Ascorbate-mediated transplasma membrane electron transport in pulmonary arterial endothelial cells

Marilyn P. Merker, Lars E. Olson, Robert D. Bongard, Meha K. Patel, John H. Linehan, and Christopher A. Dawson. Ascorbate-mediated transplasma membrane electron transport in pulmonary arterial endothelial cells. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18):L685-L693, 1998.—Pulmonary endothelial cells are capable of reducing certain electron acceptors at the luminal plasma membrane surface. Motivation for studying this phenomenon comes in part from the expectation that it may be important both as an endothelial antioxidant defense mechanism and in redox cycling of toxic free radicals. Pulmonary arterial endothelial cells in culture reduce the oxidized forms of thiazine compounds that have been used as electron acceptor probes for studying the mechanisms of transplasma membrane electron transport. However, they reduce another commonly studied electron acceptor, ferricyanide, only very slowly by comparison. In the present study, we examined the influence of ascorbate [ascorbic acid (AA)] and dehydroascorbate [dehydroascorbic acid (DHAA)] in the cell medium. The ferricyanide reductase activity could be increased 80-fold by adding DHAA to the medium, with virtually no effect on methylene blue reduction. The DHAA effect persisted after the DHAA was removed from the medium. AA also stimulated the ferricyanide reductase activity but was less potent, and the relative potencies of AA and DHAA correlated with their relative rates of uptake by the cells. The results are consistent with the hypothesis that AA is an intracellular electron donor for an endothelial plasma membrane ferricyanide reductase and that the stimulatory effect of DHAA is the result of increasing intracellular AA. Adding sufficient DHAA to markedly increase extracellular ferricyanide reduction had little effect on the plasma membrane methylene blue reductase activity, suggesting that pulmonary arterial endothelial cells have at least two separate transplasma membrane electron transport systems.

ferricyanide; cytochrome c; methylene blue; dehydroascorbic acid; endothelial cell column; ascorbic acid transport; dehydroascorbic acid transport

PULMONARY ENDOTHELIAL CELLS are capable of reducing electron acceptors at the luminal plasma membrane surface via transplasma membrane electron transport (3, 14). The role of such a system in normal vascular biology has not yet been identified. However, the existence of the high-capacity reducing system in direct contact with the blood suggests an impact on the redox status of blood constituents, with implications with regard to the physiological role of the endothelium in antioxidant defense. It is also a mechanism whereby the lungs could affect the systemic circulation by modifying the redox status of blood constituents entering the arterial system. It has been shown to be important in the pharmacokinetics of certain redox-sensitive drugs (2, 14), and it is likely to be involved in the mechanism of action and disposition of redox-cycling toxins and chemotherapeutic agents.

Transplasma membrane electron transport systems are found in various mammalian cell types (8, 10, 15, 26). Ferricyanide is commonly used as an electron acceptor in studies of transplasma membrane electron transport because neither ferricyanide nor its reduced form, ferrocyanide, enters the cells. Thus any reduction can be attributed solely to the cell surface. Ferricyanide reductase systems are present in the plasma membranes of hepatocytes (8), erythrocytes (1, 12, 17, 20, 22), and other cells (6, 8), and they may be ubiquitous. However, ferricyanide has been found to be only very slowly reduced by pulmonary arterial endothelial cells in culture in comparison with several other electron acceptors reduced on the cell surface (3).

In the process of attempting to identify the mechanisms involved in endothelial transplasma membrane electron transport, we have made the observation that the relatively inactive ferricyanide reductase activity can be stimulated by supplying dehydroascorbate [dehydroascorbic acid (DHAA)] in the cell medium. The results are consistent with the hypothesis that ascorbate [ascorbic acid (AA)] is an intracellular electron donor for this reductase and that the stimulatory effect of DHAA is the result of increasing the intracellular AA concentration. Adding sufficient DHAA to markedly increase extracellular ferricyanide reduction had no effect on plasma membrane thiazine reductase activity, suggesting that there are at least two separate transplasma membrane electron transport systems in pulmonary arterial endothelial cells.

METHODS

Reagents. Potassium ferricyanide, L-AA, ascorbate oxidase (AO; A-0157), HEPES, lactate dehydrogenase (LDH) kit 340-LD and enzyme control 2E, ferricytochrome c (from horse heart), Triton X-100, mouse anti-α-actin, tetramethylrhodamine isothiocyanate-labeled goat anti-mouse IgG, and FITC-dextran (2 × 10⁶ g/mol) were purchased from Sigma (St. Louis, MO). Dehydro-L(-)-AA dimer was also obtained from Sigma. Because the dimer dissociates on hydration, the units...
reported are for the monomeric DHAA. Experiments with DHAA were initiated within 2 min of dissolving the DHAA in the medium. α-glucose was from Fisher Scientific (Fair Lawn, NJ), and sodium hydrosulfite was from Mallinckrodt Chemical Works (St. Louis, MO). Methylene blue chloride was obtained from E. M. Science (Gibbstown, NJ). [14C]AA was from NEN Life Science Products (Boston, MA). Hanks’ balanced salt solution (HBSS), trypsin, penicillin-streptomycin, and RPMI 1640 tissue culture medium were from Gibco (Grand Island, NY). Fetal bovine serum was from Hyclone Laboratories (Logan, UT), and Biosilon beads were from A/S Nunc (Roskilde, Denmark). Type CLS2 collagenase was obtained from Worthington Biochemicals (Freehold, NJ), and diiodoindocarbocyanine-acetylated low-density lipoprotein was purchased from Biomedical Technologies (Stoughton, MA). Protein determinations were performed using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

Endothelial cell culture. The bovine pulmonary endothelial cells used in this study were obtained from two sources. One source was calf pulmonary artery obtained from a local meat-processing plant as previously described (14). The second source was cell line CPAE (CCL-209) from the American Type Culture Collection (Rockville, MD). The cells were cultured in T75 tissue culture flasks maintained at 37°C in RPMI 1640 (for the cells obtained locally) or minimal essential Eagle’s medium (for the CPAE cells). Both media contained 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 30 mg/ml L-glutamine. The endothelial cell characteristics of the cultures were confirmed by cobblestone morphology observed by phase-contrast microscopy. Cells from either source were >95% endothelial cells as determined by diiodoindocarbocyanine-acetylated low-density lipoprotein uptake observed by fluorescence microscopy and immunofluorescence staining for α-actin.

To obtain a large cell-surface area for experiments carried out in spectrophotometric cuvettes or in cell columns, the cells grown to confluence in T75 tissue culture flasks were detached from the flasks by treatment with trypsin (0.05% wt/vol) in HBSS, and ~5 × 10⁵ cells were seeded onto 3 g (dry wt) of gelatin-coated beads (0.1% gelatin). The beads were cultured in T75 tissue culture flasks maintained at 37°C in RPMI 1640 (for the cells obtained locally) or minimal essential Eagle’s medium (for the CPAE cells). Both media contained 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 30 mg/ml L-glutamine. The endothelial cell characteristics of the cultures were confirmed by cobblestone morphology observed by phase-contrast microscopy. Cells from either source were >95% endothelial cells as determined by diiodoindocarbocyanine-acetylated low-density lipoprotein uptake observed by fluorescence microscopy and immunofluorescence staining for α-actin.

To determine the efficacy of ferricytochrome c reduction as an in situ AA assay, 20 ml of H-H containing ferricytochrome c (75 µM) were placed in a beaker. AA (0.5 mM) was directly infused into the stirred cytochrome c solution at a rate of 6.8 µl/min. The cytochrome c solution absorbance (550 nm) was measured over a period of 100 min. A control was performed in a like manner using AA-free H-H infusion.

Reduction of ferricyanide and methylene blue by cells in cuvettes. The general procedure used has been described previously (3, 14). About 0.5 ml of the cell-coated beads was transferred to 10 × 10 × 55-mm polystyrene spectrophotometer cuvettes (Sarstedt, Newton, NC) and washed free of culture medium using three consecutive 3-ml volumes of HBSS containing 10 mM HEPES and 5.5 mM glucose at pH 7.4 (referred to subsequently as H-H). This procedure and subsequent experiments were carried out at room temperature, ~24°C. After the third wash, 2 ml of H-H solution containing ferricyanide (300 µM), ferricytochrome c (75 µM), or methylene blue (10 µM), each with or without DHAA (0–300 µM), were added to the cuvettes. Once the cell-coated beads had settled to the bottom (~25 s), the cuvettes were sealed, and the initial absorbance at 421, 550, or 611 nm was recorded for samples containing ferricyanide, cytochrome c, or methylene blue, respectively, using a Gilford Response spectrophotometer (Ciba Corning Diagnostics, Norwood, MA). The reduced form of ferricyanide (ferrocyanide) and reduced methylene blue do not absorb at these wavelengths. Therefore, the concentrations of ferricyanide and the oxidized form of methylene blue (MB⁺) were determined by comparing the recorded absorbance values with standard curves of known concentration prepared the day of the experiment. Reduced cytochrome c concentrations were determined using an extinction coefficient of 2.1 × 10³ cm²/mmol. The cuvette contents were gently mixed using a Nutator mixer (Clay Adams Division of Becton Dickinson, Parsippany, NJ), and at 2.5-min intervals, the cuvettes were placed in the spectrophotometer where the cell-coated beads were allowed to settle. Absorbance was measured at the appropriate wavelength(s), and the cuvettes were returned to the mixer. In some experiments, the washed cell-coated beads were mixed with H-H containing either DHAA (100 µM) or AA (100 µM) for a period of 15 min and then washed a second time using fresh H-H before the addition of 2 ml of H-H containing either ferricyanide (300 µM) or MB (10 µM). The total concentration of ferricyanide–ferrocyanide in the H-H bathing the cells was determined after the experimental period by oxidizing any ferrocyanide present with 0.1 ml of 3% H₂O₂ and 0.1 ml of 70% HClO₄/ml of H-H. Then, the difference in ferricyanide concentrations before and after the oxidation was the measure of the ferrocyanide present before the oxidation. Similarly, total cytochrome c or total methylene blue (oxidized + reduced) was determined by oxidizing any of the reduced forms present by the addition of 0.025 ml of ferricyanide (10 mM)/ml of H-H.

In each cuvette experiment, the experimental and various control cuvettes were run simultaneously with cells grown in the same flask to control for cell source and other possible cell culture effects on the reductase activity. For each redox indicator and experimental medium composition, the number of cuvettes studied and the figures showing examples of the data are as follows: ferricyanide alone, 10 cuvettes, see Figs. 1–3, 5, 6; ferricyanide after AA pretreatment, 2 cuvettes, see Figs. 1–3, 5; ferricyanide after DHAA pretreatment, 6 cuvettes, see Figs. 1 and 2; ferricyanide after AA pretreatment, 2 cuvettes, see Fig. 2; ferricyanide + AO, 1 cuvette, see Fig. 5; ferricyanide + DHAA + AO, 3 cuvettes, see Fig. 5; ferricyanide + AA + AO, 2 cuvettes, see Fig. 5; cytochrome c alone, 2 cuvettes, see Fig. 7; cytochrome c + DHAA, 2 cuvettes, see Fig. 7; methylene blue alone, 2 cuvettes, see Fig. 8; methylene blue + DHAA, 1 cuvette, see Fig. 8; and methylene blue after pretreatment with DHAA, 4 cuvettes, see Fig. 1. In each case, the examples shown are representative of the results of all experiments performed.
H-H containing FITC-dextran (0.75 µM) and either ferricyanide (200 µM) or MB⁺ (28 µM) could be introduced at the desired time without changing flow or pressure. The effluent dye concentrations were measured on-line downstream from the column at 490 nm (FITC-dextran) and at either 420 (ferricyanide) or 590 nm (MB⁺) using the custom-built optical detector described previously (16). The column experiments were also carried out at room temperature. In the cell column experiments, because of the ability to make repeated measurements, each cell column served as its own control.

Four experimental protocols were followed using the cell columns. The first was designed to determine the effect of DHAA on ferricyanide reduction. Bolus injections containing FITC-dextran and ferricyanide were made with flow rate set at 0.3, 0.7, and 1.0 ml/min (column transit times of 60, 26, and 18 s, respectively). The cells were then perfused with H-H containing 200 µM DHAA for 30 min at 1.0 ml/min. The perfusion medium was then changed back to H-H without DHAA for 10 min, and the boluses were injected at the same three flow rates as before the addition of DHAA. The second protocol was identical to the first except that the boluses contained MB⁺ instead of ferricyanide. The third protocol was used to determine the time course of the DHAA effect on ferricyanide reduction. Bolus injections of FITC-dextran and ferricyanide were made every 5 min, with cell column flow set at 1.0 ml/min. Three injections were made during perfusion with H-H alone. The cell column was then perfused with H-H containing 200 µM DHAA for 56 min. Bolus injections were made 15 min after the perfusate change and at 5-min intervals thereafter. At the end of the DHAA perfusion, the perfusate was changed back to DHAA-free H-H. After 10 min, bolus injections were again made at 5-min intervals for an additional 25 min. The fourth protocol was identical to the third except that the boluses contained MB⁺ instead of ferricyanide. The fractions of MB⁺ and ferricyanide reduced during passage of the boluses through the column were calculated by first dividing the respective indicator concentrations by their respective injected amounts (see Figs. 9 and 10). Then the areas under the fractional concentration curves were measured from the appearance time to the peak of the FITC-dextran curve. The difference in these areas under the MB⁺ or ferricyanide curves and this area under the FITC-dextran curve was the fraction of MB⁺ or ferricyanide reduced.

Cell uptake of [14C]AA and [14C]DHAA. The cell-coated beads were washed free of culture medium using H-H and dispensed (~0.25 ml) into six 1.5-ml Eppendorf tubes. A 1-ml volume of H-H containing [14C]AA (35 µM; specific activity 10 mCi/µmol) was added to the cell-coated beads in five of the tubes, and 1 ml of H-H was added to the cells in the remaining tube. The tubes were capped and gently mixed at room temperature using a Nutator mixer. Uptake was terminated at 5, 10, 15, 20, or 30 min by washing the cells with four consecutive 1.25-ml volumes of fresh H-H and then solubilizing the cells with 0.75 ml of 0.5 N NaOH containing 2% Triton X-100. The samples were mixed overnight, and radioactivity of the solubilized cells (0.5 ml) and final-wash H-H (0.5 ml) from each sample was measured in 10 ml of Bio-Safe II liquid scintillation cocktail (Research Products International, Mount Prospect, IL) using liquid scintillation spectrometry (Packard TriCarb model 3330 liquid scintillation spectrometer, Downers Grove, IL). Counting efficiencies were determined by the addition of [14C]toluene (0.01 ml; specific activity 4.4 x 10⁵ dpm/µl) to each of the vials, which were then counted a second time. The balance of the solubilized cell samples was assayed for protein. The uptake of [14C]AA expressed as nanomoles per minute per milligram of protein was calculated from the disintegrations per minute and the [14C]AA specific activity. The uptake of [14C] from [14C]DHAA was measured in a similar manner after generation of [14C]DHAA by reacting [14C]AA with AO (2 U/ml) for 5 min. The [14C] uptake was measured in 36 cuvettes: 24 with [14C]DHAA and 12 with [14C]AA added to the medium.

Assessment of the presence of AA in cell-conditioned medium. Cell-coated beads in three cuvettes were washed free of culture medium, and 2 ml of H-H containing DHAA (100 µM) and ferricyanide (300 µM) were added to one cuvette, and 2 ml of H-H containing only DHAA (100 µM) were added to the other two cuvettes. The three cuvettes were sealed, and the absorbance at 421 nm was recorded over time as described in Reduction of ferricyanide and cytochrome c and uptake of methylene blue by cells in cuvettes. The experimental period was halted when the absorbance of the ferricyanide-containing sample approached zero, and further mixing resulted in no additional decrease in absorbance (100 min). The ferricyanide-reducing capacity of the H-H contained in the ferricyanide-free cuvettes after the experimental period was determined by transferring a 1.7-ml volume of the cell-conditioned medium to a clean cuvette containing ferricyanide (300 µM). The absorbance between 350 and 450 nm was recorded in 2-nm intervals, and the resulting spectrum was compared with the spectrum of a ferricyanide solution (300 µM) prepared using fresh H-H. The H-H contained in the remaining ferricyanide-free cuvette was transferred to a quartz cuvette, and absorbance between 200 and 300 nm was recorded in 2-nm intervals using fresh H-H as the blank.

LDH and total protein determinations. The LDH activity of the cells and medium was measured as an index of cell viability for comparison with calculated LDH-reducing capacity (14). After the experimental period, the medium bathing the cells was aspirated and the cells were lysed on ice in 2.5 ml of cold deionized water using a Virsonic cell disruptor (model 16–850; Virsonic, Gardiner, NY) set to administer three 10-s exposures at 35% maximum intensity to each sample. A volume of each lysate was diluted 1:5 with 0.1 M sodium phosphate buffer (pH 7.5), and the diluted lysate and aspired medium were assayed in duplicate for LDH using the spectrophotometric method of Wroblewski and LaDue (24). Paired control LDH samples (Sigma enzyme control 2E) were run the day of each experiment to ensure assay reliability. The amounts of LDH are given in Sigma units, where one unit of LDH results in a decrease in optical absorbance (340 nm) of 0.001/min at 25°C in a 3-ml reaction volume with a 1-cm light path. The protein concentration of each sample of cell-bathing medium and cell lysate (diluted 1:20) was determined using the Bio-Rad protein assay, which is based on the Bradford dye-binding procedure (4). Paired samples were compared with standard curves prepared the day of each experiment using known concentrations of BSA. Because the typical experimental protocol used in this study involved changing the medium or the flow-through system, the cell-to-medium LDH ratio was not necessarily highly sensitive for comparisons between conditions. Therefore, the LDH-to-total cell protein ratio is also reported for assessing the possibility that
different experimental conditions might have had different effects on cell viability. The LDH found in the medium in the cuvettes as a fraction of the total (cell + medium) at the time of measurement averaged 3.8 ± 3.7% (mean ± SD), with no systematic differences detected among study conditions.

RESULTS

Figure 1 is an example of the initial observation that including DHAA in the medium surrounding the endothelial cells stimulated reduction of ferricyanide. Because DHAA itself does not reduce ferricyanide, the reduction of the ferricyanide under these conditions was cell mediated. The observation that nearly all 600 nmol of the ferricyanide added to the medium were reduced, although only 200 nmol DHAA were present (i.e., the capacity to reduce only 400 nmol of ferricyanide if each DHAA resulted in only one AA), was suggestive of a recycling process. Figure 1 also shows that the stimulatory effect of DHAA was maintained even after the DHAA had been removed from the medium.

To determine whether AA itself was capable of stimulating ferricyanide reduction, it was necessary to expose the cells to AA and then to remove the AA from the medium before adding the ferricyanide. This is because, in contrast to DHAA, AA directly reduces ferricyanide. Thus, for the experiments depicted in Fig. 2, the cells were first incubated for 15 min with AA, with AA + AO, or with DHAA. Then the medium was replaced with H-H containing ferricyanide but no other additives. In addition, a cuvette containing both DHAA and ferricyanide was included for comparison. Pretreatment with DHAA or AA stimulated ferricyanide reductase activity. However, AA was less effective than DHAA unless AO had been present in the medium. This effect of AO was presumably the result of AA conversion to DHAA.

The stimulatory effect of DHAA was found to be DHAA concentration dependent and saturable (Fig. 3). By fitting a hyperbolic function of Michaelis-Menten form to the data in Fig. 3 (solid line), the maximum rate of the DHAA-stimulated reduction of a 300 µM ferricyanide solution was estimated by nonlinear regression analysis to be 8.2 nmol·min⁻¹·mg protein⁻¹. The concentration of DHAA that produced a rate equal to...
one-half the extrapolated maximum under the conditions of these experiments was estimated to be 0.10 mM.

To determine whether DHAA and AA were in fact taken up by the cells, [14C]DHAA or [14C]AA was added to the cells under the same conditions in which the ferricyanide reduction was measured. The 14C accumulated in the cells considerably faster when it was added as [14C]DHAA than as [14C]AA, consistent with the relative potencies of DHAA and AA in stimulating ferricyanide reductase (Fig. 4).

Figure 5 provides one piece of evidence that the release of AA into the medium was not responsible for the DHAA effect on ferricyanide reduction. When AO was added to oxidize any AA that might have been released into the medium, the stimulatory effect of DHAA on ferricyanide reduction was not diminished. Controls included in this experiment demonstrated that the results with DHAA could be reproduced using AA if AO was included in the medium, thereby indicating that a relevant concentration of AO had been used. Figure 5 also shows that AO itself had no effect on ferricyanide reduction.

Additional experiments were also performed to determine whether AA released from the cells into the medium might make a significant contribution to the DHAA-stimulated ferricyanide reduction. As one approach to this question, we found that the conditioned medium removed from the DHAA-treated cells did not reduce ferricyanide (Fig. 6). In another approach, ferricytochrome c, which is directly reduced by AA but not by the cells within the relevant time frame, was added to the cells in the presence of DHAA as a colorimetric assay for AA within the cell-bathing medium (Fig. 7). There was virtually no reduction of ferricytochrome c in comparison with the amount of AA that would have been necessary to account for the reduction of ferricyanide in the presence of the DHAA-stimulated cells. As a final approach, conditioning the DHAA-containing medium by exposure to the cells did not increase the ultraviolet (UV) absorption by the medium in the 270-nm region of the spectrum, also indicating that little AA was released from the cells in comparison with that required to account for the ferricyanide reduction.

To determine whether DHAA also stimulated the extracellular reduction of other electron acceptors by the endothelial cells, the effects of DHAA on the endothelial cell uptake of methylene blue were determined (Fig. 8). In contrast to ferricyanide, which remains in the medium as ferrocyanide after reduction, when the methylene blue is reduced on the cell surface, the lipophilic reduced form enters the cells, where it becomes sequestered or autooxidized back to MB1 (14). Thus, when methylene blue was used, only the oxidized form was found in the medium. Because the first step in methylene blue uptake is reduction on the endothelial surface (3, 15), the lack of effect of DHAA on uptake...
suggested that different mechanisms are involved in the endothelial reduction of ferricyanide and MB°. However, in a cuvette experiment such as that depicted in Fig. 8, the rate of methylene blue uptake is influenced by the rates of intracellular sequestration and extracellular autooxidation as well as by reduction (14).

To further evaluate the relative effects of DHAA on endothelial cell ferricyanide and MB° reduction, the reduction of ferricyanide and MB° was studied in the cell column. Under the transient reaction conditions in the column, the rate of MB° reduction is the rate-limiting step in determining the MB° disappearance from the perfusate (15). Again, DHAA stimulated ferricyanide reduction but had little effect on MB° reduction (Figs. 9 and 10). Figure 9 shows column effluent concentration curves obtained after bolus injections of ferricyanide before DHAA was added to the perfusate and after the DHAA exposure. The amount of time the cells were exposed to the ferricyanide was varied by varying the column flow rate. The concentration curve for the reference indicator (FITC-dextran), which is convected through the column without interacting with the cells, reflects the concentration of ferricyanide that would have been measured in each sample had there been no reduction of ferricyanide on passage through the column. For clarity of presentation, only one reference indicator curve is shown because, except for small timing differences due to the fact that each curve is from a separate bolus injection, the reference curves were nearly superimposable with each other and with the ferricyanide curves obtained before treatment with DHAA. The bolus injections were carried out at three different flow rates to maximize the possibility of observing any changes in reduction rate that might occur under the assumption that if reduction rate were rate limiting, the fraction reduced would be dependent on column transit time. The time scale (x-axis) is normalized to the column mean transit time at each flow rate so that the results from the different flows can be compared on the same graph.

In the absence of DHAA, there was little separation between the ferricyanide and reference indicator (FITC-dextran) curves, indicating that little ferricyanide was reduced. After the DHAA exposure, the area under the ferricyanide curve was considerably smaller than that...
under the reference curve, indicating that the ferricyanide reduction was much greater. The fraction reduced was in fact column transit-time dependent, indicating that the reduction rate rather than the bolus delivery rate was rate limiting. The results of the similar experiment using MB as the electron acceptor are shown in Fig. 10. In contrast to the ferricyanide, substantial MB reduction occurred before the addition of DHAA, and including DHAA in the perfusate had little additional effect on MB reduction. To determine the time course and evaluate the persistence of the DHAA effect in the cell column, several boluses were injected into the cell column over a period of 105 min. The fractions of ferricyanide and MB reduced on passage through the cell column were determined before DHAA was added to the perfusate, with DHAA in the perfusate, and after the DHAA had been washed out of the perfusate (Fig. 11). The ferricyanide reduction increased with time during the DHAA perfusion and remained relatively stable after the DHAA had been washed out. The MB reduction was relatively unaffected by DHAA on the same time course.

**DISCUSSION**

These results are consistent with the hypothesis that there is a reductase on the bovine pulmonary arterial endothelial cell surface that can reduce extracellular ferricyanide and utilize intracellular AA as a direct electron donor or perhaps indirectly as a component of an intracellular electron transport sequence. According to this hypothesis, the explanation for the stimulatory effect of DHAA is that DHAA is transported into the cells where it is reduced to AA. Thus the intracellular reduction of DHAA provides AA for the reduction of the ferricyanide via a transplasma membrane electron transport system. Recycling of DHAA to AA within the cells would account for both the persistence of the DHAA effect and the ability of the cells to reduce more ferricyanide than predicted from the stoichiometry of DHAA uptake alone. The lack of effect of DHAA on the previously described (2, 3, 16) thiazine reductase activity suggests two separate electron transport systems on the endothelial cell plasma membrane, each with different electron donor and acceptor preferences.

Ferricyanide reduction via transplasma membrane electron transport has been observed in several mammalian cell types (8, 10, 15, 26), and a DHAA-stimulated ferricyanide reduction system has been observed in erythrocytes (17). Orringer and Roer (17) concluded that the DHAA stimulation of the reduction of ferricyanide in the presence of erythrocytes was the result of intracellular DHAA reduction to AA, which then left the cells, resulting in nonenzymatic reduction of the ferricyanide in the medium. Inside cells of various types that have been studied (18, 19), including endothelial cells (9), DHAA is, in fact, rapidly reduced to AA. That explanation would also be consistent with the fact that ferricyanide is rapidly reduced directly by AA. McGown et al. (13) came to a similar conclusion with respect to the ability of DHAA to stimulate extracellular methemoglobin reduction by erythrocytes. On the other hand, May et al. (12) carried out studies on erythrocytes using nitro blue tetrazolium, which is not directly reduced by AA, as the electron acceptor. They concluded that the nitro blue tetrazolium reduction by AA-loaded erythrocyte ghosts was due to the ability of intracellular AA to donate electrons to a transplasma membrane oxidoreductase. Schipfer et al. (20) also demonstrated that DHAA stimulation of a ferricyanide reductase in erythrocytes could occur without release of a reducing agent into the medium.

There are several lines of evidence suggesting that DHAA stimulation of the endothelial ferricyanide reduc-
In the present study it did not involve direct access of AA produced in the cells to the external ferricyanide. For example, the total amount of DHAA taken up by the endothelial cells was much less than the amount of ferricyanide reduced. The rate of DHAA uptake in Fig. 4 was only $\sim 3\%$ of the rate of ferricyanide reduction stimulated by the same concentration of DHAA in Fig. 3. Thus, with the assumption that AA is the electron donor, it would have to be recycled at a rapid rate to account for the observed ferricyanide reduction. The conclusion from the relatively slow rate of DHAA uptake is that the recycling must be intracellular, since the DHAA uptake rate would not be sufficient to support the rate of ferricyanide reduction if it involved oxidation of AA that had been released from the cells. This conclusion is further supported by the observation that DHAA-containing medium conditioned by exposure to the cells did not reduce ferricyanide. An AA concentration sufficient to make a significant contribution to the ferricyanide reduction would have been clearly detectable by UV absorption, but conditioning of the medium by the DHAA-treated cells did not increase the UV absorbance of the medium.

To evaluate the possibility that AA released from the cells might be too short-lived to be detectable in cell-conditioned medium removed from the cells, we took two approaches. One was to add AO to the medium to scavenge any AA that might be released into the medium bathing the DHAA-stimulated cells. The lack of effect of AO on DHAA-stimulated ferricyanide reduction suggests that AA in the medium was not involved. The other approach was to examine the ability of DHAA-stimulated cells to reduce ferricyanide. Because ferricyanide c was directly reduced by AA but not by DHAA-stimulated cells, a role for extracellular AA again seems unlikely.

The greater stimulatory effect of DHAA compared with AA is apparently due to the faster rate of DHAA accumulation by the cells (Fig. 4). Endothelial cells have been found to transport AA and DHAA (9, 23). In various cell types, AA and DHAA have been found to be transported by different mechanisms (5), and DHAA uptake has generally been faster than AA uptake (5). Wilson et al. (23) could not detect de novo synthesis of AA or a basal level of AA in cultured rat muscle microvascular endothelial cells, suggesting that endothelial cells, or at least endothelial cells in culture, do not synthesize AA, even when they are from species with hepatic AA synthesis. AA is not generally considered to be a necessary constituent of cell culture media (5), and the AA concentration in the endothelial cell growth medium used in the present study is negligible (11). The H-H buffer solution used in the study had no AA except when AA was specifically added. If there were any AA in the cells before the addition of DHAA or AA to the H-H solution, it was apparently either not sufficient or not available to allow the ferricyanide reductase to operate at a significant fraction of its capacity.

The effect of DHAA was apparently saturable (Fig. 4). Under the assumption that the stimulatory effect of DHAA involves its uptake into the cells followed by intracellular reduction to AA, this suggests that, for a given extracellular ferricyanide concentration, once the intracellular AA has reached a certain level within the cells, its role is permissive rather than rate limiting. This result is similar to that for DHAA stimulation of erythrocyte ferricyanide reduction (17). Interestingly, the concentration of DHAA in the medium that resulted in a half-maximal reduction rate by these endothelial cells of $\sim 0.1 \text{mM}$ is on the same order as the value of $\sim 0.04 \text{mM}$ that can be estimated for erythrocytes from the data of Orringer and Roer (17).

The data are consistent with the hypothesis that DHAA is reduced intracellularly to AA, which is an electron donor for the ferricyanide reductase. They are also consistent with the observation that erythrocytes can utilize intracellular AA to reduce extracellular ferricyanide (12). However, it is conceivable that DHAA stimulates the ferricyanide reductase through some other pathway. Schipfer et al. (20) noted stimulation of a ferricyanide reductase in the erythrocyte plasma membrane when DHAA was present in the medium. They concluded that NADH was the intracellular electron donor and provided no explanation for the DHAA effect.

In the cuvette experiments, the uptake of methylene blue by the endothelial cell is determined by the rates of the intracellular sequestration reaction and extracellular autooxidation in addition to the rate of reduction on the cell surface (14). On the time course of the transient passage of the bolus through the cell column, the surface reduction determines the rate of disappearance of methylene blue from the perfusate (16). The cell column results confirmed the selectivity of the DHAA-stimulated reductase activity with respect to the two electron acceptor probes ferricyanide and methylene blue, revealing that there are two distinct systems involved in the reduction of these two acceptors. One is a thiazine reductase that does not require intracellular AA, and another is a ferricyanide reductase that requires intracellular AA. Neither requires the release of AA into the medium. The ability to distinguish between the two systems does not necessarily mean that they are unrelated. Electron transport chains typically have multiple prosthetic groups, such that different electron donors and acceptors participate at different sites along the chain. Mitochondrial electron transport is prototypical in this regard. It is not clear how complex endothelial transplasma membrane electron transport systems might be. Villalba et al. (21) have begun to dissect the monoDHAA reductase of the hepatocyte plasma membrane, which appears to involve a cytochrome b$_r$ reductase on the intracellular plasma membrane surface, a coenzyme Q membrane component, and an as yet unidentified component(s) at the outer surface. Thus there is precedent for expecting a multicomponent electron transport chain within the endothelial membrane. On the other hand, several different transplasma membrane electron transport systems have been characterized in other mammalian cells (7), and there is evidence for an iodonium-sensitive NAD(P)H...
oxidase-like enzyme in the endothelial plasma membrane (25) in addition to the ferricyanide and thiazine reductases. Thus completely separate systems are also possible.

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Address for reprint requests: C. A. Dawson, Research Service 151, Zablocki VA Medical Center, 5000 W. National Ave., Milwaukee, WI 53295-1000.

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