RESEARCH COMMUNICATION

Human blood cells support the reduction of low-density-lipoprotein-associated cholesteryl ester hydroperoxides by albumin-bound ebselen

Julie CHRISTISON,* Helmut SIES,† and Roland STOCKER*‡

*Biochemistry Group, Heart Research Institute, 145 Missenden Road, Camperdown 2050, Sydney, New South Wales, Australia, and †Institut für Physiologische Chemie I, Heinrich-Heine-Universität, Düsseldorf, Federal Republic of Germany

Ebselen, a glutathione peroxidase mimic capable of reducing simple as well as complex hydroperoxides, including those of phospholipids and cholesteryl esters in intact oxidized low-density lipoprotein (LDL_{ox}), requires the presence of low-molecular-mass thiols to be active. In plasma, the drug is thought to be transported as an inactive albumin complex. As formation of LDL_{ox} is likely to occur extracellularly, we tested under which conditions ebselen can support reduction of LDL_{ox}-associated cholesteryl ester hydroperoxides outside cells. We observed that

addition of albumin-bound ebselen to whole blood, but not plasma, resulted in reduction of LDL_{ox}-associated cholesteryl linoleate hydroperoxides to the corresponding hydroxides. The observed reduction was rapid and its extent increased with increasing concentrations of ebselen. Physical contact of blood cells with LDL_{ox} was not required for this reducing activity. These results demonstrate that, in the presence of blood cells, extracellular ebselen is catalytically active. They suggest that ebselen may be considered as a drug for extracellular targets.

INTRODUCTION

The uptake of modified low-density lipoproteins (LDL) by the scavenger receptors of macrophages can lead to lipid-laden cells which are similar to 'foam' cells seen in developing atherosclerotic lesions [1,2]. It is now generally thought that oxidative modification of apolipoprotein B-100, the single protein moiety of LDL, represents a type of *in vivo* modification of LDL that contributes to atherogenesis [2]. As *in vitro* derivatization of apolipoprotein B by products derived from lipid hydroperoxides transforms the lipoprotein into a form recognized by the scavenger receptor [3], the reduction of lipid hydroperoxides can be considered a key step in the protection against formation of atherogenic, high-uptake LDL.

Human plasma contains GSH peroxidase [4–6], a selenoenzyme different from the two cellular enzymes. The three known peroxidases differ from each other in substrate specificity. The 'classical' cellular GSH peroxidase reduced H₂O₂ and organic hydroperoxides, but cannot reduce more complex lipid hydroperoxides [7,8]. The more recently discovered second cellular enzyme, phospholipid hydroperoxide GSH peroxidase [9], catalyses the reduction of a broad range of substrates, including LDL-associated hydroperoxides of cholesterol, phospholipids and cholesteryl esters [10,11]. Plasma GSH peroxidase reduces hydroperoxides of non-esterified fatty acids and phospholipids, but is inactive against hydroperoxides of cholesterol and cholesteryl esters [12,13].

Ebselen, a low-molecular-mass mimic of GSH peroxidase [14–16], may be of use in the plasma antioxidant defence against lipid peroxidation. Recent work showed that, in the presence of GSH, ebselen is capable of preventing copper-dependent LDL oxidation [17] and of reducing high-density-lipoprotein- and LDL-associated cholesteryl ester and phospholipid hydroper-

oxides [11]. At least in the case of cholesteryl ester hydroperoxides, it was shown that ebselen reduces the hydroperoxides to the corresponding hydroxides [11], as is expected from the known chemistry of ebselen [16]. However, as plasma GSH has a short half-life, and its steady-state concentration is low (about 1–2 µM [18]), the question arises as to how plasma GSH peroxidase or ebselen might be provided with sufficient reducing equivalents.

The availability of ebselen and its state of binding in plasma are also relevant. Upon administration, extracellular ebselen is present as an albumin complex [19,20], presumably as an inactive selenodisulphide, linked to the reactive cysteine residue on albumin. We therefore examined the peroxidase activity of albumin-bound ebselen in human plasma and whole blood, and observed that albumin–ebselen efficiently reduced LDL-associated cholesteryl ester hydroperoxides in the presence, but not in the absence, of blood cells.

MATERIALS AND METHODS

Materials

The azo initiators 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis-(2,4-dimethylvaleronitrile) (AMVN) were purchased from Polysciences (Warrington, PA, U.S.A.), Chelex-100 was from Bio-Rad Laboratories (Richmond, CA, U.S.A.), the PD-10 Sephadex G-25 M columns were from Pharmacia LKB Biotechnology (Uppsala, Sweden), dithiothreitol (DTT) was from Fluka (Buchs, Switzerland), BSA was from Sigma, and cholesteryl linoleate hydroxide (Ch 18:2-OH) was from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Cholesteryl linoleate hydroperoxide (Ch 18:2-OOH) standard was prepared from purified cholesteryl linoleate (Sigma) by

Abbreviations used: AAPH, 2,2'-azobis-(2-amidinopropane) dihydrochloride; AMVN, 2,2'-azobis-(2,4-dimethylvaleronitrile); Ch 18:2-OH, cholesteryl linoleate hydroxide; Ch 18:2-OOH, cholesteryl linoleate hydroperoxide; DTT, dithiothreitol; LDL, low-density lipoprotein(s); LDL_{ox}, mildly oxidized LDL.

‡ To whom correspondence should be addressed.

AMVN oxidation [21]. Ebselen was generously given by Rhône-Poulenc–Nattermann (Cologne, Germany). All other chemicals were supplied by Merck. Ethanol (Analytical Reagent) was obtained from BDH (Kilsyth, Vic., Australia), while all other organic solvents were from Mallinckrodt (Clayton, Vic., Australia) and of h.p.l.c. quality. All aqueous solutions were stored over Chelex-100 to remove trace-metal contamination before use [22].

Preparation of BSA-bound ebselen

Ebselen, dissolved in dimethylformamide at a final concentration of 4 mM, was incubated for 15 min at 37 °C with BSA (580 μ M in water). Following incubation, unbound ebselen was removed by passing the mixture over a PD-10 column and collecting the protein fraction. The absorption spectrum of the complex was then taken to determine the ebselen concentration, an ϵ_{330} value of $\sim 7700 \text{ M} \cdot \text{cm}^{-1}$ (C. E. Thomas, personal communication) being used.

Isolation of LDL

Venous blood, anticoagulated with EDTA (1 mg/ml), was obtained from healthy male or female donors (aged 22–40 years) and the plasma separated from blood cells by centrifugation for 10 min at 4 °C and at 500 *g*. The density of the plasma was then adjusted to 1.21 g/ml by addition, and dissolving, of the appropriate amount of solid KBr, and the LDL were isolated by two-step density-gradient ultracentrifugation in a TL-100 tabletop centrifuge equipped with a TL100.4 rotor (Beckman) [21]. LDL bands were aspirated, pooled, then passed through a PD-10 size-exclusion column, equilibrated with PBS, pH 7.4 (50 mM in phosphate), to remove the KBr and contaminating aqueous antioxidants. The lipoprotein concentration was estimated by protein determination [23].

Peroxy-radical-mediated oxidation of isolated LDL

Mild and controlled oxidation of the isolated gel-filtered LDL was achieved by incubation of the lipoprotein (~ 0.5 – 1.0 mg of protein/ml) at 37 °C for 2 h in the presence of the aqueous peroxy-radical generator AAPH (5 mM). Following oxidation, AAPH was removed from the mildly oxidized LDL (LDL_{ox}) by gel filtration using two consecutive PD-10 columns. Preliminary experiments showed that such treatment resulted in the complete removal of AAPH, as indicated by the absence of accelerated peroxidation upon incubation of LDL_{ox} at 37 °C. Typically, the LDL_{ox} obtained contained some 10–20 molecules of cholesteryl ester hydroperoxides/lipoprotein particle, of which the great majority were Ch18:2-OOH [24]. In agreement with our recent findings, LDL_{ox} also contained significant amounts of Ch18:2-OH, as, in isolated transition-metal-free LDL, Ch18:2-OOH is converted slowly into Ch18:2-OH [25].

Incubation of LDL_{ox} with human plasma or whole blood

LDL_{ox} (1 vol.), prepared as described above, was added to 1 vol. of fresh human EDTA-treated blood or plasma derived from it (corresponding to a final LDL_{ox} concentration of 0.2–0.4 mg of protein/ml). The resulting blood or plasma was incubated at 37 °C in the absence or presence of added albumin-bound ebselen. The final concentration of ebselen was 5 μ M unless stated otherwise. At the time points indicated, an aliquot of whole blood or plasma was withdrawn, the cells removed by centrifugation (10000 *g*, 5 min, room temperature) where required, and the samples analysed for Ch18:2-OOH and Ch18:2-OH as described below. For some experiments, LDL_{ox} and BSA–ebselen

in buffer were placed in a dialysis bag (molecular-mass cut-off 10 kDa, and sealed with a removable clip) that itself was added to whole blood and incubated at 37 °C. At various times an aliquot of LDL_{ox} was removed from the dialysis bag and the (intact) dialysis bag placed back into the blood. LDL_{ox} aliquots were extracted and analysed as described below.

Reversed-phase h.p.l.c. analysis of Ch18:2-OOH and Ch18:2-OH

Lipoprotein or plasma samples (1 vol.) were extracted with cold methanol (10 vol.) and hexane (50 vol.); the resulting organic phase was removed, evaporated under reduced pressure and resuspended in ethanol. Where samples contained whole blood, cells were removed first by centrifugation in an 5415C Eppendorf centrifuge (8000 *g* for 5–10 min at room temperature). Analysis of Ch18:2-OOH and Ch18:2-OH was performed on a 25.0 cm $\times$ 0.46 cm LC-18 column (Supelco, Bellefonte, PA, U.S.A.) eluted with acetonitrile/propan-2-ol/water (11:27:1, by vol.) at 1 ml/min and the eluate monitored at 234 nm [25]. Retention times for Ch18:2-OOH and Ch18:2-OH standards were 14.3 and 16.2 min respectively. Quantification was by comparison of the peak areas of authentic standards of Ch18:2-OOH and Ch18:2OH.

Analysis of free ebselen and ebselen–DTT adduct by h.p.l.c.

To facilitate dissociation of ebselen from its BSA complex, DTT (or buffer for the control sample) was added at a final concentration of 10 mM to a solution of BSA–ebselen (0.5 mM BSA and 0.4 mM ebselen) and incubated at 37 °C for 5 min. After incubation the aqueous samples were extracted with 5 vol. of ethyl acetate, the upper phase removed, dried under reduced pressure and resuspended in dimethylformamide. Analysis was carried out by h.p.l.c. on a 25.0 cm $\times$ 0.46 cm LC-18 column (Supelco) eluted at 1 ml/min with methanol/water/acetic acid (12:7:1, by vol.), a mobile phase previously described for the successful separation of ubiquinol-1 and ubiquinone-1 [27]. The eluant was monitored using a 1000S diode-array detector (Applied Biosystems) set at 280 and 320 nm and continuous spectral monitoring. Free ebselen was eluted at 6.2 min and the DTT adduct at 8.1 min. Peak identification was by comparison of the elution times and spectra of the eluting compounds with those of standard solutions.

RESULTS

Reduction of cholesteryl ester hydroperoxides in oxidized LDL by albumin-bound ebselen

Fresh human blood plasma used for these studies contains low-nanomolar concentrations of endogenous Ch18:2-OOH [28], while endogenous Ch18:2-OH was not detected (results not shown). Therefore the micromolar concentrations of Ch18:2-OOH and Ch18:2-OH measured in plasma upon addition of LDL_{ox} were derived almost exclusively from exogenous LDL_{ox}. LDL_{ox}-associated Ch18:2-OOH was reduced stoichiometrically to the corresponding Ch18:2-OH by BSA–ebselen when incubated in blood (Figure 1a), but not when incubated in plasma (Figure 1b) or buffer (results not shown). Separate experiments using a straight-phase h.p.l.c. assay for the detection of Ch18:2-OOH and Ch18:2-OH also showed a stoichiometric formation of the hydroxides from the corresponding hydroperoxides (results not shown), thereby confirming the results obtained with the reversed-phase h.p.l.c. method. Incubation of LDL_{ox} in whole blood for 30 min in the absence of added BSA–ebselen resulted in neither significant loss of Ch18:2-OOH nor the formation of

Figure 1 Conversion of Ch 18:2-OOH into Ch 18:2-OH during incubation of LDL_{ox} in whole blood, but not plasma, containing BSA-ebesen

LDL_{ox} (0.2–0.4 mg of protein/ml), prepared as described in the Materials and methods section, was incubated at 37 °C in either whole blood (a) or plasma (b) in the absence (open symbols) or presence (closed symbols) of 5 μM BSA-ebesen. At the time points indicated, 200 μl aliquots were removed, extracted and analysed for Ch 18:2-OOH (triangles) and Ch 18:2-OH (diamonds) as described in the Materials and methods section. The results shown are means of duplicate determinations with the variation indicated by the vertical bars.

Ch 18:2-OH (Figure 1a), demonstrating that ebisen was required for the reduction. When low-molecular-mass thiol was provided in the form of GSH or DTT to the samples incubated in plasma, there was reduction of the hydroperoxides (results not shown), in agreement with previous work on isolated LDL incubated in buffer alone [11]. DTT and GSH were able to displace ebisen from BSA; while the former appeared to form an adduct with the released ebisen, shifting the retention time and altering the absorption spectrum, GSH did not appear to form a complex with the displaced ebisen, as judged by unaltered retention time and spectrum of ebisen (results not shown).

Incubation of LDL_{ox} for 10 min in whole blood with increasing concentrations of BSA-ebisen up to 25 μM ebisen resulted in decreasing ratios of Ch 18:2-OOH to Ch 18:2-OH (Figure 2a). As can be seen from Figure 2(b), the higher the ebisen concentration the higher the initial rate of conversion of LDL Ch 18:2-OOH into Ch 18:2-OH. At higher BSA-ebisen concentrations, maximal hydroxide formation was observed within 5 min of incubation. Concomitant with Ch 18:2-OH formation was a corresponding loss of Ch 18:2-OOH (Figure 2 and results not shown).

Figure 2 Conversion of Ch 18:2-OOH into Ch 18:2-OH during incubation of LDL_{ox} in whole blood containing increasing amounts of BSA-ebesen

(a) LDL_{ox} (0.2–0.4 mg of protein/ml), prepared as described in the Materials and methods section, was incubated for 10 min at 37 °C in whole blood containing the indicated concentrations of BSA-ebisen. After incubation, aliquots (200 μl) were removed, extracted and analysed for Ch 18:2-OOH (black columns) and Ch 18:2-OH (hatched columns) as described in the Materials and methods section. Similar results were obtained in two separate experiments, and means are shown with the range indicated by the vertical bars. (b) Time course of Ch 18:2-OH formation from LDL_{ox} (0.2–0.4 mg of protein/ml) incubated for the time indicated at 37 °C in whole blood containing increasing concentrations of BSA-ebisen. The concentrations of ebisen used were 0 (○), 1.7 (◆), 5 (●), and 25 μM (■). The results shown are those obtained from a single experiment; two separate experiments gave qualitatively similar results.

Provision of reducing equivalents for the GSH peroxidase mimic reaction by human blood cells

In order to decide whether the reducing equivalents provided by the incubation in blood, and absent from the incubation in plasma, were diffusible, LDL_{ox} was placed in a dialysis bag with or without BSA-ebisen and then incubated in whole human blood. Using a dialysis bag containing 5 μM BSA-ebisen and LDL_{ox} corresponding to $26 \pm 7 \mu\text{M}$ ($n = 3$) Ch 18:2-OOH and $3.7 \pm 1.6 \mu\text{M}$ ($n = 3$), more Ch 18:2-OH was formed within 20 min than in the corresponding ebisen-free controls (Figure 3). At 25 μM initial BSA-ebisen concentration, a further 15 μM Ch 18:2-OH was produced. The amount of Ch 18:2-OH formed at 5 μM BSA-ebisen in these experiments was comparable with that seen at approx. 2 μM ebisen in the whole-blood experiments

Figure 3 Formation of Ch18:2-OH in LDL_{ox} incubated with increasing amounts of BSA-epselen during dialysis against whole blood

A solution of LDL_{ox} (0.4–0.8 mg of protein/ml) prepared as described in the Materials and methods section, containing no (○), 5 μM (●), or 25 μM (■) BSA-epselen, was dialysed against fresh whole blood for 30 min at 37 °C. At the time points indicated, an aliquot of LDL_{ox} was removed, extracted and analysed as described in the Materials and methods section. The results shown are representative of three (5 μM epselen) and two (25 μM epselen) separate experiments.

(Figure 2), possibly as the result of loss of some epselen through the dialysis membrane. This effect was less pronounced at 25 μM BSA-epselen, where the reduction was more rapid, before significant loss of epselen could occur. In all of these dialysis-bag experiments we observed a greater loss of Ch18:2-OOH than formation of Ch18:2-OH. The reason(s) for this are unknown at present, although the results do permit the conclusion that reducing diffusible equivalents were provided by the blood cells to the interior of the dialysis bag to drive the peroxidase mimic reaction.

DISCUSSION

The present results show that while fresh human plasma is incapable of providing sufficient reducing equivalents to drive the GSH-peroxidase-mimic reaction of albumin-bound epselen, presumably by displacing epselen, whole human blood is. Thus there is a cellular process providing reducing equivalents for the reduction of LDL-associated Ch18:2-OOH to Ch18:2-OH. This reduction was mediated by epselen rather than plasma GSH peroxidase, as, in the absence of the former, hydroperoxides were not reduced. These results indicate that human plasma GSH peroxidase is unable to use lipoprotein-associated cholesteryl ester hydroperoxides as substrates, as has been shown recently for the rat enzyme [13].

The results from the dialysis-bag experiments demonstrate that the reducing equivalents provided by the cells were diffusible and of low molecular mass. Many cells, including blood cells, do release various types of reductants, thereby providing a reducing extracellular environment (see, e.g., [29]). Such provision of reducing equivalents may increase under conditions of oxidative stress [30,31]. In the case of epselen-mediated reduction of LDL cholesteryl ester hydroperoxides, a likely reductant is GSH. As calculated from the slope in Figure 1, and correcting for the 1:1 (v/v) dilution of the blood with LDL_{ox}, the cells of human blood can support a rate of thiol-dependent hydroperoxide reduction of about 0.7 nmol/min per ml. As Figure 2 suggests, this may not be the maximum rate, as the rate observed with 25 μM BSA-

epselen was even higher. The use of yet higher epselen concentrations was not considered, as they may not be achieved readily following epselen administration [19].

The data also suggest that only free epselen is capable of carrying out the reduction of LDL-associated cholesteryl ester hydroperoxides, as reported in [11], and that the albumin-bound form of the drug is the one present in plasma [19,20]. The observation that a thiol-dependent reaction can be supported in human plasma by the cellular components of blood (Figure 1) is of general interest. It may explain the recently reported finding that oral administration of epselen to rats decreased their plasma levels of endogenous phosphatidylcholine hydroperoxides [32], suggesting that our *in vitro* observations with human blood are relevant to the *in vivo* situation. The support of thiol-dependent reaction(s) in plasma by cells may also be relevant for plasma GSH peroxidase, an enzyme requiring GSH for activity. Both white blood cells [33] and erythrocytes are candidate cells for providing extracellular thiols. This activity will be of interest in certain clinical conditions involving extra flux through the plasma GSH system, e.g. in inflammation, shock, acquired immunodeficiency syndrome and atherosclerosis (see e.g. [34–36]).

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