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SHORT COMMUNICATION

Antioxidation of Human Low Density Lipoprotein by Unconjugated and Conjugated Bilirubins

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ABSTRACT. We demonstrate here that both unconjugated bilirubin (Bu) and conjugated bilirubin (Bc) can protect human low density lipoprotein (LDL) against oxidation by oxyradicals generated by 2,2′-azo-bis(2-amidinopropane) dihydrochloride at 37°. The oxidation was assessed by agarose gel electrophoresis and was further corroborated by assaying the malondialdehydes and lipid peroxides formed throughout oxidation. On a per mole basis, Bu and less so Bc was more effective than ascorbate in preventing LDL oxidation. Since oxidative modification of human LDL was implicated in plaque formation in blood vessels leading to atherogenesis, the data suggested that either bile pigment may help reduce the risk of atherogenesis. BIOCHEM PHARMACOL 51;6:859–862, 1996.

KEY WORDS. bilirubin; low density lipoprotein; antioxidant

Oxidative modification of LDL† has been suggested to be a risk factor for the development of atherosclerosis [1]. Oxidatively modified LDL are taken up by the scavenger receptors of the intimal macrophages [2], which contribute to formation of lipid-rich foam cells [1]. Treatment with antioxidants such as probucol was noted to prevent the progression of atherosclerosis in the Watanabe heritable rabbit [3], which is an animal model for familial hypercholesterolemia [4]. The latter finding suggested that, *in vivo*, antioxidants may protect LDL from oxidation. The LDL isolated from individuals supplemented with vitamin E exhibits a higher resistance to oxidation than LDL from subjects without vitamin supplementation [1]. Recent findings also indicated that prolonged intake of vitamin E decreases the chance of atherosclerosis in humans [5]. To date, little is known of the effect of endogenous antioxidants on LDL oxidation.

Among endogenous antioxidants, bilirubin is one reported to protect phosophlipids in multilamellar liposomes and rat liver microsomes [6, 7]. Actually, total bilirubin in serum comprises multiple subfractions [8]—principally Bu and its sugar conjugates Bc. Recently, we found [9] that Bu effectively inhibits the oxidation of LDL mediated by Cu^{2+}. In this study, we further tested whether Bu or Bc can protect LDL from oxidation by oxyradicals *in vitro*. Peroxyl radicals were produced by AAPH in our human LDL incubations. Stocker *et al.* [7] have shown that Bu and Bc can protect human serum lipids against peroxidative attack by scavenging the peroxyl radicals formed *in vitro*. We tested whether the scavenging activities of Bu and Bc also exist in our LDL system.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals were supplied by the Sigma Chemical Co. (St. Louis, MO, U.S.A.). AAPH was obtained from Polysciences Inc. (Warrington, PA, U.S.A.). Ditaurobilirubin (Bc) was purchased from Porphyrin Products (Logan, UT, U.S.A.).

LDL Preparations

Human LDL (d = 1.019 to 1.063 g/mL) from either the Sigma Chemical Co. or purified by preparative ultracentrifugation of normal human serum was used. Both preparations gave similar results in oxidation experiments. LDL was dialyzed against 10 μM EDTA in PBS immediately before use. The protein content in LDL was estimated by using a protein assay kit from the Sigma Chemical Co.

Preparations of Bu and Bc Solutions

Since Bu and Bc are light sensitive, all experiments were carried out in reaction tubes covered with aluminum foil. To prepare a stock solution of Bu, solid Bu was wetted on all surfaces with 100 μL of 0.5 M sodium hydroxide, brought up to

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† *Abbreviations:* AAPH, 2,2′-azo-bis(2-amidinopropane) dihydrochloride; Bc, conjugated bilirubin; Bu, unconjugated bilirubin; CAD, coronary artery disease; LDL, low density lipoprotein; and MDA, malondialdehyde.

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volume with 0.05 M sodium phosphate buffer, pH 7.4, containing 0.9% NaCl (PBS), and re-adjusted to pH 7.4 with HCl. The solution should exhibit a bright reddish-yellow colour without any particulate matter. It should be diluted to the desired concentration and be used preferably within 2–3 hr. Any solution that has changed colour or appearance should be discarded. Commercially available Bc, i.e. ditaurobilirubin, was dissolved directly into PBS.

Oxidation of LDL

Prior to oxidation, the LDL preparations were dialyzed extensively against PBS containing 10 μM sodium EDTA. LDL oxidation was performed by AAPH incubation. LDL (150–250 μg/mL) was incubated in PBS containing 4 mM AAPH and 1 μM EDTA for 20 hr at 37° [7] with or without bilirubins. Ascorbate was used for comparison.

LDL Electrophoresis

LDL electrophoresis was carried out at pH 8.6 in 0.05 M barbital buffer on 0.6% agarose gels as described [10]. The gels were stained with Sudan Black B.

MDA Equivalents of Oxidized LDL

The extent of LDL oxidation was estimated as thiobarbituric acid-reactive substance using the method of Steinbrecher *et al.* [11]. LDL was mixed with 1.5 mL of 0.67% thiobarbituric acid and 1.5 mL of 20% trichloracetic acid. After heating at 100° for 30 min, the reaction product was assayed fluorometrically using a Shimadzu RF-5000 spectrofluorometer with excitation at 515 nm and emission at 553 nm. Freshly diluted tetraethoxypropane, which yields MDA, was used as a standard. Results are expressed as nanomoles of "MDA equivalents." The sensitivity of the assay was 0.1 nmol of MDA.

Lipid Peroxides in Oxidized LDL

The method of El-Saadani *et al.* [12] was followed. Two hundred microliters of LDL (300–500 μg protein) was mixed with 950 μL of CHOD-iodide reagent obtained from a CHOD iodide kit (Merck Co., Darmstadt, F.R.G.) and 50 μL of 10 mM butyrated hydroxytoluene and incubated at 25° for 60 min. The increase of absorbance at 365 nm after incubation was recorded with a spectrophotometer (model UV-160, Shimadzu, Kyoto, Japan). The molar absorptivity of I_3^- (formed by the oxidation of iodide into I_2 by lipid peroxides) is 2.46×10^4 M^{-1} cm^{-1} [12]. The result was expressed as nanomoles lipid peroxides per milligram protein.

Statistical Analysis

All data are expressed as means ± SD. Differences between groups were evaluated by ANOVA, followed by Duncan's multiple range test. $P < 0.05$ between groups was considered to be statistically significant.

RESULTS

Figure 1 shows the relative electrophoretic mobility of LDL after various treatments. LDL incubated with 4 mM AAPH at 37° for 20 hr resulted in the oxidative modification of LDL as evidenced by an increase in negative charge (column 2 vs column 1). However, when LDL was incubated with AAPH in the presence of 75 μM Bu (column 7), 100 μM Bu (column 8), 75 μM Bc (column 10) or 100 μM Bc (column 11), the electrophoretic mobilities of these LDL preparations were partially reversed, indicating that Bu or Bc at 75–100 μM could exhibit partial protection of LDL against oxidation. However, in the presence of lower concentration of Bu or Bc, the findings on electrophoretic mobilities of the LDL preparations suggested that they were oxidized and could not be protected by these concentration of bilirubins. For comparison, ascorbate at concentrations as high as 200–400 μM did not protect LDL oxidation (columns 3–5).

Figure 2 demonstrates that the MDA equivalents of LDL increased drastically after oxidation. In the presence of 100 μM Bu, the MDA levels of oxidized LDL were reduced significantly ($P < 0.05$). However, Bc at a concentration of 100 μM only exhibited a small change of MDA equivalents compared with oxidized LDL, and the difference was not statistically significant. Ascorbate treatments did not show any effects.

Figure 3 shows the lipid peroxides of LDL under various treatment conditions. Treatment of oxidized LDL with Bu at either 75 or 100 μM and Bc at 100 μM resulted in significant reductions in their values of lipid peroxides compared with untreated oxidized LDL ($P < 0.05$ in both cases). Ascorbate treatments still did not show any effects.

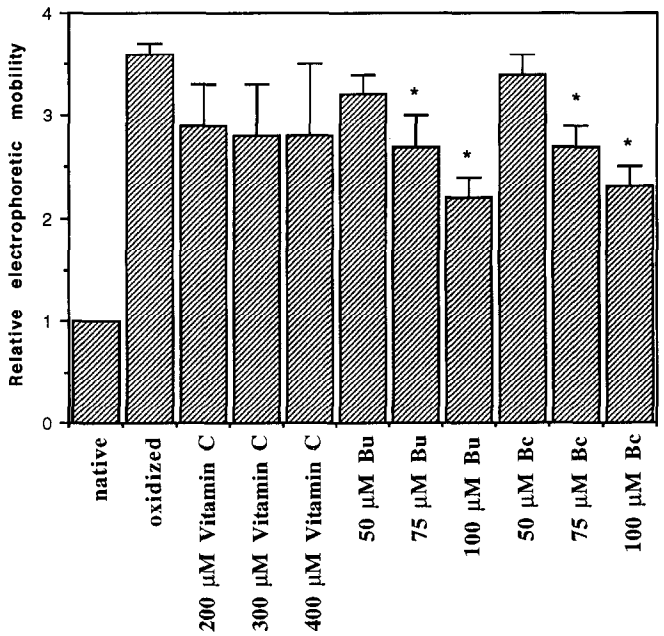

FIG. 1. Relative electrophoretic mobility of LDL under various treatment conditions. LDL was treated with AAPH in the absence or presence of Bu, Bc, or ascorbate as described in Materials and Methods. Data are expressed as means ± SD (N = 4). Key: (*) $P < 0.05$ compared with oxidized LDL.

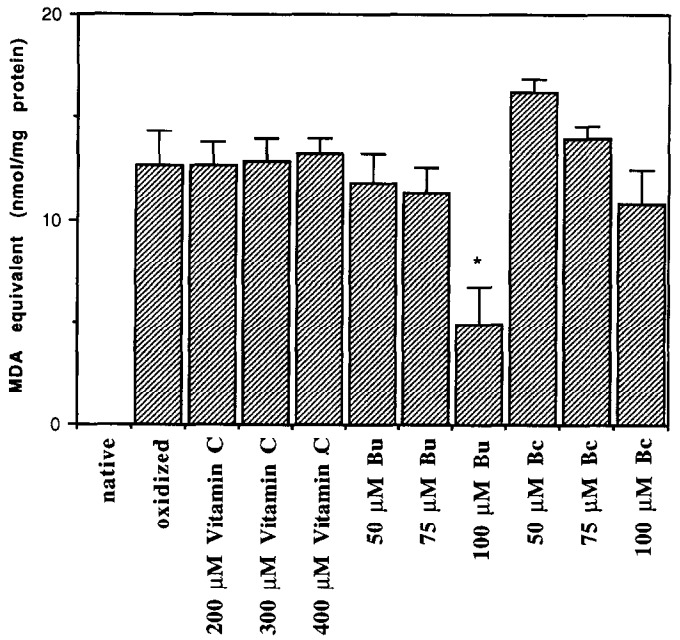

FIG. 2. MDA equivalents of LDL under various conditions. LDL was treated with AAPH in the absence or presence of Bu, Bc, or ascorbate as described in Materials and Methods. Data are expressed as means ± SD (N = 4). Key: (*) $P < 0.05$ compared with oxidized LDL.

DISCUSSION

The data in this study indicate that either Bu or Bc can inhibit the oxidation of LDL *in vitro* against artificially generated peroxyl radicals using an azo-initiator, AAPH, at 37°. The data

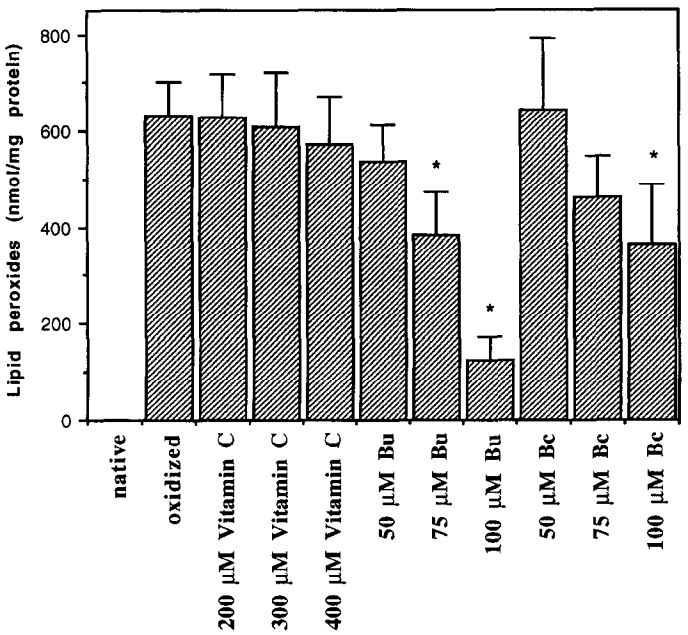

FIG. 3. Lipid peroxides of LDL under various conditions. LDL was treated with AAPH in the absence or presence of Bu, Bc, or ascorbate as described in Materials and Methods. Data are expressed as means ± SD (N = 4). Key: (*) $P < 0.05$ compared with oxidized LDL.

are consistent with that in a previous paper [9] showing, at lower (e.g. 17–34 μM) bilirubin concentrations, that Bu also protects LDL against copper-induced oxidations.. It is noteworthy that the oxyradical generating system used here is a drastic one, so that even ascorbate at 400 μM did not seem to protect LDL against oxidation. Stocker *et al.* [7, 13] reported that Bu or Bc protects serum lipids against damage inflicted by *in situ*-generated peroxyl radicals. In 1991, we observed that Bu protects human erythocytes, cultured rat hepatocytes, and rat livers from oxyradical damage [14, 15]. The protection elicited was substantially higher than that given by equimolar levels of ascorbate and mannitol. Similarly, the bilirubins are also more effective protectors of human ventricular myocytes than these antioxidants [16]. Results of all these experiments support the inference that bilirubin is an antioxidant. However, the precise molecular mechanism of this action remains to be elucidated. When LDL is exposed to AAPH at 37° for 20 hr, its apolar lipid components, namely cholesteryl esters and triglycerides, will transfer into an isotropic oily state [17]. The latter is susceptible to oxidation mediated by free radicals [18]. Most recently, Schuster *et al.* [18] demonstrated that in order to display resistance to oxidative stress *in vivo*, LDL should exist in core lipid structure, i.e. cholesteryl ester and triglyceride molecules are organized in two concentric layers. Since Bu or Bc are amphiphilic, they may dissolve in the lipid components of LDL and assist the apolipoprotein to rearrange the disrupted isotropic oily state of LDL at 37° into core lipid structure, resulting in protecting LDL oxidation.

Our present data on the antioxidant activities of Bu and Bc are in concert with those of Schwertner *et al.* [19], who recently reported that serum bilirubin behaves as an inverse and independent risk factor for CAD. They examined serum bilirubin as a possible risk factor for angiographically confirmed CAD in 619 naval men with complete data for all risk factors and in 258 men with incomplete data for risk factors. From statistical analyses of the data, they deduced that a 50% decrease in serum bilirubin was associated with a 47% increase in the probability of being in a more severe CAD category. They proposed that bilirubin, as a known antioxidant, may prevent oxidation of LDL and hence reduce cholesterol plaque formation *in vivo*.

In this work, we have directly verified that either Bu or Bc alone is capable of *partially* preventing peroxyl radical-mediated oxidation of LDL as gauged by electrophoresis and the quantitation of its presumptive oxidation products. If so, then serum bilirubin as a family of bile pigments may subserve at least two functions [20]: one, to partially protect against LDL oxidation and possible atherogenesis, and two, as a novel risk factor for CAD (plus any other myocardial disorders). Among ongoing work, we are studying the role of serum delta bilirubin (Bd) in this aspect.

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