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Requirement for GSH in recycling of ascorbic acid in endothelial cells

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Abstract

Ascorbic acid may be involved in the defense against oxidant stress in endothelial cells. Such a role requires that the cells effectively recycle the vitamin from its oxidized forms. In this work, we studied the ability of cultured bovine aortic endothelial cells (BAECs) to take up and reduce dehydroascorbic acid (DHA) to ascorbate, as well as the dependence of ascorbate recycling on intracellular GSH. BAECs took up and reduced DHA to ascorbate much more readily than they took up ascorbate. Although BAECs in culture did not contain ascorbate, ascorbate accumulated to concentrations of 2–3 mM in BAECs following incubation with 400 μM DHA. Extracellular ferricyanide oxidized intracellular ascorbate, which was recycled by the cells. Reduction of DHA, either when added to the cells or when generated in response to ferricyanide, caused significant decreases in intracellular GSH concentrations. Depletion of intracellular GSH with 1-chloro-2,4-dinitrobenzene, diethylmaleate, and diamide almost abolished the ability of the cells to reduce DHA to ascorbate. DHA reduction by thioredoxin reductase was evident in dialyzed cell extracts, but occurred at rates far lower than direct GSH reduction of DHA. These results suggest that maximal rates of DHA reduction, and thus recycling of ascorbate from DHA, are dependent upon GSH in these cells.

Keywords: Ascorbic acid; GSH; Ferricyanide; Dehydroascorbic acid; Bovine aortic endothelial cells

1. Introduction

Endothelial cells are exposed in the vascular bed to a variety of oxidant stresses of both endogenous and exogenous origin [1]. Endogenous oxidants include superoxide and H_2O_2 generated in the mitochondria [2], as well as the product of superoxide and nitric oxide reaction, peroxynitrite [3]. Exogenous oxidants include serum-derived oxidized LDL [1,4] and peroxides and hypochlorous acid generated by inflammatory cells in areas of atherosclerosis [5]. To protect against such oxidant stress, endothelial cells use enzymes such as superoxide dismutase, GSH peroxidase, and catalase [6,7]. They also rely on low molecular weight antioxidants, including GSH [8–10] and possibly ascorbic acid. With regard to ascorbate, Martin and Frei [11] reported that cultured human aortic endothelial cells and HUVECs loaded with millimolar concentrations of ascorbate have lower rates of LDL oxidation than control cells. They also showed that this effect of ascorbate likely relates to decreased H_2O_2 generation by the cells [11]. On the other hand, ascorbate-loading provides little protection of HUVECs against lipid peroxidation initiated by exogenous H_2O_2 [12].

Whereas an antioxidant role for ascorbate in endothelial cells must be confirmed, results from numerous clinical studies suggest that ascorbate can reverse endothelial dysfunction in a variety of conditions associated with oxidant stress. These conditions include hypercholesterolemia [13], hypertension [14], smoking [15], diabetes [16], and atherosclerosis [17,18]. The mechanism of the ascorbate effect has not been established, but is considered by most to relate to the removal of superoxide before it reacts with endothelial cell-generated nitric oxide to form peroxynitrite [19].

If ascorbate is important to endothelial cells as an antioxidant, then they should be able to maintain relatively high intracellular ascorbate concentrations. A key mechanism used by other cell types to conserve ascorbate involves reduction of its oxidized forms back to ascorbate. These forms include the AFR and DHA, which are one- and two-electron-oxidized derivatives of ascorbate, respec-

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Abbreviations: AFR, ascorbate free radical; BAECs, bovine aortic endothelial cells; CDNB, 1-chloro-2,4-dinitrobenzene; DHA, dehydroascorbic acid; HUVECs, human umbilical vein endothelial cells; KRH, Krebs-Ringer HEPES; and LDL, low density lipoprotein.

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tively. DHA uptake and reduction have been demonstrated for HUVECs, resulting in intracellular concentrations as high as 5–10 mM [12]. How endothelial cells carry out the reduction or recycling of DHA to ascorbate is unknown. Both GSH-dependent and -independent mechanisms of ascorbate recycling have been demonstrated in other cell types. For example, human erythrocytes [20,21] and lens epithelium [22] require GSH for optimal reduction of DHA to ascorbate. On the other hand, neither HL-60 cells [23] nor human keratinocytes [24] require GSH to reduce DHA to ascorbate. Since GSH is important in protecting cultured endothelial cells from oxidant stress induced by H_2O_2 [8] and oxidized LDL [9,25], it is necessary to consider whether or not these cells require GSH to maintain ascorbate.

The present studies were carried out in BAECs to determine whether these cells use GSH to reduce DHA to ascorbate. The results show that BAECs take up DHA much more rapidly than they take up ascorbate, that BAECs rapidly recycle DHA to ascorbate, and that this recycling requires GSH for optimal activity.

2. Materials and methods

2.1. Materials

Amersham Pharmacia Biotech provided the 3-*O*-[^{14}C] methyl-d-glucose (53 mCi/mmol), and New England Nuclear Life Science Products Inc. supplied the l-[^{3}H]glucose (14.6 Ci/mmol). Cell culture medium was prepared by the Cell Culture Core of the Vanderbilt Diabetes Research and Training Center. Ascorbate 2-phosphate was purchased from the Wako Chemical Co. Analytical reagents, including aurothioglucose, diethylmaleate, CDNB, diamide, ferricyanide, selenocystine, and tetrapentylammonium bromide were obtained from the Sigma Chemical Co./Aldrich Chemical Co. Diethylmaleate and CDNB were initially dissolved in dimethyl sulfoxide, such that final concentrations of the latter were 0.8% (v/v) at 2 mM diethylmaleate, and 0.2% (v/v) at 20 mM CDNB. These were the highest concentrations of the reagents used for the experiments. In control experiments not shown, dimethyl sulfoxide at 1% had no effect on intracellular ascorbate concentrations following DHA loading, or on ferricyanide reduction.

2.2. Cell culture and preparation for assays

BAECs were isolated and characterized both morphologically and immunocytochemically as described previously [26]. BAECs were cultured at 37° under 5% CO_2 in Dulbecco's Minimal Essential Medium that contained 20 mM HEPES, 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL of penicillin, and 100 U/mL of streptomycin. The culture medium was changed every 2–3 days, and experiments were carried out using confluent cultures between passages 8 and 12. Cells were cultured in 12-well plates and, unless noted otherwise, experiments were initiated by gently removing the culture medium and rinsing three times with 1 mL of 37° KRH that consisted of 20 mM HEPES, 128 mM NaCl, 5.2 mM KCl, 1 mM NaH_2PO_4, 1.4 mM $MgSO_4$, and 1.4 mM $CaCl_2$, pH 7.4.

2.3. Measurement of intracellular water space

Intracellular water space was taken to correspond to the distribution of 3-*O*-[^{14}C]methylglucose, corrected for trapped extracellular water using l-[^{3}H]glucose. Cells in KRH were incubated with 0.2 μCi of 3-*O*-[^{14}C]methylglucose and 0.5 μCi of l-[^{3}H]glucose for 10 min at 37°. After removal of an aliquot of the buffer for the counting of radioactivity, the cells were rinsed twice with 1 mL of ice-cold KRH and lysed in 1 mL of 80% methanol (v/v) that contained 1 mM EDTA. The cell lysate was assessed for radioactivity on a Packard CA-2000 liquid scintillation spectrometer, with windows set for dual-label counting. The intracellular water space was calculated as follows. The ratio of ^{14}C disintegrations per minute in the cells to the disintegrations per minute in the total volume of buffer was determined, and from this was subtracted the corresponding ratio of ^{3}H disintegrations per minute. The latter was used to provide an estimate of the extracellular space remaining in the washed cells. Corrected volume in a confluent well of cells was 1.35 μL/mg cell protein (0.11 $\pm$ 0.04 μL/well of cells), which is in agreement with previous calculations [11].

2.4. Measurement of intracellular ascorbic acid and GSH

After incubation as indicated, the medium was aspirated, and the cells were gently rinsed twice with 2 mL of ice-cold KRH. The medium was removed, and the monolayer was treated with 0.5 mL of 80% methanol:water (v/v) containing 1 mM EDTA. The cells were scraped from the plate, the methanol treatment was repeated, and the methanol fractions were combined. Debris was pelleted in a microfuge, and the supernatant was taken for the assay of ascorbate as previously described [27], except that tetrapentylammonium bromide was used as the ion pair reagent. GSH was assayed by the method of Hissin and Hilf [28]. This assay specifically measures GSH and not GSSG or other low molecular weight thiols. Intracellular concentrations of ascorbate and GSH were calculated based on the intracellular water space present in a well of confluent cells, measured as described above.

2.5. Assay of ferricyanide reduction

Ferricyanide reduction was measured as the generation of ferrocyanide in the incubation medium of BAECs. Due to their size and charge, neither ferricyanide nor ferrocyanide crosses the plasma membrane [29]. After the indicated pretreatments, BAECs were rinsed twice in 1 mL of warm

KRH and incubated at 37° in KRH that contained 1 mM ferricyanide and 5 mM d-glucose, except where noted. After 30 min, duplicate aliquots of the incubation medium were taken for the assay of ferrocyanide by the method of Avron and Shavit [30], using *o*-phenanthroline as the detecting agent.

2.6. Measurement of DHA reduction in dialyzed cell extracts

Cells were scraped from 12 wells of a plate, suspended in 1.5 mL PBS, frozen in dry ice-acetone, and allowed to thaw on ice. The lysate was microfuged at 16,000 *g* for 5 min at 3°, and the resulting supernatant was centrifuged at 100,000 *g* for 1 hr at 3° in a Beckman T-100 ultracentrifuge. The supernatant was dialyzed overnight against three changes of Tris-EDTA buffer in 3500 molecular weight cut-off dialysis tubing. The dialysate was used immediately for the assay of DHA reduction, as previously described [31].

2.7. Other methods

Protein was determined by the method of Bradford [32]. Statistical comparisons were made using SigmaStat 2.0 software (Jandel Scientific). Differences between treatment groups were assessed by two-way analysis of variance with post-hoc testing using the Tukey test.

3. Results

Without supplementation, ascorbate was not detected in BAECs in culture. When BAECs were incubated with DHA or ascorbate in KRH at 37°, accumulation of ascorbate in the cells was linear for at least 40 min (results not shown), so that a time point of 30 min was chosen for the concentration–response studies. The ability of BAECs to take up increasing concentrations of ascorbate, DHA, and ascorbate 2-phosphate is shown in Fig. 1. Intracellular ascorbate concentrations increased very little following incubation of BAECs with ascorbate (Fig. 1). This contrasts with the uptake and reduction of DHA to ascorbate, which increased linearly with increasing extracellular DHA concentrations. Following incubation with 400 μM DHA, intracellular ascorbate reached concentrations of 1.5 to 3 mM, although there was variability between different cultures in the response attained (Fig. 1). To determine whether intracellular ascorbate concentrations could be increased by supplementation in culture, cells were incubated overnight with ascorbate 2-phosphate. As also shown in Fig. 1, this resulted in a linear increase in intracellular ascorbate up to about 200 μM ascorbate 2-phosphate, above which there was no further uptake. The maximal intracellular ascorbate concentration achieved following overnight incubation with ascorbate 2-phosphate was 0.7 ± 0.03 mM. To determine whether loading BAECs with ascorbate affects their ability to respond to an extracellular oxidant stress, the effects of ferricyanide were studied, with the results shown in Fig. 2.

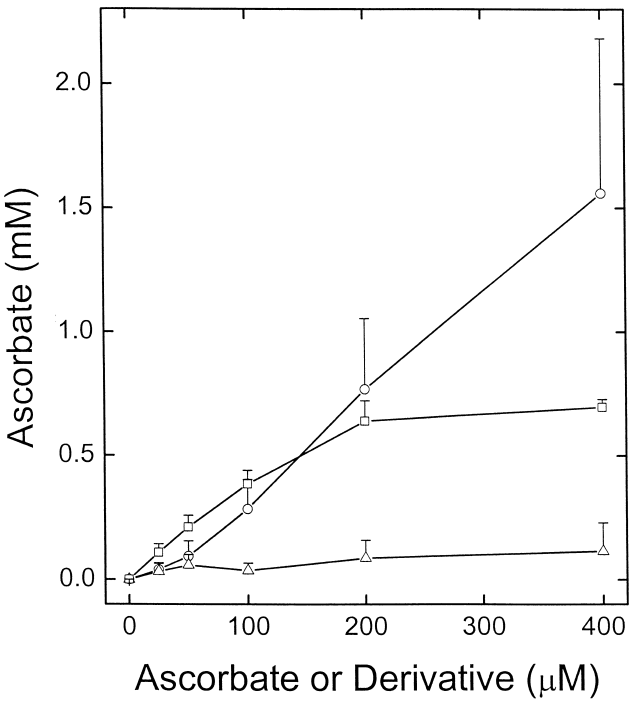

Fig. 1. Concentration-dependence of the uptake of ascorbate and its derivatives into BAECs. For the uptake of DHA (circles, N = 6 experiments) and ascorbate (triangles, N = 4 experiments), rinsed cells were incubated for 30 min at 37° in KRH that contained 5 mM d-glucose before determination of ascorbate. Cellular ascorbate was determined in cells treated in culture with ascorbate 2-phosphate for 16–18 hr (squares, N = 3 experiments). All values are shown as means ± SEM.

Incubation with either DHA or ascorbate at the concentrations used in the studies shown in Fig. 1 increased the ability of BAECs to reduce extracellular ferricyanide (Fig. 2). The effect of DHA was several-fold greater than that of ascorbate, in accord with differing intracellular ascorbate concentrations, as measured in the studies shown in Fig. 1. In the absence of ferricyanide, ascorbate was undetectable in extracellular medium during DHA treatment over this incubation period (results not shown). This indicates that DHA was not reduced on the outer surface of the cells, and that ascorbate did not leak in appreciable amounts from the cells. The marked increase in intracellular ascorbate concentrations and ferricyanide reduction due to loading with DHA also suggests that BAECs have efficient reduction mechanisms for DHA. The extent to which these are dependent on GSH was assessed using several approaches.

First, the effect of increasing concentrations of ferricyanide on intracellular concentrations of ascorbate and GSH was determined in cells that had been preloaded with ascorbate by incubation with 400 μM DHA. As expected, DHA treatment increased the rate of ferricyanide reduction (Fig. 3A), which was nearly maximal at 0.2 mM ferricyanide. Intracellular ascorbate concentrations decreased to 28% of the levels found in ascorbate-loaded cells not treated with

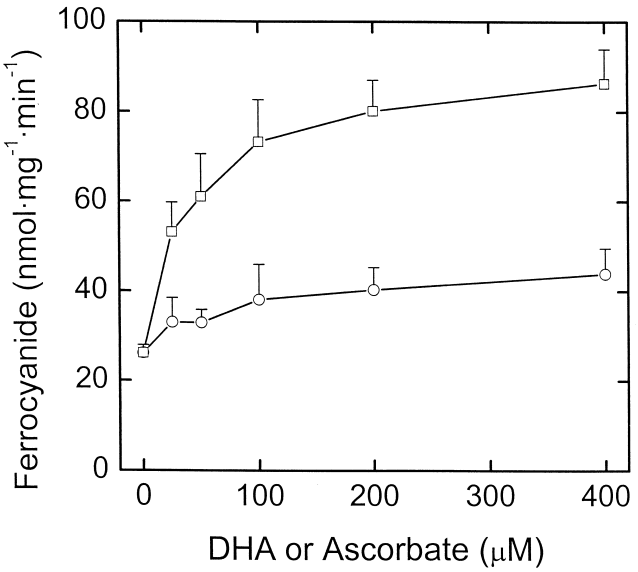

Fig. 2. Ascorbate-dependent enhancement of extracellular ferricyanide reduction by BAECs. Rinsed cells were incubated for 30 min at 37° in 1 mL KRH that contained 5 mM d-glucose and the indicated concentration of DHA (squares) or ascorbate (circles) before the assay of ferricyanide reduction. Results are shown as means ± SEM from 3 experiments.

ferricyanide (Fig. 3B). Since the amount of ferricyanide added was in excess of that reduced, the plateau in ferricyanide reduction could have been related, in part, to ascorbate depletion. The total amount of ferricyanide reduced by the cells at 0.2 mM ferricyanide ($\sim$ 60 nmol $\cdot$ mg^{-1} $\cdot$ min^{-1}) that was ascorbate-dependent (i.e. in excess of basal amounts) was almost three orders of magnitude greater than was the amount of ascorbate oxidized ($\sim$ 0.07 nmol $\cdot$ mg^{-1} $\cdot$ min^{-1}) at a ferricyanide concentration of 0.2 mM. This indicates that the cells were recycling ascorbate to maintain the observed rates of ferricyanide reduction and intracellular ascorbate concentrations. GSH concentrations decreased significantly with increasing extracellular ferricyanide, but only by 12% at 1 mM ferricyanide (Fig. 3C). These results suggest that BAECs have a high capacity to recycle ascorbate and that GSH is affected by this reduction, albeit to a small extent.

The role of GSH in DHA reduction was investigated further by assessing whether DHA loading of the cells affects intracellular GSH concentrations. Incubation of BAECs with relatively high concentrations of DHA caused a small but progressive depletion of intracellular GSH, which was significant at DHA concentrations of 1 mM and higher (Fig. 4). Omission of glucose from the incubations did not affect the relative decreases in intracellular GSH (results not shown). The DHA-induced decreases in intracellular GSH suggest that reduction of high DHA concentrations requires GSH, either directly to provide reducing equivalents, or in response to DHA-induced oxidant stress in the cells. If GSH is required for DHA reduction by BAECs, then selective depletion of GSH should diminish

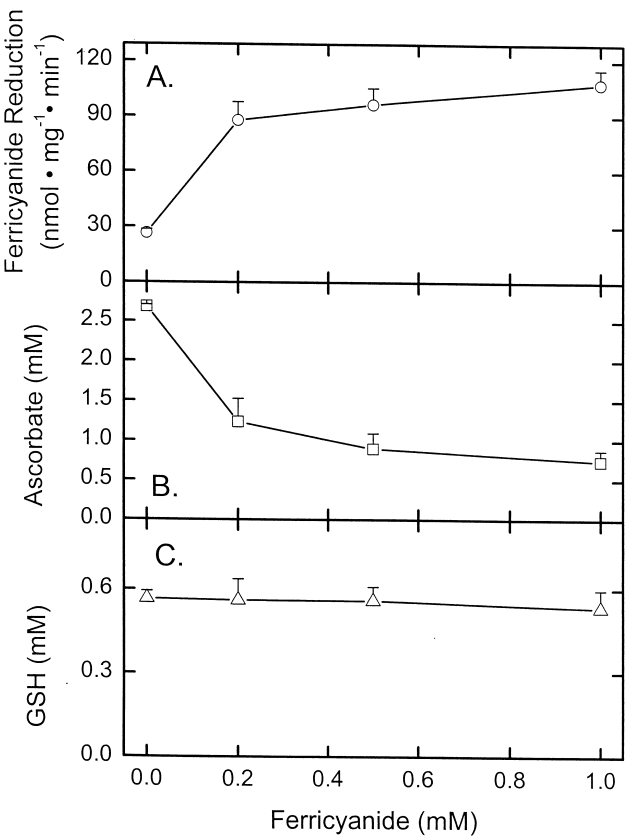

Fig. 3. Effects of increasing ferricyanide concentrations on BAECs. Rinsed cells were incubated for 10 min at 37° in KRH that contained 5 mM d-glucose and 0.4 mM DHA. Ferricyanide was then added to the indicated concentration, and incubations were continued at 37°. At 30 min, aliquots of the buffer were removed for the assay of ferrocyanide (panel A), and the cells were rinsed twice in 1 mL KRH before determination of intracellular ascorbate (panel B) and GSH (panel C). Results are shown as means ± SEM from 3 experiments. Linear regression of the GSH results generated a line with a significant decrease in slope ($P < 0.01$).

the ability of the cells to reduce DHA. This was tested with several different GSH-depleting reagents.

Preincubation of cells with increasing concentrations of diethylmaleate (Fig. 5). or CDNB (Fig. 6) progressively depleted cellular GSH to about 20% of control values at the highest concentrations of the agents used. These reagents deplete GSH by serving as substrates for conjugation of GSH by glutathione *S*-transferase [33,34]. Associated with GSH depletion was a corresponding but more profound decrease in the ability of the cells to reduce DHA to ascorbate, such that no ascorbate was generated at the highest concentrations of the agents used (Figs. 5 and 6).

We also tested the effects of diamide on intracellular GSH and on the ability of BAECs to reduce DHA to ascorbate. Diamide crosses the cell membrane and oxidizes GSH to GSSG [35]. In this experiment, cells were treated sequentially with diamide, followed by the addition of DHA to 400 μM. In response to increasing concentrations of diamide, intracellular GSH decreased to about 50% of that present in cells treated with diamide alone (Fig. 7). How-

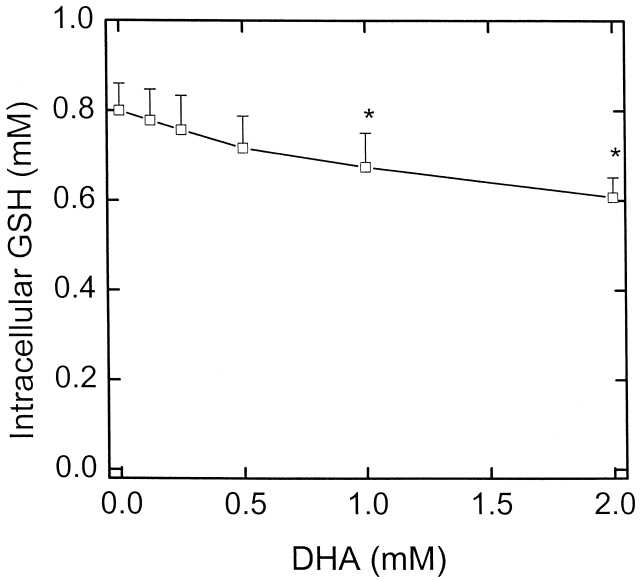

Fig. 4. Effects of DHA on intracellular GSH concentrations in BAECs. Rinsed cells were incubated for 30 min at 37° in KRH that contained 5 mM d-glucose and the indicated concentration of DHA. Following two rinses with 1 mL KRH, intracellular GSH was determined. Results are shown as means ± SEM from 4 experiments, with an asterisk (*) indicating $P < 0.05$ compared with cells incubated in the absence of DHA.

ever, the ability of cells to subsequently reduce added DHA to ascorbate was markedly blunted at even 0.1 mM diamide. To the extent that these agents specifically deplete GSH,

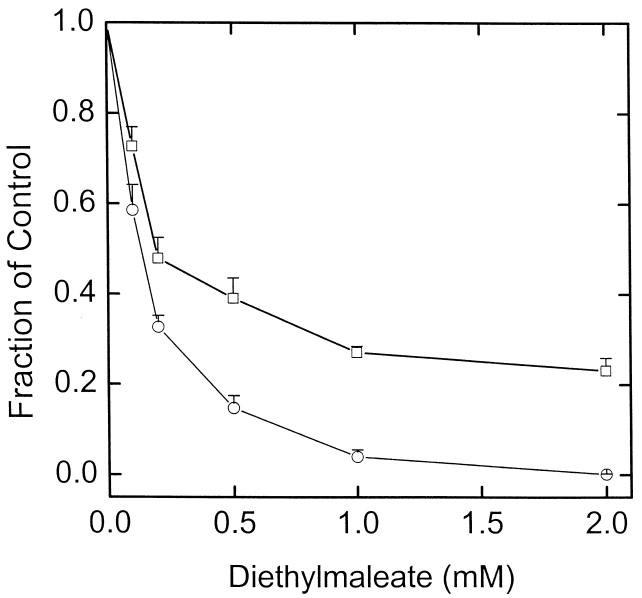

Fig. 5. Effects of diethylmaleate on ascorbate and GSH concentrations in BAECs. Rinsed cells were incubated at 37° in KRH that contained 5 mM d-glucose and the indicated concentration of diethylmaleate. After 10 min, DHA was added to a concentration of 400 μM, and the incubation was continued for another 30 min before the cells were rinsed twice in ice-cold KRH and assayed for ascorbate (circles) and GSH (squares). Results (means ± SEM from 3 experiments) are shown as a fraction of the initial value in cells incubated with only DHA. Initial ascorbate and GSH concentrations were 1.9 ± 0.7 and 0.63 ± 0.16 mM, respectively.

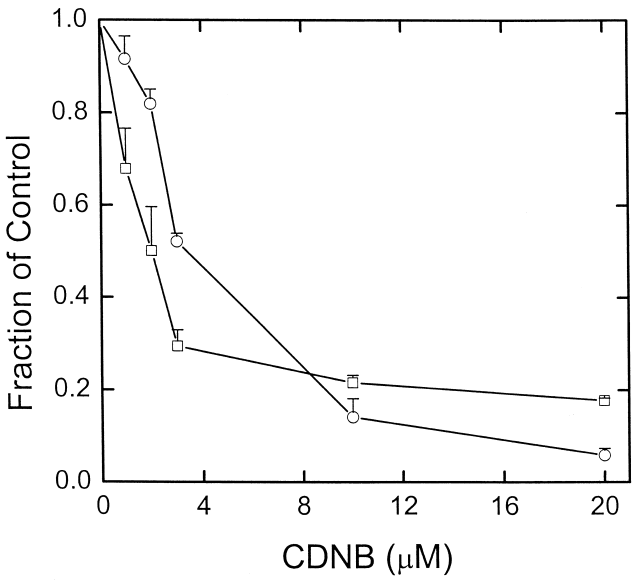

Fig. 6. Effects of CDNB on ascorbate and GSH concentrations in BAECs. The conditions of incubation were the same as those described in the legend to Fig. 5, except that the initial incubation was carried out with the indicated concentration of CDNB, followed by the assay of ascorbate (circles) and GSH (squares). Results (means ± SEM from 3 experiments) are shown as a fraction of the initial value in cells incubated only with DHA. Initial ascorbate and GSH concentrations were 2.0 ± 0.8 and 0.64 ± 0.16 mM, respectively.

these results suggest that DHA reduction to ascorbate is strongly dependent upon the intracellular concentration of GSH in BAECs.

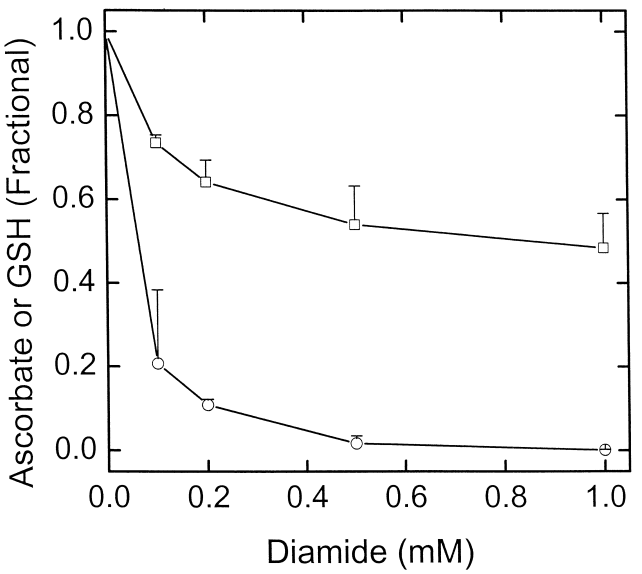

Fig. 7. Effect of diamide on the content of ascorbate and GSH in BAECs. Cells were incubated at 37° in KRH with the indicated concentration of diamide, followed by the addition of DHA to 400 μM and further incubation for 15 min before the assay of ascorbate (circles) and GSH (squares) in the cells. Results (means ± SEM from 3 experiments) are shown as a fraction of the initial value in cells treated with DHA alone. Initial ascorbate and GSH contents were 2.1 ± 0.1 and 0.53 ± 0.1 mM, respectively.

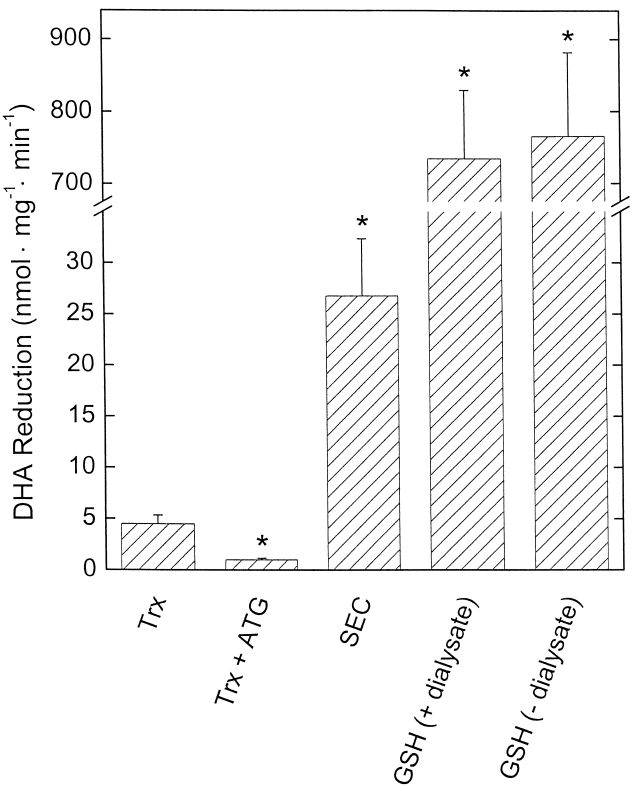

Fig. 8. DHA reduction in overnight-dialyzed extracts of BAECs. After preparation and dialysis as described under "Materials and methods," extracts of BAECs (0.3 to 0.4 mg/mL protein) were incubated at 37° in phosphate-buffered saline (12.5 mM sodium phosphate, 140 mM NaCl, pH 7.4) that contained: 0.4 mM NADPH and 5 μM thioredoxin (Trx); 0.4 mM NADPH, 5 μM thioredoxin, and 10 μM aurothioglucose (Trx + ATG); 0.4 mM NADPH and 100 μM selenocystine (SEC); 1 mM GSH (GSH + dialysate). The last bar shows the results of incubation with GSH, but no dialysate [GSH (– dialysate)]. The reaction was started by the addition of 0.4 mM DHA to each sample and followed for 10 min before the assay of ascorbate in the incubation. Results are shown as means ± SEM from 5 experiments, with an asterisk indicating $P < 0.05$ compared with the first bar.

Whereas GSH appears to be important for the reduction of DHA by BAECs, we also tested whether BAECs also might use thioredoxin reductase to reduce DHA to ascorbate [31]. To do this, extracts of BAECs were dialyzed overnight to remove low molecular weight cofactors such as NAD(P)H and GSH. As shown in Fig. 8, these extracts reduced DHA to ascorbate in an NADPH-dependent manner. Most of this activity was due to thioredoxin reductase, since it was inhibited 75% by 10 μM aurothioglucose, which at this concentration is specific for the enzyme [36]. Further, NADPH-dependent DHA reduction was enhanced 6-fold by selenocystine. The latter facilitates the action of thioredoxin reductase [37]. These incubations were also carried out in the presence of 1 mM GSH without NADPH, with the results shown in the last two bars of Fig. 8. It is apparent that direct reduction of DHA by GSH occurred at a rate more than two orders of magnitude greater than that of NADPH-dependent reduction in these dialysates (Fig. 8, next-to-last bar). Further, the rate of GSH reduction of DHA in buffer alone was the same as observed in the presence of dialysates (Fig. 8, compare last two bars). This suggests that GSH-dependent enzymes in the dialysate contribute little to DHA reduction under these conditions.

4. Discussion

BAECs take up DHA and reduce it to ascorbate to a greater extent than they take up ascorbate itself (Fig. 1). DHA enters cells primarily on the GLUT-type glucose transporter and, once reduced by cellular metabolism, it is trapped in the cell as ascorbate [38]. When normalized to cellular protein, the rates of DHA uptake in BAECs in the current study are similar to those reported by Ek *et al.* [12] in HUVECs. Although Martin and Frei [11] found no ascorbate uptake in BAECs, in our study at relatively high ascorbate concentrations there was a small increase in ascorbate entry (Fig. 1) that was associated with increased ferricyanide reduction (Fig. 2). This may reflect slow uptake of ascorbate on a sodium-dependent ascorbate transporter [39], or oxidation of ascorbate outside the cells with subsequent DHA uptake and reduction to ascorbate. Ascorbate uptake also may be regulated by the stage of the cell cycle, and is decreased in confluent compared with exponentially growing cells [40]. In contrast to BAECs, HUVECs [12], muscle capillary endothelial cells [41], and human aortic endothelial cells [11] efficiently transport ascorbate.

Overnight incubation of BAECs with ascorbate 2-phosphate increased intracellular ascorbate, although only to about 0.7 mM (Fig. 1). Ascorbate 2-phosphate is stable (i.e. not an antioxidant) until hydrolyzed by exofacial phosphatases to ascorbate and inorganic phosphate [42,43]. This slow hydrolysis provides a continuous supply of ascorbate to the cells. Although DHA loading can acutely increase intracellular ascorbate concentrations to more than 1.5 mM, BAECs apparently cannot maintain such levels in culture. On the other hand, human aortic endothelial cells were able to maintain ascorbate concentrations of 3–8 mM in culture for up to 24 hr in the presence of ascorbate [11]. This may reflect differences in the capacity of species to recycle ascorbate, or possibly that hydrolysis of ascorbate 2-phosphate is limiting.

BAECs reduce extracellular ferricyanide in an ascorbate-dependent manner (Fig. 2) that is associated with the oxidation of ascorbate within the cells (Fig. 3). This shows that BAECs have a trans-plasma membrane ferricyanide oxidoreductase activity such as that described in several other cell types, including erythrocytes [44], pulmonary arterial endothelial cells [45], and cultured tumor cells [46,47]. Although the physiologic function of this oxidoreductase activity has not been determined, its activation by ferricyanide provides a convenient measure of the capacity of the cells to recycle ascorbate [48]. The capacity of BAECs to recycle ascorbate that has been oxidized by extracellular

ferricyanide is more than two orders of magnitude greater than required to maintain the intracellular ascorbate concentration in the absence of the oxidant. Results of several different types of experiments in BAECs suggest that such recycling is at least partially GSH-dependent.

First, the oxidation of intracellular ascorbate by increasing concentrations of ferricyanide overwhelms the capacity of BAECs to regenerate and maintain ascorbate (Fig. 3B). This is associated with a small but detectable decrease in GSH content (Fig. 3C). Second, the incubation of BAECs with low millimolar concentrations of DHA lowers intracellular GSH by about 25% (Fig. 4), whether or not glucose is present. The failure of GSH to fall more drastically in the ferricyanide- and DHA-loading experiments could reflect a substantial capacity for BAECs to recycle GSH. However, it could also indicate the presence of other mechanisms of DHA reduction by the cells, as discussed below. Third, agents that deplete GSH impair the ability of BAECs to reduce DHA to ascorbate. Both diethylmaleate and CDNB depleted GSH in BAECs, and this was associated with a corresponding but more pronounced fall in the ability of the cells to reduce DHA to ascorbate (Figs. 5 and 6). These agents irreversibly decrease intracellular GSH when they are conjugated with GSH through the action of glutathione *S*-transferase [49,50]. Guaiquil *et al.* [23] found that GSH depletion with diethylmaleate and buthionine sulfoximine did not affect the ability of HL-60 cells to reduce DHA to ascorbate. The discordant results with diethylmaleate in the present study and those of Guaiquil *et al.* [23] suggest that there are intrinsic differences in the mechanism of DHA reduction between BAECs and HL-60 cells. Diamide, which oxidizes intracellular GSH to GSSG [51], also impaired the ability of BAECs to reduce DHA to ascorbate (Fig. 7). Although none of these agents is entirely specific for GSH, GSH lowering is the common element associated with the impaired ability of the cells to reduce DHA to ascorbate. This and the finding that DHA itself lowers GSH suggest that GSH does play a role in ascorbate recycling in BAECs. GSH-dependent reduction of DHA in BAECs may reflect a direct two-electron transfer from GSH [52], or may be mediated by one or more GSH-dependent enzymes, such as glutaredoxin or protein disulfide isomerase [53].

In their dependence on GSH for reducing DHA, BAECs resemble erythrocytes [20] and lens epithelial cells [22], but differ from cultured tumor cells [23] and keratinocytes [24], which do not require GSH for DHA reduction. This discrepancy could reflect different capacities for GSH recycling, or more likely, different primary mechanisms for DHA reduction. With regard to the latter, NADPH-dependent DHA reduction has been described in liver [31,54] and in erythrocytes [55]. We also observed NADPH-dependent reduction of DHA in dialyzed extracts of BAECs that could be attributed to thioredoxin reductase (Fig. 8). However, this activity was much lower than the direct reduction of DHA by 1 mM GSH under the same conditions. Although we could show that part of the GSH-dependent reduction of DHA was dependent on cellular enzymes in erythrocytes [55], this was not evident in BAECs. Therefore, the direct reduction by GSH appears to be a major mechanism for DHA recycling in BAECs.

It is also necessary to consider the extent to which ascorbate is recycled by AFR reductases present in microsomes and mitochondria. Our finding of only a small decrease in GSH concentrations following ferricyanide treatment of BAECs (Fig. 3C) could reflect such a mechanism. Ferricyanide initially causes a one-electron oxidation of ascorbate to the AFR [44], which will provide substrate for NADH-dependent AFR reductases. Reduction at the AFR stage would also make sense from the standpoint that any DHA generated within the cells might irreversibly ring-open or leak out of the cells before it could be reduced. It remains to be seen whether endothelial cells exposed to an oxidant stress recycle ascorbate from the AFR as well as from DHA.

To the extent that ferricyanide treatment or GSH depletion induces an oxidant stress in BAECs, our results suggest that ascorbate is a sensitive indicator of such stress. Although the culture of endothelial cells with ascorbate or its more stable derivatives may not enhance the ability of the cells to withstand a strong oxidant stress [12], intracellular ascorbate can serve as an indicator of such stress, and it may be involved in the redox regulation of endothelial nitric oxide synthase [56].

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