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

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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 syndrome; GS) are protected from atherosclerosis, cardiovascular disease, 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 ($\leq 250\ \mu M$) 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-acetyllysine 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 was also reduced after MPO oxidation in plasma supplemented with UCB ($P<0.05$; 25 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 hyperbilirubinemic 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.

Introduction

Unconjugated bilirubin (UCB), a potentially toxic by-product of heme 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 hemolytic 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 [16] and 2,2′-azobis (2,4-dimethylvaleronitrile)) [17] and inhibits copper (Cu^{2+})-induced lipid oxidation [18].

Whether bilirubin and related compounds protect from oxidation mediated by biologically relevant myeloperoxidase (MPO)

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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 for 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]. The reaction of HOCl with amine groups on proteins gives rise to chloramines, which decompose to form protein carbonyls [28,29] and can also induce the oxidation of low-density lipoproteins (LDLs), 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 oxidized 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 considering that persons with Gilbert 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 against protein and lipid oxidation induced by reagent and enzymatically generated HOCl. We hypothesized that bilirubin would protect from protein oxidation by quenching HOCl-induced chloramine formation. These data may help to rationalize the association between bilirubin and protection from CVD.

Methods

Human subjects

Forty-two subjects (21 GS; 21 controls) were recruited and matched for gender, age, and body mass index. 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 (i.e., 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 before commencement of experimentation.

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 (Specialty Feeds, Glen Forrest, Australia) and fresh water. Female homozygous Gunn rat offspring were assumed to possess jaundice at birth, were ear-tagged, and were housed together with female littermate (nonjaundiced) 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 serum vacutainers and prepared for analysis (see next section). All procedures were approved by the Griffith University Animal Ethics Research Committee before commencement of experimentation (MSC/06/12).

Sample preparation

Whole blood was centrifuged (Thermo Scientific 5810 R, Australia) at 2500 *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 general biochemistry, antioxidant activities, and HOCl oxidation, whereas EDTA plasma samples were used for MPO oxidation and 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.

Serum biochemistry

Serum samples from animals were diluted 1:1 with distilled water and analyzed 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 analyzer (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). Total nonthiol antioxidant capacity, including ferric reducing ability of plasma (FRAP) [34], was also assessed on an open channel of the COBAS Integra analyzer. All analyses were conducted in duplicate.

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 a C18 reserve-phase HPLC guard and analytical column (4.6 × 150 mm, 3 μm; Phenomenex, Australia) that was perfused at 1 ml/min. Extracted samples were injected (100 μl) with a run time of 15 min in duplicate. Unconjugated bilirubin (Frontier Scientific, USA; 0–100 μM) served as an external standard and was detected at 450 nm.

Induction of HOCl oxidation

Human quality control samples (serum) were thawed to room temperature and centrifuged at 2500 *g* for 5 min and diluted (1:10 for reagent HOCl oxidation) with deionized water. Sodium phosphate buffer (0.1 M, pH 7.4) was prepared and treated with Chelex resin before use, to minimize contamination with transition metal ions [36]. Buffer was stored at room temperature and used as a