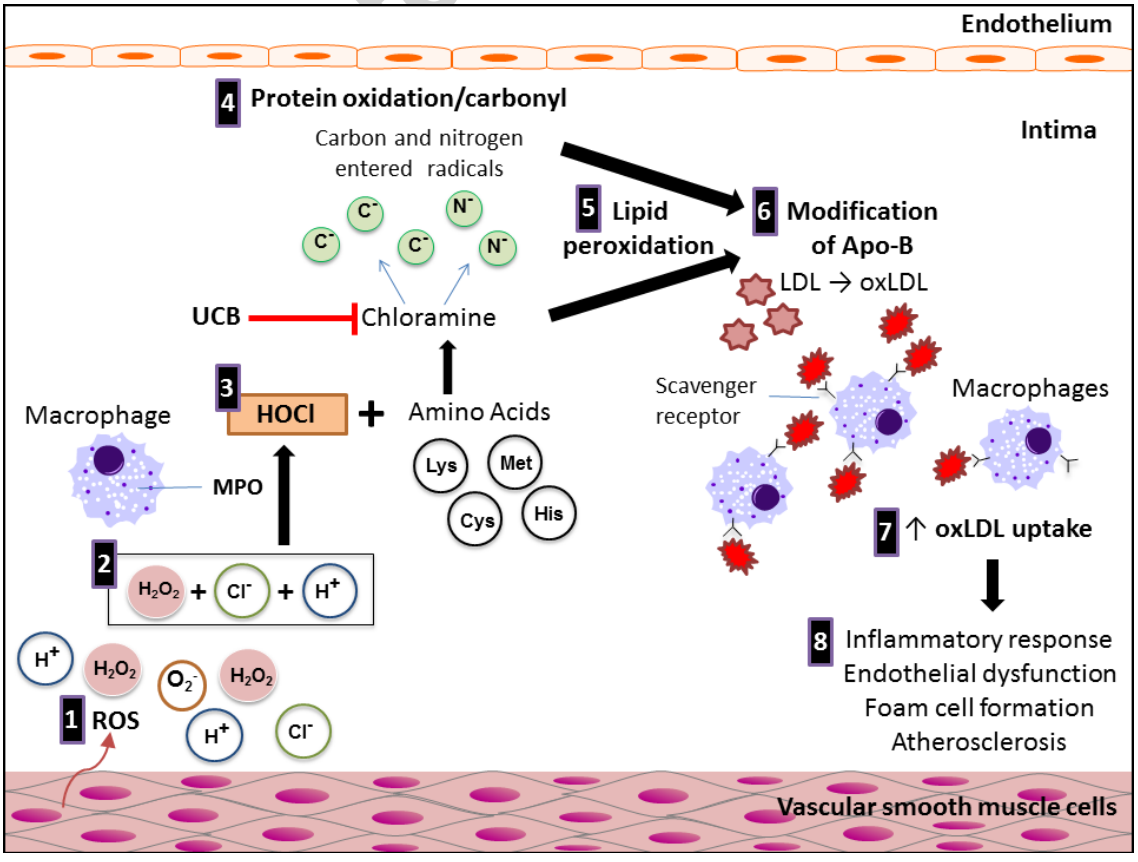

Figure 7. Summary of bilirubin's protective mechanism, which inhibits MPO/HOCl oxidation, and the potential importance of this within the context of atherogenesis. (1) Vascular smooth muscle cells stimulate the production of reactive oxygen species (ROS) (eg. hydrogen peroxide [H_2O_2] superoxide [O_2^-]), which are released into the interstitium. (2) Myeloperoxidase (MPO) is abundantly expressed by monocytes/macrophages and produces powerful oxidants, hypochlorous acid (HOCl) in the presence of H_2O_2, hydrogen (H^+) and chloride ions (Cl^-). (3) Hypochlorous acid reacts readily with amino groups on proteins including methionine (Met), lysine (Lys), histidine (His) and cysteine (Cys) resulting in chloramine formation. (4) Chloramines, carbon (C^-) and nitrogen (N^-) centred radicals derived from chloramine can propagate protein and (5) lipid peroxidation. (6) These radicals derived from chloramine can initiate the modification of Apo-B associated with LDL. (7) Macrophages express scavenger receptors that bind and endocytose oxidized LDL (oxLDL), (8) which can initiate inflammatory responses and augment foam cell formation. Bilirubin (UCB) is a potential antioxidant and may directly scavenge intermediate chloramine formation, protecting from MPO/HOCl induced protein and lipid oxidation.

6. Acknowledgements

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7. Supplementary figures

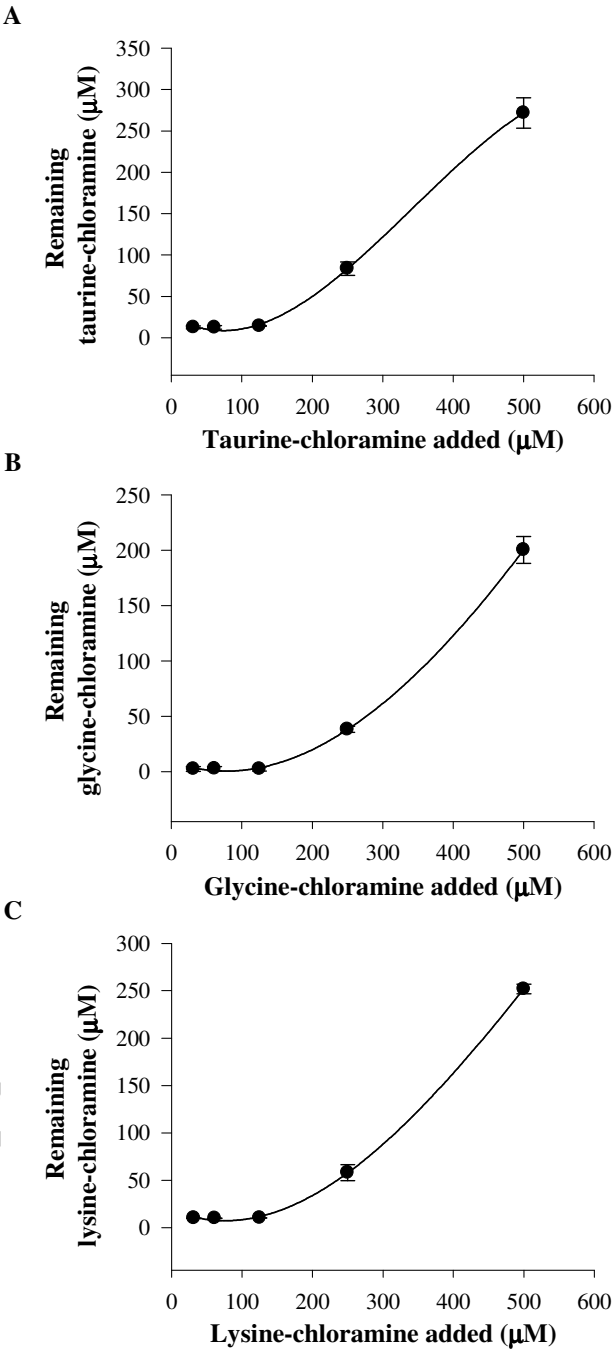

Figure S1. Remaining chloramine (y-axis) in the presence of BSA (250 μM), 5 mins after the addition of 31.2-500 μM (x-axis; A) taurine-, (B) glycine- and (C) N-α-acetyl-lysine-chloramine. Based upon these results, 250 μM chloramine was selected to test whether albumin-bound UCB could quench unreacted chloramine.

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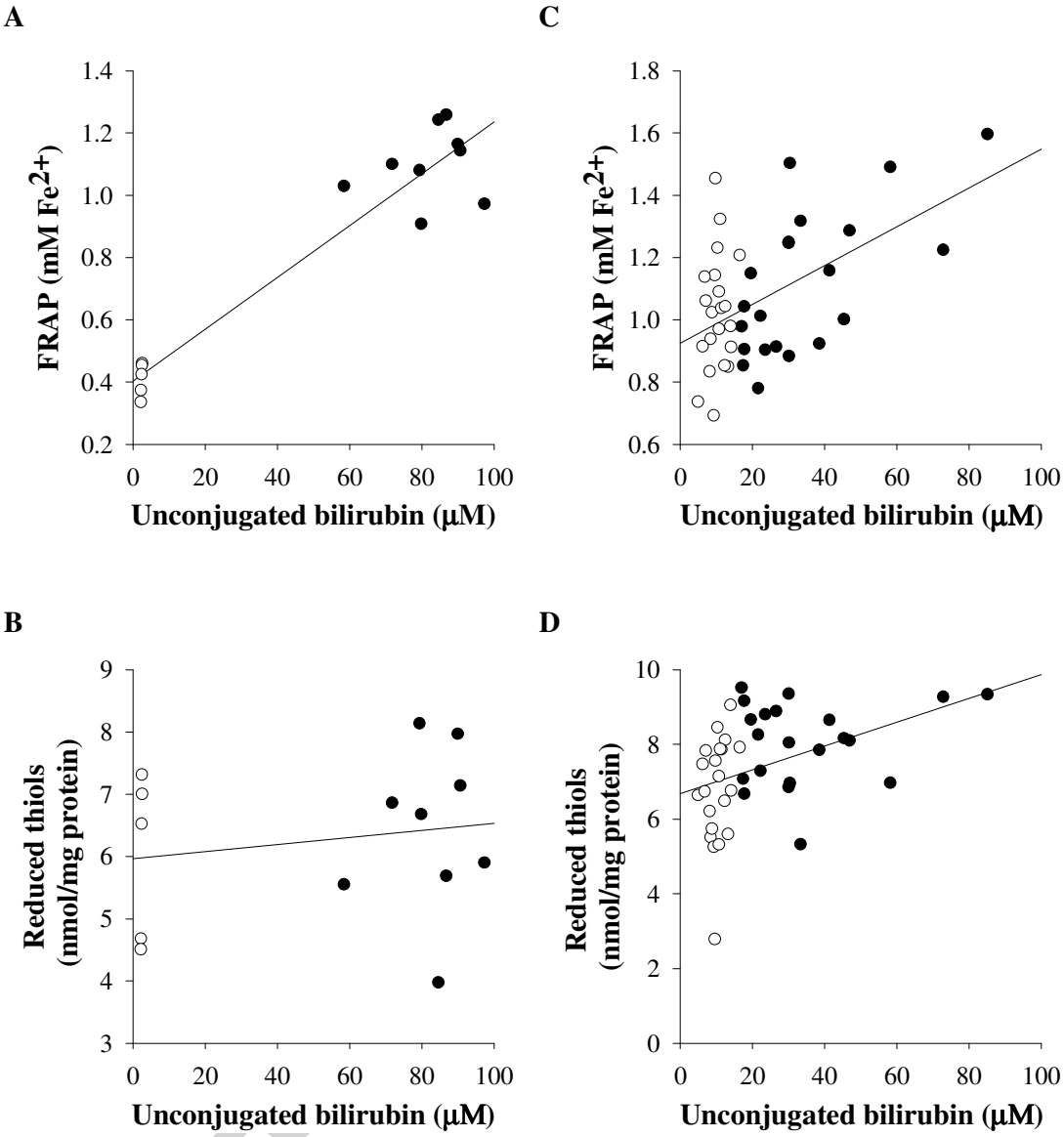

Figure S2. Correlation between unconjugated bilirubin and A) FRAP (r=0.950; *P*<0.001) and B) reduced thiol (r=0.181; *P*=0.537) in hyperbilirubinemic in Gunn rats (●; n=9) and littermate controls (○; n=5). Correlation between unconjugated bilirubin and C) FRAP (r=0.521; *P*<0.01) and D) reduced thiols (r=0.403; *P*<0.01) in hyperbilirubinemic in GS (●) and control (○) subjects (n=21 per group).

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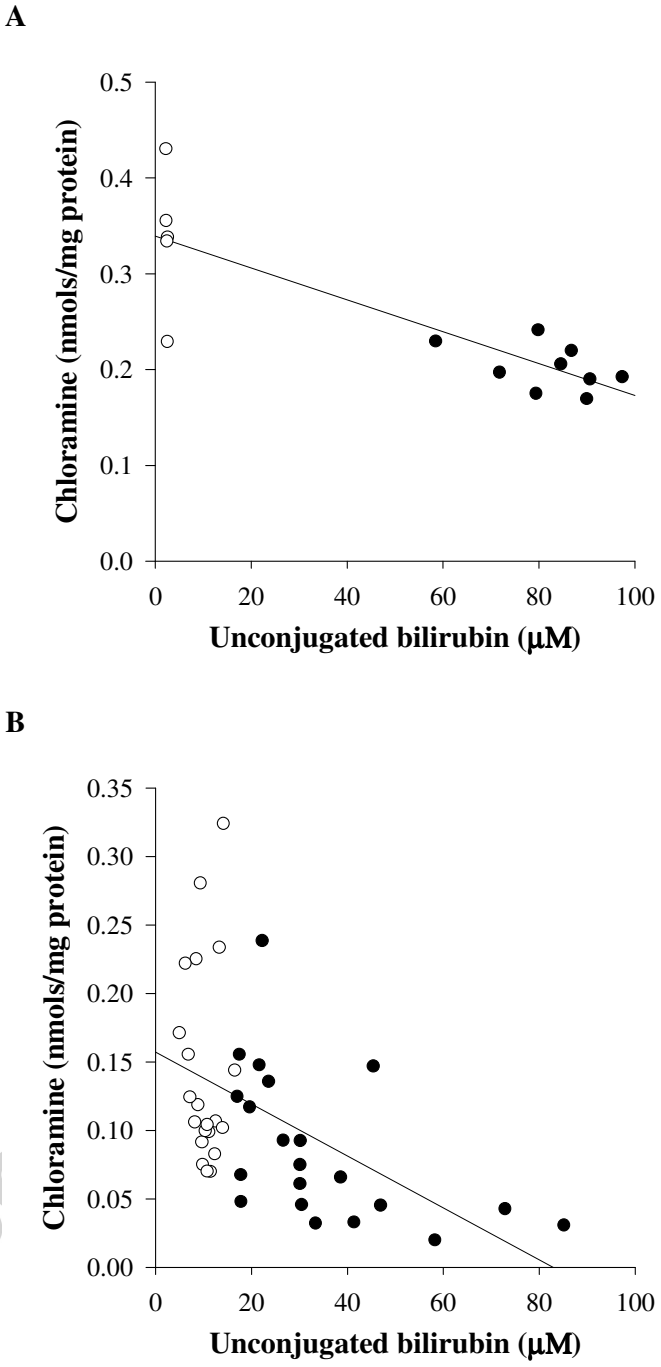

Figure S3. Correlation between MPO/H_2O_2 (50 μM)-induced chloramine formation and unconjugated bilirubin concentrations in hyperbilirubinemic A) Gunn rats (●; n=9) and littermate controls (○; n=5; r=-0.826, P<0.001) and B) GS (●) and control (○) subjects (n=21 per group: r=-0.486; P<0.01).

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Highlights

- Endogenous hyperbilirubinemia is associated with protection from atherosclerosis
- Unconjugated bilirubin appears to directly quench biologically relevant chloramines
- Plasma from hyperbilirubinemic humans and rodents was protected from myeloperoxidase generated chloramines
- Inhibition of chloramine formation in plasma supplemented with bilirubin was associated with protection of proteins and lipids from oxidation

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