Dehydroascorbic acid uptake in a human keratinocyte cell line (HaCaT) is glutathione-independent

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Vitamin C plays an important role in neutralizing toxic free radicals formed during oxidative metabolism or UV exposure of human skin. This study was performed to investigate the mechanisms that regulate the homoeostasis of vitamin C in HaCaT cells by identifying the events involved in the transport and in the reduction of dehydroascorbic acid. Dehydroascorbic acid accumulated to a greater extent and faster compared with ascorbic acid; its transport appeared to be mediated by hexose transporters and was entirely distinct from ascorbic acid transport. Dehydroascorbate reductase activity was unaffected by glutathione depletion, although it was sensitive to thiol protein reagents. These observations, as well as the subcellular distribution of this enzymic activity and the cofactor specificity, indicate that thioredoxin reductase and lipoamide dehydrogenase play an important role in this reduction process. HaCaT cells were able to enhance their dehydroascorbic acid reductase activity in response to oxidative stress.

Key words: dehydroascorbic acid reductase, lipoamide dehydrogenase, thioredoxin reductase.

INTRODUCTION

The skin provides the first line of defence against oxidative damage induced by environmental factors. The epidermis is composed mainly of keratinocytes, which are rich in enzymes such as superoxide dismutase, catalase, thioredoxin reductase and glutathione reductase, and in low-molecular-mass antioxidant molecules such as tocopherol, glutathione and ascorbic acid (AA), and thus provides effective protection against reactive oxygen species [1]. Upon reaction with free radicals, AA is oxidized to ascorbyl free radical, which then rapidly disproportionates to AA and dehydroascorbic acid (DHA). We have shown recently that human non-tumoural HaCaT cells (a human keratinocyte cell line), which behave phenotypically like normal keratinocytes in terms of growth and differentiation [2], possess very efficient mechanisms for intracellular AA accumulation and ascorbyl free-radical reduction, and that this ability results in protection against cell death induced by UV-B irradiation [3]. In pathological conditions, such diabetes [4], skin inflammatory diseases (lupus erythematosus, psoriasis, contact dermatitis) [1] or wound healing [5], high amounts of extracellular AA are oxidized to DHA, which may then be taken up by cells and reduced back to AA. Several studies have demonstrated that the uptake and intracellular reduction of DHA from the surrounding interstitial space contributes to the maintenance of intracellular concentrations of AA. Transport studies have revealed that there are at least two different systems involved in the cellular uptake of vitamin C in mammalian cells; a Na^+-dependent co-transporter for AA [6], and a facilitative glucose transporter for DHA [7]. Following its uptake, DHA can be reduced to ascorbate in an enzymic or non-enzymic manner through a direct chemical reaction with reduced glutathione [8]. The exact nature of the systems involved in DHA reduction in human cells is still under debate, though a tissue-specific difference appears to be implicated. Many studies indicate that different enzymic systems are involved in DHA reductase activity, although current experimental evidence does not specify the extent to which this occurs *in vivo*. Enzymic systems of mammalian tissues include: (i) glutathione-dependent DHA reductases, such as glutaredoxin, protein disulphide-isomerase [9] and glutathione peroxidase [10]; (ii) NADPH-dependent DHA reductases, such as thioredoxin reductase [11] and 3α-hydroxysteroid dehydrogenase [12]; and (iii) NADH-lipoic-acid-dependent lipoamide dehydrogenase [13].

This study aims to clarify the events involved in DHA transport and reduction by HaCaT cells. To investigate whether this cell line is able to transport both AA and DHA and to establish the similarity, if any, of these carrier mechanisms, a kinetic analysis of AA and DHA uptake by HaCaT cells was performed. The effect of: (i) extracellular Na^+ concentration; (ii) putative competitive inhibitors such as glucose or deoxyglucose; and (iii) facilitated hexose-transport inhibitor, such as cytochalasin B, was also examined. Furthermore, to understand the contribution of glutathione-dependent and -independent systems in DHA reduction in HaCaT cells, changes in DHA reductase activity after selective treatment were investigated. Buthionine sulphoximine, an irreversible inhibitor of glutathione synthesis [14], and ethacrynic acid or diethyl maleate, covalent and irreversible reagents for free cysteines [15,16], were used to investigate the role of intracellular glutathione. Since almost all enzymes involved in DHA reduction are characterized by the presence of a redox-active disulphide, *p*-chloromercuribenzoate and iodoacetamide were used as specific thioprotein inhibitors. Finally, quercetin was used as an inhibitor of 3α-hydroxysteroid dehydrogenase [12]. The results of this study indicate that HaCaT cells take up DHA by a mechanism distinct from AA, and that DHA is reduced to AA through a highly efficient DHA reductase activity, which is mainly glutathione-independent.

EXPERIMENTAL PROCEDURES

Reagents

Modified minimal essential medium and Ham's F-12 were obtained from Life Technology (Merelbeke, Belgium). PBS, bicarbonate, L-glutamine, non-essential amino acids, Hepes buffer, catalase, rotenone, succinic acid disodium salt, PMSF, sodium L-ascorbate, α-oxoglutaric acid, L-glutathione (GSH), EDTA,

Abbreviations used: AA, ascorbic acid; DHA, dehydroascorbic acid; DTT, dithiothreitol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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NADH, NADPH, diethyl maleate, dithiothreitol (DTT), ethacrynic acid, quercetin, iodoacetamide, *p*-chloromercuribenzoate, L-buthionine-[*S*,*R*]-sulphoximine, lipoic acid, glucose, deoxyglucose, [1,2-^{3}H]2-deoxy-D-glucose, cytochalasin B, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), DMSO, scintillation cocktail, lactate dehydrogenase-UV test and HPLC-grade methanol were obtained from Sigma (St. Louis, MO, U.S.A.). Trypsin and fetal calf serum were obtained from Hyclone (Logan, UT, U.S.A.); Tris (hydroxymethyl)-aminomethane, HCl, sucrose, D-mannitol, choline chloride, trichloroacetic acid, iodonitrotetrazolium chloride, potassium dihydrogen phosphate and dipotassium hydrogen phosphate were obtained from Fluka (Buchs, Switzerland); tetrabutylammonium hydroxide was obtained from Nova Chimica (Milan, Italy); ascorbate oxidase was obtained from Boehringer Mannheim (Mannheim, Germany); DHA was obtained from ICN (Aurora, OH, U.S.A.) and [^{14}C]AA was obtained from Amersham (Rainham, Essex, U.K.).

Cell culture

The HaCaT cell line was kindly provided by Professor N. E. Fusenig (German Cancer Research Center, Heidelberg, Germany). Cells were grown in a mixture of minimal essential medium and Ham's F-12 medium supplemented with 10 % (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 1.2 g/l bicarbonate, non-essential amino acids (1 %, v/v) and 15 mM Hepes in a humidified atmosphere with 5 % (v/v) CO_2 at 37 °C. Cell viability was measured by the Trypan Blue exclusion assay in a Neubauer haematocytometer chamber. Lactate dehydrogenase leakage was measured spectrophotometrically using a lactate dehydrogenase-UV test from Sigma.

Subcellular fractionation

Mechanically removed cells were washed twice in PBS. The following preparations were performed on ice: cells were suspended 1:5 (w/v) in buffer (50 mM Tris/HCl, pH 7.4, containing 70 mM sucrose, 1 mM EDTA, 210 mM D-mannitol, 30 mg/ml catalase, 1 mM PMSF) and homogenized with a motor-driven pestle (RW 16 Basic JKA equipped with Potter–Elvehjem homogenizer with Teflon pestle and glass tube; clearance, 0.1 mm) at 1000 rev./min for 6 min. Unbroken cells and nuclei were removed by centrifugation for 5 min at 5 °C and 3000 ***g***. Mitochondria were sedimented by centrifugation for 20 min at 5 °C and 8000 ***g*** and washed once with lysis buffer. The supernatant was centrifuged for 60 min at 5 °C and 100000 ***g*** to obtain microsomal and soluble fractions. Mitochondria and microsomes were resuspended in 50 mM Tris/HCl, pH 7.4.

The purity of the obtained fractions was guaranteed by marker-enzyme analysis [3]. Succinate-iodonitrotetrazolium chloride reductase served as a mitochondrial marker [17], NADH-cytochrome *c* reductase (rotenone-insensitive) was used as a microsomal marker [18] and 5′-nucleotidase served as a plasma-membrane marker [19].

Drug treatments

Drug treatments were performed in culture medium at 37 °C under continuous flow of 95 % O_2/5 % CO_2. HaCaT cells were incubated with 1 mM buthionine sulphoximine, 0.2 mM ethacrynic acid or 1 mM diethyl maleate for different times to induce maximal glutathione depletion. Concentrations and incubation times for cell treatments with iodoacetamide, *p*-chloromercuribenzoate and quercetin were chosen to avoid cellular damage, as determined in preliminary experiments. Ethacrynic acid and quercetin were dissolved in ethanol (final concentration in the medium did not exceed 0.1 %), iodoacetamide and *p*-chloromercuribenzoate were dissolved in PBS, and lipoic acid and diethyl maleate were dissolved in 0.1 M phosphate buffer (pH 6.0).

HPLC measurements

Cells (2.5×10^6 in a 25-cm^2 flask) were incubated in 7 ml of culture medium with AA or DHA at 37 °C under a continuous flow of 95 % O_2/5 % CO_2. After incubation at 37 °C, the reaction was stopped by washing with 10 ml of PBS, and the cells were directly extracted with 1 ml of ice-cold 70 % methanol containing 1 mM EDTA. Preliminary experiments showed that cells released AA upon treatment with 70 % methanol and the additional disruption of cells only decreased the recovery of this unstable compound. After AA was extracted with methanol, the remaining cell monolayer was detached mechanically in 0.6 ml of PBS and analysed for protein content by the method of Bradford [20] using BSA as a standard.

Intracellular AA content was measured by HPLC with the UV-detection wavelength set at 265 nm, i.e. the absorbance peak of AA. Since DHA does not absorb at this wavelength, it was quantified by treating the sample with 10 mM DTT for 10 min. The extracted samples were filtered through a 0.22-mm filter (Millipore) and analysed immediately, or were frozen at −70 °C for later analysis. The HPLC consisted of a chromatograph PE NELSON model LC 1022 Plus equipped with a series 200 pump and LC-295 UV/VIS detector (Perkin–Elmer). AA was separated on a INERTSIL ODS-2 C_{18} packing column (5 mm; 150 × 4 mm internal diameter) with an ODS 2-I guard column (40 mm; 20 × 4.6 mm internal diameter) from SGE (Ringwood, Victoria, Australia). The mobile phase consisted of two solutions: 10 mM tetrabutylammonium hydroxide/10 mM potassium dihydrogen phosphate/0.5 % methanol, adjusted to pH 6 (solution A) and 50:50 (v/v) doubly distilled water/methanol (solution B). The flow rate was 0.5 ml/min. The elution conditions were as follows: isocratic solution A (0–15 min), linear gradient of solutions A → B (15–25 min), linear gradient of solutions B → A (25–35 min) and re-equilibration with solution A (35–60 min). Under these conditions, and at room temperature, the retention time of AA was about 11 min. We confirmed that this peak is derived from AA by incubating the samples with ascorbate oxidase before extraction with methanol. AA was quantified using a calibration curve. The detection limit was 2 pmol. Intracellular AA concentration was calculated as pmol/mg of cell protein and then converted into molar amounts, assuming a HaCaT cellular volume of 1.43×10^{-6} μl [21].

To investigate a possible efflux of AA or DHA from the cells, after incubation for 10 min with 0.05 mM DHA, cells were washed and 1 ml of fresh culture medium was added. At 5, 10 and 30 min, 50 μl of the medium was removed and extracted with 70 % methanol containing 1 mM EDTA. DHA was quantified, as above, by treating the sample with DTT.

To characterize AA or DHA transport, glucose, deoxyglucose or cytochalasin B was added to the culture medium simultaneously with AA or DHA. To analyse the effect of Na^+ on AA or DHA transport, an incubation medium consisting of 5 mM KCl/1.9 mM KH_2PO_4/5.5 mM glucose/0.3 mM $MgSO_4$/1 mM $MgCl_2$/0.3 mM $CaCl_2$/10 mM Hepes/147 mM NaCl/1.1 mM Na_2HPO_4 (pH 7.4) was used. For a sodium-free medium, NaCl was replaced by choline chloride and Na_2HPO_4 by K_2HPO_4.

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Scintillation spectrometry measurements

Cells (8×10^5 in a 9-cm^2 dish) were incubated in 0.6 ml of culture medium or sodium-free transport medium containing 40 μl of 1.43 mM [^{14}C]AA (7 mCi/mmol) or 40 μl of 1.43 mM [^{14}C]DHA. The latter compound was prepared by incubating [^{14}C]AA with 2 units of ascorbate oxidase immediately before being added to the incubation medium; complete oxidation was verified by HPLC. For deoxyglucose uptake, the incubation medium contained 80 μl of 2 mM [1,2-^{3}H]2-deoxy-D-glucose (50 Ci/mmol); uptake was stopped by adding 10 vol. of ice-cold PBS. The cells were washed with ice-cold PBS, harvested by mechanical scraping and disrupted by two cycles of freezing–thawing with liquid nitrogen. An aliquot of the cell lysate was used for protein determination and the remainder was added to the scintillation cocktail. The radioactive content of the cells was measured by liquid-scintillation spectrometry.

Glutathione content

Intracellular reduced and oxidized glutathione content was measured using a DTNB-glutathione reductase recycling assay, according to the method of Anderson [22].

Assays of DHA reductase activity

The samples (containing 0.2–0.3 mg of protein) were incubated at 37 °C for 20 min in 200 μl (final volume) of 50 mM Tris/HCl, pH 7.5, containing 1 mM EDTA and 1 mM DHA in order to measure basal DHA reductase activity. The basal activity was calculated after correction for non-enzymic reduction of DHA by a concentration of GSH corresponding to its cellular content. To test cofactor specificity for DHA reductase activity, GSH, NADPH/NADH or lipoic acid was added to the reaction mixture; in the mitochondrial fraction, lipoic acid-dependent DHA reductase activity was also tested in the presence of succinic acid or α-ketoglutaric acid. The reaction was stopped by adding ice-cold 70% (v/v) methanol containing 1 mM EDTA. After incubation for 5 min on ice, the samples were centrifuged to remove precipitated proteins and the supernatant was assayed by HPLC for ascorbate content.

Statistics

Statistical analysis of means $\pm$ S.D. was conducted with the program StatView 4.02 for the Macintosh (Abacus Concept, Berkeley, CA, U.S.A.).

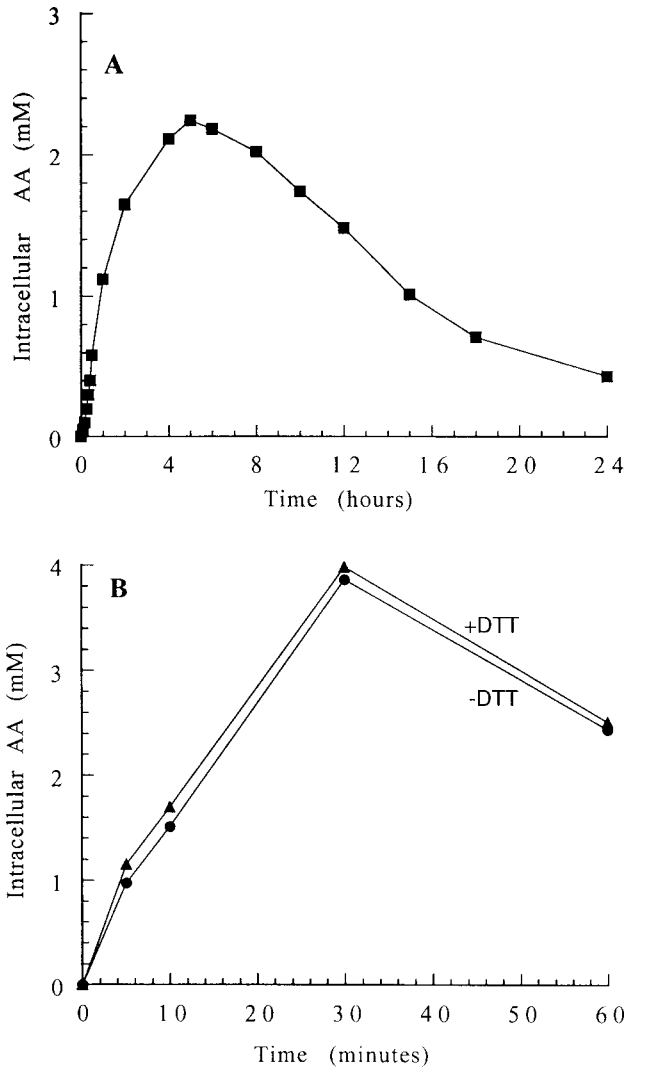

Figure 1 Time course of AA and DHA uptake by HaCaT cells

(**A**) Time-dependent uptake of 0.05 mM AA. (**B**) Time-dependent uptake of 0.05 mM DHA. Intracellular AA content was determined by HPLC. For DHA uptake, the intracellular AA content was measured both in the absence (−DTT) and in the presence (+DTT) of 10 mM DTT. Conditions were as reported in the Experimental Procedures section. Values shown are the means of three independent experiments (S.D. $\leqslant$ 9%).

RESULTS

DHA and AA transport

HaCaT cells accumulate AA when incubated with DHA, as shown by experiments reported in Figures 1 and 2. DHA was probably transported across the cell membrane and then reduced to AA upon entry into the cell. Preliminary experiments were thus performed to differentiate transport of DHA from that of AA and to distinguish the DHA-transport step from its reduction.

Kinetic analysis showed two patterns of intracellular AA and DHA uptake. When HaCaT cells were incubated with 50 μM AA, a concentration in the range of that found physiologically in human plasma [23], the AA accumulated to a maximum value of about 2.2 mM (i.e. about 44-fold) after 5 h (Figure 1A). When HaCaT cells were incubated with 50 μM DHA, AA accumulated to higher intracellular concentrations (about 80-fold), reaching a maximum after 30 min; the vitamin C was all present in the reduced form, as indicated by DTT treatment (Figure 1B). Imported DHA was completely reduced by HaCaT cells when incubated with DHA up to a concentration of 0.1 mM, indicating that transport, and not reduction, was the rate-limiting step. However, when HaCaT cells were incubated with DHA at concentrations > 0.1 mM, intracellular reduction was the rate-limiting process, and vitamin C accumulated both in its oxidized and reduced forms; for example, when cells were incubated with 2 mM DHA, the intracellular concentration of DHA was equal to that of AA (Figure 2).

To verify the different transport mechanisms of AA and DHA, the effects of Na^+, of hexose-transport competitors (glucose and deoxyglucose) [24] and of a specific hexose-transport inhibitor (cytochalasin B) [25] on the vitamin C transport were examined. AA uptake was Na^+-dependent although unaffected by hexose-transport inhibitors/competitors, whereas DHA uptake was Na^+-

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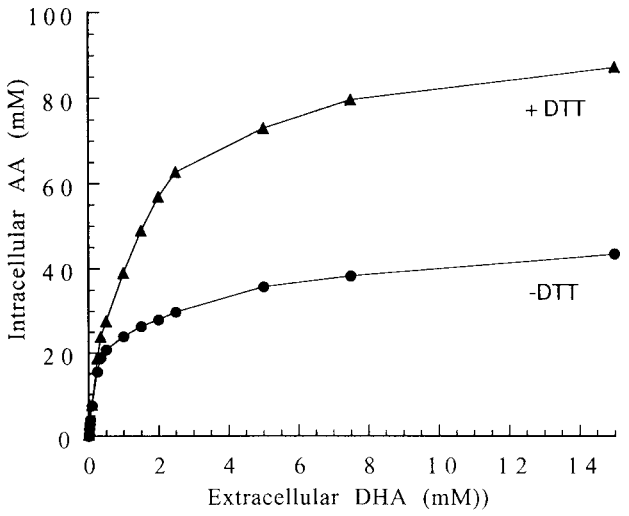

Figure 2 Concentration-dependence of DHA uptake by HaCaT cells

HaCaT cells were incubated with different concentrations of DHA (1 μM–15 mM) for 30 min. Intracellular AA concentration was determined by HPLC in the absence (−DTT) and in the presence (+DTT) of 10 mM DTT. Values are the means of five independent experiments (S.D. $\leqslant$ 11%).

Table 1 Effect of Na^+, sugars or inhibitor on [^{14}C]AA and [^{14}C]DHA transport

The radioactive contents of the cells were determined by liquid-scintillation spectrometry. In the control cells, the mean intracellular content of [^{14}C]DHA was 1.7 mM, whereas the mean intracellular content of [^{14}C]AA was 0.12 mM (S.D. = 5%), after 10 min of incubation (see the Experimental Procedures section for details). Glucose, deoxyglucose and cytochalasin B were added simultaneously with [^{14}C]DHA or [^{14}C]AA. Data are the means of four independent experiments (S.D. $\leqslant$ 8%). Similar results were also obtained with HPLC measurements.

	[^{14}C]DHA (% over control)	[^{14}C]AA (% over control)
10 mM Glucose	72.2	87.7
10 mM Deoxyglucose	51.9	100.9
10 μM Cytochalasin B	2.9	86.6
Na^+ depletion	97.9	3.3

independent although affected by cytochalasin B and deoxyglucose (Table 1).

DHA reductase activity

HaCaT-cell homogenate showed a basal DHA reductase activity of 1.76 nmol/min per mg of protein. Glutathione was derived only from cell homogenate, corresponding to about 0.1 mM in the reaction mixture since the intracellular glutathione content of HaCaT cells was 8–12 mM. Basal DHA reductase activity was not modified in the presence of exogenous GSH, but increased in the presence of exogenous NADPH, NADPH plus GSH or NADH plus lipoic acid, as summarized in Table 2. The subcellular distribution of DHA reductase-specific activity in the presence of these cofactors is reported in Figure 3. The addition of NADPH, cofactor for thioredoxin reductase and 3α-hydroxysteroid dehydrogenase, increased both mitochondrial and cytosolic DHA reductase. GSH and NADPH, cofactors for glutaredoxin, protein disulphide-isomerase and glutathione peroxidase, increased cytosolic activity with no effect on mitochondrial activity. NADH and lipoic acid, cofactors for mitochondrial lipoamide dehydrogenase, increased mitochondrial DHA reductase specific activity, but did not affect cytosolic activity. The role of lipoamide dehydrogenase in DHA reduction was investigated further by analysing the effects of α-oxoglutarate and succinic acid, the substrate and the product, respectively, of the multi-enzyme complex α-oxoglutarate dehydrogenase. The addition of α-oxoglutarate further increased NADH-lipoic acid-dependent mitochondrial activity whereas the addition of succinic acid had an inhibitory effect (Table 3).

Table 2 Cofactor specificity for DHA reductase activity in HaCaT cells

Corrections were made for blanks when appropriate. In the reaction mixture, only the glutathione derived from cell homogenate was present. The basal mixture was composed of 50 mM Tris/HCl, pH 7.5, containing 1 mM DHA, 1 mM EDTA and 0.2 mg of cell homogenate protein. The basal activity was calculated after correction for non-enzymic reduction of DHA by a concentration of GSH corresponding to its cellular content. This chemical reaction corresponded to 2–4% of the enzymic activity. The experiment was performed using at least three different homogenates with similar results (S.D. $\leqslant$ 5%).

Assay composition	DHA reduction (nmol/min per mg of protein)	Relative activity (%)
Basal	1.76	100
Basal + 2 mM GSH	1.76	100
Basal + 0.4 mM NADPH	2.22	126
Basal + 2 mM GSH + 0.4 mM NADPH	5.16	293
Basal + 0.4 mM NADH	1.76	100
Basal + 0.5 mM lipoic acid	1.78	101
Basal + 0.5 mM lipoic acid + 0.4 mM NADH	3.04	173
Basal + 0.5 mM lipoic acid + 0.4 mM NADPH	2.24	127

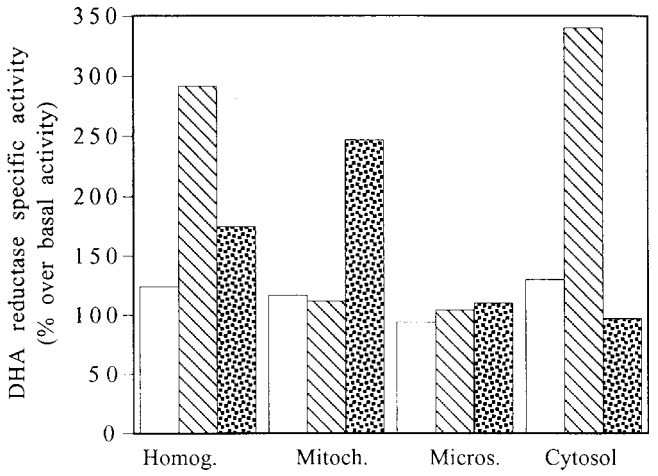

Figure 3 Cofactor specificity for DHA reductase activity in subcellular fractions from HaCaT cells

DHA reductase activities in subcellular fractions were measured in the presence of 0.4 mM NADPH (white columns), 0.4 mM NADPH + 2mM GSH (hatched columns) and 0.5 mM lipoic acid + 0.4 mM NADH (dotted columns). The basal DHA reductase activities were: 1.9 nmol/min per mg of protein measured in cell homogenate (1.32 mg of total protein; Homog.); 0.7 nmol/min per mg of protein measured in mitochondria (0.28 mg of total protein; Mitoch.); 0.3 nmol/min per mg of protein measured in microsomes (0.19 mg of total protein; Micros.); and 3.3 nmol/min per mg of protein measured in cytosol (0.65 mg of total protein). Values are the means of three independent experiments (S.D. $\leqslant$ 12%).

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Table 3 Lipoic acid-dependent DHA reductase activity in mitochondria from HaCaT cells

Corrections were made for blanks when appropriate. The basal mixture containing 0.2 mg of mitochondrial protein was as shown in Table 2. The experiment was performed using four different mitochondrial fractions (S.D. $\leqslant$ 5%).

Assay composition	DHA reduction (nmol/min per mg of protein)	Relative activity (%)
Basal + 0.4 mM NADH + 0.5 mM lipoic acid	1.75	100
Basal + 0.4 mM NADH + 0.5 mM lipoic acid + 6.4 mM succinic acid	0.87	49.7
Basal + 0.4 mM NADH + 0.5 mM lipoic acid + 3.2 mM α-ketoglutarate	1.96	112

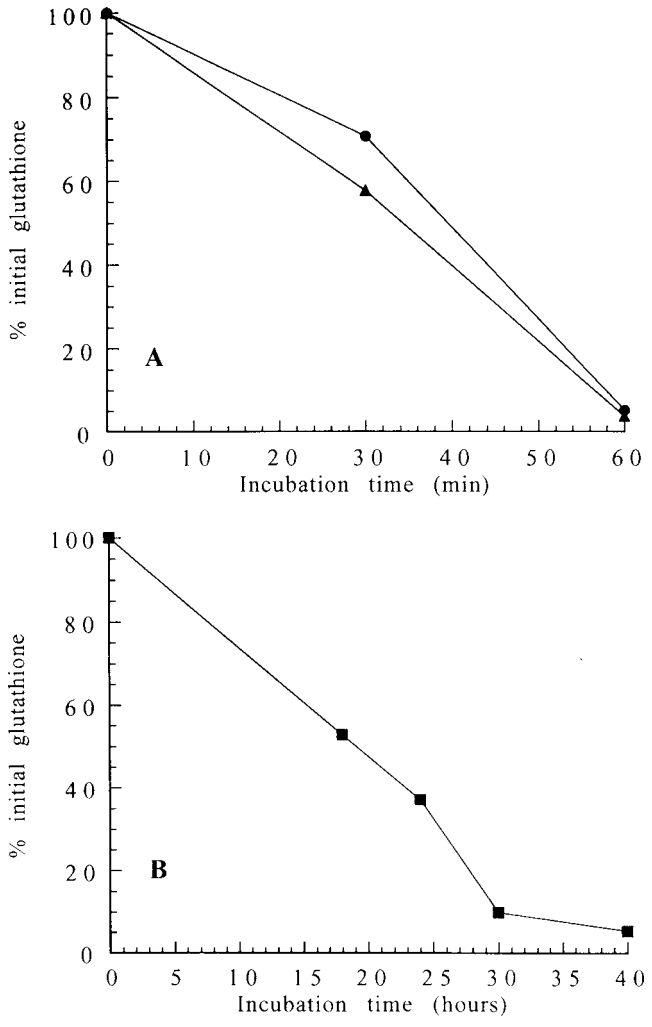

Figure 4 Depletion of HaCaT-cell glutathione by different treatments

(**A**) Time-dependent effects of 0.2 mM ethacrynic acid (●) and 1 mM diethyl maleate (▲) on total intracellular glutathione content. (**B**) Time-dependent effect of 1 mM buthionine sulphoximine on total intracellular glutathione content. Values are the means of three independent experiments (S.D. $\leqslant$ 8%).

To obtain more information on the enzymic systems involved in DHA reduction, DHA uptake after cellular glutathione depletion was analysed. Three different procedures were used to obtain glutathione depletion (Figure 4); buthionine sulphoximine was used as an irreversible inhibitor of γ-glutamylcysteine synthetase, the first enzyme of glutathione synthesis; diethyl maleate was used as a covalent and irreversible reagent for free cysteines; and ethacrynic acid was used as a penetrating thiol reagent to deplete even mitochondrial glutathione. The data on DHA uptake obtained by HPLC measurements were consistent with those obtained by scintillation spectrometry using [^{14}C]DHA (Table 4). To check for possible cell damage, cell viability and lactate dehydrogenase release were tested; no cell damage was observed in any of the reported experiments. Impairment of the DHA-transport system was checked by measuring [1,2-^{3}H]2-deoxy-D-glucose transport. To test for possible AA or DHA extracellular release, cells preloaded with DHA were incubated with a fresh medium that was analysed for AA content at 5, 10 and 30 min. No AA or DHA efflux was observed in any of the reported experiments.

Table 4 Effect of different treatments on [1,2-^{3}H]2-deoxy-D-glucose and [^{14}C]DHA transport in HaCaT cells

HaCaT cells were preincubated in the presence of 1 mM buthionine sulphoximine for 40 h, 0.2 mM ethacrynic acid for 1 h, 1 mM diethyl maleate for 1 h, 0.5 mM iodoacetamide for 15 min, 0.5 mM *p*-chloromercuribenzoate for 15 min or 0.2 mM quercetin for 1 h. Transport experiments were initiated by the addition of 0.1 mM [^{14}C]DHA or 0.26 mM [1,2-^{3}H]2-deoxy-D-glucose. After 10 min of incubation the radioactive contents of the cells were determined. Values represent the means of four independent experiments (S.D. $\leqslant$ 7%).

Treatment	[1,2-^{3}H]2-Deoxy-D-glucose (% over control)	[^{14}C]DHA (% over control)
Buthionine sulphoximine	97.7	144.2
Ethacrynic acid	98.8	42.8
Diethyl maleate	61.8	52.5
Iodoacetamide	86.4	33.0
p-Chloromercuribenzoate	99.4	32.1
Quercetin	46.5	41.3

In cells depleted of glutathione ($\approx$ 95%) by 1 mM buthionine sulphoximine treatment for 40 h, the intracellular vitamin C level after DHA incubation was about 150% higher with respect to control cells. Vitamin C was present in its reduced form, as indicated by DTT treatment (Figure 5). No effect on [1,2-^{3}H]2-deoxy-D-glucose transport was observed (Table 4). DHA reductase activity was enhanced in cells treated with buthionine sulphoximine (Table 5) and this increase may have caused the higher AA cellular trapping. The analysis of subcellular DHA reductase activity showed that both mitochondrial and cytosolic fractions contributed to the increase in enzymic activity (Figure 6).

In cells depleted of glutathione ($\approx$ 95%) by 5 mM diethyl maleate treatment for 1 h, the intracellular level of vitamin C, all of which was in its reduced form, was about half that found in control cells (Figure 5). [1,2-^{3}H]2-Deoxy-D-glucose transport was impaired (Table 4). DHA reductase activity, both basal or in the presence of exogenous 0.1 mM GSH, decreased by about 50% (Table 5). Similarly, in cells depleted of glutathione ($\approx$ 95%) by 0.2 mM ethacrynic acid treatment for 1 h, the intracellular level of vitamin C after DHA incubation decreased by about 50% with respect to control cells (Figure 5), although about 30% was still in the oxidized form. [1,2-^{3}H]2-Deoxy-D-glucose transport was not altered (Table 4), whereas DHA reductase activity, both basal or in the presence of 0.1 mM exogenous GSH, decreased by about 50%. To assess the direct effect of diethyl maleate or ethacrynic acid, DHA reductase activity of homogenate from

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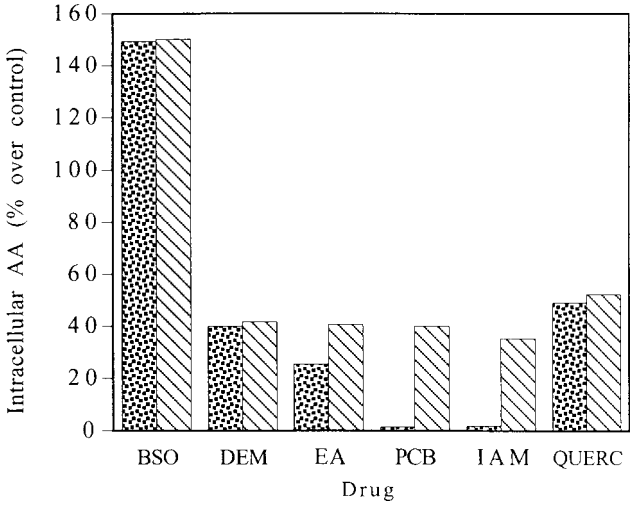

Figure 5 DHA uptake after different HaCaT-cell treatments

All values are expressed as a percentage of DHA uptake by untreated cells (measured as intracellular AA content). DHA uptake was evaluated after specific cell treatments consisting of the addition to the medium of: 1 mM buthionine sulphoximine (BSO) for 40 h, 5 mM diethyl maleate (DEM) for 1 h, 0.2 mM ethacrynic acid (EA) for 1 h, 0.5 mM iodoacetamide (IAM) for 15 min, 0.2 mM *p*-chloromercuribenzoate (PCB) for 15 min and 0.2 mM quercetin (QUERC) for 1 h, before the addition of 0.1 mM DHA. The intracellular AA content was measured after 10 min of incubation. For all samples the intracellular AA content was measured both in the absence (dotted columns) and in the presence (hatched columns) of 10 mM DTT. Values are the means of four independent experiments (S.D. $\leqslant$ 7%).

Table 5 Effects of different treatments on DHA reductase activity in HaCaT cells

Conditions for HaCaT-cell treatments were the same as those reported in Table 4. DHA reductase activities of untreated cell homogenates were measured after a 5-min preincubation of the cell homogenates with the different drugs. The final concentrations of the drugs in the incubation mixtures were the same as those used to treat the cells, as reported in Table 4. Values are the means of five independent experiments (S.D. $\leqslant$ 9%).

	DHA reductase activity (% over control)	
Treatment	Treated cell homogenates	Untreated cell homogenates
Buthionine sulphoximine	147.8*	100.0
Ethacrynic acid	45.1*	50.1
Diethyl maleate	56.3*	59.4
Iodoacetamide	3.4	8.9
p-Chloromercuribenzoate	2.6	12.6
Quercetin	96.7	99.3

* DHA reductase activity measured in the presence of 0.1 mM exogenous glutathione.

untreated cells was measured after the compounds were added to the assay mixture. As shown in Table 5, both compounds immediately inhibited this activity.

Ethacrynic acid treatment mainly affected intracellular reduction since the transport system was not impaired and intracellular vitamin C was partially oxidized. At variance with AA, which was slowly transported, DHA showed rapid transport kinetics (Figure 1) and thus, within the range of our experimental times, the amount of DHA that exits the cell could be significant, if not immediately reduced to AA. In any case, DHA efflux was not found in our experiments, so if DHA reduction did not

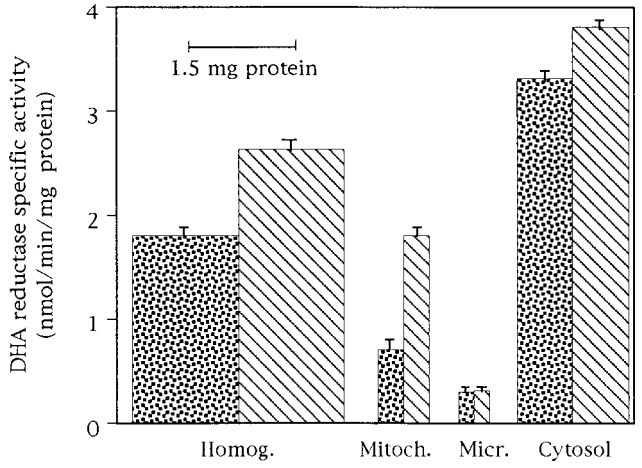

Figure 6 Effect of buthionine sulphoximine treatment on DHA reductase activity in different subcellular fractions from HaCaT cells

DHA reductase activities in different subcellular fractions (for abbreviations see Figure 3) from untreated cells (dotted columns) and from BSO-treated cells (hatched columns) are shown. Values are the means $\pm$ S.D. of three independent experiments.

occur, this unstable compound was likely to be degraded, thus explaining the low levels of intracellular vitamin C found in cells with altered DHA-reducing activity. Similarly to ethacrynic acid, diethyl maleate affected enzymic DHA reduction. However, it also impaired DHA transport, an observation which precludes any conclusions about the effect of this drug. On the other hand, the use of less experimentally severe conditions, such as low levels of diethyl maleate or a short incubation time, did not lead to satisfactory gluthathione depletion. In any case, the effects of both ethacrynic acid and diethyl maleate treatment could not be explained by glutathione depletion (5% residual glutathione versus 50% residual DHA reduction), and were probably due to the ability of these compounds to react with protein thiol groups, in particular with the disulphide/dithiol present in the redox-active site of thioredoxin reductase and lipoamide dehydrogenase, both GSH-independent enzymes.

To verify this hypothesis, the effects of iodoacetamide and *p*-chloromercuribenzoate, two selective thiol protein reagents, on DHA uptake were analysed. For both iodoacetamide (0.5 mM) and *p*-chloromercuribenzoate (0.2 mM), a 15-min treatment drastically decreased the intracellular levels of vitamin C, which was all in its oxidized form (Figure 5). The compounds severely affected DHA reductase activity (Table 5), although they did not induce cell damage and only slightly impaired [1,2-^{3}H]2-deoxy-D-glucose transport (Table 4).

To determine whether HaCaT cells also possess DHA reductase activity that corresponds to the GSH-independent 3α-hydroxysteroid dehydrogenase, the effect of quercetin, a potent inhibitor of this enzyme, on DHA uptake was analysed. The results of these experiments showed that in quercetin-treated cells, the level of intracellular vitamin C decreased, and all the vitamin was present in its reduced form (Figure 5). No significant effect on enzymic DHA reductase activity was observed in treated cells or in untreated cell homogenate directly supplemented with quercetin (Table 5), indicating that 3α-hydroxysteroid dehydrogenase was not involved in DHA reduction in this cell line. Instead, a drastic effect on [1,2-^{3}H]2-deoxy-D-glucose was observed (Table 4). This is in agreement with a

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recent report showing that some flavonoids, i.e. quercetin and genistein, can inhibit vitamin C transport in human cells [26].

DISCUSSION

The ability of cells to accumulate AA when incubated with DHA depends on the efficiency of the transport and reduction processes. The present study was performed to characterize DHA uptake by HaCaT cells. We have demonstrated previously that this cell line accumulates AA when incubated with a stable derivative of AA, ascorbic acid 2-phosphate [3]. The present study showed that HaCaT cells are able to accumulate AA to the same extent when incubated with DHA, indicating that HaCaT cells are able to transport and to reduce DHA, leading to a rapid increase of intracellular AA also in the absence of AA. In HaCaT cells DHA transport is entirely distinct from that of AA. DHA transport is Na^+-independent and mediated by facilitative hexose transporters.

In this cell line, DHA reduction is a limiting step in DHA uptake when the extracellular DHA concentration is higher than 0.1 mM. Therefore, an increase in DHA caused by stress conditions, such as prolonged UV exposure or chronic skin disorders, to a concentration greater than physiological plasma levels (1–2 μM) [23] should be well tolerated in epithelial cells, given that up to 0.1 mM both the transport and the reduction systems work at maximum efficiency.

HaCaT cells possess both cytosolic and mitochondrial DHA reductase activities. DHA reduction in HaCaT cells was not affected by glutathione depletion and thus, glutathione and the GSH-dependent enzymes glutaredoxin, protein disulphide-isomerase and glutathione peroxidase have a secondary role in this process. On the other hand, DHA reduction was greatly affected by thiol reagents, suggesting that one or more enzymes with redox-active disulphide/dithiol are involved in this reaction. The putative enzymic systems were found to be the NADPH-dependent thioredoxin reductase system, consistent with the known inhibition of this enzyme by *p*-chloromercuribenzoate [27], and the NADH-lipoic acid system, consistent with the known inhibition of lipoamide dehydrogenase by iodoacetamide [13]. The results reported in Table 3 provide further evidence that mitochondrial DHA reductase activity may largely be mediated by lipoamide dehydrogenase. In any case, the extent to which these NAD(P)H-dependent activities predominate in HaCaT cells is difficult to determine.

Previous studies have indicated that glutaredoxin is responsible for the 80% DHA reduction in neutrophils [28], and that both GSH-dependent and GSH-independent systems are responsible for DHA reduction in erythrocytes [29]. At variance with this is that only GSH-independent systems are operative in HL-60 cells [30] and in astrocytes [31]. These results suggest that the enzymes involved in DHA reduction differ depending on the cell type and on the metabolic requirements. It has been shown that selenoproteins and, in particular, thioredoxin reductase, play an important role in the protection of human skin against UV-B radiation [32]. This protective effect may also be linked with a role in AA recycling.

The higher DHA uptake by cells in which glutathione synthesis was inhibited by buthionine sulphoximine may be explained as a response of the cell to oxidative stress induced by buthionine sulphoximine, either directly, through the induction of enzymes with DHA reductase activity, or indirectly, through the induction of glucose-6-phosphate deydrogenase, which is responsible for the generation of NADPH required in many detoxifying reactions [33]. Expression of the thioredoxin/thioredoxin reductase system is significantly induced under oxidative conditions in lymphoid and keratinocyte cells [34]; a similar induction might also occur in HaCaT cells. Our results also suggest that DHA reductase activity may be part of an inducible mechanism that is activated in the cell in response to altered redox status.

Efficient DHA uptake by HaCaT cells is consistent with the important role that this pathway plays in detoxifying the skin during oxidative stress. Skin damage is a rapid event, and vitamin C prevents such damage only when present in relevant concentrations during oxidative stress [35]. Impaired AA recycling can result in a rapid vitamin C depletion and decreased antioxidant defence. It has been shown in disorders such as insulin-dependent diabetes mellitus, which involve impaired DHA reductase activity, that the cutaneous sensitivity to oxidative injury increases [36]; moreover it has been demonstrated recently that hepatocytes from older rats, with significantly decreased AA-recycling ability compared with cells from younger rats, have a declining ability to respond to increased oxidative stress [37]. In this study, it is demonstrated clearly that the ability of HaCaT cells to reduce DHA corresponds to intracellular AA level. In light of the relationship between DHA reductase activity and vitamin C status in HaCaT cells, further characterization of the enzymes involved in AA recycling in the skin would be of great interest.

We thank Mariarosaria De Stefano for excellent technical assistance in parts of this work. This research was supported by grants from the Italian MURST (Ministero dell'Università e della Ricerca Scientifica e Tecnologica) and from the Italian CNR (Consiglio Nazionale delle Ricerche).

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Received 3 August 1999/11 October 1999; accepted 10 November 1999

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