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THE UPTAKE OF ASCORBIC ACID INTO HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS AND ITS EFFECT ON OXIDANT INSULT

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Abstract—Intracellular reduced ascorbate (AA) levels in confluent cultures of human umbilical vein endothelial (HUVE) cells, grown under conventional conditions, were shown to be very low, ranging between undetectable, <0.1 nmol/mg protein, and 0.3 nmol/mg protein. Reduced ascorbate was accumulated into the endothelial cells from M199 culture medium in time- and concentration-dependent manners, and was saturated at medium concentrations related to the normal plasma concentrations of the antioxidant (i.e. between 50 μM and 100 μM). Cells derived from different individuals demonstrated considerable inter-individual variation in these AA uptake parameters. The uptake of AA was sensitive to temperature and the presence of the structural analogue iso-ascorbate in the medium, indicating the involvement of an active transport mechanism. A role for the glucose transporter is, however, not indicated, as AA uptake was not sensitive to phloretin, an inhibitor of the cellular glucose transporter, nor greatly enhanced by depletion of glucose from the medium. Incubation of HUVE cells with dehydroascorbate (DHAA) caused a dose-dependent, but transient increase in intracellular AA. This indicates that HUVE cells are both competent in the uptake and intracellular reduction of oxidised ascorbate, and may resecrete AA into the medium. Indeed, reduced ascorbate in the medium was shown to be preferentially maintained in the presence of cells. The uptake of AA was not sensitive to the presence of DHAA in the medium, perhaps indicating different transporters for reduced and oxidised forms of ascorbate in these human cells. Pre-loading HUVE cells with AA was shown to protect control cells only weakly from the acute, sub-lethal toxicity of H_2O_2 generated by xanthine oxidase (1 U/mL or 10 U/mL). Protection was optimal at intracellular levels of 3–4 nmol AA/mg protein, with higher concentrations lacking a protective effect. Additionally, the presence of the iron chelator, desferoxamine, significantly protected GSH-depleted HUVE cells only in response to the peroxide, but did not potentiate the protective action of intracellular AA in either control or GSH-depleted cells. This indicates that ascorbate-driven redox-cycling of the Fe^{2+}/Fe^{3+} does not hamper the intracellular protective function of ascorbate during hydrogen peroxide-derived oxidative stress. These results are discussed in terms of the central role of endothelial cells in the distribution of AA to the tissues of the body, the use of the HUVE cell system for model studies of the toxicity of oxidants in the human endothelium, and the balance between the antioxidant and pro-oxidant actions of AA.

Key words: ascorbic acid; dehydroascorbic acid; uptake; human; endothelial cells; glutathione; hydrogen peroxide; oxidative stress

The human endothelium represents a tissue that is constantly exposed to oxidants generated both internally, by the action of xanthine oxidase in the endothelial cells [1], and externally by activated blood granulocytes such as neutrophils [2]. Under normal conditions, the intracellular milieu is protected by the concerted action of a network of antioxidative principles [3], of which ascorbic acid has been proposed as one of the major, water-soluble components [4].

AA† reacts readily with a variety of ROMs, including superoxide radical anion, singlet oxygen, hydroxyl radical, and water-soluble peroxyl radicals [4]. Moreover, it has been reported that AA and α-tocopherol act synergistically in the prevention of lipid peroxidation, as AA readily reduces the tocopheroxyl radical at the membrane interface [5]. The efficacy of AA as an antioxidant is enhanced by its regeneration from ascorbyl radical or DHAA through the activity of glutathione (GSH)- and NADPH-dependent enzymatic processes [6]. However, it is pertinent to consider that as a redox-active substance, AA can also act as a pro-oxidative stimulus, especially in the presence of transition metal ions such as Fe^{2+}/Fe^{3+} [5].

The transport of AA across the plasma membrane of cells is an active process against a steep concentration gradient [7], requiring the function of specific transport proteins [8]. Since AA structurally resembles glucose, evidence has suggested that AA is transported on the glucose transporter of various cells, including neutrophils [9], hepatocytes [10], and oocytes [11]. Additional evidence suggests that DHAA is transported on the glucose transporter into neutrophils and fibroblasts [12] and erythrocytes [13]. It should be remembered, however, that other transport mechanisms may be operative in cells, and that there is a possibility that the reduced and oxidised forms of ascorbate are simultaneously transported into cells by different systems. Indeed, the uptake of DHAA into most cells appears to be more rapid and extensive than that of AA [13, 14]; however, the DHAA is rapidly reduced to AA, thus maintaining low intracel-

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† Abbreviations: AA, reduced ascorbate; DHAA, dehydroascorbate; GSH, reduced glutathione; ROMs, reactive oxygen metabolites; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; mBBr, monobromobimane; HUVE, human umbilical vein endothelial; PBS, phosphate-buffered saline; Gox, glucose oxidase; BSA, bovine serum albumin; and DETAPAC, diethylenetriaminepentaacetic acid.

lular concentrations of DHAA [7]. Recently, Lam and co-workers proposed that DHAA is carried into cells by a low-affinity transporter distinct from the AA carrier, although both activities were simulated by extracellular sodium, which was proposed to alter the affinity of the transporters for their respective substrates [8].

Human cells lack the ability to synthesise AA [4], and rely on a constant supply of ascorbate derived from the diet. Normal plasma concentrations of AA vary between 50 μM and 200 μM [6, 7], and it is thought that this circulatory reservoir serves to supply the tissues with this essential nutrient. Thus, the endothelial cells lining the blood vasculature play a potentially vital role in the absorption of AA from the circulation and in its distribution to the underlying tissues. Despite this, there have been no reports of the mechanisms by which endothelial cells absorb AA or DHAA from the circulation. In addition, human endothelial cells [15–17] and endothelial cells from other species [18–20] are sensitive to oxidative insult from ROMs. This toxicity is thought to be central in the pathophysiological processes seen during the development of human diseases such as the adult respiratory distress syndrome [21]. Despite this, nothing is known of the potential antioxidative role of intracellular AA in human endothelial cells, although several studies have shown that large concentrations of extracellular AA clearly provide protection during neutrophil-endothelial cell interactions [19, 22]. In contrast, several studies have clearly established an important role for intracellular GSH in the protection of endothelial cells from oxidant toxicity [15, 20]. In the present study we describe, for the first time, the uptake kinetics of AA and DHAA into confluent cultures of HUVE cells, and have investigated the potential role of the cellular glucose transporter in the uptake of AA. Additionally, as preliminary experiments had demonstrated that HUVE cells lack detectable AA when grown under conventional conditions, we have investigated the influence of pre-loading the cells with AA on the acute cytotoxicity response to the naturally-occurring oxidant, H_2O_2. The results are discussed in terms of the potential function of AA uptake into these cells, and the use of this cell type for model studies of oxidant toxicity in the human endothelium.

MATERIALS AND METHODS

Chemicals

L-Ascorbate, L-isoascorbate (isoAA), phloretin, GSH, DETAPAC, and MTT were all obtained in >99% purity from Sigma Chemical Co., St. Louis, MO. Desferoxamine (Desferal™) was obtained from Ciba Geigy AG, Basel, Switzerland. DHAA (99%) was obtained from Aldrich-Chemie, Steinheim, Germany. Hydrogen peroxide (30% aqueous solution) was from Riedel-de-Haen, Seeize, Holland. Glucose oxidase (Gox) and BSA (fraction V, lyophilised and fatty-acid free) were obtained from Boehringer Mannheim, Mannheim, Germany. Monobromobimane (mBBr) was from Calbiochem, La Jolla, CA, and all materials for the isolation and culture of HUVE cells were purchased from Nord Cell AB, Stockholm, Sweden. Human serum was obtained from the donor center of Sabbatsberg Hospital, Stockholm, Sweden. All other chemicals and reagents were obtained in the highest grade possible from local suppliers.

Cell cultures and incubations

The HUVE cells were prepared from umbilical cords less than 6 hours post parturitum by the collagenase perfusion method described by Jaffe *et al.* [23], with minor modifications [24]. The cells were cultured to primary confluency in M199 medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM) and 20% human serum (henceforth termed "control medium"). For uptake studies, the cells derived from individual cords were passaged by conventional trypsinisation and kept apart to determine any interindividual variation in the biochemical processes. For toxicity experiments, the cells were passaged as above but pooled at the P1 stage before further subcloning, since interindividual differences in the susceptibility of HUVE cells to peroxide-induced toxicity is minimal [17]. Cells of the third passage were used throughout the experiments, seeded onto 35 mm dishes (Falcon) for uptake experiments and 12-well plates (Falcon) for the assay of toxicity.

For the determination of the uptake kinetics, HUVE cell cultures were incubated in control medium supplemented with either AA or DHAA at a variety of concentrations from 10 μM to 200 μM. After various times the medium was removed, the cells washed with PBS, and the cells and medium analysed for their content of AA, as described below. Control experiments were performed to determine the optimal number of cellular washes for removing AA from loose association with the cells. In another series of experiments, the effects of varying temperature between 4°C and 37°C and of co-incubation of structurally-related compounds and inhibitors of glucose transport on control AA uptake were tested. Finally, the effect of varying the glucose concentration from zero to 5 mM on the uptake of AA at different medium concentrations was tested. Glucose-free M199 medium was used for this experiment.

For the assessment of the effect of intracellular AA on the sensitivity of HUVE cells to the sub-lethal toxicity of peroxides, the cells were preincubated with control medium supplemented with AA (200 μM) for between 30 minutes and 24 hours. The AA content of the cells was assayed, and parallel cultures washed and resupplied with control medium lacking AA serum and phenol red, but supplemented with BSA (1%) and Gox (1 U/mL or 10 U/mL) for 30 minutes. The cells were then washed twice with PBS, and the toxicity determined by their ability to reduce MTT as described below. Control experiments were performed to determine the rates of accumulation of H_2O_2 in the Gox-containing media. In some instances, cellular GSH was depleted prior to the loading of AA by a 24-hour preincubation in control medium lacking sulfur amino acid precursors (M199-medium) [24]. This depletion medium was also used to perform the toxicity incubations in GSH-depleted cells. Control incubations were performed to determine if AA uptake was affected by the prior depletion of intracellular GSH. Some experiments were also performed with control and GSH-depleted HUVE cells, pre-loaded with AA, in the presence of the iron chelator Desferal.

Biochemical analyses

Reduced ascorbate was assayed in cells and medium according to Honegger *et al.* [25] following extraction of the samples with TCA (1.25%). The molecule was sep-

arated by hplc (Waters Associates) on a supelcosil L-18 column (150 mm × 4.5 mm, 3 μm) and quantitated by electrochemical detection using an Antec Model CU-03 detector, with the electrode set in the oxidative mode at +0.5 V. Samples were analysed as soon after generation as possible. Intracellular GSH was analysed following *in situ* derivatisation of the cells with the membrane-permeable thiol reagent mBBr, exactly as described previously [26]. Hydrogen peroxide was analysed spectrophotometrically in media according to the method of Ovenston and Parker [27]. Cellular protein was determined by the method of Peterson [28].

Assay of cytotoxicity

The sub-lethal, acute cytotoxicity of H_2O_2 was assessed in the HUVE cells by monitoring changes in their ability to reduce MTT [29]. Briefly, following insult and washing, the cells were incubated in control medium containing MTT (0.5 mg/mL) for 2 hours. The cells were then washed (2 × 2 mL) with PBS, dissolved with DMSO (1 mL), and the absorbance of this extract determined at 540 nm. The results are expressed as the fractional change in absorbance of the test sample against the appropriate control.

Statistical appraisals

Sets of data were compared for differences using the Student's *t*-test for unpaired observations. Significance occurs at the level of the two-tailed value of $P < 0.05$.

RESULTS

Reduced ascorbate was found to be unstable in the control medium used for cellular incubations. Figure 1 shows that when AA was spiked into control medium at 200 μM and incubated in culture plates under exactly the same conditions used for cell experiments, levels fell rapidly, with nearly 50% lost after 6 hours and >90% depletion by 24 hours. Interestingly, when this experiment was repeated in the presence of confluent monolayers of HUVE cells, the levels of AA in the medium were maintained above medium-only controls at most time points. Thus, the presence of cells induced a significant increase in AA after 6 hours (76% ± 13% vs 55% ± 5%, $P < 0.05$, $n = 4$) as well as after 24 hours (38% ± 5% vs 10% ± 8%, $P < 0.001$, $n = 4$). Furthermore, the inclusion of the iron chelator (DETAPAC, 100 μM) in the medium in the absence of cells also significantly protected the levels of AA, to a similar extent as HUVE cells (Fig. 1).

One of the initial steps in establishing the uptake kinetics for AA in cultures of HUVE cells was to determine the optimal washing protocol for removing extracellular AA following incubation. Cells derived from one umbilical cord and incubated with 200 μM AA for 2 hours had 15.3 nmol ± 1.1 nmol AA/mg protein ($n = 3$) associated with them if the medium was carefully removed and the cells directly assayed without washing. If these cells were washed up to three times with PBS, the levels of AA associated with the cells fell to 7.9 ± 0.3, 5.3 ± 2.4, and 5.0 ± 0.7 nmol/mg protein, respectively ($n = 3$ on all points). Thus, 3 washes with PBS was chosen for subsequent experiments on AA uptake.

The incubation of HUVE cells with AA resulted in time- and concentration-dependent accumulation of AA in the cells (Fig. 2). Four noteworthy observations

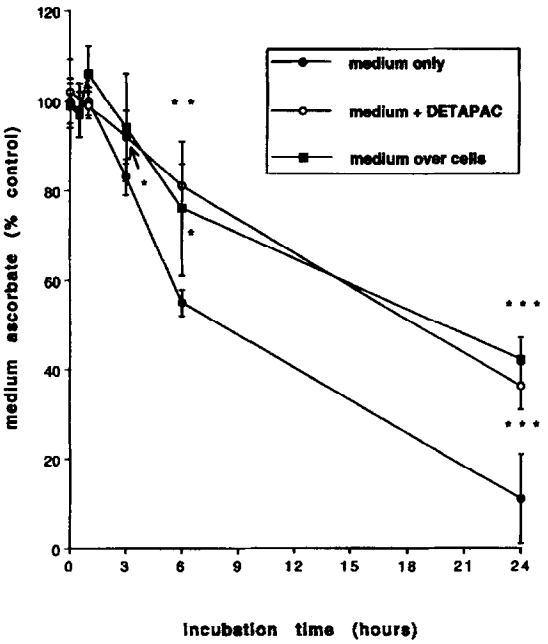

Fig. 1. *The stability of reduced ascorbate in control M199 medium in the absence and presence of an iron chelator or confluent HUVE cells.* Reduced ascorbate (AA) was spiked into control M199 medium and incubated in a humidified CO_2 incubator in 35-mm culture dishes in the absence and presence of either the Fe^{2+} chelator DETAPAC (100 μM) or confluent monolayers of HUVE cells of the third passage, derived from one batch of primary cells. At various times up to 24 hours, aliquots of medium were removed for the determination of AA as described in Materials and Methods. $N = 4$ on all observations. * and *** denote significant ($P < 0.05$ and 0.001, respectively) elevations in medium ascorbate levels from controls.

emerge from this figure. Firstly, under the conditions employed, the uptake increased with increasing concentrations between 10 μM, and 200 μM of AA in the medium. It appears that the uptake may be saturated between 100 μM and 200 μM, yet this is not certain due to the considerable interindividual variability in the response of the cells. Secondly, the uptake seems to be fairly linear at each concentration of AA tested over the 2-hour time period studied. Additionally, other experiments using one individual clone of cells revealed that AA continued to concentrate in the cells over a 24-hour period, with levels of 8.4 ± 1.4 and 19.3 ± 3.1 nmol/mg protein accumulated after 6 or 24 hours of incubation, respectively, in control medium supplemented with AA (200 μM) ($N = 4$ on both observations). Moreover, it will be noted that 10 μM AA in the medium was unable to sustain a rise in intracellular AA past 60 minutes of incubation. Thirdly, interindividual variation in the ability of the cells to take up AA was clearly evident at all time points and concentrations of AA used. This variability lay between 20% and 100% in the four individual cell batches tested, hence the omission of error data from the figure. Lastly, the control preincubation levels of cellular AA are extremely low, varying from <0.1 nmol/mg protein to circa 0.3 nmol/mg protein.

When similar experiments were performed on the effect of incubating the cells with DHAA over a 2-hour period on intracellular levels of AA, levels of AA rose from <0.1 nmol/mg protein to between 0.20 ± 0.03 and

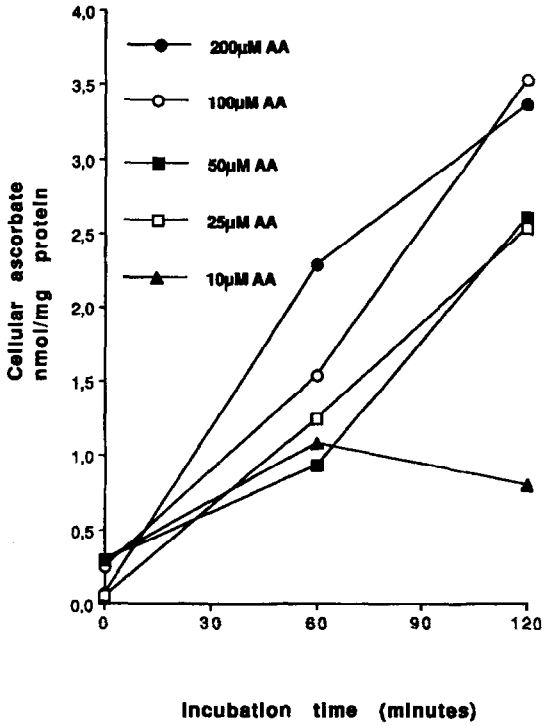

Fig. 2. *Concentration dependency of the accumulation of reduced ascorbate in HUVE cells from ascorbate-spiked control medium.* Confluent HUVE cells at passage 3, derived from 4 individual batches of primary cells, were incubated on 35-mm culture plates with control M199 medium supplemented with various concentrations of AA between 10 μM and 200 μM. After set times, the medium was removed and the cells washed with prewarmed (37°C) PBS (3 × 2 mL). The cells were then analysed from their AA content/mg cell protein as described in Materials and Methods. Interindividual differences in the extent of accumulation of each time point with each concentration of AA were large (between 20% and 100%), and have been omitted from the figure for clarity.

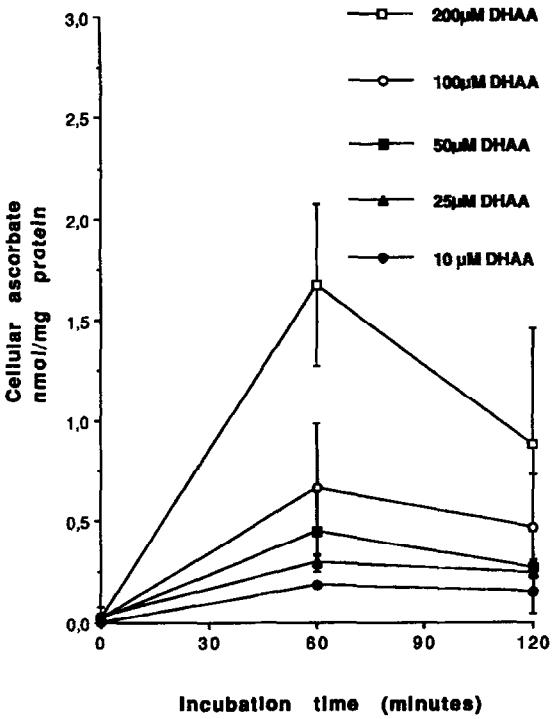

Fig. 3. *Concentration dependency of the accumulation of reduced ascorbate in HUVE cells from control medium spiked with dehydroascorbate.* All details as in Fig. 2 except that the cells were incubated in control medium spiked with dehydroascorbate.

1.72 ± 0.45 nmol/mg protein by 60 minutes of incubation, dose-dependently (Fig. 3). Again, interindividual variation was noted among the four individual cell batches tested, but this was generally less than that obtained with AA. Further, by 2 hours the levels sustained by each concentration of AA decreased in a convergent manner. When one individual batch of cells incubated with 200 μM DHAA was followed beyond 2 hours, it was noted that levels of intracellular AA continued to fall to 0.25 ± 0.1 nmol/mg protein (n = 3) by 6 hours, a level maintained at 24 hours (0.22 ± 0.09, n = 3).

In another series of experiments, cells from 3 individual batches were loaded with AA by incubation with control medium supplemented with AA (200 μM) for 4 hours, washed, and then reincubated in control medium minus AA. Intracellular AA levels were shown to decline rapidly in the absence of extracellular AA. Thus, cellular AA fell from control levels of 7.8 ± 2.0 nmol/mg protein to 6.6 ± 1.3, 6.2 ± 2.0, 4.8 ± 0.9, and 3.0 ± 1.0 nmol/mg protein at 5, 10, 15, and 30 minutes of incubation, respectively. Again, the data confirm interindividual variability in loading of AA, but also indicate extensive variability in the loss of AA from the cells.

Preliminary experiments designed to probe the nature of the uptake of AA into HUVE cells demonstrated that AA uptake from medium supplemented with AA (200 μM) for 2 hours was temperature sensitive, falling from 3.1 ± 0.3 nmol/mg protein at 37°C to 1.9 ± 0.5 nmol/mg protein and 1.3 ± 0.4 nmol/mg protein at 20°C and 4°C, respectively (n = 3 with one individual batch of cells). Under the same conditions, the control accumulation of AA into HUVE cells was unaffected by coincubation with either DHAA (1 mM) or phloretin (100 μM), but significantly ($P < 0.001$) inhibited to 22 ± 4% (n = 3) of control by coincubation with isoAA (1 mM). In another series of experiments, HUVE cells were incubated with AA (50 μM–200 μM) for 2 hours in glucose-free M199 medium supplemented with 0–5 mM glucose. It can be seen from Fig. 4 that AA was accumulated in the cells in the absence of glucose, with 1.2 ± 0.2 nmol/mg protein, 1.8 ± 0.3 nmol/mg protein, and 2.8 ± 0.3 nmol/mg protein accumulated from medium supplemented with 50 μM, 100 μM, and 200 μM AA. Similarly, at all AA concentrations tested, the inclusion of glucose at 1 mM or 2.5 mM stimulated the accumulation of AA in the cells. At concentrations above 2.5 mM, a trend towards decreased accumulation was evident. Again, as the data were derived from cells derived from three individual primary cultures, considerable variation was evident in the effect of glucose on AA uptake.

When control HUVE cells were incubated with Gox (1 U/mL or 10 U/mL) for 30 minutes in M199 medium lacking serum, but supplemented with BSA (1%), a dose-dependent loss of MTT-reducing ability (to 60.7% ± 1.3% and 50.9% ± 1.7% of controls, respectively) was detected in the cells (Fig. 5). On the other hand, when the cells were pre-loaded with AA from control medium

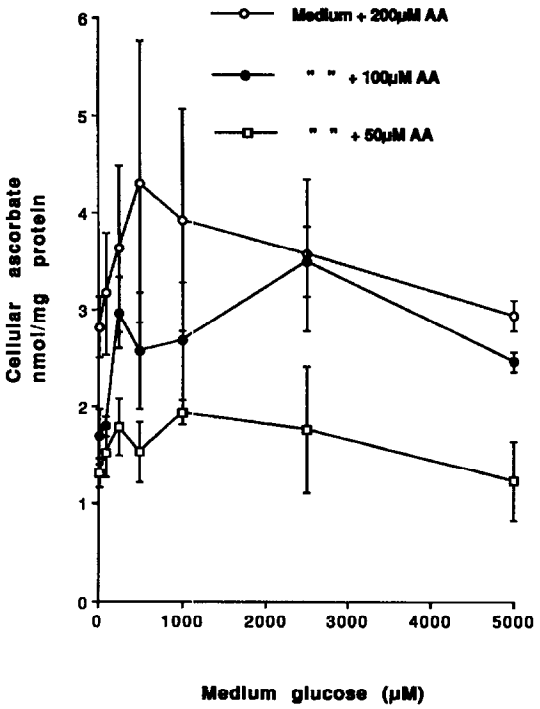

Fig. 4. *The effect of variation of medium glucose concentration on the accumulation of reduced ascorbate from ascorbate-spiked medium.* Confluent HUVE cells from the third passage of cells derived from 3 individual primary cultures were incubated for 2 hours in glucose-free medium supplemented with various concentrations of glucose (from 0–5 mM) and ascorbate (from 50 μM to 200 μM). The content of cellular AA was then determined as described in Materials and Methods.

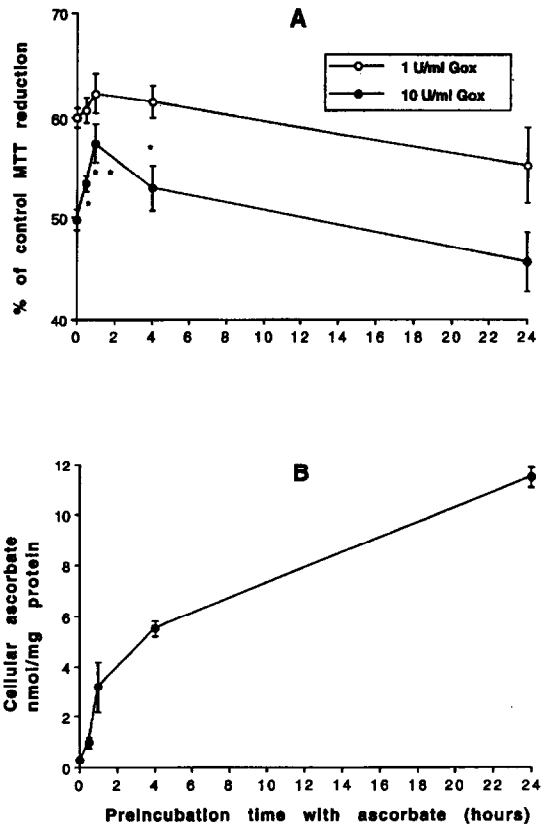

Fig. 5. *The effect of variation of intracellular ascorbate levels on the acute, sub-lethal toxicity response of HUVE cells to hydrogen peroxide.* Confluent control HUVE cells at passage three from one batch of primary cells were pre-loaded with ascorbate in control M199 medium supplemented with AA for increasing times between 30 minutes and 24 hours. Before the exposure of the cells to Gox, parallel plates (35 mm) were assayed for the extent of cellular accumulation of AA. The cells were then exposed to M199 lacking AA and serum, but supplemented with BSA (1%) and glucose oxidase (Gox, 1 U/mL, or 10 U/mL) for 30 minutes on 12-well plates. Thereafter, the cells were washed (2 × 2 mL) with PBS and their viability determined by their incubation in control medium supplemented with MTT, as described in Materials and Methods (n = 4 on all points). The accumulation of H_2O_2 at 5, 10, and 30 minutes amounted to 110, 220, and 300 μM, and 280, 310, and 330 μM for 1 U/mL and 10 U/mL Gox, respectively. * and ** denote significant ($P < 0.05$ and $P < 0.01$, respectively) differences between values obtained without pre-loading with AA and those obtained with pre-loading.

supplemented with 200 μM AA for between 30 minutes and 24 hours, dose-dependent protection from the toxicity of the peroxide was noted, which was significant (between $P < 0.05$ and $P < 0.01$ for 1–4 h pre-loading) when 10 U/mL Gox was utilised. It was, however, evident that an optimal intracellular concentration of AA of circa 3 nmol/mg protein was necessary to elicit protection, with increasingly higher intracellular concentrations diminishing or totally removing the protective efficacy of the antioxidant.

Finally, to assess if the availability of intracellular Fe^{2+} limits the protective capacity of AA, due to eventual pro-oxidative redox-cycling, both control cells and cells >90% depleted of their intracellular GSH (from 32 ± 4 nmol/mg protein) were treated with Gox (1 or 10 U/mL) for 30 minutes, in the absence or presence of the iron chelator Desferal (100 μM), following a 4-hr pre-loading with AA. This pre-loading period was chosen because it provides intracellular AA concentrations greater than those for optimal protection. Additionally, it will be noted that the AA uptake kinetics were not affected by the depletion of cellular GSH (data not shown). It can be seen from Fig. 6a that pre-loading the cells with AA only elicited significant ($P < 0.05$) protection in control cells when 10 U/mL Gox was used. This was not potentiated by coincubation of such cells with Desferal. On the other hand, the depletion of GSH from the cells significantly ($P < 0.01$) potentiated a sub-acute toxicity of both 1 and 10 U/mL Gox, and revealed significant ($P < 0.05$–$P < 0.01$) protective effects for both pre-incubation with AA and coincubation with Desferal (Fig. 6b). When the treatments were combined, however, an additive or synergistic protective effect was not observed.

DISCUSSION

Many studies have highlighted the antioxidant properties of AA in biological systems [4–6, 9–11]. Ascorbic acid is an essential nutrient in humans, distributed throughout the body via the circulation. Thus, the endothelial barrier of the human circulation occupies a potentially critical position in the control of distribution of ascorbate throughout the body. At the same time, it is of interest to probe the potential antioxidant function of AA in human endothelial cells, as they represent one of the

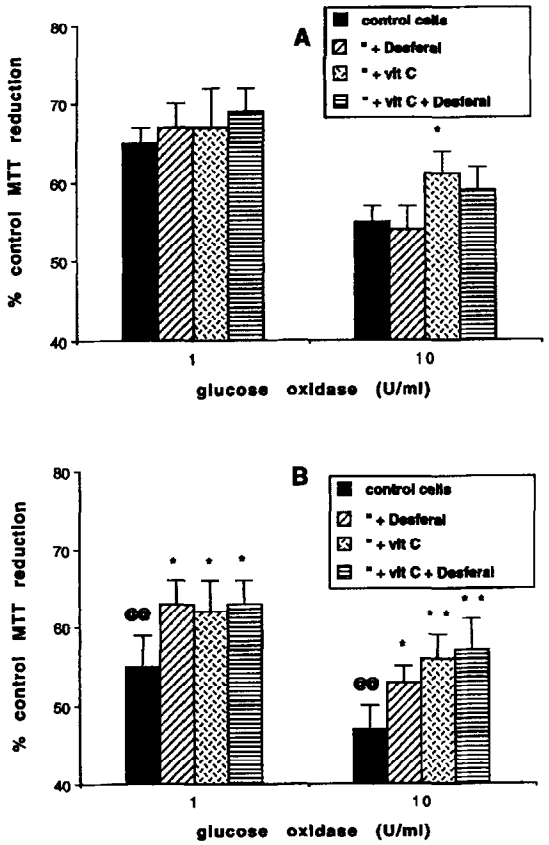

Fig. 6. *The effect of the iron chelator Desferal on the acute, sub-lethal toxicity of hydrogen peroxide in control and ascorbate pre-loaded HUVE cells.* Confluent control HUVE cells (A) at passage three from one batch of primary cells, or similar cells depleted of their GSH (B) by a 24-hour preincubation in M199 medium lacking sulfur amino acids, were pre-loaded with ascorbate for 4 hours from control medium supplemented with 200 μM of the antioxidant. These cells were then washed (2 × 2 mL) with PBS and exposed to M199 medium lacking AA and serum, but supplemented with BSA (1%) and glucose oxidase (Gox, 1 U/mL, or 10 U/mL) in the absence or presence of desferoxamine (Desferal, 100 μM), for 30 minutes on 12-well plates. The cells were then washed (2 × 2 mL) with PBS, and the viability of the cells determined by their incubation in control medium supplemented with MTT, as described in Materials and Methods ($n = 4$ on all points). Control GSH levels (32 ± 4 nmol/mg protein, $n = 4$) were depleted by >90% by preincubation in M199-medium. Control accumulation of AA (4.8 ± 0.5 nmol/mg protein, $n = 3$) was not affected by GSH depletion. All other details as in Fig. 5, except that @ @ denotes a significant (<0.01) difference between the toxicity of H_2O_2 in control and GSH-depleted cells at each concentration of Gox.

primary targets for the induction of oxidative stress and oxidant-related toxicity [15–17, 21]. Despite this, an appreciation of the biochemical processes underlying the transport of AA into human endothelial cells is lacking.

In this communication we clearly show that when cultured under conventional conditions, confluent monolayers of HUVE cells possess very low levels of reduced ascorbate, between 0.1 nmol/mg protein and 0.3 nmol/mg protein. Indeed, these often lie below the detection limit of the assay for AA in the manner employed (i.e. below circa 0.1 nmol/mg protein). It will be noted that AA is included in M199 medium, but is unstable and is usually undetectable in the control media used. Thus, when HUVE cells are supplied with medium supplemented with AA, the cells accumulated AA in a time-dependent manner. Experiments on the concentration-dependency on this uptake process revealed that the process is optimal at extracellular concentrations of between 50 μM and 100 μM (Fig. 2), matching the affinity of the carrier mechanism to the normal levels of AA in human plasma/serum [7]. However, further experiments are required to determine the exact carrier affinity for AA uptake into HUVE cells. This might involve measuring the initial velocity of the uptake of ^{14}C-labelled AA into the cells. The affinity and activity of the HUVE cell AA carrier may vary among individuals, as large interindividual differences in the concentration-dependent accumulation of AA were noted for cells derived from individual umbilical cord primary cultures. Interindividual differences in GSH-dependent parameters have also been noted for HUVE cells from different donors [17].

The nature of the carrier mechanism for AA in HUVE cells is as yet unknown. However, the fact that the uptake was shown to be temperature-sensitive and inhibitable with the structural analogue isoAA indicates an active receptor/transporter mechanism. Many cells have been shown to rely on the activity of their glucose transporter for the uptake of AA [9–11]. However, it is clear that phloretin, a potent inhibitor of glucose transport [10], is completely without inhibitory effects in HUVE cells. Additionally, lowering the glucose concentration of the incubation from the control levels of 5 mM did not greatly affect the uptake of AA from the medium at concentrations between 50 μM and 200 μM (Fig. 4). If glucose had competed with AA for uptake into the cells, one might expect that lowering the glucose content would stimulate the uptake of the antioxidant. Finally, it is interesting to note that AA uptake occurred in the total absence of glucose in the medium. Further work is required to characterise the nature of the AA carrier in these human cells. Preliminary data indicate that the uptake of AA is sensitive to the presence of other structurally-related sugar derivatives, particularly glucuronic acid, glucuronamide, and various glucuronides (authors' unpublished observations), perhaps indicating common transport pathways for these agents and AA in HUVE cells.

When DHAA was supplied to the extracellular medium, it was apparent that AA accumulated in the cells in a dose-dependent manner (Fig. 2). However, the steady-state levels of AA achieved were much lower than those obtained with corresponding concentrations of AA in the extracellular medium. Moreover, it was apparent that after an initial peak of AA in the cells, levels converged to a steady state niveau <0.5 nmol AA/mg protein over the entire range of DHAA tested (10 μM–200 μM). Irrespective of the kinetics of appearance of AA in the cells, the data clearly indicate uptake of DHAA into HUVE cells and the activity of a reductase able to generate AA from intracellular DHAA. Despite this, the activity of the transporter and/or reductase may be limited, due to the low levels of AA maintained in the cells. Once again, the exact nature of the DHAA carrier is uncertain from our experiments; however, it may be that AA and DHAA are carried on separate carriers, as our results indicate that DHAA did not inhibit the uptake of AA from the external medium. Here, work with radiolabelled DHAA is indicated to determine the extent

of DHAA uptake in the cells and the efficiency of its reduction to AA.

Following the removal of AA from the extracellular medium, the intracellular levels of AA were shown to fall rapidly within the first hour of re-incubation in AA-free medium. This indicates either that AA undergoes rapid breakdown/oxidation in the cells and/or is released back into the medium. Some evidence for the latter process may have been provided by the data in Fig. 1, which demonstrate that AA levels in control medium were maintained more efficiently in the presence of the HUVE cells themselves. Such "cycling" of AA across the membrane, coupled with the ability to transport and reduce DHAA, may have physiological relevance to the maintenance of AA in a largely reduced state in the plasma. Additionally, it remains to be seen whether AA is released on the basal side of the endothelial layer, thus providing AA to underlying cell types, such as smooth muscle cells. Further work with HUVE cell cultures on porous filter supports may provide insight into these processes. An alternative explanation for the protective effects of HUVE cells on medium AA may, however, lie in the cells' ability to chelate extracellular Fe^{2+}. Thus, the inclusion of the chelator DETAPAC in the medium was shown to protect the levels of AA to an extent similar to that of the HUVE cells (Fig. 1).

It is clear from the data obtained from control HUVE cells grown under conventional conditions that intracellular AA concentrations are very low, variably below the limit of detection of the assay or in the order of 0.5%–1.5% that of GSH, which generally lie around 20 nmol/mg protein, or circa 3 mM [24]. This implies that intracellular AA concentrations lie considerably under 50 μM in such cultures. This presents potential ramifications for the use of this cell type in model studies on the role of intracellular oxidative stress in the toxicity of ROMs, such as H_2O_2 [15–17]. However, the data presented in Fig. 5 and 6 clearly show that the presence of intracellular AA has minimal protective effect on H_2O_2-induced sub-lethal injury in the cells, as assessed by the use of the MTT reduction assay. It is interesting to note that a weak protective effect was coupled with an optimal intracellular AA level of circa 3 nmol/mg protein. Here it must be remembered that the actual levels of AA in the endothelial cells *in vivo* are unknown. It may be that above these levels AA begins to function in a pro-oxidant manner, a possibility clearly indicated by previous studies [5].

To test the possibility that the net protective effect of intracellular AA is a compound of its pro- and anti-oxidative properties, experiments were performed in which both control and GSH-depleted cells were incubated with the iron chelator Desferal. It will be noted that DETAPAC was unsuitable in these toxicity experiments, as it was shown to interfere in the MTT assay of cell viability. It can be seen from Fig. 6a that a 4-hour preloading with AA only marginally protected control cells against the toxicity of the 10 U/mL Gox, and that this was not potentiated by the inclusion of Desferal. This was further supported in GSH-depleted cells (Fig. 6b), where the toxicity of H_2O_2 was significantly potentiated. This has been noted previously [15, 17] and may be due to a compromised ability of the cells to detoxify H_2O_2 via GSH-peroxidase-dependent processes. It must be stated, however, that the method used to deplete GSH in the present experiments may also deplete other low molecular weight thiol-containing compounds and affect the stability of intracellular AA in an indirect manner. Despite this, in these GSH-depleted cells, both Desferal or AA pre-loading protected the cells from cytotoxicity, but the coincubation of AA-pre-loaded cells with Desferal did not elicit an additive or synergistic protective effect. This suggests that AA-driven intracellular redox cycling with Fe^{2+}/Fe^{3+}, with the generation of hydroxyl radicals via a Fenton reaction [30], does not limit the overall, rather weak antioxidative efficacy of HUVE cell AA under the conditions employed.

In conclusion, HUVE cells possess transport mechanisms for both AA and DHAA, with the activity of the former transporter optimised to normal plasma levels of the antioxidant. This transport mechanism probably does not involve the cellular glucose carrier. Under normal conditions, AA levels in HUVE cells are much lower than those of GSH, but loading the cells with AA prior to exposing them to peroxides has little or no protective influence on the resultant toxicity. These results have implications both for the role of such endothelial cells in the disposition of circulatory AA in the body and for the use of the cells in model studies of the mechanisms of oxidant toxicity.

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REFERENCES

1. Varani J, Phan SH, Gibbs DF, Ryan US, and Ward PA, H_2O_2-mediated cytotoxicity in rat pulmonary endothelial cells: Changes in purine products and effects of protective intervention. *Lab Invest* **63,** 683–689, 1990.
2. Weiss SJ and Lobuglio AF, Biology of disease: Phagocyte-generated oxygen metabolites and tissue injury. *Lab Invest* **47,** 5–18, 1982.
3. Cotgreave IA, Moldéus P and Orrenius S, Host biochemical antioxidant defence mechanisms against prooxidants. *Ann Rev Pharmacol Toxicol* **28,** 189–212, 1988.
4. Halliwell B. and Gutteridge JMC, The antioxidants of extracellular fluids. *Arch Biochem Biophys* **280,** 1–8, 1990.
5. Niki E, Selected vitamins, minerals and functional consequences of maternal malnutrition. In: *World Rev Nutr Diet,* pp. 1–30. Karger, Basel, 1991.
6. Halliwell B and Gutteridge JMC, In: *Free Radicals in Biology and Medicine,* pp. 100–103. Clarendon Press, Oxford, 1989.
7. Dhariwal KR, Hartzell WO, and Levine M, Ascorbic acid and dehydroascorbic acid measurements in human plasma. *Am J Clin Nutrit* **54,** 712–716, 1991.
8. Lam KW, Yu HS, Glickman RD and Lin T, Sodium-dependent ascorbic and dehydroascorbic acid uptake in SV-40 transformed retinal pigment epithelial cells. *Ophthamol Res.* **25,** 100–107, 1993.
9. Washko P and Levine M, Inhibition of ascorbic acid transport in human neutrophils by glucose. *J Biol Chem* **269,** 23568–23574, 1992.
10. Cornu MC, Moore GA, Nakagawa Y and Moldéus P, Ascorbic acid uptake by isolated rat hepatocytes. *Biochem Pharmacol* **46,** 1333–1338, 1993.
11. Vera JC, Rivas CI, Fischbarg J and Golde DW, Mammalian facilitated hexose transporters mediate the transport of dehydroascorbate. *Nature* **364,** 79–82, 1993.
12. Bigley R, Wirth M, Layman D, Riddle M and Stankova L, Interactions between glucose and dehydroascorbate transport in human neutrophils and fibroblasts. *Diabetes* **32,** 545–548, 1983.

13. Mann GV and Newton P, The membrane transport of ascorbic acid. *Ann NY Acad Sci* **258,** 243–252, 1975.
14. Bode AM, Vanderpool SS, Carlson EC, Meyer DA and Rose RC, Ascorbic acid uptake and metabolism by the corneal epithelium. *Invest Ophthamol Vis Sci* **32,** 2266–2271, 1991.
15. Andreoli SP, Mallet CP and Bernstein JM, Role of glutathione in protecting endothelial cells from hydrogen peroxide oxidant injury. *J Lab Clin Med* **108,** 190–198, 1986.
16. Shingu M, Yoshioka K, Nobunga M and Yoshida K, Human vascular smooth muscle cells and endothelial cells lack catalase and are susceptible to hydrogen peroxide. *Inflammation* **9,** 309–319, 1985.
17. Tu B, Wallin A, Moldéus P and Cotgreave IA, Individual, culture-specific alterations in the human endothelial glutathione system: Relationships to oxidant toxicity. *Pharmacol Toxicol* **75,** 82–90, 1994.
18. Weiss SJ, Young J, Lobuglio AF, Slivka A and Nimeh M, Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells. *J Clin Invest* **68,** 714–721, 1981.
19. Martin WJ, Neutrophils kill pulmonary endothelial cells by a hydrogen peroxide-mediated pathway. *Am Rev Respir Dis* **130,** 209–213, 1984.
20. Suttorp N, Toepfer W and Roka L, Antioxidant defense mechanisms of endothelial cells: Glutathione redox cycle versus catalase. *Am J Physiol* **251,** C671–C680, 1986.
21. Repine JE, Neutrophils, oxygen radicals and the Adult Respiratory Distress Syndrome. In: *The Pulmonary Circulation and Acute Lung Injury.* (Ed. Said S), pp. 249–282. Futura Publishing, Armonk, NY, 1985.
22. Jonas E, Dwenger A and Hager A, *In vitro* effects of ascorbate on neutrophil-endothelial cell interactions. *J Biolumin Chemilumin* **8,** 15–20, 1993.
23. Jaffe EA, Nachman RL, Becker CG and Minick LR, Culture of human endothelial cells derived from umbilical cord vein. *J Clin Invest* **52,** 2745–2756, 1973.
24. Cotgreave IA, Constantin D and Moldéus P, Nonxenobiotic manipulation and sulfur amino acid precursor specificity of human endothelial cell glutathione. *J Appl Physiol* **70,** 1220–1227, 1991.
25. Honneger CG, Langemann H, Krenger W and Kempf A, Liquid chromatographic determination of common water-soluble antioxidants in biological samples. *J Chromatog* **487,** 463–468, 1989.
26. Cotgreave IA and Moldéus P, Methodologies for the application of monobromobimane to the simultaneous analysis of soluble and protein thiol components of biological systems. *J Biochem Biophys Meths* **13,** 231–249, 1986.
27. Ovenston TCJ and Parker CA, Some notes on the reaction of ferric and thiocyanate ions. *Anal Chem* **3,** 277–284, 1949.
28. Peterson GL, A simplification of the protein assay method of Lowry, which is more generally applicable. *Analyt Biochem* **83,** 346–350, 1977.
29. Carmichael J, De Graff WG, Gazdar AF, Mina JD and Mitchell JB, Evaluation of a tetrazolium-based, semi-automated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res* **47,** 936–942, 1987.
30. Sies H, Hydroperoxides and thiol oxidants in the study of oxidative stress in intact cells and tissues. In: *Oxidative Stress* (Ed. Sies H), pp. 73–90. Academic Press, New York, 1985.