Efflux of hepatic ascorbate: a potential contributor to the maintenance of plasma vitamin C

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Ascorbate (AH, the reduced form of vitamin C) is an important radical scavenger and antioxidant in human plasma; the resulting ascorbyl radical can disproportionate to AH and dehydroascorbic acid (DHA). Here we address potential maintenance mechanism(s) for extracellular AH by examining the ability of cells to convert extracellularly presented DHA to AH. DHA was rapidly transported into human liver (HepG2), endothelial and whole blood cells *in vitro* by plasma membrane glucose transporters and reduced intracellularly. Liver cells displayed the highest capacity to release the intracellularly accumulated AH. The proteins responsible for DHA uptake and AH release could be distinguished by inhibitor studies. Thus, unlike DHA uptake, AH efflux was largely insensitive to cytochalasin B and thiol-reactive agents but was inhibited by phloretin, 4,4'-di-iso-

thiocyanostilbene-2,2'-disulphonate and isoascorbate. Efflux of AH from cells was temperature-sensitive and saturable with a low affinity (millimolar, intracellular) for AH. In addition to isolated liver cells, perfusion of intact rat and guinea-pig liver with DHA resulted in AH in the circulating perfusate. Our results show that hepatocytes take up and reduce DHA and subsequently release part of the AH formed, probably via a membrane transporter. By converting extracellular DHA to extracellular AH, the liver might contribute to the maintenance of plasma AH, a process that could be important under conditions of oxidative stress.

Key words: antioxidant, liver, metabolism, transport.

INTRODUCTION

The reduced form of vitamin C, ascorbate (AH), in addition to being essential for various hydroxylation reactions, has a major role as a powerful, water-soluble, non-enzymic antioxidant [1]. AH can reduce a variety of oxidizing species, including alkoxyl and peroxy radicals, to eliminate harmful free-radical reactions in aqueous compartments within and outside cells [2]. AH also efficiently maintains α -tocopherol (vitamin E) *in vitro* by reducing α -tocopheroxyl radicals [3]. Thus AH effectively protects plasma lipids against peroxidation by reducing both aqueous [4] and lipid-associated radicals [5]. Extracellular reduction and elimination of reactive oxygen species by AH might be important in several diseases such as atherosclerosis, in which lipoprotein lipid peroxidation is thought to be an important early event [6].

Ascorbyl radical, the one-electron oxidation product of AH, disproportionates to dehydroascorbic acid (DHA) and AH. Increased plasma levels of DHA, the two-electron oxidation product of AH, have been reported in diseases such as diabetes [7] and might be formed under acute inflammatory conditions in which phagocytes become activated and release oxidants [8]. However, in normal subjects, plasma vitamin C is primarily found in its fully reduced, antioxidant form (AH) [9]. Thus it seems that, under normal physiological conditions, effective mechanism(s) exist that eliminate DHA. Whether the formation and subsequent elimination of DHA is coupled to a replenishment of AH remains largely unknown. A number of potential recycling mechanisms for extracellular AH have been described. These include the disproportionation [1] or extracellular reduction [10] of ascorbyl radicals via a transplasma membrane electron transport system [11], and the recycling of AH by human blood cells

[12]. Such mechanisms might be important in humans, incapable of synthesizing vitamin C.

In addition to the above recycling pathways, the liver might also contribute to extracellular AH. Thus, as the primary tissue for AH formation in mammals that synthesize vitamin C, the liver is likely to contain one or more mechanisms to release AH. In support of this, increased plasma AH levels are found in mice after intravenous injection of bolus DHA, whereas the addition of a comparable amount of DHA to murine whole blood does not alter plasma AH [13]. We hypothesized that the liver, although incapable of vitamin C synthesis in humans, might retain a mechanism that allows AH secretion into plasma, i.e. it performs a (re)absorption function. To test this, we examined various cells for their ability to form AH extracellularly after incubation with DHA. Here we show that hepatocytes have the capacity to release AH in its reduced form, most probably via a membrane protein distinct from the glucose/DHA transporter.

MATERIALS AND METHODS

Materials

AH, isoascorbate, D,L-homocysteine, dithiothreitol, EDTA disodium salt, cytochalasins B and D, phloretin, 4,4'-di-iso-thiocyanostilbene-2,2'-disulphonate (DIDS), *N*-ethylmaleimide, *p*-chloromercuribenzyldisulphonate (*p*-CMBS), Hanks balanced salt solution (HBSS, with 5 mM D-glucose, lacking Ca²⁺, Mg²⁺, bicarbonate and Phenol Red, pH 7.4), choline chloride, dibutylphthalate and phthalic acid bis-(2-ethylhexylester) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DHA was purchased from ICN Biomedicals (Costa Mesa, CA, U.S.A.). L-

Abbreviations used: AH, ascorbate; DHA, dehydroascorbic acid; DIDS, 4,4'-di-iso-thiocyanostilbene-2,2'-disulphonate; HBSS, Hanks balanced salt solution; HUVEC, human umbilical-vein endothelial cells; PBMC, peripheral blood mononuclear cells; *p*-CMBS, *p*-chloromercuribenzyldisulphonate; PMN, polymorphonuclear leucocytes.

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[carboxyl- ^{14}C]AH (622 MBq/mmol), $^3\text{H}_2\text{O}$ (37 MBq/ml) and [U- ^{14}C]sucrose (22.8 GBq/mmol) were from Amersham (Little Chalfont, Bucks., U.K.). [^{14}C]DHA was prepared by exposure of [^{14}C]AH to bromine and typically converted at least 80% AH to DHA. Subsequently, the sample was flushed with nitrogen to eliminate excess bromine. Q12 ion-pair cocktail was from Regis Chemical Co. (Morton Grove, IL, U.S.A.) and sodium acetate was from Merck (Darmstadt, Germany). All solvents were of HPLC grade (Malinkrodt, Victoria, Australia); Ultima-Gold and Ultima-Flow M scintillants were from Packard Instruments (Meriden, CT, U.S.A.). Before use, nanopure water (Millipore Systems, Australia) and buffers were treated with Chelex-100 resin (Bio-Rad, Hercules, CA, U.S.A.) to remove transition-metal ions, as verified by the AH auto-oxidation method [14].

Cell preparations

HepG2 (human hepatoma) cells were cultured with standard techniques in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (heat-inactivated) and 2 mM L-glutamine. Cells were maintained in 12-well plates (Falcon, Lincoln Park, NJ, U.S.A.) in air/ CO_2 (19:1) at 37 °C and used at 80% confluence or more. For some efflux experiments, non-adherent HepG2 cells were utilized: HepG2 cells, maintained in T150 cm^2 flasks (Falcon), were harvested by treatment with trypsin and washed once in serum-containing Dulbecco's modified Eagle's medium. Human umbilical-vein endothelial cells (HUVEC) were obtained from fresh tissue [15], grown to confluence in gelatin-coated 12-well plates and used on the third passage. The human endothelial cell line ECV 304 was maintained in six-well plates in M199 medium containing 10% (v/v) fetal bovine serum (heat-inactivated). All cultured cells were washed three times in HBSS before experimentation and were devoid of endogenous AH, as described for various cells maintained in minimal medium.

Whole blood was obtained from normal, healthy volunteers. Before the isolation of polymorphonuclear leucocytes (PMN) and peripheral blood mononuclear cells (PBMC), erythrocytes were sedimented with dextran sulphate (500 kDa) and the resulting supernatant was subjected to Percoll gradients to separate PMN and PBMC, as described [16]. Erythrocytes were obtained from whole blood by centrifugation (500 g, 15 min) and washing in HBSS (three times) with removal of the buffy coat between washes. The purity of the different cell populations was determined by staining (DiffQik Stain; Lab Aids, Sydney, Australia) and detected by light microscopy.

Cellular uptake of DHA and efflux of AH

Both unlabelled and ^{14}C -labelled DHA were freshly prepared, typically as a 1 mM stock solution, before addition to cells in HBSS. Unless noted otherwise, incubations were performed at 37 °C. For adherent cell experiments, DHA was added in 1 or 2 ml of HBSS to cells in twelve-well or six-well plates respectively. Aliquots of the medium were removed at various times and centrifuged (16000 g, 15 s); the supernatant was analysed for AH and DHA by HPLC (see below). For blood cell suspensions, aliquots were removed at various times and centrifuged (300 g, 5 min) to obtain a clear supernatant for analysis. To determine intracellular AH, cells were washed three times in HBSS to remove extracellular AH and DHA. Cells were extracted with ice-cold 90% (v/v) methanol/1 mM EDTA followed by centrifugation (16000 g, 2 min), as described [17]. The clear supernatant was removed and stored on solid CO_2 for less than 8 h before analysis. For some experiments, both DHA uptake and AH efflux by HepG2 cells were performed in Na^+ -deficient

medium. In such studies, HBSS was replaced with 50 mM potassium phosphate buffer, pH 7.4, containing 5 mM D-glucose and 140 mM choline chloride.

For characterization of DHA uptake and AH efflux in HepG2 cells, cells harvested by trypsin were washed and incubated with [^{14}C]DHA at 37 °C. For [^{14}C]DHA uptake, aliquots (100 μl) of the cell suspension were removed at various times and placed in an Eppendorf tube over 150 μl of a phthalate oil mixture (density 1.03 g/ml), prepared by combining dibutylphthalate and phthalic acid bis-(2-ethylhexylester) (1:1, v/v). Uptake was stopped by centrifugation (16000 g, 15 s) to separate the extracellular medium (aqueous layer above the oil layer) from the pelleted cells, as described [18]. For efflux assays, aliquots of cells (0.5–1 ml) were removed at various times, the cells were washed once in 10 vol HBSS to remove residual DHA and AH, then resuspended in HBSS to the original volume and incubated further. Aliquots (100 μl) were removed at various times and laid over the phthalate oil as described for the uptake assays, followed by centrifugation to halt efflux. Radiolabel in the aqueous layer was measured by mixing 50 μl with 5 ml of Ultima Gold scintillant followed by liquid-scintillation counting.

The total pellet volume was determined by mixing $^3\text{H}_2\text{O}$ with cells before centrifugation. To measure contaminating extracellular radiolabel, [^{14}C]sucrose was added before centrifugation and the radiolabel was extracted as follows. Triton X-100 [1% (w/v), 0.4 ml] was added to solubilize the pellet followed by ice-cold HClO_4 [5% (v/v), 0.75 ml] to precipitate cell protein. After centrifugation (16000 g, 2 min) an aliquot (1 ml) of the clear supernatant was removed and added to 5 ml of scintillant for liquid-scintillation analysis.

For kinetic analysis of AH efflux, cells (approx. $10^6/\text{ml}$) were incubated with 0–200 μM [^{14}C]DHA in HBSS for 15 min at 37 °C to allow the uptake and intracellular accumulation of [^{14}C]AH at various intracellular concentrations. Cells were washed once in 10 vol. of cold HBSS and resuspended in the original volume of warm buffer. Aliquots (100 μl) were removed immediately and centrifuged over oil to determine the initial intracellular [^{14}C]AH. The remaining cell suspensions were incubated at 37 °C for a further 15 min to allow [^{14}C]AH efflux. Efflux was halted by centrifugation of the cells over oil. Both extracellular and intracellular [^{14}C]AH were measured as described above.

Determination of DHA and AH by HPLC

Unlabelled DHA was determined indirectly by chemical reduction to AH. Freshly prepared DHA (approx. 100 μM , 100 μl in HBSS) was added to 1% (w/v) homocysteine (25 μl) and incubated at room temperature for 30 min followed by the electrochemical detection of AH as described [19]. For [^{14}C]AH and [^{14}C]DHA detection, this HPLC system was combined with UV (265 nm) and a Flo-One/Beta A-100 radiometric detector (Packard Instruments) containing a 1.5 ml flow cell. The ratio of scintillant (Ultima-Flow M) to mobile-phase flow was 4. AH concentrations were determined by a comparison of peak areas with those of authentic standards.

Liver perfusion experiments

Liver perfusions were performed by the method of [20] except that perfusion in a recirculating mode was used. Male rats (250 g, Wistar) and guinea-pigs (330 g) were used for these studies. The flow rate (35 ml/min for both species) was set so that one pass of the perfusate through the liver and tubing was completed in 1 min. [^{14}C]DHA was prepared freshly by bromine oxidation of [^{14}C]AH in nanopure, Chelex-treated water. In brief, bromine

(8 μ l, 1:8 (v/v) in nanopure water) was added to 3.3 mM [14 C]AH (in 350 μ l). This solution was mixed thoroughly, excess bromine was removed by nitrogen purging, then the solution was diluted immediately in the perfusate buffer (35 ml, Krebs–Henseleit buffer containing 5 mM D-glucose, pH 7.4, 37 °C) to yield a final concentration of 20–40 μ M [14 C]DHA. Samples of the circulating perfusate (200 μ l) were collected at various times (0–30 min) and fresh perfusate buffer (37 °C, devoid of any additives) was added to maintain a constant volume. The dilution effect was accounted for in the results. The perfusate samples were frozen immediately on solid CO₂ then maintained at –80 °C (for not more than 24 h) until analysed by HPLC with on-line radiometric detection (see above).

Transplasma membrane electron transport activity

Transplasma membrane electron transport activity in cells was assessed by the reduction of ferricyanide to ferrocyanide; ferrocyanide was determined by the method of Avron and Shavit [21]. In brief, non-adherent HepG2 cells (at least 3×10^6 cells) were incubated in 1 ml of HBSS containing 0.4 mM ferricyanide at 37 °C. After 30 min, cells were pelleted by centrifugation (16000 g, 1 min). The resulting supernatant (0.7 ml) was then mixed with 0.1 ml of 3 M sodium acetate (pH 6.5), 0.1 ml of 0.2 M citric acid, 0.05 ml of 3.3 mM FeCl₃ (in 0.1 M acetic acid) and 0.05 ml of 1% (w/v) 1,10-phenanthroline (in ethanol). The solution was incubated at 25 °C for 5 min for full colour development. Ferrocyanide was quantified spectrophotometrically from a molar absorption coefficient (ϵ_{510}) of 10 500 M⁻¹·cm⁻¹ [21].

RESULTS

In contrast with the millimolar intracellular concentration of AH in freshly isolated hepatocytes, HepG2 cells cultured under standard conditions did not contain endogenous AH. We first examined whether HepG2 cells accumulated and reduced DHA and whether part of the resulting AH was subsequently released into the extracellular space. As shown previously for other cell types (see, for example, [13,22–24]), incubation of adherent HepG2 cells *in vitro* with 80 μ M DHA at 37 °C resulted in detectable intracellular AH. The concentration of AH increased time-dependently to 5 nmol/10⁶ cells over 1 h, concurrently with a decrease in the concentration of DHA in the extracellular medium (Figure 1A). HepG2 cells incubated with DHA also released significant amounts of AH to the extracellular medium. Up to 6 μ M AH was released in 1 h, corresponding to approx. 8% of the DHA added and representing approx. 50% of the total vitamin C present both intracellularly and extracellularly at 1 h. In contrast, AH was not observed in the medium of control cells incubated in HBSS in the absence of DHA (Figure 1B) or during incubation of DHA in the absence of cells (results not shown). Cellular permeability did not change during the time course, as judged by Trypan Blue exclusion, the absence of detectable lactate dehydrogenase in the medium and a lack of [14 C]sucrose uptake by cells (results not shown).

Previous studies on the uptake of extracellular DHA and its subsequent intracellular conversion to AH have commonly used erythrocytes [13,22–24]. It has been suggested that erythrocytes release AH [22]; however, the detection of AH efflux from erythrocytes resuspended at 37 °C in HBSS was hampered by haemoglobin leakage (results not shown), as this gives rise to inadvertent oxidation of AH during sample preparation [25]. When incubated in homologous plasma at 37 °C, freshly isolated whole blood cells (white cells and erythrocytes, $[4.3 \pm 2.7(\text{S.D.})] \times 10^9$ cells/ml) released approx. 5 μ M ($n = 4$) of

Figure 1 HepG2 cells convert extracellular DHA into extracellular AH

Adherent HepG2 cells in 12-well plates ($(1 \pm 0.1) \times 10^6$ cells in 1 ml per well) were incubated with DHA (80 μ M) at 37 °C. At times, aliquots of the extracellular medium were removed; extracellular DHA (A, $\square$) and AH (B, $\bullet$) were analysed by HPLC as described in the Materials and methods section. The cells were washed three times, lysed and analysed for intracellular AH (A, $\blacksquare$). Cells not exposed to DHA did not contain endogenous intracellular AH (results not shown) and consequently did not release AH extracellularly (B, $\circ$). Results are means $\pm$ S.D. for four individual experiments.

their endogenous AH per hour. In contrast, HepG2 cells released AH at a more than 1000-fold greater rate than whole blood cells (Table 1), when compared on a cellular basis.

To determine further whether cells found in human whole blood other than erythrocytes might release intracellular AH, we incubated isolated PMN and PBMC, as well as primary (HUVEC) endothelial cells and the endothelial cell line ECV304, with DHA. When compared with HepG2 cells, these cell types released significantly less AH into the surrounding medium, although all cells accumulated AH after incubation with DHA (Table 1). The apparently high capacity of HepG2 cells to release AH compared with the other cell types could not be explained by a large variation in cell volumes (see Table 1 legend). Although AH accumulation was largest in ECV304 cells, these cells released significantly less AH than HepG2 cells. ECV304 cells did, however, seem to release greater amounts of AH than the corresponding primary cells, HUVEC. The reasons for these differences are not clear at present although it remains possible that glucose and vitamin C transport might be inherently different in the two cell types and/or potential differences exist in their ability to reduce intracellular DHA. AH was stable in HBSS in the presence of all cells for at least 4 h at 37 °C, demonstrating that the absence of extracellular AH was not due to its auto-oxidation. In addition, all cells (except erythrocytes in buffer alone), remained viable (at least 95%) for up to 3 h at 37 °C in

Table 1 Human liver cells efficiently maintain AH extracellularly

Human PMN and PBMC separated from whole blood, and HepG2, HUVEC and ECV304 cells, were incubated with DHA (55–110 μM) in HBSS at 37 °C. After 1 h, extracellular and intracellular AH were determined by HPLC as described in the Materials and methods section. The initial DHA concentrations (in μM) incubated with the individual cell types are shown below. The individual cell volumes ranged from approx. 300 fl (HepG2, ECV304 and HUVEC) to approx. 450 fl (PMN and PBMC). Results are means $\pm$ S.D. for four individual experiments for each cell type. PMN and PBMC were at least 95% pure.

Cell type	Initial [DHA] (μM)	$10^{-6} \times$ Cell density (ml^{-1})	Extracellular AH (μM)	Intracellular AH (nmol/ 10^6 cells)	Extracellular AH (nmol/ 10^6 cells)
HepG2	70	0.80 ± 0.10	5.65 ± 0.50	5.04 ± 2.32	7.06
PMN	67	14.8 ± 9.2	0.55 ± 0.36	0.38 ± 0.25	0.04
PBMC	66	1.88 ± 1.17	0.82 ± 0.57	0.57 ± 0.40	0.44
HUVEC	55	0.77 ± 0.31	0.24 ± 0.17	1.42 ± 1.34	0.31
ECV304	113	0.50 ± 0.08	0.92 ± 0.37	34.9 ± 8.5	1.84

Figure 2 DHA uptake by glucose transporters is required for DHA reduction by, and AH efflux from, HepG2 cells

Non-adherent cells [$(1 \pm 0.1) \times 10^6/\text{ml}$] were incubated with [^{14}C]DHA (80 μM) in the absence (solid lines) or presence (broken lines) of 20 μM cytochalasin B at 37 °C. At various times aliquots were removed and the cells were pelleted through oil. Extracellular [^{14}C]DHA (A), intracellular [^{14}C]AH (B), extracellular [^{14}C]AH (C), and total (intracellular plus extracellular) vitamin C (D) were determined by HPLC with on-line radiometric detection as described in the Materials and methods section. [^{14}C]DHA was not found intracellularly at any time point. Results are means $\pm$ S.D. for two individual and duplicate experiments.

the presence of 50 μM DHA in HBSS as judged by Trypan Blue exclusion (results not shown).

As liver cells released most AH, HepG2 cells were used for further studies of the putative efflux mechanism involved. We first explored the possibility that extracellular AH was produced by extracellular reduction of DHA or ascorbyl radical. For this,

non-adherent HepG2 cells were incubated with [^{14}C]DHA in the presence or absence of cytochalasin B, a potent inhibitor of glucose [26] and DHA transport in mammalian cells (Figure 2). Extracellular [^{14}C]DHA was transported rapidly into HepG2 cells in the absence of cytochalasin B (Figure 2A) and significant amounts of [^{14}C]AH accumulated intracellularly (Figure 2B). In

Table 2 Cytochalasin B does not affect the transplasma electron transport system in HepG2 cells

HepG2 cells were pretreated with or without 20 μM cytochalasin B for 15 min at 37 $^{\circ}\text{C}$ in 1 ml of HBSS. Ferricyanide was then added (0.4 mM final concentration) and reduction of this reagent was measured as described in the Materials and methods section. Results are means $\pm$ S.D. derived from two experiments with separate cell preparations and performed in duplicate and triplicate respectively.

$10^{-6} \times \text{Cell density}$ (ml^{-1})	Ferricyanide reduced (nmol/ 10^6 cells)	
	Control	+ 20 μM cytochalasin B
3	10.8 $\pm$ 0.1	11.1 $\pm$ 0.9
22.5	4.5 $\pm$ 0.3	4.4 $\pm$ 0.1

the presence of cytochalasin B the rate of intracellular [^{14}C]AH accumulation decreased markedly (Figure 2B) and correspondingly larger amounts of [^{14}C]DHA remained present extracellularly (Figure 2A). This suggested an effective blockage of DHA uptake by cytochalasin B. Concomitantly with this, the presence of cytochalasin B also strongly inhibited the accumulation of AH outside HepG2 cells (Figure 2C), suggesting that, at least under the experimental conditions used, significant extracellular reduction of DHA to AH by HepG2 cells did not take place. At pH 7.4, DHA is unstable at 37 $^{\circ}\text{C}$ and in the absence of cellular uptake (i.e. in the presence of cytochalasin B) it degraded extracellularly to an unknown, single product that was not taken up by HepG2 cells and accounted for the apparent loss of stoichiometry in Figure 2(D).

Although the above results suggest that DHA uptake was required for extracellular AH accumulation, the possibility nevertheless remained that cytochalasin B disrupted the transplasma membrane electron transport system [11] that might transduce extracellular reduction. The activity of transplasma membrane electron transport in HepG2 cells was therefore determined by the rate of ferricyanide reduction, as described previously [11]. In contrast with its effects on DHA uptake, cytochalasin B had no effect on extracellular ferricyanide reduction by HepG2 cells (Table 2), as has been demonstrated for HL-60 cells [27] and erythrocytes [28].

Together the above experiments established that liver cells were capable of releasing AH. To exclude the possibility that this was due to an inherent property of immortal cell lines and also to relate the observations *in vitro* to physiology, we performed liver perfusion experiments *in situ* with rat and guinea-pig livers. In contrast with rats, guinea-pigs (and humans) are unable to synthesize AH *de novo*. [^{14}C]DHA (43 μM) added to the buffer perfusing rat liver disappeared rapidly (Figure 3A). After this, 2.3 μM [^{14}C]AH, corresponding to approx. 5% of the added [^{14}C]DHA, appeared in the perfusate over 30 min (Figure 3A). In addition to radiolabelled AH, comparably larger amounts of unlabelled, endogenous AH were secreted from rat liver when perfused with buffer in the presence or absence of [^{14}C]DHA (Figure 3B). Similarly to the rat, guinea-pig liver released [^{14}C]AH (Figure 4A) and considerable amounts of unlabelled AH (Figure 4B) after perfusion with 19 μM [^{14}C]DHA. These results demonstrate that AH release from the liver can be observed, independently of whether the animal is capable of vitamin C synthesis. Bile samples obtained from each species contained neither AH nor radioactivity (results not shown).

The transport of DHA into, and AH efflux from, HepG2 cells was characterized further. Uptake and release of the two forms

Figure 3 AH appears in the circulating perfusate of rat liver after a bolus of DHA

Rat liver was perfused *in situ* with Krebs–Henseleit buffer containing [^{14}C]DHA ($43.1 \pm 3.4 \mu\text{M}$) for 30 min at 37 $^{\circ}\text{C}$. (A) The concentrations of [^{14}C]DHA ($\square$) and [^{14}C]AH ($\bullet$) in the perfusate were determined by removing aliquots (200 μl) at various times for analysis by HPLC with on-line radiometric and UV detection. (B) Unlabelled endogenous AH in the perfusate before ($\square$) and after ($\blacktriangle$) the addition of [^{14}C]DHA. Results are means and extents of variation (error bars) for two separate animals.

of vitamin C were found to be differentially affected by various reagents (Table 3), suggesting two separate transport mechanisms. In agreement with previous studies [29], DHA uptake was inhibited by reagents known to block mammalian glucose transport, including cytochalasin B and phloretin (Table 3). Importantly, cytochalasin D did not affect transport, precluding a non-specific interaction of the cytochalasins with the cell cytoskeleton to affect DHA uptake or AH release. *N*-Ethylmaleimide and *p*-CMBS strongly inhibited DHA transport, probably by reacting with sensitive thiols of the glucose transporter (Table 3). Inhibition of DHA uptake was determined as a decrease in intracellular AH accumulation compared with control cells; this method might inadvertently measure an effect on intracellular DHA reduction. However, none of the reagents used inhibited DHA reduction by HepG2 cell lysates (results not shown).

In contrast with DHA uptake, cytochalasin B had little effect on AH release from cells (Table 3). Phloretin significantly inhibited AH efflux, whereas AH release from cells seemed to be relatively insensitive to *N*-ethylmaleimide (permeable) or *p*-CMBS (impermeable), both of which blocked DHA transport by more than 95%. These results suggest that AH efflux probably does not involve sensitive thiol groups and is different from the

Figure 4 AH is released from guinea-pig liver after a bolus of DHA

The experiment was performed as described for rat liver in Figure 3 except that $19 \pm 1.5 \mu\text{M}$ [^{14}C]DHA was added to the perfusion buffer. The symbols are as described in the legend to Figure 3. Results are means and extents of variation (error bars) for two separate animals.

glucose transporter. DIDS, which interacts with anion transporters [30], inhibited AH release from cells. Dissipation of the plasma membrane Na^+ gradient by ouabain or deletion of Na^+

Figure 5 AH efflux from HepG2 cells is temperature-sensitive

Non-adherent HepG2 cells [$(2.5 \pm 0.7) \times 10^6$ in 1 ml] were incubated with [^{14}C]DHA ($100 \pm 15 \mu\text{M}$) for 15 min at 37 °C. Cells were then washed three times with cold HBSS, resuspended in two equal portions (2 ml) of cold buffer and incubated further at 37 °C (●) or 4 °C (○). Aliquots (100 μl) were removed at various times and AH efflux was halted by pelleting the cells through an oil layer as described in the Materials and methods section. Extracellular [^{14}C]AH in the aqueous layer was determined by liquid-scintillation analysis. Results are means $\pm$ S.D. for three individual, duplicate assays.

from the medium had little effect on DHA uptake and AH efflux (Table 3, and results not shown), suggesting that neither transport process was Na^+ -dependent. Isoascorbate, the stereoisomer of AH, had little effect on DHA uptake but inhibited AH release (Table 3). 6-Chloroascorbate, an analogue previously shown to inhibit AH transport effectively [24], affected only DHA uptake. None of the reagents tested significantly altered the stability of AH at 37 °C in buffer devoid of cells (results not shown).

The differential effect on AH release from HepG2 cells by inhibitors suggested the involvement of a facilitated transporter. To characterize this putative transport mechanism, the temperature dependence of AH efflux by HepG2 cells pretreated with DHA was tested. AH release was significantly lower at 4 °C than at 37 °C (Figure 5), suggesting facilitated, but not energy-

Table 3 Effect of various reagents on DHA uptake and AH release from HepG2 cells

For DHA uptake studies, various reagents were preincubated with cells (approx. 10^6 in 1 ml of HBSS) for 15 min at 37 °C. DHA (80 μM) was then added and uptake was monitored by analysing the intracellular AH levels at 30 min as described in the Materials and methods section. AH release from cells was performed by preincubating the cells with DHA (80 μM) for 30 min at 37 °C followed by three washes to remove residual DHA and any AH formed extracellularly. Inhibitors were then added to the cells (in 1 ml of HBSS) and extracellular AH was determined after 15 min at 37 °C. Results are representative of means $\pm$ S.D., derived from four individual experiments with different cell preparations. Control cells were incubated with DHA for uptake and efflux studies as described above but were not treated with any other reagent. The 100% values shown represent DHA transport and AH release in control cells.

Reagent	Reagent concentration (μM)	DHA uptake (%)	AH efflux (%)
Control		100	100
Cytochalasin B	20	13.8 ± 0.6	89.0 ± 6.7
Cytochalasin D	20	107 ± 3.9	87.4 ± 3.1
Phloretin	500	4.4 ± 0.2	25.7 ± 7.1
<i>p</i> -CMBS	500	17.4 ± 0.7	130.5 ± 25.7
<i>N</i> -Ethylmaleimide	200	4.3 ± 0.2	62.9 ± 5.7
DIDS	500	83.0 ± 1.5	45.8 ± 14.0
Ouabain	500	80.2 ± 1.2	73.1 ± 13.6
Isoascorbate	500	85.5 ± 2.9	37.9 ± 7.0
6-Cl-ascorbate	500	61.9 ± 0.6	89.5 ± 3.5

Figure 6 Kinetic analysis of AH efflux from HepG2 cells

Non-adherent cells were incubated with various concentrations of [14 C]DHA (0–200 μ M) for 15 min at 37 °C to achieve various intracellular [14 C]AH concentrations. The cells were washed three times in cold HBSS and resuspended in HBSS. Aliquots (50 μ l) were removed immediately (zero time), and after incubation for 15 min at 37 °C, from the extracellular medium and the cells were pelleted through an oil layer. Extracellular [14 C]AH was determined from the aqueous (upper) layer by liquid-scintillation analysis. Intracellular [14 C]AH at zero time was determined by analysing radiolabel in the cell pellet as described in the Materials and methods section. Results are derived from two separate experiments, performed in triplicate with two individual cell preparations [$(0.64 \pm 0.1) \times 10^6$ cells/ml].

dependent (Table 3), transport into the extracellular medium. In addition, the rate of AH efflux from HepG2 cells containing various intracellular concentrations of AH was saturable with a K_m of 2.4 mM (intracellular) and a V_{max} of 0.17 μ M AH released/min (Figure 6).

DISCUSSION

This study addressed whether cells present in, or exposed to, plasma vitamin C could facilitate the maintenance of the vitamin in its reduced, antioxidant-active form by the release of cellular AH. HepG2 cells (hepatocytes) released AH after incubation with the two-electron oxidized form of the vitamin, DHA. In contrast, various cells associated with whole blood (PMN, PBMC and endothelial cells) were significantly less effective in maintaining extracellular AH. Further, perfusion studies *in situ* revealed that livers obtained from both rats and guinea-pigs released AH into the circulating perfusate. In agreement with previous studies [29], DHA uptake was mediated by glucose transporters in HepG2 cells. However, AH release from HepG2 cells seemed to be mediated by a separate temperature-sensitive mechanism that was susceptible to inhibition by phloretin and DIDS but was not affected by the glucose transport inhibitor cytochalasin B. Together, the results demonstrate that hepatocytes convert extracellular DHA into extracellular AH and that AH efflux probably involves facilitated transport. The results presented here suggest that the liver might be involved in the maintenance of extracellular AH.

Our findings, that liver cells and intact liver accommodate a mechanism to export AH, are novel. Although vitamin C synthesis, where it occurs in mammals, is performed in the liver, a transport mechanism that mediates AH efflux from this organ for delivery to other tissues has not been characterized. Despite this it is not unreasonable to suggest that such a mechanism exists and remains even in hepatocytes of non-synthesizing species. Indeed, we were able to confirm that AH efflux from human hepatocytes and intact liver of the rat (synthesizing) or

the guinea-pig (non-synthesizing) occurs. Together with (re)absorptive processes, AH efflux from the liver is likely to be important in overall AH homeostasis in all species. In this context, it might be interesting to test whether liver disease and/or partial hepatectomy adversely affect whole-body vitamin C levels.

In comparison with the liver, the transport of vitamin C in organs important for (re)absorption is well understood (reviewed in [31,32]). Thus transport of AH across brush-border membranes in both intestinal and renal epithelia is Na^+ -dependent, whereas AH efflux across the corresponding basolateral membrane is independent of Na^+ ions. In agreement with this, the AH efflux described here was also found to be Na^+ -independent, because ouabain, which dissipates Na^+ gradients in cells, and the deletion of Na^+ from the medium by replacement with choline ions (Table 3, and results not shown), were without effect on AH efflux from HepG2 cells. Our results, and those of others (reviewed in [31,32]), suggest that AH uptake and efflux from cells are mediated via distinct membrane transport systems.

At physiological pH, AH is present predominantly as an anion; there is little evidence that either DHA or AH traverses cell membranes via simple diffusion. As mentioned above, in cells in which AH efflux has been studied (intestinal and renal epithelia), transport has been described as being mediated via a facilitated transporter. Our results also support facilitated AH efflux from HepG2 cells because this process was sensitive to temperature and was differentially inhibited by various reagents, thus showing some degree of specificity. Although AH efflux from HepG2 cells seemed to be facilitated, the transport mechanism involved was clearly distinguished from mammalian glucose/DHA transport (Table 3). The inhibition of AH efflux from HepG2 cells by phloretin and DIDS agrees with previous studies on other cells [33–36]. The putative AH transport mechanism might have characteristics in common with DIDS-sensitive anion channels but further work is required to support this hypothesis. Similarly to the AH efflux described here, hepatocytes can also release glucose via a cytochalasin-B-insensitive temperature-dependent pathway [37], although this transporter has not been characterized further.

Further evidence for facilitated AH efflux included saturable rate constants. The affinity of the putative transporter for AH seemed to be low and in the millimolar range. Various cells contain a low-affinity AH transporter (K_m 2–7 mM) that has been primarily described for AH influx [36,38–40]. In addition, cells accumulate millimolar levels of AH intracellularly [23,38,41]. Thus an intracellular millimolar K_m for AH efflux in hepatocytes might be physiologically relevant.

In comparison with AH efflux from cells, more is known of DHA uptake into cells, largely because DHA formation outside cells is expected and has been shown to occur under conditions of oxidative stress [8]. DHA uptake into cells is accompanied by intracellular reduction to AH catalysed by one or more intracellular proteins [42,43]. However, despite this rapid and complete intracellular reduction of DHA in a wide variety of cells, including whole blood cells, AH release from these cells has not been reported. Furthermore, frog oocytes transfected with the mammalian glucose (GLUT 1) transporter isoform take up DHA but specifically fail to release intracellularly reduced AH [29]. Thus cells need to be specifically equipped with a mechanism to mediate AH efflux, a process that seems to be biochemically and biophysically distinct from the transport of DHA (oxidized vitamin C).

The concentration of AH in human plasma is probably determined by several factors, including dietary intake, renal loss, absorption by tissues and redox processes. Uptake of DHA

and subsequent release of AH by the liver could be important under conditions of oxidative stress. Consistent with this is the observation that reactive oxygen species can activate glucose transport in cells [44]. Increased glucose transport could represent an adaptive antioxidant response that bolsters energy levels (and cellular reducing agents including NADH/NADPH) as well as enhancing the recycling of extracellular AH. Further, as mentioned above, AH recycling is stimulated by oxidative stress, suggesting that various cells have a large capacity to handle oxidized vitamin C.

It has been suggested that erythrocytes, carrying up to 30 % of the total whole-blood vitamin C, are responsible for maintaining AH and enhancing the antioxidant reserve of human whole blood [12]. Several studies [12,27,45] have suggested that AH and NADH act as intracellular electron donors for transplasma membrane electron transport systems [11] that transfer electrons to extracellular acceptors and hence might participate in the maintenance of extracellular AH. In addition, electrons might be transferred to plasma membrane monodehydroascorbate reductases, thereby maintaining extracellular AH under oxidative conditions that produce ascorbyl radicals [46]. It is not known whether DHA can accept electrons via such systems to form AH; however, the results shown here suggest that this is unlikely for the following reasons. First, AH was released from cells after the removal of DHA from the extracellular medium. Secondly, blockage of DHA uptake, but not ferricyanide reduction, significantly decreased extracellular AH concentration. Thirdly, little AH was maintained extracellularly in the presence of DHA and human whole blood cells, although these cells have an equivalent, if not a higher, capacity to reduce ferricyanide than HepG2 cells [47].

In conclusion, this study has identified that hepatic AH can be released. In intact liver (Figures 3 and 4) this release does not require, though it is enhanced by, DHA uptake, suggesting that it might boost the extracellular antioxidant reserve of vitamin C. We propose that AH release by the liver is a hitherto unrecognized mechanism in vitamin C homeostasis. Together with adequate dietary intake, renal absorption and ascorbyl radical/DHA reducing systems, the release of AH by the liver might be important in the bioavailability of AH in plasma in its reduced form for cellular and antioxidant roles.

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