

# Vitamin C Is an Essential Antioxidant That Enhances Survival of Oxidatively Stressed Human Vascular Endothelial Cells in the Presence of a Vast Molar Excess of Glutathione\*

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Cellular glutathione levels may exceed vitamin C levels by 10-fold, generating the question about the real antioxidant role that low intracellular concentrations of vitamin C can play in the presence of a vast molar excess of glutathione. We characterized the metabolism of vitamin C and its relationship with glutathione in primary cultures of human endothelial cells oxidatively challenged by treatment with hydrogen peroxide or with activated cells undergoing the respiratory burst, and analyzed the manner in which vitamin C interacts with glutathione to increase the antioxidant capacity of cells. Our data indicate that: (i) endothelial cells express transporters for reduced and oxidized vitamin C and accumulate ascorbic acid with participation of glutathione-dependent dehydroascorbic acid reductases, (ii) although increased intracellular levels of vitamin C or glutathione caused augmented resistance to oxidative stress, 10-times more glutathione than vitamin C was required, (iii) full antioxidant protection required the simultaneous presence of intracellular and extracellular vitamin C at concentrations normally found *in vivo*, and (iv) intracellular vitamin C cooperated in enhancing glutathione recovery after oxidative challenge thus providing cells with enhanced survival potential, while extracellular vitamin C was recycled through a mechanism involving the simultaneous neutralization of oxidant species. Therefore, in endothelial cells under oxidative challenge, vitamin C functions as an essential cellular antioxidant even in the presence of a vast molar excess of glutathione.

Human cells contain two important water soluble antioxidants, vitamin C and the tripeptide glutathione (L- $\gamma$ -glutamyl-L-cysteinyl-glycine). Vitamin C plays an important physiological role in cells as a reducing agent and antioxidant, free radical scavenger, and enzyme cofactor (1, 2). Glutathione is the most abundant non-protein thiol in mammalian cells and participates in multiple functions central to the physiology of cells, acting as a reducing agent, antioxidant, and free-radical scavenger and is involved in the metabolism and detoxification of xenobiotics, and alterations in GSH levels and metabolism have been associated with different human diseases (3, 4). Glutathione and vitamin C show a strong functional interdependence *in vivo*. Disruption of glutathione metabolism *in vivo* in rats and guinea pigs by treatment with buthionine-(S,R)-sulfoximine (BSO),<sup>5</sup> a potent and specific glutathione synthesis inhibitor, revealed that the dysfunction and mortality associated with glutathione deficiency can be ameliorated by vitamin C supplementation (3, 5). Inversely, glutathione ester supplementation can protect or delay the effects of a vitamin C-free diet in newborn rats and guinea pigs unable to synthesize vitamin C (3, 6).

Although a functional relationship between glutathione and vitamin C has been clearly established in rats and guinea pigs, we know little about how they cooperate in providing human cells with potent antioxidant defense mechanisms and to which degree this cooperation is affected by their different metabolism. Glutathione is found in cells predominantly as reduced glutathione (GSH), with very low levels of oxidized glutathione (GSSG) present in cells under physiological conditions. Moreover, all human cells possess the capacity to synthesize glutathione from its constitutive amino acids with participation of the enzymes  $\gamma$ -glutamylcysteine synthetase and GSH synthetase, and oxidized glutathione can be recycled back to glutathione by enzymes with glutathione reductase activity (4). On the other hand, humans are one of the few species that lack the

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<sup>5</sup> The abbreviations used are: BSO, L-buthionine-(S,R) sulfoximine; AA, ascorbic acid; DHA, dehydroascorbic acid; GLUT, facilitative glucose transporter; HUVEC, human umbilical vein endothelial cells; HUTEc, human tonsils endothelial cells; SVCT, Na<sup>+</sup>-vitamin C transporter; DEM, diethylmaleate; DOG, 2-deoxy-D-glucose; PDI, protein-disulfide isomerase; PMA, phorbol myristate acetate.

capacity to synthesize vitamin C and therefore the vitamin must be provided from external sources through the diet (7, 8). In solution, vitamin C exists in two forms; the reduced form, L-ascorbic acid (ascorbic acid, AA), and the oxidized form, dehydro-L-ascorbic acid (dehydroascorbic acid, DHA). The plasma concentration of vitamin C (in the form of AA) is  $\sim 50 \mu\text{M}$ , with intracellular and tissue levels several orders of magnitude higher (8), indicating that vitamin C is concentrated in the cellular compartment of target tissues. Cells possess two complementary and overlapping mechanisms for the acquisition of vitamin C. AA is transported into cells by the  $\text{Na}^+$ -ascorbate co-transporters (SVCTs), which correspond to high affinity AA transporters that transport their substrate down the electrochemical sodium gradient (7, 9, 10). DHA transport is mediated by members of the facilitative glucose transporter family (GLUTs) down the substrate concentration gradient (11, 12). Importantly, transport of DHA allows the recycling of the DHA generated in oxidative reactions associated to the normal metabolism of the cells, and may be central to the low daily requirements of vitamin C in humans (13–15). Glutathione has been implicated in the cellular accumulation of AA and the maintenance of the vitamin in its reduced state intracellularly. Thus, mammalian cells express a number of glutathione-dependent DHA reductases involved in DHA metabolism such as glutaredoxin, protein-disulfide isomerase and glutathione S-transferase omega, but they also express the NADPH-dependent DHA reductases thioredoxin reductase and aldo-keto reductases (8).

Remarkably, cellular glutathione levels may exceed vitamin C by more than one order of magnitude on a molar basis (3), generating the question about the real antioxidant role that low intracellular concentrations of vitamin C can play in the presence of a vast molar excess of glutathione. In this regard, in animals made glutathione deficient by treatment with BSO, the glutathione deficiency was accompanied by a marked decrease in the tissue levels of ascorbic acid, indicating either a role for glutathione in the metabolism of vitamin C or that vitamin C is used in reactions that normally use GSH. Interestingly, when the GSH-deficient animals were supplemented with ascorbic acid, besides the expected rise in the tissue ascorbate levels there was also a remarkable increase in cellular glutathione (5). These experiments suggest that ascorbic acid has an important role in the maintenance of adequate cellular levels of glutathione, although the data cannot be used to differentiate between a glutathione sparing effect or an effect on the *de novo* synthesis of glutathione. We addressed this question in *in vitro* experiments using as models primary cultures of endothelial cells from human umbilical vein (HUVECs) and human tonsils (HUTECs). We characterized the metabolism of vitamin C in the endothelial cells, including the manner in which vitamin C cooperates with glutathione to increase the antioxidant capacity of the cells. Our data indicate that vitamin C plays an essential role as an antioxidant in cells containing a vast excess of glutathione, cooperating with glutathione to provide the endothelial cells with the capacity to survive under conditions of oxidative stress, and that full antioxidant protection requires the simultaneous presence of intracellular and extracellular vitamin C at concentrations that are normally observed *in vivo*.

## EXPERIMENTAL PROCEDURES

**Cell Culture**—Human umbilical vein endothelial cells (HUVECs) were obtained from human umbilical cords by collagenase dissociation, cultured in HE-SFM medium supplemented with 20% fetal calf serum, L-glutamine, penicillin, streptomycin, and fungizone and used at passages 3–4 (16). Human tonsil endothelial cells (HUTECs) (17) and HL-60 neutrophils (18) were grown in M199 and RPMI 1640 medium, respectively, supplemented with 10% (v/v) fetal bovine serum.

**Uptake Studies**—Transport of radiolabeled ascorbic acid, dehydroascorbic acid, 2-deoxy-D-glucose (deoxyglucose), or 3-O-methyl-D-glucose (methylglucose) was performed as described (19–20). NaCl was replaced with choline chloride ( $\text{Na}^+$ -free buffer) to measure the effect of  $\text{Na}^+$  on ascorbate transport. Ascorbic acid uptake assays contained 0.1–0.4  $\mu\text{Ci}$  of L-[ $^{14}\text{C}$ ]ascorbic acid (specific activity 8.2 mCi/mmol, PerkinElmer Life Sciences), and a final concentration of 0.05–15 mM ascorbic acid. For dehydroascorbic acid uptake, 0.1–10 units of ascorbate oxidase (50 units/mg protein, Sigma) were added to the ascorbic acid mix. Hexose uptake assays contained 1  $\mu\text{Ci}$  of 3-O-[methyl- $^3\text{H}$ ]-D-glucose (specific activity 10 Ci/mmol, PerkinElmer Life Sciences) and 0.3–20 mM methylglucose, or 1  $\mu\text{Ci}$  of 2-[1,2- $^3\text{H}$ (N)]-deoxy-D-glucose (specific activity 26.2 Ci/mmol, PerkinElmer Life Sciences) and 0.3–20 mM deoxyglucose. For co-culture uptake experiments, the endothelial cells were grown to subconfluency in 6-well plates ( $\sim 2 \times 10^5$  cells/well). On the day of the experiment, HL-60 neutrophils ( $0.1\text{--}10 \times 10^6$  cells/well) were added to wells containing the adherent cells, followed by the addition of 0.2  $\mu\text{M}$  phorbol myristate acetate (PMA) and radiolabeled ascorbic acid. After uptake, the adherent cells were washed twice with cold phosphate-buffered saline, and the incorporated radioactivity was assayed by scintillation counting. Cell volumes were calculated by incubating cells with 1 mM radiolabeled methylglucose, a non-metabolizable glucose analog, until equilibrium was reached, and the amount of methylglucose inside the cells at equilibrium was used to estimate the internal volume of the cells that is free to exchange with the extracellular medium. All cells reached equilibrium within 2 h and the estimated exchangeable intracellular water volumes were 2 and 3  $\mu\text{l}$  per million cells for HUVECs and HUTECs, respectively. When necessary, these values were used to normalize the data from uptake experiments in term of intracellular concentrations.

**Identification of Intracellular Vitamin C by HPLC**—For HPLC, cells were lysed in 60% methanol, 1 mM EDTA (pH 8.0) at 4 °C. HPLC analysis were performed using a Whatman strong anion exchange column (Partisil 10 SAX, 4.6 mm  $\times$  25 cm, 10- $\mu\text{m}$  particle) (15).

**Glutathione Depletion**—Cells were incubated (1–24 h) with graded concentrations of L-buthionine-(S,R) sulfoximine and diethyl maleate. For total glutathione determination, cells were washed twice with normal saline and lysed with 0.25 ml of 0.4% Triton X-100, and the supernatant was processed using the recycling procedure and 5,5'-dithio-bis-(2-nitrobenzoic acid) (21).

**RT-PCR**—The amplification reactions were performed using cDNA prepared from mRNA extracted from HUVECs and

## Vitamin C Function in Endothelial Cells

HUTECS. The RNAs were prepared using the micro poly(A) pure kit (Ambion), and reverse transcription was performed using the rapid RT-PCR kit (Clontech). For amplification of the ascorbic acid transporters, each primer pair used corresponded to the region of the highest specificity within the nucleotide sequences of the transporters (10). For amplification of the glucose transporters, we designed primer pairs specific for each of the isoforms from GLUT1 to GLUT12 (12). For amplification of enzymes with DHA reductase activity, we designed primer pairs for the enzymes glutaredoxin (Grx) 1, 2a, and 2b (22), protein-disulfide isomerase (23), glutathione *S*-transferases omega (GSTO) 1 and 2 (24), thioredoxin reductase (TrxR) 1 and 2 (25), and aldo-keto reductase (AKR) 1C1, 1C2, and 1C3 (26). Amplification of  $\beta$ -actin was used as an internal control of the RT-PCR procedure and as a control of the structural integrity of the different samples. A Biometra TGradient<sup>TM</sup> thermoblock was programmed for a starting amplification protocol consisting of an initial step of 2 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, and a final extension period of 7 min at 72 °C. For each of the 25 primer pairs, the PCR conditions were optimized for Mg<sup>2+</sup> concentrations (from 1 to 4 mM) and annealing temperature (from 52 to 62 °C). The PCR products were separated by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide. PCR amplification products were extracted and purified from agarose gels with the QIAx kit (Qiagen), cloned in pBluescript II KS (Stratagene), submitted to automatic sequencing and analyzed by BLAST.

**Immunocytochemistry**—Cells fixed in 4% *p*-formaldehyde were processed for immunocytochemistry with antibodies against SVCT1, SVCT2, and GLUT1 to -GLUT6 (Alpha Diagnostics), as described (18, 27–28).

## RESULTS AND DISCUSSION

**Endothelial Cells Transport AA and DHA but Accumulate Exclusively AA**—Endothelial cells can synthesize and contain millimolar concentrations of glutathione but cannot synthesize vitamin C. We characterized the mechanism by which endothelial cells acquire and accumulate vitamin C and examined whether glutathione influences the capacity of the cells to accumulate the vitamin. Our data revealed that endothelial cells acquire vitamin C using two different mechanisms, specific for AA or DHA, respectively. Although uptake of AA has been demonstrated in human, rat, bovine, and pig endothelial cells, it has been also reported that they lack the capacity to transport AA (29–31). Our transport studies revealed that the endothelial cells expressed a single AA transporter with the expected functional properties of SVCT2, including activation by Na<sup>+</sup> in a cooperative and specific manner ( $nH = 1.9$ ;  $Na^+50 = 55$  mM) (Fig. 1, A–C and Table 1) and a transport  $K_m$  of 16  $\mu$ M (Fig. 1B and Table 1). RT-PCR, quantitative real-time PCR and immunolocalization experiments confirmed expression of SVCT2 in endothelial cells and were negative for SVCT1 (Fig. 1D and data not shown). The controls used were CaCo-2 cells for SVCT1 and SVCT2, rat hepatocytes for SVCT1 and human melanoma cells for SVCT2 (20, 28).

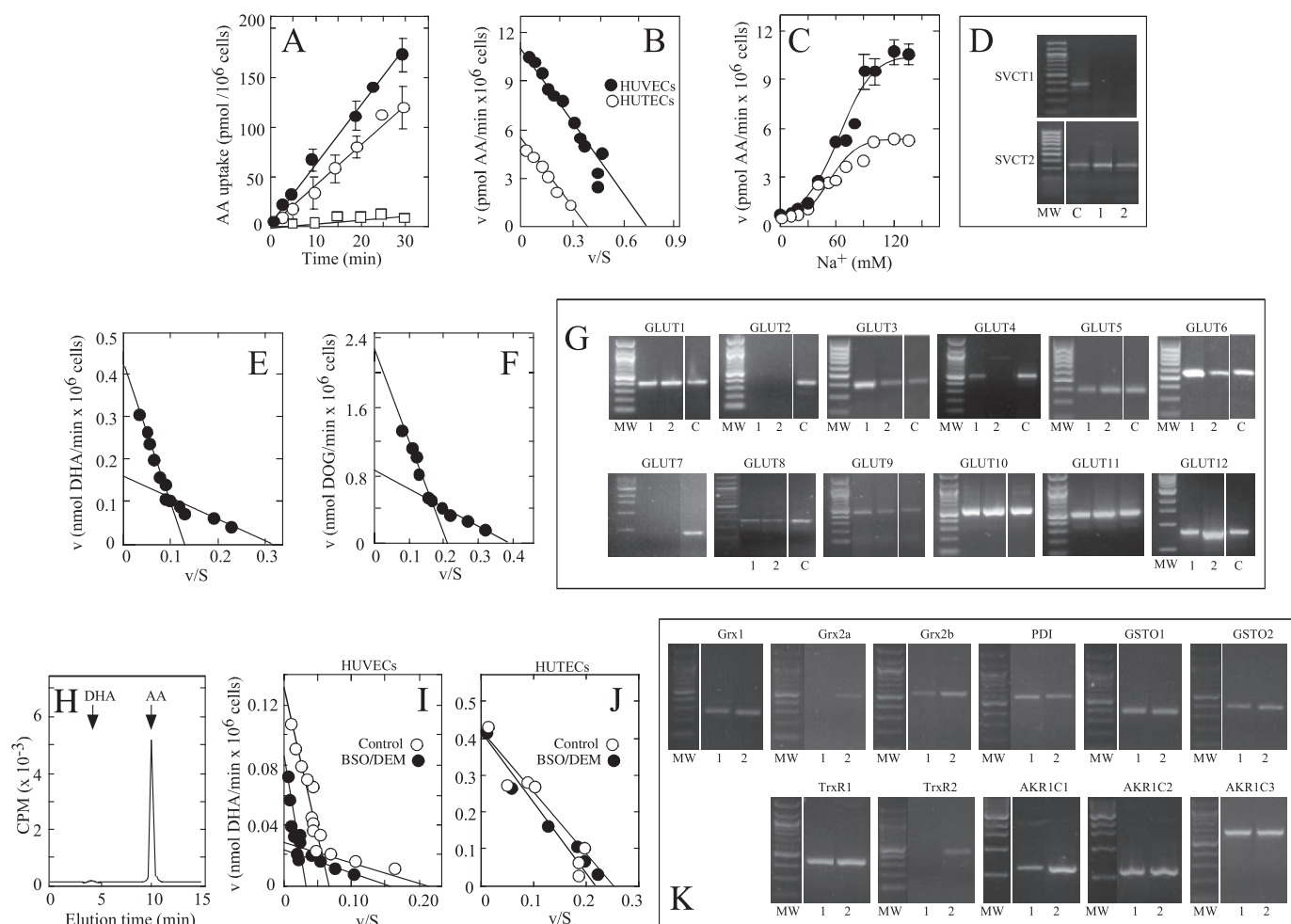
Three members of the glucose transporter family, GLUT1, GLUT3, and GLUT4 are also DHA transporters (11, 32–33).

Previous studies indicated that endothelial cells take up DHA, but no data are available on the kinetic properties or the molecular identity of the transporters involved. Our transport data indicated that primary endothelial cells express at least two transporters of different affinity with the capacity of transporting DHA ( $K_m \approx 0.7$  and 4 mM) and DOG/OMG ( $K_m \approx 2.2$ –3.3 and 13.0–24.4 mM) (Fig. 1, E and F and Table 1). The results of RT-PCR experiments revealed that the endothelial cells expressed several members of the glucose transporter family, and confirmed expression of GLUT1, GLUT3, and GLUT4 (Fig. 1G). However, they lacked expression of GLUT2 and expressed the transporters GLUT5, GLUT6, and GLUT8 to GLUT12, results that were validated using as controls RNA from CaCo-2 cells (for GLUT1 to GLUT5) and total human brain RNA (for GLUT6 to GLUT12) (Fig. 1G). Immunolocalization experiments confirmed expression of GLUT1 and absence of GLUT2 in the endothelial cells, and revealed no GLUT3 and GLUT4 immunostaining under conditions in which they showed positive immunoreactivity in cells expressing the respective transporters (data not shown). Therefore, the PCR, immunolocalization, and functional data ( $K_m$  DHA  $\approx 0.7$  mM,  $K_m$  DOG/OMG  $\approx 3$ –4 mM) support the conclusion that GLUT1 is the higher affinity DHA transporter expressed by human endothelial cells, a conclusion that is in line with the observation that GLUT1 is expressed at high levels in cerebral microvessel endothelial cells (34–35). What is the identity of the low affinity DHA ( $K_m \approx 4$  mM) and DOG/OMG transporter ( $K_m \approx 20$  mM) expressed by endothelial cells? Although GLUT2 and GLUT6 are low affinity glucose transporters, our data indicate that only GLUT6 is expressed by endothelial cells. We have evidence from expression studies in *Xenopus laevis* oocytes that GLUT6 is a low affinity DHA transporter.<sup>6</sup> The preliminary identification of GLUT6 as the low affinity DHA transporter expressed in endothelial cells is consistent with studies indicating expression of a low affinity glucose transporter in primary endothelial cells (36–37). Are other GLUTs expressed by endothelial cells (GLUT8 to GLUT12) capable of transporting DHA? The isoforms GLUT8, GLUT10, and GLUT12 belong to the same class as GLUT6; they transport glucose and therefore are potential DHA transporters (11–12, 32–33). On the other hand, GLUT9 and GLUT11 belong to the same class as GLUT5, a fructose transporter unable to transport DHA, and therefore they are not expected to be DHA transporters (12, 32).

We used glutathione-depleted cells to assess the role of glutathione on vitamin C accumulation. Endothelial cells contained 3 mM glutathione, and treatment with BSO and diethylmaleate lowered cellular glutathione levels to less than 100  $\mu$ M without viability loss (data not shown). HPLC analysis revealed that greater than 98% of the vitamin C accumulated in control and glutathione-depleted cells corresponded to AA (Fig. 1H), indicating that the endothelial cells accumulate and recycle AA independently of their glutathione content. On the other hand, the absence of glutathione affected accumulation capacity in HUVECs but not in HUTECS (Fig. 1, I–J). AA accumulation in

<sup>6</sup> C. G. Guzman, L. K. Azócar, C. I. Rivas, J. C. Vera, and J. G. Cárcamo, manuscript in preparation.





**FIGURE 1. Vitamin C transport and accumulation in endothelial cells.** A–C, ascorbic acid transport in HUVECs (●, □), and HUTECS (○). A, time course of AA uptake by endothelial cells incubated in medium containing NaCl (●, ○), or choline chloride (□). B, Eadie-Hofstee plots of the substrate dependence for AA transport. C, dose response analysis of the effect of Na<sup>+</sup> on AA transport. D, RT-PCR analysis of SVCT1 (upper gel) and SVCT2 (lower gel) expression. PCR analysis was performed using cDNAs prepared from control CaCo-2 cells (labeled C), HUVECs (lane 1), and HUTECS (lane 2). The left lane (labeled MW) contains a 100-bp DNA ladder. E, DHA transport. Eadie-Hofstee plot of the substrate dependence for DHA transport by HUVECs. F, DOG transport. Eadie-Hofstee plot of the substrate dependence for DOG transport by HUVECs. G, RT-PCR analysis of GLUT1 to GLUT12 expression. PCR analysis was performed using cDNAs prepared from HUVECs (lane 1), HUTECS (lane 2). Control cDNAs (lane 3) were prepared from RNA obtained from CaCo-2 cells for GLUT1 to GLUT5 and from a commercially available human brain cDNA (Clontech) for GLUT7 to GLUT12. The left lane in each gel (labeled MW) contains a 100-bp DNA ladder. H–J, ascorbic acid accumulation in endothelial cells. H, HPLC of intracellular vitamin C accumulated in BSO/DEM-treated HUVECs. Treated cells were incubated for 10 min with 1 mM radiolabeled DHA, homogenized and the extract fractionated by HPLC. DHA and AA eluted at 4.5 and 10 min, respectively. I and J, Eadie-Hofstee plots of the DHA dependence for AA accumulation in control (○) and in BSO/DEM-treated (●) HUVECs (I) and HUTECS (J). K, expression analysis by RT-PCR of GSH-dependent (upper gels) and NADPH-dependent (lower gels) DHA reductases in HUVECs (lane 1) and HUTECS (lane 2). The left lane in each gel contains a 100-bp DNA ladder. For each reductase, the result of a single agarose gel is shown and the differences in background intensity in different lanes are due to rearrangement of the lanes after the electrophoresis in the photographic image for presentation purposes. For transport, cells were plated in 12-well plates and uptake experiments were performed at 37 °C for AA and at room temperature for DHA and DOG. Data represent the mean ± S.D. of experiments performed in triplicate. For RT-PCR, the amplification products were fractionated on a 2% agarose gel and stained with ethidium bromide. *GSTO1* and *GSTO2*, glutathione S-transferases omega 1 and omega 2; *TrxR1* and *TrxR2*, thioredoxins R1 and R2; *AKR1C1*, *AKR1C2*, and *AKR1C3*, aldoketo reductases 1C1, 1C2, and 1C3; *Grx1*, *Grx2a*, and *Grx2b*, glutaredoxins 1, 2a, and 2b.

HUVECs was mediated by two components of different affinity (Fig. 1I and Table 1), with only the lower affinity component showing a 50% decrease in  $V_{max}$  in the glutathione-depleted cells (Table 1). Absence of glutathione did not affect AA accumulation in HUTECS (Fig. 1J and Table 1), a process that was characterized by a single functional component (Table 1). The previous data indicate that the endothelial cells express at least three DHA reductase activities involved in the intracellular reduction of DHA to AA, of which one was glutathione dependent and the other two were active in the absence of glutathione. The results of RT-PCR experiments confirmed that the endothelial cells express glutathione-dependent (glutaredoxin, pro-

tein-disulfide isomerase, and glutathione S-transferase omega) as well as NADPH-dependent DHA reductases (thioredoxin and aldo-keto reductase) (Fig. 1K). Glutaredoxin shows a  $K_m$  for DHA of 1.5 mM and shows a cytoplasmic localization (23), and therefore may correspond to the low affinity, glutathione-dependent DHA reductase activity detected in HUVECs. On the other hand, thioredoxin reductase shows a  $K_m$  for DHA of 1.5 mM and shows a cytoplasmic localization (38), and therefore may correspond to the low affinity, glutathione-independent DHA reductase activity detected in the endothelial cells. No evidence is currently available for a cytoplasmic, glutathione independent reductase with a  $K_m$  for DHA of 0.15 mM similar to

TABLE 1

Kinetic parameters of vitamin C transport and accumulation in human endothelial cells

Transport: for ascorbic acid, dehydroascorbic acid and 2-deoxy-D-glucose transport, values correspond to three determinations each carried in triplicate. For 3-O-methyl-D-glucose transport, values are from one experiment performed in triplicate. Accumulation: endothelial cells were left untreated (controls) or treated for 24 h with BSO and then incubated in the presence of graded concentrations of dehydroascorbic acid. Values are from one experiment of two performed in triplicate.

| Transported substrate | Transport     |               | Cells  | Accumulation                     |           |                                  |           |
|-----------------------|---------------|---------------|--------|----------------------------------|-----------|----------------------------------|-----------|
|                       | HUVECs        | HUTECS        |        | Control cells                    |           | BSO-treated cells                |           |
|                       | $K_m$         |               |        | $K_m$                            | $V_{max}$ | $K_m$                            | $V_{max}$ |
|                       |               |               | mM     | pmol/min × 10 <sup>6</sup> cells | mM        | pmol/min × 10 <sup>6</sup> cells |           |
| Ascorbic acid         | 16.0 ± 3 μM   | 17.0 ± 2.0 μM | HUVECs | 0.15                             | 30        | 0.15                             | 30        |
| Dehydroascorbic acid  | 0.6 ± 0.1 mM  | 0.8 ± 0.1 mM  | HUTECS | 1.8                              | 100       | 1.8                              | 50        |
|                       | 4.1 ± 0.3 mM  | 3.8 ± 0.7 mM  |        | 1.8                              | 420       | 1.7                              | 420       |
| 2-Deoxy-D-glucose     | 3.3 ± 0.6 mM  | 2.2 ± 1.2 mM  |        |                                  |           |                                  |           |
|                       | 15.0 ± 1.5 mM | 13.0 ± 3.0 mM |        |                                  |           |                                  |           |
| 3-O-Methyl-D-glucose  | 2.9 mM        | 3.2 mM        |        |                                  |           |                                  |           |
|                       | 24.4 mM       | 20.5 mM       |        |                                  |           |                                  |           |

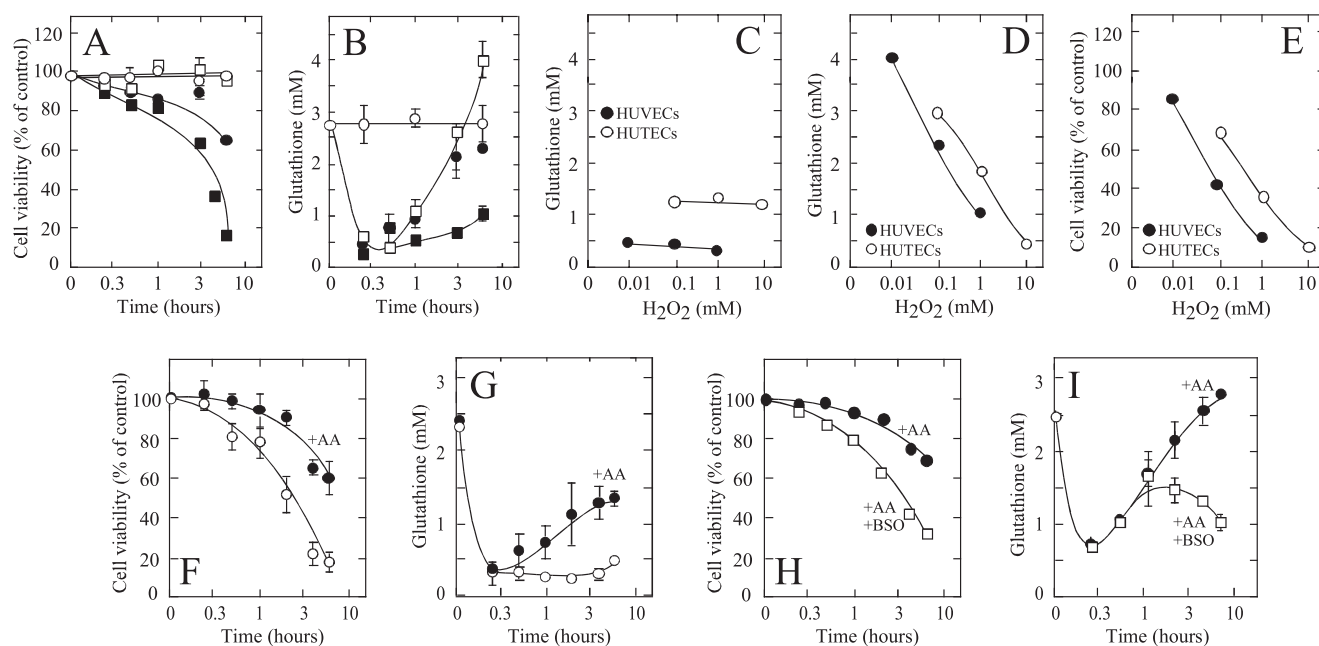
the glutathione independent, higher affinity component detected in HUVECs.

Previous studies using cultured bovine aortic endothelial cells revealed that glutathione is required for the recycling of AA in endothelial cells, with pharmacological depletion of intracellular glutathione almost completely abolishing the ability of the cells to reduce DHA to AA (39), and glutathione-dependent mechanisms are also involved in the recycling of vitamin C in a cell line derived from human umbilical vein endothelial cells (40). One important difference between the previous analyses and the present studies is that we used endothelial cells that were almost completely deprived of glutathione (intracellular concentration <30 μM) by treatment with BSO/DEM, which is an important consideration because most of the enzymes that use glutathione as an electron donor show  $K_m$  values for glutathione in the 1–3 mM concentration range (8). Thus, any reductase activity detected in BSO/DEM-treated cells represents by definition a glutathione-independent enzyme. Moreover, our data revealed that the response of the endothelial cells to glutathione depletion was cell-specific, with glutathione-depleted HUVECs experimenting a marked decrease in their capacity to accumulate AA, while HUTECS were unaffected. The functional differences between the two primary cells may reflect their different origins and the differentiation state and function of the endothelium. Thus, HUVECs may have a more undifferentiated phenotype due to its fetal origin and are directly involved in the mother-fetus exchange, while HUTECS correspond to highly differentiated endothelial cells from a secondary lymphoid organ specialized in the migration of cells from the host defense system. Morphologically, HUVECs correspond to typical planar peripheral endothelial cells (16), while HUTECS correspond to high endothelial cells with characteristics of cubic epithelial cells (17, 41).

*Vitamin C Is Required for Survival of Endothelial Cells Containing Elevated Glutathione Concentrations*—To investigate the role of glutathione and vitamin C in the defense of endothelial cells against oxidative stress, we treated the cells with graded concentrations of hydrogen peroxide and studied the effect on cell survival, the content of glutathione and the role of vitamin C in the process. In a typical experiment, we exposed the cells to graded concentrations of hydrogen peroxide for 15 min, washed the cells in hydrogen peroxide-free medium, and continued the incubation for 6 h while monitoring the changes

in cell viability and glutathione content. There was a cell-, dose-, and time-dependent decrease in cell viability as a result of the treatment with hydrogen peroxide. HUVECs were highly sensitive to the treatment, with about 15 and 60% cells surviving treatment with 1 and 0.1 mM hydrogen peroxide, respectively (Fig. 2A). HUTECS were more resistant, with 10% cells surviving treatment with 10 mM hydrogen peroxide (data not shown). Interestingly, treatment of the endothelial cells with hydrogen peroxide was associated with a time-dependent biphasic change in the content of glutathione, with an initial phase characterized by a rapid decrease in the glutathione content, followed by a secondary, slower glutathione recovery phase (Fig. 2B and data not shown). For HUVECs and HUTECS, the content of glutathione at the end of the initial period of glutathione loss was unrelated to the concentration of hydrogen peroxide used (Fig. 2C). The secondary glutathione recovery phase was more informative; cells capable of recovering their glutathione content were able to survive the oxidative challenge, while those in which the glutathione levels remained low or increased slowly showed the lowest viability. For both cell types, there was a good correlation between the final glutathione content of the cells at the end of the assay and their viability, with higher concentrations of glutathione associated with augmented viability (Fig. 2, D and E), indicating that the ability of cells to survive the oxidative challenge was directly related to the capacity of recovering their glutathione content.

The above results suggest that a treatment that preserves the content of glutathione in oxidatively challenged endothelial cells could increase cell survival. Because of the described functional relationship between glutathione and AA, we asked whether supplementation with intracellular AA could spare glutathione and increase cell survival. When AA-containing HUVECs were treated with hydrogen peroxide, there was increased survival (Fig. 2F) associated with an increased recovery of the glutathione content of the cells (Fig. 2G). However, and confirming our observations on the lack of correlation between the initial decrease in cellular glutathione and cell survival, intracellular AA did not affect the time course or the extent of the loss of glutathione during the early response to hydrogen peroxide (Fig. 2G). We then asked whether blocking the capacity of the cells to synthesize glutathione would increase their sensitivity to hydrogen peroxide. In HUVECs preloaded with vitamin C at concentrations which caused



**FIGURE 2. Effect of intracellular AA and treatment with BSO on the glutathione content and the viability of endothelial cells treated with hydrogen peroxide.** *A* and *B*, time course of the effect of 0 (○), 0.01 (□), 0.1 (●), and 1 mM (■) hydrogen peroxide on the viability (*A*) and glutathione content (*B*) of HUVECs. Cells were incubated for 15 min with hydrogen peroxide, washed and cultured for up to 6 h with hydrogen peroxide-free culture medium, and the viability and glutathione content of the cells were measured at different times. *C*, glutathione content of HUVECs (●) and HUTECS (○) 15 min after treatment with graded concentrations of hydrogen peroxide. *D* and *E*, glutathione content (*D*) and viability (*E*) of HUVECs (●) and HUTECS (○) 6 h after treatment with graded concentrations of hydrogen peroxide. *F* and *G*, time course of the effect of hydrogen peroxide on the viability (*F*) and glutathione content (*G*) of control HUVECs containing 2 mM glutathione (○) or HUVECs containing 2 mM glutathione and 1.5 mM AA (●). *H* and *I*, time course of the effect of hydrogen peroxide on the viability (*H*) and glutathione content (*I*) of HUVECs containing 2 mM glutathione and 1.5 mM AA (○) or HUVECs containing 2 mM glutathione and 1.5 mM AA and cultured in the presence of 1 mM BSO after the treatment with hydrogen peroxide (●). Data represent the mean  $\pm$  S.D. of at least two experiments performed in triplicate.

increased glutathione recovery and augmented viability, incubation with BSO concentrations that effectively blocked the synthesis of glutathione as evidenced by a continuous decrease in the cellular content of glutathione in the endothelial cells (Fig. 2*I*), caused a corresponding decrease in cell viability after hydrogen peroxide treatment (Fig. 2*H*). Moreover, treatment with BSO did not affect the time course or the extent of the loss of glutathione during the early response to hydrogen peroxide (Fig. 2*I*). Essentially identical results were obtained when these experiments were repeated with HUTECS (data not shown). We conclude that endothelial cell survival to oxidative challenge with hydrogen peroxide is related to the capacity of the cells to recover their glutathione content and that the recovery process requires synthesis of glutathione, a process that is accelerated in the presence of intracellular AA.

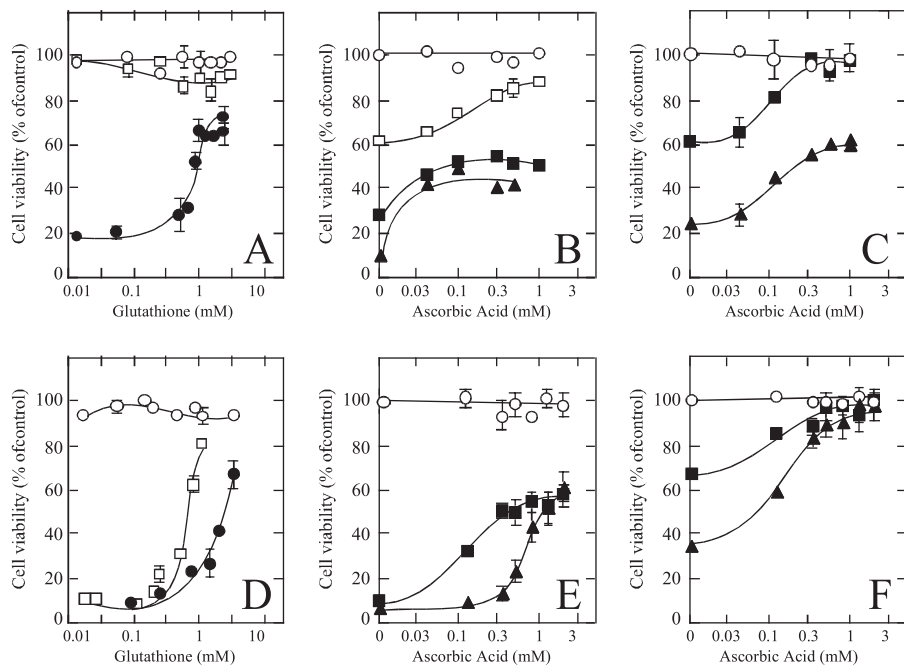
From the previous results we concluded that glutathione is required to protect endothelial cells from oxidative challenge, a process that is made more efficient in the presence of vitamin C. To directly test the role of glutathione and vitamin C in survival to oxidative challenge, we obtained endothelial cells containing graded concentrations of glutathione or vitamin C, or glutathione + vitamin C, and examined the resistance to hydrogen peroxide. Cells lacking glutathione and vitamin C were highly sensitive to hydrogen peroxide, and an increased glutathione content was associated with increased resistance (Fig. 3, *A* and *D*). Maximal protection was observed at glutathione concentrations greater than 1–2 mM, with no protection observed at 0.5 mM or less (Fig. 3, *A* and *D*). In cells lacking glutathione but containing graded concentrations of AA, resistance to oxida-

tive stress increased with increasing AA concentrations, with maximal protection observed at 0.1–0.3 mM AA (Fig. 3, *B* and *E*). On the other hand, endothelial cells simultaneously containing glutathione (2 mM) and graded concentrations of AA (0–2 mM) showed the greatest capacity to survive treatment with hydrogen peroxide (Fig. 3, *C* and *F*). These experiments revealed that, even in the presence of a vast molar excess of glutathione, low concentrations of AA provided endothelial cells with a dose-dependent increase in the resistance against oxidative stress, with a clear protective effect observed starting at 0.1 mM AA and a maximal effect observed at concentrations lower than 0.5 mM AA (Fig. 3, *C* and *F*).

The previous data were obtained using cells oxidatively stressed by treatment with hydrogen peroxide. We asked whether glutathione and vitamin C were also cooperating in providing a potent antioxidant capacity in endothelial cells interacting with activated blood cells undergoing respiratory burst. To test this issue, we co-cultured endothelial cells with activated HL-60 neutrophils, a cell line that undergoes respiratory burst when activated with PMA and in many respects behaves like human neutrophils (15). Co-culturing the endothelial cells with increasing numbers of PMA-activated HL-60 cells differentially decreased the viability of the endothelial cells in a manner dependent on the number of activated cells present during the assay (Fig. 4*A*). Thus, in the presence of one million activated HL-60 cells, the viability of HUVECs at the end of the assay was less than 5%, compared with viabilities of 60% for HUTECS (Fig. 4*A*). The previous experiments were performed using endothelial cells grown under standard culture condi-



## Vitamin C Function in Endothelial Cells



**FIGURE 3. Effect of intracellular glutathione or AA, alone or in combination, on the viability of endothelial cells treated with hydrogen peroxide.** Endothelial cells were left untreated (○), or treated with 0.01 (□), 0.1 (●), 1 (■), or 10 mM (▲) hydrogen peroxide for 15 min, washed, and cultured for 6 h before determining cell viability. A–C, HUVECs. D–F, HUTECS. Cells contained: (i) graded concentrations of glutathione and no AA (A and D), (ii) graded concentrations of AA and glutathione concentrations of less than 30  $\mu$ M (B and E), and (iii) 2 mM glutathione and graded concentrations of AA (C and F). Data represent the mean  $\pm$  S.D. of two experiments performed in triplicate.

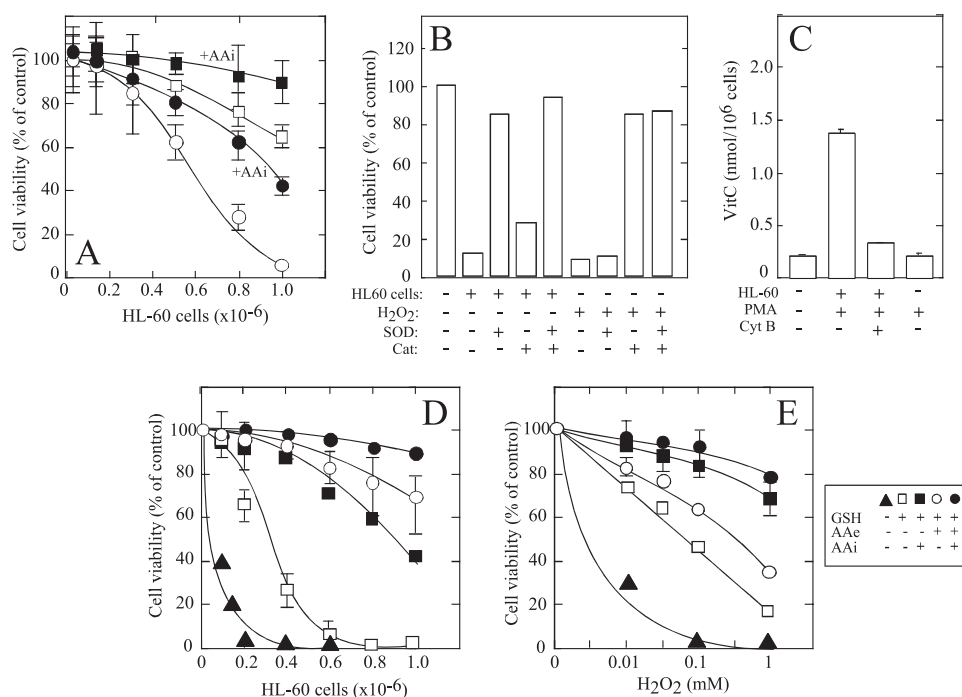
tions and containing 2.5 mM glutathione but lacking AA. Supplementation with AA prior to exposing the cells to activated HL-60 increased the viability of HUVECs to 40% and of HUTECS to 90%, indicating a potent protection by intracellular AA (Fig. 4A). To identify the oxidant molecules produced by activated HL-60 cells, we tested the effects of saturating amounts of the enzymes superoxide dismutase and catalase, alone or in combination, on the viability of HUVECS and HUTECS co-cultured with activated HL-60 cells or challenged with hydrogen peroxide. As expected, catalase, but not superoxide dismutase, blocked the effect of hydrogen peroxide on the endothelial cells (Fig. 4B and data not shown). On the other hand, superoxide dismutase, but not catalase, blocked most of the effect of activated HL-60 cells on HUVECs (Fig. 4B), a result that is consistent with the production of superoxide radicals by activated HL-60 cells. Co-incubation with activated HL-60 in presence of extracellular AA resulted in a large (>5-fold) increase in the uptake of vitamin C by HUVECs (Fig. 4C) and HUTECS (data not shown). This increase was markedly decreased when cytochalasin B, a potent inhibitor of the glucose transporters that does not block the oxidative burst, was present in the coculture during the uptake assay (Fig. 4C), indicating that HUVECs take up the DHA generated from extracellular AA during respiratory burst. Control experiments revealed that treatment of HUVECs with control, untreated HL-60 cells, or with PMA in the absence of HL-60 cells, failed to increase their uptake of vitamin C (Fig. 4C and data not shown).

The above results suggest that vitamin C may protect cells via a 2-fold mechanism, by acting simultaneously as an extracellular as well as an intracellular scavenger of oxidant species. We

tested this hypothesis directly by analyzing the effect of extracellular and intracellular AA, alone or in combination with glutathione, on the resistance of HUVECs coincubated with PMA-activated HL-60 cells as a source of superoxide (Fig. 4D), or treated with graded concentrations of hydrogen peroxide (Fig. 4E). HUVECs lacking glutathione and AA were highly sensitive to the presence of activated HL-60 cells, with no viable endothelial cells observed in assays containing as low as 200,000 activated HL-60 cells (Fig. 4D). Control, glutathione-containing cells showed a greater resistance, with 30% viability observed in assays containing 400,000 activated HL-60 cells (Fig. 4D). The presence of AA increased the resistance against oxidative stress of the glutathione-containing endothelial cells in the order extracellular AA < intracellular AA < extracellular + intracellular AA (Fig. 4D). Maximal resistance to oxidative stress was observed when AA was present

simultaneously in the extracellular (50  $\mu$ M AA) and intracellular (1.5 mM AA) compartments; the glutathione-containing endothelial cells survived the co-culture with the PMA-activated HL-60 cells (Fig. 4D). When HUVECs were exposed to graded concentrations of hydrogen peroxide, the resistance increased in the order cells lacking glutathione < cells containing glutathione < cells containing glutathione + intracellular AA < cells containing glutathione + extracellular AA < cells containing glutathione + extracellular AA + intracellular AA (Fig. 4E). Therefore, a maximal level of antioxidant protection requires that the endothelial cells contain appropriate intracellular levels of glutathione and AA in addition to extracellular AA, which is the normal situation encountered under *in vivo* conditions.

Our data shed light on the role of the interactions between activated host defense cells and endothelial cells from the perspective of the antioxidant potential of endothelial cells. The interaction of activated neutrophils with endothelial cells and the associated oxidative burst has been classically viewed as a mechanism leading to decreased endothelial cell survival (42, 43). However, our data indicated that the interaction of endothelial cells with activated cells resulted in a marked increase in the content of AA in endothelial cells. More important, elevated AA concentrations in the endothelial cells are related to increased antioxidant defense mechanisms as revealed by an important decrease in cell death induced by the interaction with activated cells. The increased AA content of the endothelial cells is accomplished by the transport of DHA (through the glucose transporters) produced locally from extracellular AA as the respiratory burst proceeds, followed by its immediate intra-



**FIGURE 4. Effect of intracellular and extracellular AA on the resistance of endothelial cells to oxidative stress induced by activated neutrophilic cells or hydrogen peroxide.** A, effect of intracellular glutathione and AA on the viability of endothelial cells co-cultured with increasing numbers of activated HL-60 cells. HUVECs (○, ●), or HUTECS (□, ■) were co-cultured for 60 min with the indicated numbers of PMA-activated HL-60 cells. Cells contained 2 mM glutathione and no AA (○, □), or 2 mM glutathione and 1.5 mM AA (●, ■, labeled +AAi). B, effect of superoxide dismutase (100 units) and catalase (100 units), alone or in combination, on the viability of HUVECs co-cultured for 60 min with  $1 \times 10^6$  HL-60 cells activated with 0.2  $\mu$ M PMA, or incubated with 1  $\mu$ M hydrogen peroxide. C, uptake of vitamin C in HUVECs incubated with  $1 \times 10^6$  HL-60 cells in the absence or in the presence of PMA or cytochalasin B in medium containing 100  $\mu$ M radiolabeled ascorbic acid. D, viability of HUVECs incubated for 60 min with  $1 \times 10^6$  PMA-activated HL-60 cells, washed, and cultured for 6 h before measuring viability. The following conditions were used: (i) BSO/DEM-treated cells containing glutathione concentrations lower than 30  $\mu$ M and no AA (▲); (ii) cells containing 2 mM glutathione and no AA (□); (iii) cells containing 2 mM glutathione and 1.5 mM AA (■); (iv) cells containing 1.5 mM AA in the presence of 50  $\mu$ M extracellular AA (○); and (v) cells containing 2 mM glutathione and 1.5 mM AA in the presence of 50  $\mu$ M extracellular AA (●). Data represent the mean  $\pm$  S.D. of two experiments performed in triplicate. E, viability of HUVECs incubated for 60 min with 0.1 mM hydrogen peroxide, washed, and cultured for 6 h before measuring viability. Other conditions similar as in D. Data represent the mean  $\pm$  S.D. of two experiments performed in triplicate. SOD, superoxide dismutase; Cat, catalase; CytB, cytochalasin B; AAe, extracellular AA; AAi, intracellular AA.

cellular reduction to AA. We have named this process the bystander mechanism for the acquisition and recycling of vitamin C (15). This process allows the efficient recycling and salvage of vitamin C by avoiding the irreversible loss of vitamin C through the hydrolysis of DHA, which occurs very rapidly in solution with a  $t_{1/2} < 1$  min (44). Our present data, indicating that maximal protection against oxidative stress requires extracellular AA in addition to the intracellular pair glutathione-AA, unveils an additional role for the bystander mechanism of AA acquisition, by identifying extracellular vitamin C as a central component of the antioxidant machinery that protects endothelial cells from oxidative stress. Thus, the interaction of endothelial cells with activated cells undergoing respiratory burst may not necessarily result in endothelial dysfunction in the presence of vitamin C at the intracellular and extracellular compartments, having instead a 3-fold positive effect by simultaneously scavenging oxygen reactive species, avoiding the loss of oxidized vitamin C and increasing intracellular AA through the recycling of DHA.

**Conclusions**—Human endothelial cells acquire and maintain elevated intracellular concentrations of AA through a combina-

tion of overlapping mechanisms that directly impact their interaction with blood cells and their resistance against oxidative stress. Our present data provide definitive evidence for functional cooperation between vitamin C and glutathione in providing endothelial cells with strong antioxidant defenses. Maximal antioxidant protection was observed only when both antioxidants were simultaneously present, indicating that both antioxidants are needed for cell survival against oxidative stress. Most importantly, AA concentrations in the micromolar range were fully effective in increasing antioxidant protection in the presence of a 10-fold glutathione excess. This is an important finding because any analysis about the role of vitamin C in antioxidant defense should consider the evidence that the content of glutathione of most human cells and tissues exceeds, sometimes by more than one order of magnitude the respective concentrations of vitamin C. The main conclusions of this work are: (i) human endothelial cells obtain vitamin C using two complementary mechanisms: they acquire AA through the sodium ascorbic acid transporter SVCT2 and acquire DHA through the glucose transporters GLUT1 and GLUT6, although additional transporters of

the GLUT family may be involved; (ii) endothelial cells of different origin show particular requirements of glutathione for vitamin C accumulation and express several glutathione- and NADPH-dependent DHA reductases, but they accumulate the vitamin only in the form of ascorbic acid; (iii) although in isolation, intracellular AA or glutathione can partially protect endothelial cells from oxidative stress, with increased protection associated to increased antioxidant content, higher concentrations of glutathione were required to provide antioxidant protection compared with vitamin C. Thus, while maximal glutathione-dependent protection was observed at glutathione 2 mM or higher, maximal AA-dependent protection was observed at 300  $\mu$ M AA; (iv) when present simultaneously, intracellular AA and glutathione interact in a complementary manner to provide endothelial cells with increased antioxidant protection, with vitamin C protecting cells by two complementary mechanisms: by directly acting as an antioxidant in isolation and by improving the capacity of the endothelial cells to recover their glutathione content after the oxidative challenge; and (v) maximal protection against oxidative stress is observed only in cells containing glutathione in the simultaneous pres-



ence of intracellular and extracellular AA at concentrations normally encountered *in vivo*, with extracellular AA preferentially protecting the endothelial cells from the deleterious effect of superoxide.

These results can be explained in terms of the fate of glutathione in oxidative challenged cells. Treatment of endothelial cells with hydrogen peroxide resulted in a dose- and time-dependent decrease in cell viability, an effect that was preceded by a massive loss of intracellular glutathione that was, however, unrelated to the concentration of hydrogen peroxide or cell viability. In contrast, the ability of the cells to survive the oxidative challenge was directly related to their capacity to recover their content of glutathione. The recovery process requires synthesis of glutathione, as revealed by the experiments showing blockade by BSO, and is accelerated in the presence of low intracellular AA concentrations. Our data are therefore consistent with AA functioning as an efficient scavenger of radicals and oxidants that can spare or recycle glutathione. An additional physiological function of AA is to maintain active site metal ions of several enzymes in reduced state. In this context, another reason why AA is needed even in the presence of large amounts of GSH could be due to the fact that GSH might be incapable of replacing AA in keeping active site metals of enzymes in reduced state under oxidative stress, which is essential for cell survival.

We conclude from our *in vitro* data that vitamin C is an essential antioxidant that is required to protect endothelial cells from oxidative challenge even in the presence of a vast molar excess of glutathione. Our present findings may change the manner in which we view the functional interrelationships between vitamin C and glutathione *in vivo*. Altered antioxidant redox cellular machinery is implicated in the development of cardiovascular disease in humans, including atherosclerosis (45). The endothelial cells that cover the walls of blood vessels are continuously exposed to biological oxidants from endogenous and exogenous origin, including superoxide and hydrogen peroxide generated in the mitochondria, oxidized LDL, and peroxides and hypochlorous acids generated by inflammatory reactions in areas of atherosclerosis. Endothelial cells interact with blood cells such as neutrophils, an interaction that is greatly increased under inflammatory conditions and has been classically associated with decreased endothelial cell viability (42–43, 45). Vitamin C protects endothelial cells from oxidative stress by neutralizing the effects of oxidative species and decreasing blood cell-endothelial cell interactions, while glutathione modulates the redox properties of vitamin C in endothelial cells. Clinical studies have revealed that vitamin C can reverse endothelial dysfunction under different pathological conditions such as hypercholesterolemia, hypertension, smoking, diabetes, and atherosclerosis (1, 45).

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**Metabolism and Bioenergetics:**  
**Vitamin C Is an Essential Antioxidant That  
Enhances Survival of Oxidatively Stressed  
Human Vascular Endothelial Cells in the  
Presence of a Vast Molar Excess of  
Glutathione**

Viviana Montecinos, Paula Guzmán, Valeria Barra, Marcelo Villagrán, Carola Muñoz-Montesino, Kirsty Sotomayor, Elizabeth Escobar, Alejandro Godoy, Lorena Mardones, Paula Sotomayor, Catherine Guzmán, Osmán Vásquez, Victoria Gallardo, Brigitte van Zundert, María Rosa Bono, Sergio A. Oñate, Marcelo Bustamante, Juan G. Cárcamo, Coralia I. Rivas and Juan Carlos Vera

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