informa
healthcare

ORIGINAL ARTICLE

Ascorbic acid prevents increased endothelial permeability caused by oxidized low density lipoprotein

JAMES M. MAY & ZHI-CHAO QU

Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232-0475, USA

Abstract
Mildly oxidized low density lipoprotein (mLDL) acutely increases the permeability of the vascular endothelium to molecules that would not otherwise cross the barrier. This study has shown that ascorbic acid tightens the permeability barrier in the endothelial barrier in cells, so this work tested whether it might prevent the increase in endothelial permeability due to mLDL. Treatment of EA.hy926 endothelial cells with mLDL decreased intracellular GSH and activated the cells to further oxidize the mLDL. mLDL also increased endothelial permeability over 2 h to both inulin and ascorbate in cells cultured on semi-permeable filters. This effect was blocked by microtubule and microfilament inhibitors, but not by chelation of intracellular calcium. Intracellular ascorbate both prevented and reversed the mLDL-induced increase in endothelial permeability, an effect mimicked by other cell-penetrant antioxidants. These results suggest a role for endothelial cell ascorbate in ameliorating an important facet of endothelial dysfunction caused by mLDL.

Keywords: *Paracellular transport, oxidative stress, endothelial dysfunction*

Abbreviations: *BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester); DHA, dehydroascorbic acid; Hepes, N-2-hydroxyethylpiperazine-NN-2-ethanesulphonic acid; KRH, Krebs-Ringer Hepes; mLDL, oxidized low density lipoprotein*

Introduction

Dysfunction of the vascular endothelium is an early stage of atherosclerosis that can be induced by oxidized low density lipoprotein [1,2]. One of the hallmarks of early endothelial dysfunction due to oxidized LDL is increased vascular permeability to serum proteins, which has been observed both *in vivo* [3,4] and *in vitro* [5,6]. Oxidized LDL increases endothelial permeability by causing a cytoskeleton-dependent contraction of the endothelial cells and opening of gaps between adjacent cells, which then allow large molecules such as albumin to diffuse across the endothelial barrier [5,7].

Given that at least part of the cytotoxicity of oxidized LDL is due to oxidative damage to the endothelial cells, small molecule antioxidants such as vitamins C and E have been tested and found in most studies

to protect endothelial cells in culture from oxidized LDL-induced oxidative damage and death [8,9]. This was especially evident for vitamin C or ascorbic acid, which prevented both cytotoxicity to the cells and further oxidative modification of LDL [10,11]. Whether this might extend to protection against oxidized LDL-induced increases in endothelial barrier permeability is unknown. Ascorbate was shown to increase barrier function in cultured endothelial cells treated with daily additions of 10–100 μM ascorbate, although the effect was attributed for the most part to increased collagen deposition induced by ascorbate over 5 days in culture. We recently found, however, that intracellular ascorbate rapidly (over 15–60 min) increased the barrier function of both immortalized (EA.hy926) and human dermal capillary endothelial cells cultured on porous membrane supports [12]. Barrier function was

Correspondence: Dr James M. May, 7465 Medical Research Building IV, Vanderbilt University School of Medicine, Nashville, TN 37232-0475, USA. Tel: (615) 936-1653. Fax: (615) 936-1667. Email: james.may@vanderbilt.edu

RIGHTSLINK

measured as transfer of radiolabelled inulin (molecular weight 5000–5500) and of ascorbate itself across the cell layer and membrane filter. The effect was not due to collagen deposition and was prevented by the microtubule agent colchicine, suggesting that it involved acute cytoskeletal changes.

In the present work we tested whether intracellular ascorbate can prevent the acute increase in endothelial permeability induced by mildly oxidized LDL. Again, we studied EA.hy926 endothelial cells, which are a hybridoma line derived from human umbilical vein endothelial cells [13]. In addition to providing a permeability barrier to inulin and ascorbate when cultured on semi-porous membrane supports [12], they also manifest several other features of endothelial cells [13–15], including oxidative modification of LDL [14]. We found that minimally oxidized LDL increased permeability of the endothelial barrier to both inulin and ascorbate, an effect that was completely reversed by intracellular ascorbate, likely through an antioxidant mechanism.

Materials and methods

Materials

Reagent chemicals, including ascorbate, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), dehydroascorbic acid (DHA) and N-2-hydroxyethylpiperazine N′-2-ethanesulphonic acid (Hepes) were supplied by Sigma-Aldrich Chemical Co. (St. Louis, MO). BAPTA-AM was initially dissolved in a small amount of dimethylsulphoxide and then diluted with culture medium such that the final dimethylsulphoxide concentration was 0.8%. Perkin-Elmer Life and Analytical Sciences, Inc. (Boston, MA) supplied the [carboxyl-14C]inulin (molecular weight range 5000–5500, 2 mCi/g, ~10 Ci/mol).

Cell culture

EA.hy926 cells were originally provided by Dr Cora Edgell (University of North Carolina, Chapel Hill, NC). They were cultured to confluence at 37°C in humidified air containing 5% CO_2 in Dulbecco's minimal essential medium and 10% (v/v) heat-inactivated foetal bovine serum, which contained 20 mM *D*-glucose and HAT media supplement (Sigma-Aldrich Chemical Co., St. Louis, MO).

Preparation of minimally oxidized LDL

LDL-enriched lipoproteins were obtained from a volunteer with familial hypercholesterolemia at the time of plasma apheresis to lower LDL. In this procedure, apolipoprotin B-containing particles were removed during apheresis from heparinized plasma using a dextran sulphate-cellulose adsorbent column of a packed volume of 150 ml (LA-15, Liposorber, Kaneka Corp., Osaka, Japan). Apolipoprotein B-enriched lipoproteins were eluted from the column resin with 70 ml of 690 mM sodium chloride. The lipoprotein solution had a protein concentration of 2–4 mg/ml and was not concentrated before subsequent storage or oxidation. It was either stored at 3°C or immediately oxidized by incubation of 2 mg/ml LDL with 5 µM copper sulphate at 23°C for 24 h under nitrogen [15]. The reaction was terminated by addition of EDTA to 5 mM. This preparation was dialysed under nitrogen for 24 h at 3°C against three changes of phosphate buffered saline (12.5 mM sodium phosphate, 140 mM sodium chloride, pH 7.4) to remove the copper and EDTA. It was stored for up to 3 weeks before use under nitrogen in an opaque plastic tube at 3°C. Gel electrophoresis showed that the oxidized LDL preparation consisted largely of lipoproteins migrating in the beta range, corresponding to LDL [16]. Copper oxidation did not affect electrophoretic migration, but doubled the thiobarbituric acid-reactive substances content of the preparation when measured by HPLC as malondialdehyde [16]. This suggests that, although lipids underwent peroxidation, apolipoproteins were not damaged significantly, indicating that the oxidized LDL preparation was minimally oxidized LDL (mLDL). The amount of mLDL in experiments was determined as its protein concentration in aqueous solution [11] using the Bradford reagent.

Assay of trans-endothelial ascorbate and inulin transfer

EA.hy926 cells were cultured either on standard 6-well culture plates or on polyethylene terephthalate cell culture inserts (6-well, 0.4 micron pores at a density of $2 \pm 0.2 \times 10^6$ pores per cm^2, Falcon BD Biosciences, Franklin Lakes, NJ). Cells were cultured to confluence and thereafter for 6–8 days with 1.7 ml of medium in the upper well and 2.8 ml of medium in the lower well. Medium was changed every 3 days. After incubations as described, either ascorbate (0.3 mM) or [carboxyl-14C]inulin (10 µM) was added above the cells/filter and incubation was carried out at 37°C for 1 h, except where noted. For assay of ascorbate transfer, medium below the cells/filter was sampled taken for ascorbate assay as described below.

Assay of [carboxyl-14C]inulin permeability was determined as previously described [17] with minor modifications [12]. The permeability coefficients for [carboxyl-14C]inulin were calculated, including adjustment for the rate of [carboxyl-14C]inulin transfer across filters after removal of cells [18]. This corrects in each well for any changes in permeability due to deposition of the matrix laid down by the cells during culture and the experiment. The permeability

of inulin was calculated as the volume or space occupied by the inulin that was transferred from above the cells to below the cells over 1 h, divided by the surface area of the filter, giving units of cm/h.

Assay of ascorbate, GSH and lipid peroxidation

For determination of ascorbate transferred across the cells and filter, ascorbate was measured in culture medium as follows. An aliquot of medium (0.1 ml) taken from below the cells was added to 0.1 ml of 25% metaphosphoric acid (w/v), mixed, neutralized with 0.35 ml of the above phosphate/EDTA buffer and centrifuged to remove any precipitated solids before assay of ascorbate as described below.

For assay of intracellular ascorbate, after removal of the medium, cells on the 6-well plate or filter were rinsed 3-times with Krebs-Ringer Hepes buffer (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. After removal of the last rinse, the cell monolayer was treated with 0.1 ml of 25% (w/v) metaphosphoric acid, detached from the filter with a rubber spatula and then treated with 0.35 ml of 0.1 M Na_2HPO_4 and 0.05 mM EDTA, pH 8.0. The lysate was removed and centrifuged at 3°C for 1 min at $13\,000 \times g$ and the supernatant was taken for assay of ascorbate. Assay of ascorbic acid was performed in duplicate by high performance liquid chromatography as previously described [19]. Intracellular GSH was measured in duplicate by the method of Hissin and Hilf [20] using fluorometric detection of a conjugate of *o*-phthalaldehyde with GSH on extract derived from the same samples as ascorbate. Intracellular concentrations of ascorbate and GSH were calculated based on the intracellular distribution space of 3-*O*-methylglucose in EA.hy926 cells, which was previously measured to be 3.6 ± 1.2 μl/mg protein [21].

Lipid hydroperoxides in mLDL present in the cell incubation medium was measured using the FOX-2 assay with *tert*-butyl hydroperoxide as a standard [22].

Data analysis

Results are shown as mean + standard error. Statistical comparisons were made using SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA). Differences between treatments were assessed by two-way analysis of variance with post-hoc testing using Tukey's test.

Results

mLDL generates acute oxidant stress in EA.hy926 cells

Although EA.hy926 cells do not contain appreciable ascorbate in culture, incubation of these cells with 0.5 mM DHA for 15 min raised the intracellular ascorbate concentration to almost 2 mM (Figure 1A, time zero). This increase was due to uptake of DHA on glucose transporters and intracellular reduction to ascorbate [23]. Treatment of cells with 0.2 mg/ml mLDL caused a 50% depletion of ascorbate by ~90 min that persisted to 180 min of incubation at 37°C (Figure 1A). Intracellular GSH did not change significantly (Figure 1B, circles) and lipid hydroperoxides in the mLDL increased by a small amount at the 120 and 180 min time points (Figure 1C, circles). On the other hand, when cells were not loaded with ascorbate prior to incubation with the mLDL, intracellular GSH progressively decreased, with a significant fall by 90 min, reaching ~50% of the time zero value at 180 min (Figure 1B, squares). Further, lipid hydroperoxides in the mLDL progressively increased in the absence of intracellular ascorbate (Figure 1C, squares). Thus, although intracellular ascorbate decreased in response to treatment of the cells with
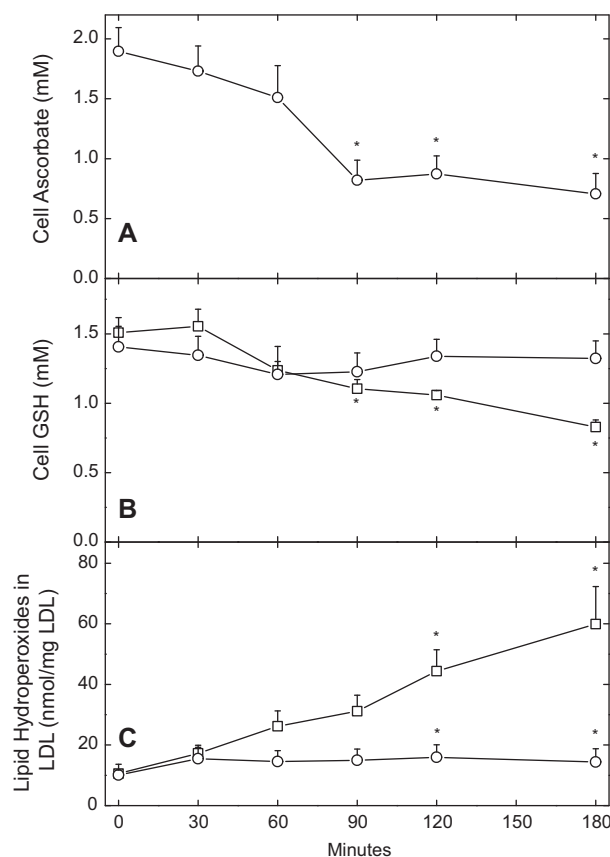

Figure 1. Time dependence of oxidative stress due to mLDL in EA.hy926 cells. Cells were rinsed three times in KRH and incubated at 37°C in KRH containing 5 mM D-glucose in the absence (squares) or presence (circles) of 0.3 mM DHA. After 15 min, 0.2 mg/ml mLDL was added and the incubation was continued for the times indicated, followed by removal of an aliquot of the medium for determination of lipid hydroperoxides (C). The cells were rinsed three times in KRH and taken for determination of ascorbate (A) and GSH (B). Results are shown from four-to-six experiments for each assay, with an * indicating $p < 0.05$ compared to the zero time sample.

mLDL, ascorbate spared intracellular GSH and largely prevented the rise in lipid hydroperoxides in the mLDL due to incubation with the cells. The same concentration of LDL that had not been oxidized had no effect on intracellular ascorbate or GSH over 180 min (results not shown).

To assess the concentration dependence of mLDL-induced ascorbate depletion, and whether changes in extracellular ascorbate contributed to changes in intracellular ascorbate in response to mLDL, cells loaded with ascorbate by DHA treatment were incubated for 120 min at 37°C with increasing amounts of mLDL and the ascorbate and GSH contents of the cells and the ascorbate concentration in the medium were measured. As shown in Figure 2, extracellular ascorbate was ~4 μM and did not significantly change with increasing concentrations of mLDL. On the other hand, intracellular ascorbate decreased such that a significant difference from control was seen at mLDL concentrations of 0.1 mg/ml and 0.4 mg/ml decreased intracellular ascorbate to ~40% of normal. Intracellular GSH did not change with increasing mLDL (Figure 2), in agreement with the time course experiment of Figure 1B for cells loaded to ~2 mM ascorbate and treated with 0.2 mg/ml mLDL for 120 min. Thus, the mLDL-induced decrease in intracellular ascorbate depended on the concentration of mLDL and could not be accounted for by changes in extracellular ascorbate.

mLDL increases endothelial barrier permeability

Incubation of EA.hy926 cells in culture on semi-porous membrane supports with 0.2 mg/ml mLDL
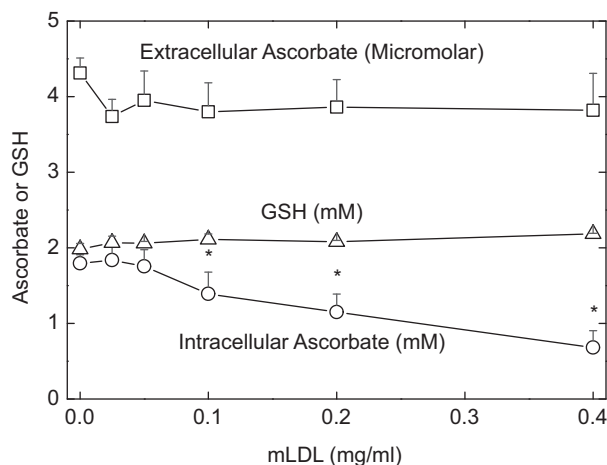

Figure 2. Effects of mLDL on extracellular ascorbate in DHA-loaded cells. EA.hy926 cells were rinsed three times in KRH and incubated at 37°C in KRH containing 5 mM D-glucose and 0.3 mM DHA. After 15 min, the indicated concentration of mLDL was added and the incubation was continued for an additional 2 h. Aliquots of the medium were sampled for ascorbate (squares) and intracellular ascorbate (circles) and GSH (triangles) were measured after removal of the cells. Results are shown from four experiments, with an * indicating $p < 0.05$ compared to the sample not exposed to mLDL.

gradually increased transfer of radiolabelled inulin across the cells and filter such that there was a significant difference by 45 min (Figure 3). This is shown for each time point as inulin transfer. As depicted in the inset to Figure 3, in a separate experiment, this increase persisted out to 140 min of incubation. Subsequent experiments were carried out after 1–2 h of incubation with mLDL. The increase in endothelial barrier permeability due to mLDL was concentration-dependent, as shown for both inulin and ascorbate transfer in Figure 4. This treatment did not cause morphologic changes evident on light microscopy (results not shown). Similar results have been found with mLDL prepared from different severely hypercholesterolemic donors (results not shown).

Permeability of the endothelial cell layer to radiolabelled inulin over a total of 90 min of incubation increased significantly at an mLDL concentration of 0.05 mg/ml and almost doubled at 0.35 mg/ml
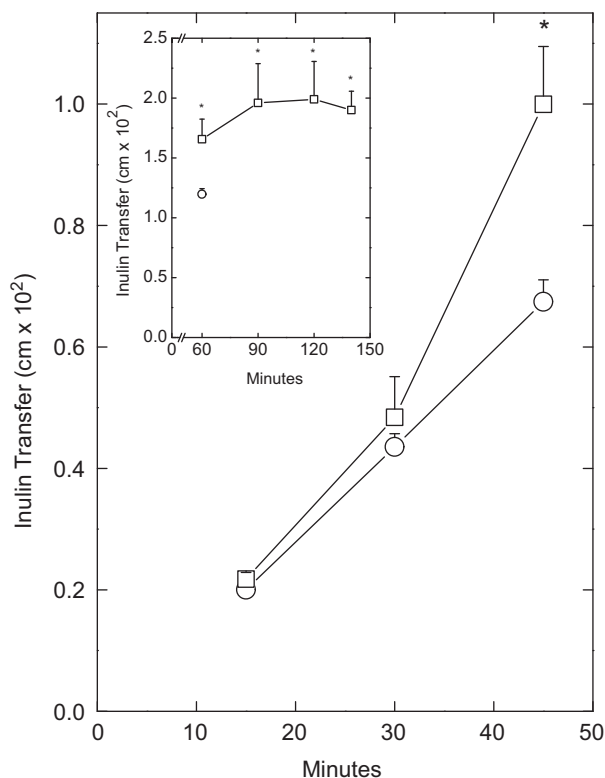

Figure 3. Time course of mLDL-induced increases in endothelial permeability. Cells cultured membrane filters in culture medium were treated without (circles) or with 0.2 mg/ml mLDL (squares) as well as 10 μM [carboxyl-^{14}C]inulin at 37°C. At the times indicated, the experiment was terminated and the extent of radiolabelled inulin transfer across the cells and filter was determined as described in Materials and methods. Results are shown from four experiments, with an * indicating $p < 0.05$ compared to the sample at the same time point that did not receive mLDL. Inset: Longer time course of inulin transfer increase due to mLDL (squares) compared to the amount transferred at 60 min in the absence of mLDL treatment (circle). Results are shown from six experiments, with the asterisk indicating $p < 0.05$ compared to the 60 min sample without mLDL.
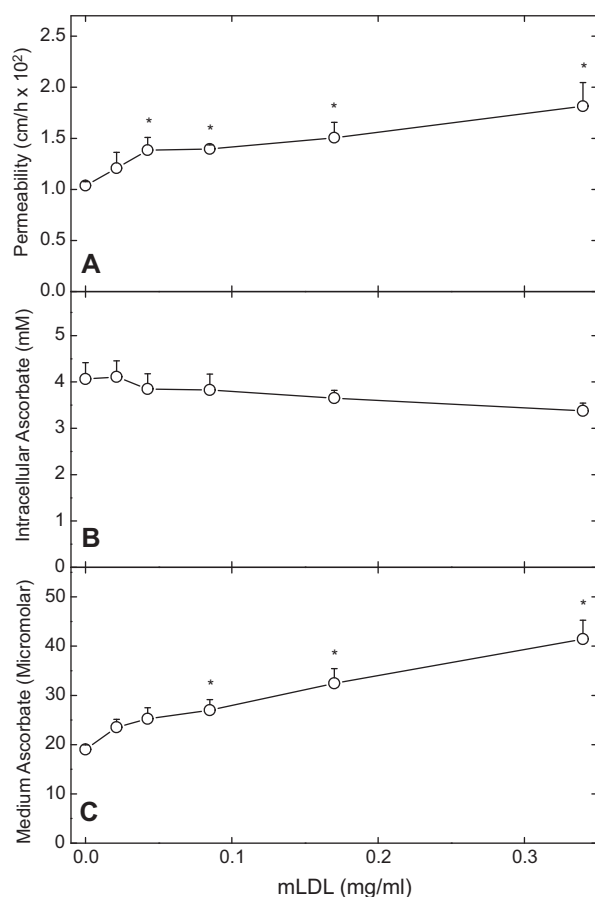
Figure 4. Concentration dependence of mLDL-induced increases in endothelial permeability. Cells cultured on filters were incubated in culture medium at 37°C for 30 min with the indicated concentration of mLDL, followed by a 60 min assay of permeability of 10 μM [carboxyl-14C]inulin (A) or of transfer of 0.3 mM ascorbate (C). Intracellular ascorbate was also measured (B). Results are shown from seven experiments for inulin transfer and from six experiments for ascorbate transfer, with an * indicating $p < 0.05$ compared to the sample not treated with mLDL.

(Figure 4A). Similar results were seen when ascorbate transfer was measured, determined as the concentration in the medium below the cells after 60 min of transfer of 0.3 mM ascorbate from above the cells (Figure 4C). Cells accumulated ascorbate under these conditions to concentrations of ~4 mM or twice that seen with DHA loading (Figures 1 and 2). This uptake against a concentration gradient was greater than seen with DHA due to function of the SVCT2, which likely mediates energy- and sodium-dependent ascorbate transport in these cells [24]. Increasing mLDL caused a significant downward trend in intracellular ascorbate concentrations ($p = 0.045$ by linear regression analysis), although the decrease was less than that seen in DHA-loaded cells (Figure 2), probably because of the availability of additional sources of reducing equivalents in culture medium compared to KRH containing 5 mM D-glucose alone.

To determine whether changes in the cytoskeleton were responsible for the increased permeability due to mLDL, EA.hy926 cells were incubated with 10 μM colchicine for 30 min followed by 0.2 mg/ml mLDL for 1 h and then by the radiolabelled inulin transfer assay. As shown in Figure 5, whereas colchicine alone had no effect on basal radiolabelled inulin transfer, it completely prevented the increase due to mLDL.

To assess whether the increase in endothelial barrier permeability due to mLDL was affected by intracellular calcium, cells were incubated with increasing concentrations of the intracellular calcium chelator BAPTA-AM for 30 min, followed by 0.2 mg/ml mLDL for 60 min and used for assays of permeability by both radiolabelled inulin and ascorbate. As shown in Figure 6, although there was a non-significant trend downward in radiolabelled inulin transfer (Figure 6A), this was not seen for the mLDL-induced increase in transfer of ascorbate (Figure 6B). BAPTA-AM alone at concentrations up to 30 μM had no effect on transfer of either radiolabelled inulin or ascorbate (data not shown). These results show that the ability of mLDL to increase endothelial cell permeability was unaffected by chelation of intracellular calcium.

Prevention of mLDL-induced increases in endothelial barrier permeability by ascorbate

To test whether ascorbate alone can prevent mLDL-induced increases in endothelial barrier permeability, EA.hy926 cells cultured on semi-porous filters were treated simultaneously with 0.2 mg/ml mLDL and varying concentrations of ascorbate for 60 min, followed by assay of inulin permeability. As shown in Figure 7, 0.3 mM ascorbate alone had the
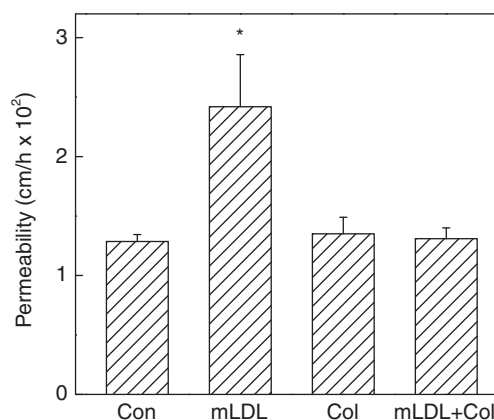

Figure 5. Prevention of mLDL-induced increases in endothelial permeability by colchicine. EA.hy926 cells cultured on filters were treated in culture medium at 37°C for 30 min without (Con) or with 10 μM colchicine (Col) followed by 0.2 mg/ml mLDL as indicated for 2 h followed by the radiolabelled inulin transfer assay. Results are from four experiments, with an * indicating $p < 0.05$ compared to the other treatments.
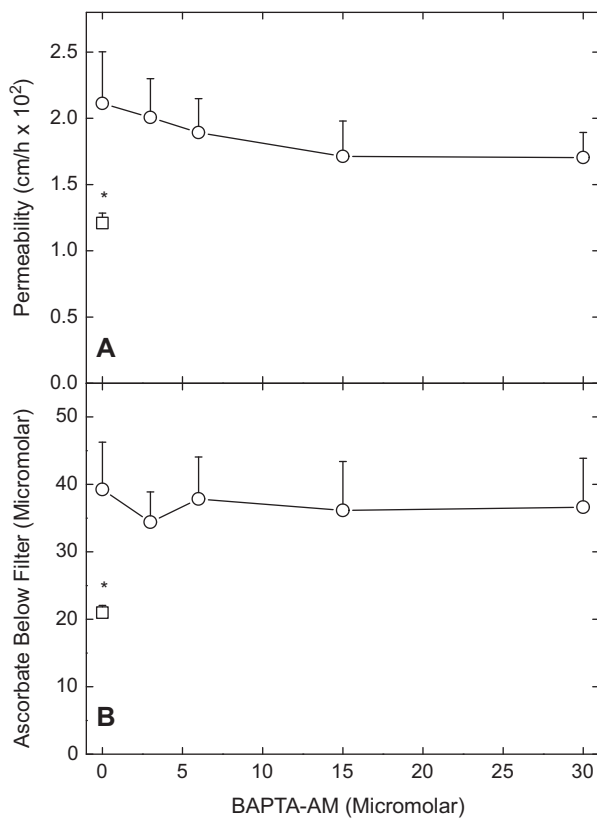
RIGHTS LINK

Figure 6. Failure of intracellular calcium chelation to affect the mLDL-induced increase in endothelial permeability to radiolabelled inulin or ascorbate. Cells cultured on filters were treated in culture medium at 37°C for 30 min with the indicated concentration of BAPTA-AM followed by 0.2 mg/ml mLDL (circles). A control sample (squares) received neither agent. After 60 min, either 10 μM [carboxyl-^{14}C]inulin (A) or 0.3 mM ascorbate (B) were added above the cells followed after 60 min by assay of inulin permeability (A) or measurement of the concentration of ascorbate in the well below the cells and filter (B). Results are shown from six experiments for inulin transfer and five experiments for ascorbate transfer, with an asterisk indicating $p < 0.05$ compared to the sample that received mLDL alone.

expected effect to decrease inulin transfer, while mLDL alone doubled the rate of transfer. All three ascorbate concentrations tested completely prevented the mLDL-induced increase in transfer. In addition, transfer rates were suppressed to levels no different than either untreated cells or cells treated with ascorbate alone. Although not shown, the ascorbate concentration above the cells was unaffected by mLDL under these conditions (ascorbate alone: 0.32 ± 0.01 mM vs mLDL + ascorbate: 0.33 ± 0.01 mM, $n = 2$).

Intracellular ascorbate was shown in our earlier work to tighten the endothelial barrier to transfer of both radiolabelled inulin and ascorbate itself [12]. To assess whether intracellular ascorbate (as opposed to extracellular ascorbate) might affect the mLDL-induced increase in endothelial barrier permeability, cells were either loaded first with ascorbate by a 15 min incubation with DHA followed by addition of 0.2 mg/ml mLDL for 60 min (Figure 8, open bars) or
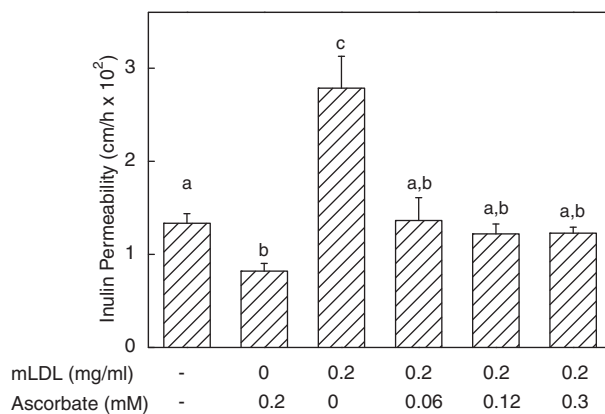

Figure 7. Inhibition of the mLDL-induced increase in endothelial permeability by ascorbate. Ea.hy926 cells in culture on microporous filters were treated with mLDL or ascorbate above the cells as noted for 60 min at 37°C, followed by addition of 10 μM [carboxyl-^{14}C]inulin and determination of inulin permeability after an additional 60 min. Results are shown from six experiments with $p < 0.05$ differences for bars not sharing the same letters.

they were treated first for 60 min with 0.2 mg/ml mLDL followed by loading ascorbate with DHA for 60 min (Figure 8, hatched bars). This different order of addition tested whether intracellular ascorbate could both prevent and reverse the increase in endothelial permeability due to mLDL. Transfer of both inulin (Figure 8A) and ascorbate (Figure 8B) were measured for each order of addition. As expected, intracellular ascorbate alone decreased transfer of both radiolabelled inulin (Figure 8A) and ascorbate (Figure 8B) by 30–50%. As also expected, mLDL alone increased the permeability of both inulin (Figure 8A) and ascorbate (Figure 8B). When DHA was added first, the mLDL-induced increase in permeability was decreased to control levels for both inulin and ascorbate transfer by initial DHA concentrations of 60 μM and greater (Figure 8, last three open bars). When mLDL was added before DHA, a significant decrease in transfer of both agents required addition of a concentration of 120 mM DHA (Figure 8, last three hatched bars). Thus, intracellular ascorbate can both prevent and reverse the increase in endothelial permeability due to mLDL, although not to levels seen with ascorbate loading alone.

To assess whether the effect of ascorbate to prevent the mLDL-induced increase in endothelial membrane permeability might be due to an antioxidant effect, responses to several different antioxidants were examined. EA.hy926 endothelial cells that had been exposed to mLDL for 60 min were treated above the filters with the indicated concentrations of DHA, Tempol, dithiothreitol and lipoic acid. After another 30 min, the inulin transfer assay was carried out, with the results shown in Figure 9. Of the agents tested, only DHA and dithiothreitol decreased basal endothelial permeability (Figure 9A). On the other hand, when cells pre-treated with mLDL were then exposed
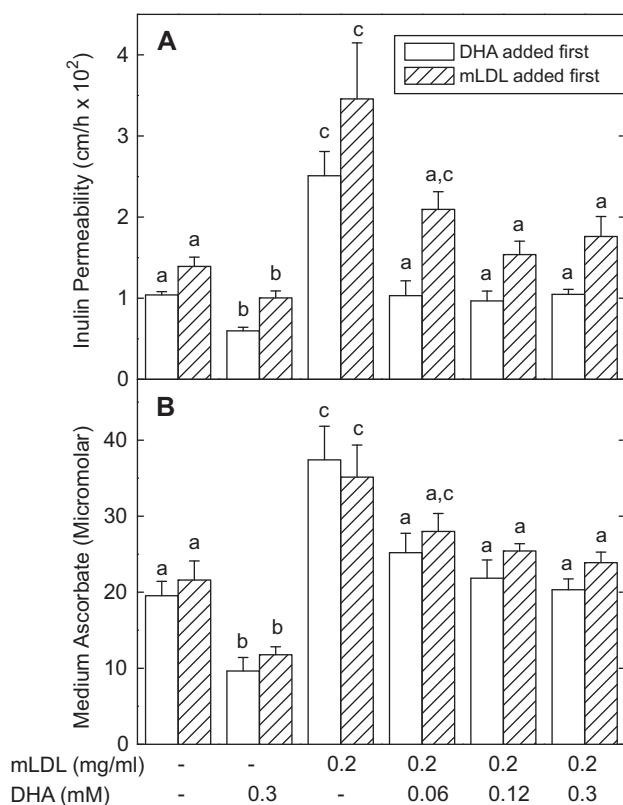
Figure 8. Inhibition of the mLDL-induced increase in endothelial permeability by intracellular ascorbate. Ea.hy926 cells in culture on filters were either treated first for 15 min with DHA followed by a 60 min treatment with mLDL (open bars) or were treated first with mLDL for 60 min followed by a 15 min treatment with DHA (hatched bars). This was followed by the [carboxyl-14C] inulin transfer assay (A) or by measurement of transfer of 0.3 mM ascorbate from above to below the cells. Results are shown from five-to-seven experiments, with $p < 0.05$ differences for bars of the same type not sharing the same letters in the same panels.

to the antioxidants, each of them prevented the mLDL-induced increase in membrane permeability, although not below control rates (Figure 9B). These results suggest that the effect of mLDL to increase endothelial permeability is due to increases in cellular oxidant stress.

Discussion

Treatment of endothelial cells for 90–180 min with minimally oxidized LDL caused an oxidative stress that depleted the intracellular antioxidants ascorbate and GSH, activated the cells to further oxidize the LDL and increased permeability of the endothelial barrier to both inulin and ascorbate. When cells exposed to mLDL were loaded with ascorbate, GSH was preserved, there was minimal further oxidation of mLDL and the increase in endothelial barrier permeability due to mLDL was both prevented and reversed.

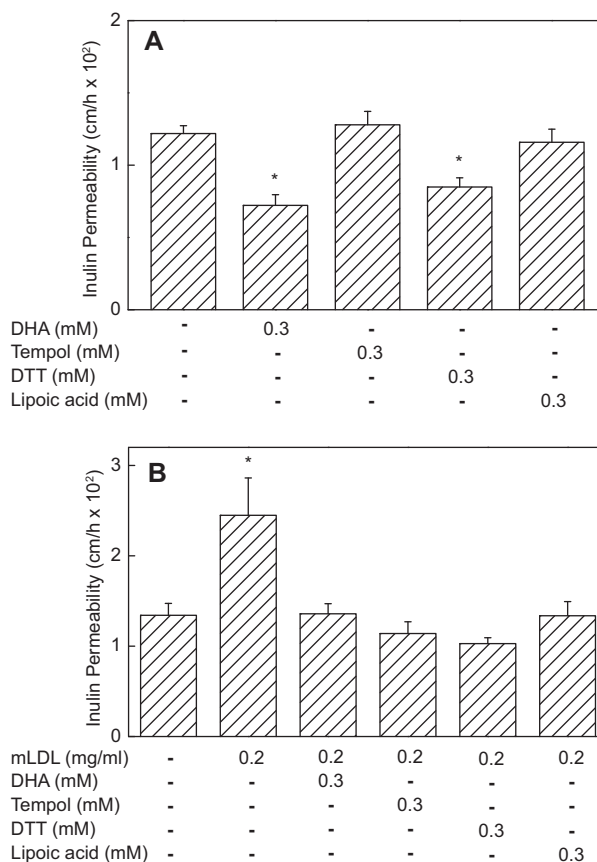The increase in endothelial permeability to large molecules caused by mLDL is due to cytoskeletal

Figure 9. Inhibition of mLDL-induced increases in endothelial permeability by antioxidants. (A) EA.hy926 cells in culture on microporous filters were treated for 60 min above the cells with the agents noted, followed by the 60 min inulin transfer assay. Results are shown from six assays, with an * indicating $p < 0.05$ compared to samples receiving no additions. (B) Cells were treated for 60 min without or with the indicated concentration of mLDL as noted, followed by addition above the cells of the antioxidants noted. After 30 min of incubation, 10 μM [carboxyl-14C]inulin was added and the 60 min inulin transfer assay was performed. Results are shown from five assays, with an * indicating $p < 0.05$ compared to all other bars in (B).

rearrangement with cellular contraction and opening of pores between the cells through which otherwise impermeant molecules can pass [5,7,25,26]. That this mechanism was operative in the present studies is supported by the finding that the mLDL-induced increase in endothelial permeability was prevented by colchicine, a microtubule inhibitor.

The cellular mechanism by which exposure to mLDL for minutes to several hours causes endothelial cell contraction and loss of the permeability barrier may be multi-factorial. It is likely due initially to an increase in oxidative stress generated by further oxidation of mLDL in mitochondria [27] or lysosomes [28]. The effect has also been linked to mLDL-dependent increases in intracellular calcium in both cultured arterial and venous endothelial cells [26,29]. Similar acute increases in intracellular calcium following cell treatment with thrombin and histamine are known to increase endothelial barrier permeability

[30]. Increased calcium in turn activates calmodulin-dependent myosin light chain kinase to phosphorylate myosin light chain, leading to cytoskeletal rearrangement and cell contraction [30–32]. Subsequent studies in cultured human umbilical vein endothelial cells failed to show an mLDL-induced increase in intracellular calcium; rather, the mLDL-induced increase in permeability was due to activation of Rho/Rho kinase causing phosphorylation and inhibition of myosin light chain phosphatase. The resulting increase in myosin light chain phosphorylation then caused increases in endothelial permeability [5]. The same group showed that a similar mechanism may also occur in response to thrombin stimulation [31]. Additional support for this mechanism derives from the observation that oxidized LDL activates the receptor for advanced glycation end products (RAGE) *in vivo* [32]. RAGE has recently been shown in endothelial cells to activate Rho/Rho kinase [33]. The results of the present study, that the mLDL-induced increase in endothelial barrier permeability was not reversed by chelation of intracellular calcium with BAPTA, also support the Rho/Rho kinase mechanism of activation of myosin light chain kinase with subsequent cytoskeletal rearrangement and cell contraction.

The major finding in the present study was that ascorbate prevented the mLDL-induced increase in endothelial barrier permeability. To assess whether this effect was due to intracellular ascorbate, cells were treated for 15 min or more with DHA, which is taken up on glucose transporters and rapidly reduced to ascorbate within the cells. Under these conditions, extracellular ascorbate concentrations were low (~4 µM) and stable for 2 h during mLDL treatment. Ascorbate loading of cells using this approach both prevented and reversed the mLDL-induced increase in endothelial barrier permeability. The effect of ascorbate was evident at extracellular ascorbate concentrations of 60–120 µM and at intracellular concentrations of 0.5–2.0 mM, both of which are likely to be in the physiologic range for endothelial cells *in vivo* [34]. These results also suggest that endothelial cell ascorbate is a factor in baseline endothelial barrier function and should be considered in studies of endothelial dysfunction due to mLDL.

The mechanism by which ascorbate alone increases endothelial barrier function is under study, but it appears that an antioxidant effect could mediate its ability to antagonize mLDL-induced increases in endothelial barrier permeability. In this scenario, oxidized LDL would generate radicals through mitochondrial [27] or lysosomal [28] activation that would in turn cause oxidative stress in the cells [35]. That direct oxidative stress can increase endothelial barrier permeability has been demonstrated for H_2O_2 [36] and menadione [37] in brain microvascular endothelial cells. H_2O_2 directly induces oxidative

stress, whereas menadione does so by redox cycling within cells [38]. Two results of this work support an antioxidant effect of ascorbate. First, it preserved intracellular GSH, either by directly reacting with intracellular radicals or by repairing protein radicals [39] before they could react with GSH. Second, several cell-penetrant antioxidants with different mechanisms of action prevented the effect of mLDL. These included Tempol, a radical species able to donate a single electron to scavenge other radicals, as well as two thiol reagents, dithiothreitol and lipoic acid. Dithiothreitol is a small 4-carbon cell-penetrant molecule with 2-thiol groups that acts directly as a reducing agent. Lipoic acid, on the other hand, is an 8-carbon fatty acid with a disulphide link between sulphur atoms on carbons 6 and 8. It must first enter the cell and be reduced to dihydrolipoic acid [40], which has two thiol groups on each molecule and is a potent antioxidant. The thiols would be expected to compensate for the mLDL-induced decrease in GSH as well as directly scavenge radical species.

Although a decrease in oxidative stress due to ascorbate is likely involved in its prevention and reversal of the mLDL effect on endothelial permeability, it may not do so directly. Endothelial cell activation by mLDL has been shown to generate lysophosphatidic acid from the mLDL [7], which through binding to its cellular receptor caused endothelial stress fibre and gap formation in cultured human umbilical vein endothelial cells [7] and a decrease in trans-endothelial resistance in brain endothelial cells [41]. Our finding that intracellular ascorbate decreased endothelial cell-dependent oxidation of mLDL could result in less release of lysophosphatidic acid from the mLDL, thus decreasing the subsequent cellular response.

In conclusion, the finding that intracellular ascorbate both prevents and reverses the mLDL-induced impairment in endothelial barrier function suggests that ascorbate could help to maintain endothelial barrier permeability *in vivo* and thus prevent endothelial dysfunction in response to mLDL. Although the cellular and molecular mechanisms of the ascorbate effect remain to be determined, the present results support a mechanism involving decreased oxidative stress in cells containing ascorbate.

References

[1] Ross R. Atherosclerosis–an inflammatory disease. N Engl J Med 1999;340:115–126.

[2] Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 1989;320: 915–924.

[3] Liao L, Aw TY, Kvietys PR, Granger DN. Oxidized LDL-induced microvascular dysfunction. Dependence on oxidation procedure. Arterioscler Thromb Vasc Biol 1995;15: 2305–2311.

[4] Rangaswamy S, Penn MS, Saidel GM, Chisolm GM. Exogenous oxidized low-density lipoprotein injures and alters the barrier function of endothelium in rats *in vivo*. Circ Res 1997;80:37–44.

[5] Essler M, Retzer M, Bauer M, Heemskerk JW, Aepfelbacher M, Siess W. Mildly oxidized low density lipoprotein induces contraction of human endothelial cells through activation of Rho/Rho kinase and inhibition of myosin light chain phosphatase. J Biol Chem 1999;274:30361–30364.

[6] Orr AW, Stockton R, Simmers MB, Sanders JM, Sarembock IJ, Blackman BR, Schwartz MA. Matrix-specific p21-activated kinase activation regulates vascular permeability in atherogenesis. J Cell Biol 2007;176:719–727.

[7] Siess W, Zangl KJ, Essler M, Bauer M, Brandl R, Corrinth C, Bittman R, Tigyi G, Aepfelbacher M. Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. Proc Natl Acad Sci USA 1999;96:6931–6936.

[8] Negre-Salvayre A, Mabile L, Delchambre J, Salvayre R. α-Tocopherol, ascorbic acid, and rutin inhibit synergistically the copper-promoted LDL oxidation and the cytotoxicity of oxidized LDL to cultured endothelial cells. Biol Trace Elem Res 1995;47:81–91.

[9] Mabile L, Fitoussi G, Periquet B, Schmitt A, Salvayre R, Nègre-Salvayre A. α-tocopherol and trolox block the early intracellular events (TBARS and calcium rises) elicited by oxidized low density lipoproteins in cultured endothelial cells. Free Radic Biol Med 1995;19:177–187.

[10] Martin A, Frei B. Both intracellular and extracellular vitamin C inhibit atherogenic modification of LDL by human vascular endothelial cells. Arterioscler Thromb Vasc Biol 1997;17: 1583–1590.

[11] Sabharwal AK, May JM. Alpha-Lipoic acid and ascorbate prevent LDL oxidation and oxidant stress in endothelial cells. Mol Cell Biochem 2008;309:125–132.

[12] May JM, Qu ZC, Qiao H. Transfer of ascorbic acid across the vascular endothelium: mechanism and self-regulation. Am J Physiol Cell Physiol 2009;297:C169–C178.

[13] Bauer J, Margolis M, Schreiner C, Edgell CJ, Azizkhan J, Lazarowski E, Juliano RL. *In vitro* model of angiogenesis using a human endothelium-derived permanent cell line: contributions of induced gene expression, G-proteins, and integrins. J Cell Physiol 1992;153:437–449.

[14] Pech-Amsellem MA, Myara I, Pico I, Maziere C, Maziere JC, Moatti N. Oxidative modifications of low-density lipoproteins (LDL) by the human endothelial cell line EA.hy 926. Experientia 1996;52:234–238.

[15] Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc Natl Acad Sci USA 1984;81:3883–3887.

[16] Chi X, May JM. Oxidized lipoprotein induces the macrophage ascorbate transporter (SVCT2): protection by intracellular ascorbate against oxidant stress and apoptosis. Arch Biochem Biophys 2009;485:174–182.

[17] Siflinger-Birnboim A, del Vecchio PJ, Cooper JA, Blumenstock FA, Shepard JM, Malik AB. Molecular sieving characteristics of the cultured endothelial monolayer. J Cell Physiol 1987; 132:111–117.

[18] Utoguchi N, Ikeda K, Saeki K, Oka N, Mizuguchi H, Kubo K, Nakagawa S, Mayumi T. Ascorbic acid stimulates barrier function of cultured endothelial cell monolayer. J Cell Physiol 1995;163:393–399.

[19] May JM, Qu Z-C, Mendiratta S. Protection and recycling of α-tocopherol in human erythrocytes by intracellular ascorbic acid. Arch Biochem Biophys 1998;349:281–289.

[20] Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 1976;74:214–226.

[21] Jones W, Li X, Perriott LM, Whitesell RR, May JM. Uptake, recycling, and antioxidant functions of α-lipoic acid in endothelial cells. Free Radic Biol Med 2002;33:83–93.

[22] Wolff SP. Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. Methods Enzymol 1994;233:182–189.

[23] May JM, Qu ZC, Li X. Requirement for GSH in recycling of ascorbic acid in endothelial cells. Biochem Pharmacol 2001;62:873–881.

[24] May JM, Qu ZC. Transport and intracellular accumulation of vitamin C in endothelial cells: relevance to collagen synthesis. Arch Biochem Biophys 2005;434:178–186.

[25] Zhao B, Zhang Y, Liu B, Nawroth P, Dierichs R. Endothelial cells injured by oxidized low density lipoprotein. Am J Hematol 1995;49:250–252.

[26] Zhao B, Ehringer WD, Dierichs R, Miller FN. Oxidized low-density lipoprotein increases endothelial intracellular calcium and alters cytoskeletal f-actin distribution. Eur J Clin Invest 1997;27:48–54.

[27] Mabile L, Meilhac O, Escargueil-Blanc I, Troly M, Pieraggi MT, Salvayre R, Negre-Salvayre A. Mitochondrial function is involved in LDL oxidation mediated by human cultured endothelial cells. Arterioscler Thromb Vasc Biol 1997;17: 1575–1582.

[28] Wen Y, Leake DS. Low density lipoprotein undergoes oxidation within lysosomes in cells. Circ Res 2007;100: 1337–1343.

[29] Negre-Salvayre A, Fitoussi G, Reaud V, Pieraggi MT, Thiers JC, Salvayre R. A delayed and sustained rise of cytosolic calcium is elicited by oxidized LDL in cultured bovine aortic endothelial cells. FEBS Lett 1992;299:60–65.

[30] Ehringer WD, Edwards MJ, Miller FN. Mechanisms of alpha-thrombin, histamine, and bradykinin induced endothelial permeability. J Cell Physiol 1996;167:562–569.

[31] Essler M, Amano M, Kruse HJ, Kaibuchi K, Weber PC, Aepfelbacher M. Thrombin inactivates myosin light chain phosphatase via Rho and its target Rho kinase in human endothelial cells. J Biol Chem 1998;273:21867–21874.

[32] Sun L, Ishida T, Yasuda T, Kojima Y, Honjo T, Yamamoto Y, Yamamoto H, Ishibashi S, Hirata K, Hayashi Y. RAGE mediates oxidized LDL-induced pro-inflammatory effects and atherosclerosis in non-diabetic LDL receptor-deficient mice. Cardiovasc Res 2009;82:371–381.

[33] Hirose A, Tanikawa T, Mori H, Okada Y, Tanaka Y. Advanced glycation end products increase endothelial permeability through the RAGE/Rho signaling pathway. FEBS Lett 2010;584:61–66.

[34] May JM, Qu ZC. Ascorbic acid efflux and re-uptake in endothelial cells: maintenance of intracellular ascorbate. Mol Cell Biochem 2009;325:79–88.

[35] Lum H, Roebuck KA. Oxidant stress and endothelial cell dysfunction. Am J Physiol Cell Physiol 2001;280:C719–C741.

[36] Lee HS, Namkoong K, Kim DH, Kim KJ, Cheong YH, Kim SS, Lee WB, Kim KY. Hydrogen peroxide-induced alterations of tight junction proteins in bovine brain microvascular endothelial cells. Microvasc Res 2004;68:231–238.

[37] Lagrange P, Romero IA, Minn A, Revest PA. Transendothelial permeability changes induced by free radicals in an *in vitro* model of the blood-brain barrier. Free Radic Biol Med 1999;27:667–672.

[38] McCord JM, Fridovich I. The utility of superoxide dismutase in studying free radical reactions. II. The mechanism of the mediation of cytochrome *c* reduction by a variety of electron carriers. J Biol Chem 1970;245:1374–1377.

[39] Domazou AS, Koppenol WH, Gebicki JM. Efficient repair of rotein radicals by ascorbate. Free Radic Biol Med 2009;46: 1049–1057.

[40] Handelman GJ, Han D, Tritschler H, Packer L. α-lipoic acid reduction by mammalian cells to the dithiol form, and release into the culture medium. Biochem Pharmacol 1994; 47:1725–1730.

[41] Schulze C, Smales C, Rubin LL, Staddon JM. Lysophosphatidic acid increases tight junction permeability in cultured brain endothelial cells. J Neurochem 1997;68:991–1000.