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Comparison of oxidized low-density lipoprotein toxicity on EA.hy 926 cells and human vein endothelial cells: influence of antioxidant systems

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Abstract. We compared the susceptibility to oxidized LDL cytotoxicity of primary human umbilical vein endothelial cells (HUVEC) and EA.hy 926 cells. EA.hy 926 endothelial cells were more susceptible than HUVEC. To determine the basis of this difference, we evaluated the enzymatic antioxidant machinery in the two cell types. The antioxidant enzyme activities of superoxide dismutase, catalase and glutathione peroxidase were significantly lower in EA.hy cells than in HUVEC: 54%, 71% and 8% of the HUVEC enzyme activities respectively. Pre-incubation of the EA.hy 926 endothelial cells with glutathione peroxidase (100 IU/ml) inhibited the cytotoxic effect of oxidized LDL. Superoxide dismutase (300 or 600 IU/ml) and catalase (300 or 600 IU/ml) had no effect. Compared to HUVEC, the higher susceptibility of EA.hy 926 cells to oxidized LDL–induced injury may be associated with lower antioxidant defences, in particular with lower glutathione peroxidase activity which is known to eliminate lipid hydroperoxides and thereby to prevent the formation of damaging peroxyl radical intermediates.
Key words. Oxidized-LDL; endothelial cells; EA.hy 926; antioxidant enzymes; glutathione peroxidase; cytotoxicity; atherosclerosis.

Abbreviations. LDL = low-density lipoproteins, apo B = apolipoprotein B, ox-LDL = oxidized LDL, GPx = selenium-glutathione peroxidase, SOD = superoxide dismutase, GR = glutathione reductase, HUVEC = human umbilical vein endothelial cells, MTT = methyl thiazol tetrazolium salt, TBARS = thiobarbituric acid-reactive substances, FCS = fetal calf serum, MEM = Eagle's minimum essential medium.

A high serum concentration of low-density lipoproteins (LDL) is a major risk factor for atherosclerosis [1]. Oxidized LDL (ox-LDL) have been detected in atherosclerotic lesions and are thought to play a major role in the initiation and progression of atherosclerosis [2, 3]. In vitro LDL oxidation can be induced by reactive oxygen species generated by monocytes/macrophages, smooth muscle cells and endothelial cells, in the presence of trace amounts of transition metals [4]. This oxidative modification of LDL leads to lipid peroxidation and the formation of hydroperoxides, lipoperoxide decomposition end products, and apolipoprotein B (apo B) modifications [3, 4]. The oxidized lipoproteins are taken up by the scavenger receptor of macrophages and form lipid-laden foam cells [4, 5]. Ox-LDL are toxic to various cell types including vascular endothelial cells [6–8].

The incubation of endothelial cells with ox-LDL induces a loss of intracellular glutathione and lipid peroxidation of the cellular membrane, thereby implicating radical activities [9, 10]. Cell sensitivity to these phenomena is related to enzymatic antioxidant systems: glutathione peroxidase (GPx) cooperates with superoxide dismutase (SOD) and catalase to maintain the redox potential within the endothelial cells [11–13].

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Human umbilical vein endothelial cells (HUVEC) are a good model for studying the mechanisms of ox-LDL cytotoxicity and the cytoprotective effect of various molecules on the vascular endothelium [14]. However, the use of HUVEC is complicated by batch-to-batch variability of the cells and the difficulty of culturing them: they only survive for a limited number of passages and require special growth factors [15, 16].

A human permanent EA.hy 926 endothelial cell line, obtained by hybridizing HUVEC with human lung tumor cells (A 549), has been used to study some metabolic effects of native and ox-LDL [17–19]. Easier to handle than HUVEC, this cell line expresses for more than 100 passages several characteristics common to endothelial cells, such as von Willebrand factor and prostacyclin [17, 20]. Nevertheless, the antioxidant equipment of this cell line has never been studied. Important variations in the enzymatic antioxidant machinery between cell types are usually observed [11–13]. As radical activity is implicated in the ox-LDL cytotoxic effect [6, 9, 21], the use of a cell line may lead to problems in drawing conclusions on the mechanisms of the cytotoxicity.

The aim of this study was to compare the sensitivity to the ox-LDL cytotoxicity and the enzymatic antioxidant equipment of HUVEC and EA.hy 926 endothelial cells.

Materials and methods

Chemicals

Hank's balanced salt solution (HBSS), medium 199 (M199), Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (MEM), HEPES, trypsin-EDTA solution, L-glutamine, penicillin and streptomycin were obtained from Gibco (Cergy Pontoise, France). The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), DNA standards and chemicals used for enzyme assays were obtained from Sigma (L'Isle d'Abeau-Chesnes, France). HAT (100 μM hypoxanthine/0.4 μM aminopterin/16 μM thymidine), fetal calf serum (FCS) and Dispase (grade II from *Bacillus polymyxa*) were obtained from Boehringer (Mannheim, Germany). Other chemicals were provided by Merck (Chelles, France).

Cell culture

The human EA.hy 926 endothelial cell line was a kind gift from Dr C.-J. S. Edgell (University of North Carolina, USA) [17]. The cells were cultured in 75 cm^2 flasks (Costar), in DMEM containing 10% (v/v) FCS, L-glutamine (2 mmol/l), penicillin (100 units/ml), streptomycin (100 μg/ml) and HAT. The cells were sub-cultured twice a week at a ratio of 1:4.

The HUVEC were prepared within 24 h post parturitum from umbilical cords from healthy women. The cells were detached from the umbilical vein by treatment for 30 min at 37 °C with dispase. The freshly isolated cells were cultured in 25 cm^2 flasks (T-25, Falcon Plastics) and the adherent HUVEC grown to confluency (within 3–5 days). The culture complete medium consisted of M 199 supplemented with 10% (v/v) FCS, 1.5% (v/v) HEPES, L-glutamine (8 mmol/l), penicillin (100 units/ml) and streptomycin (100 μg/ml). When confluent, the cells were trypsinized and resuspended in complete medium. We did not detect any selenium (Se) in the two cell types culture media, using electrothermal atomic absorption spectrometry (ETAAS) with a Perkin-Elmer 5100 Pc spectrometer and Zeeman-effect background correction (characteristic mass of 23 pg).

LDL isolation

In the presence of EDTA, LDL (d 1.019–1.063 g/ml) was isolated from pooled human plasma by sequential ultracentrifugation in a Kontron TGA-55 centrifuge (TFT 65.38 rotor) as described by Havel [22]. LDL was dialysed against 0.01 mol/l Tris-HCl buffer, pH 7.4, containing 1 mmol/l EDTA, and stored at 4 °C. Cholesterol and triglyceride contents of the LDL were determined enzymatically (Boehringer, Mannheim). The apo B concentrations were measured before and after native and ox-LDL filtration using a BNA Behring analyser.

LDL copper oxidation

LDL (2 mg apo B/ml) was incubated with 5 μmol/l CuSO4 at 37 °C for 24 h. The native and ox-LDL were extensively dialysed against phosphate buffered saline (PBS, 10 mM, pH 7.4). After experiments, the oxidation was stopped by adding 1 mmol/l EDTA and 0.02 mmol/l butylated hydroxytoluene (BHT).

Thiobarbituric acid-reactive substances (TBARS) were measured in dialysed samples according to the method of Yagi [23] and lipid hydroperoxides (LOOH) assayed by the iodometric method [24]. The increased negative charge-modified LDL fractions were separated by ion exchange liquid chromatography analysis as described by Vedie et al., adapted to HPLC on chromatographic equipment manufactured by Waters [25]. With this system, the LDL with unmodified apo B corresponds to the arbitrarily named A fraction, and the other HPLC fractions, named B, C and D, correspond to LDL with modified apo B with increasing electronegative charge.

Native LDL had no detectable TBARS, little hydroperoxide (37 ± 10 nmol/mg) and the apo B was not modified (isolated A fraction). After dialysis, ox-LDL contained 6.9 ± 2.7 nmol/mg TBARS, 1529 ± 70 nmol/mg hydroperoxide, and the apo B was modified (isolated C fraction).

Cell cytotoxicity

EA.hy 926 cells were seeded at a density of 25,000 cells/well and HUVEC at 35,000 cells/well in 96-microwell tissue culture plates (Costar) so that they reached confluence in two days. Once confluent, the culture medium was removed, replaced by MEM supplemented with 2% (v/v) FCS, and then native LDL and ox-LDL were added to the following final concentrations: 50, 100, 150 and 200 μg apo B/ml, and incubated for 24 h. The cell viability in MEM with 2% FCS was assessed by Trypan blue exclusion. It was around 95% for the two cell types.

When the antioxidant enzymes were tested, the cells were pre-incubated with SOD (300 or 600 IU/ml), catalase (300 or 600 IU/ml) and GPx (100 IU/ml) in complete medium for 16 h. The medium was then removed and the cells rinsed twice with MEM. The cells were then incubated for 24 h with 100 μg/ml native or ox-LDL in fresh MEM supplemented with 2% FCS without enzyme.

After exposure to LDL, the cells were washed twice with HBSS and their viability was determined by a modification of the method of Mosmann [26]. The cells were incubated with 0.5 mg/ml methyl thiazol tetrazolium (MTT) for 4 h at 37 °C. The formazan crystals resulting from the MTT reduction were dissolved by adding 0.04 N HCl in isopropanol and agitating gently overnight. The absorbance of the supernatant was mea-

Table 1. Comparative antioxidant enzyme activities of glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase in human vein endothelial cells (HUVEC) and in EA.hy 926 endothelial cells.

Cell type	GPx (10^{-3} units/mg protein)	GR (10^{-3} units/mg protein)	SOD (10^{-3} units/mg protein)	Catalase (10^{-3} units/mg protein)
HUVEC (12)	148.4 ± 47.9**	74.5 ± 12.1	5.1 ± 2.1*	28.0 ± 7.3
EA.hy 926 (12)	11.95 ± 6.7	74.6 ± 6.7	2.74 ± 0.64	20.0 ± 3.5

The HUVEC and the EA.hy 926 endothelial cells were seeded at a density of 10^6/25 cm^2 culture flask and cultured until confluence. Cells were then trypsinized, and total protein, glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase activities were assayed in the supernatant of the sonicated cell pellet obtained by centrifugation as described in Materials and methods. Enzyme activities are given as 10^{-3} units/mg of protein. Data are means $\pm$ 1 SD.
*$p < 0.05$ and ** $p < 0.01$: significant and highly significant difference between EA.hy 926 cells and HUVEC.

sured spectrophotometrically in an ELISA reader at 570/630 nm (Diagnostic Pasteur LP400). The results are expressed as a percentage of the value for cells incubated in the medium without LDL.

Cell antioxidant systems

The cells were plated in T-25 culture flasks at an initial density of 10^6/flask. Once confluent, endothelial monolayers were rinsed twice with PBS.

Total glutathione. The endothelial cells were scraped in 0.5 ml 5% (w/v) sulfosalicylic acid. Samples were centrifuged at 9500 g for 10 min. The pellets and the supernatants were separated and kept at -80 °C until use. The total glutathione content in the acid-soluble supernatant was determined by a kinetic assay involving the continuous reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) by NADPH as described by Tietze [27]. The glutathione is expressed in nmol/mg of DNA. The DNA content of the cell pellet was determined by the fluorimetric assay of Fiszer-Szafarz [28].

Antioxidant enzyme activities. The endothelial cells were trypsinized, counted, centrifuged at 2000 g for 10 min and frozen at -80°C. The cell pellet was resuspended in 0.5 ml of triethanolamine buffer 0.1 M, pH 7.6, containing 1 mmol/l EDTA and 0.01 mol/l glutathione, sonicated on ice using a Vibra-cell sonicator (Bioblock 72405). The sonicated cells were centrifuged for 30 min at 125,770 g (Beckman TL-100 Ultracentrifuge) and enzymatic activities in the supernatant of sonication were determined using a Cobas Fara centrifuge analyser (Roche).

GPx activity was determined according to the method of Beutler using a twofold concentration of NADPH and glutathione [29]. Glutathione reductase (GR) was determined according to Calberg [30]. Catalase activity was determined by the Johansson method, based on its peroxidative action on methanol in the presence of hydrogen peroxide (H_2O_2) [31]. SOD activity was determined as the inhibition of formazan reduction by the superoxide anion ($O_2^{\cdot -}$), generated by the xanthine-xanthine oxidase system (Randox Laboratories Assay). The total protein concentrations in supernatants were determined by the method of Bradford [32]. All enzymatic activities are expressed in 10^{-3} units/mg of protein, as conventionally used [11, 12, 16]. We correlated the protein content in the two cell types to the cell number with $r = 0.693$ for HUVEC ($n = 12$) and $r = 0.769$ for the EA.hy 926 cells ($n = 25$).

Statistics

Data were analysed using a one-way or two-way analysis of variance (ANOVA). The protected least-significant-difference test was performed using Fisher's test to analyse differences. Statistical probability was considered significant with *$p < 0.05$ or **$p < 0.01$.

Results

Cytotoxic effect of oxidized LDL on EA.hy 926 endothelial cells and on HUVEC

EA.hy 926 cells and HUVEC were incubated with 50 to 200 μg/ml of native LDL and ox-LDL (fig. 1). Native LDL at 100, 150 and 200 μg/ml had a significant proliferative effect on both cell types ($p < 0.05$). The effects of ox-LDL on the two cell types differed. At 150 μg/ml of ox-LDL the cell viability expressed as 'MTT%' of EA.hy 926 endothelial cells was 30% (fig. 1a). In contrast, ox-LDL had no significant cytotoxic effect on HUVEC at any concentration tested (fig. 1b). The ox-LDL had been extensively dialysed after copper oxidation, but we nevertheless checked that 5 μmol/l copper did not have deleterious effects on endothelial cells.

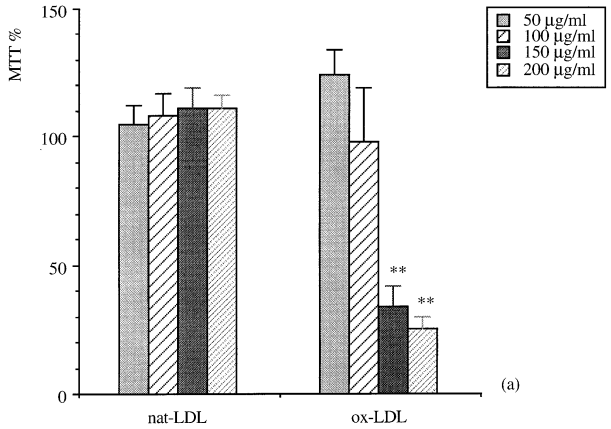
Glutathione levels, glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase activities

The activities of the intracellular antioxidant enzymes were assayed in EA.hy 926 cells and HUVEC homogenates ($n = 12$) (table 1). There was no significant difference in GR activity between the two cell types. The activities of catalase, SOD and in particular GPx were significantly lower in the EA.hy 926 cells than in HUVEC: the GPx activity of EA.hy 926 cells was 8% of that in HUVEC, SOD activity 54% and catalase 71%. The basal total glutathione level was significantly higher (2.8-fold) in EA.hy 926 cells (5.51 ± 0.36 nmol/mg DNA) than in HUVEC (0.89 ± 0.33 nmol/mg DNA).

The DNA content was significantly higher in EA.hy 926 cells compared to HUVEC ($p < 0.05$), respectively 16.0 ± 0.9 μg/10^6 cells and 12.4 ± 0.6 μg/10^6 cells.

The protective effect of cellular antioxidant enzymes

To determine whether antioxidant enzymes could protect cells from the cytotoxic effect of ox-LDL, EA.hy 926 endothelial cells were pre-incubated overnight with exogenous SOD, catalase and GPx. The culture medium was replaced and native or ox-LDL added. Ox-LDL-induced cytotoxicity was totally antagonized by pre-incubating the cells with GPx (100 IU/ml) whereas SOD and catalase (300 IU/ml or 600 IU/ml) had no effect. Figure 2 represents the results obtained by pre-incubating the cells with 100 IU/ml GPx, 600 IU/ml SOD or catalase. The total cellular GPx activity was 211.9 ± 9.9 10^{-3} units/mg protein (18-fold increased) after pre-incubation with GPx. Heat-inactivated GPx was ineffective in protecting the cells (data not shown), indicating that the specific enzymatic activity was required for the protective effect.



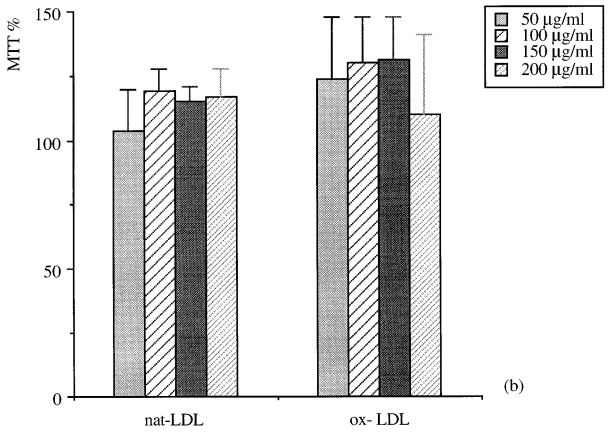


Figure 1. Dose-dependent sensitivity of human vein endothelial cells and EA.hy 926 cells to the cytotoxic effect of oxidized LDL. Confluent EA.hy 926 cells (*a*) and human umbilical vein endothelial cells (HUVEC) (*b*) in 96-well plates were incubated in MEM supplemented with 2% (v/v) FCS containing each of a series of concentrations of native LDL (nat-LDL) or oxidized LDL (ox-LDL). After 24 h incubation, cell viability was assessed by the MTT assay. The results are expressed as a percentage of absorbance for cells incubated in medium without LDL. Means ± SEM were calculated from three separate experiments, each in triplicate. The decrease was significant as compared to control cells with ** $p < 0.01$.

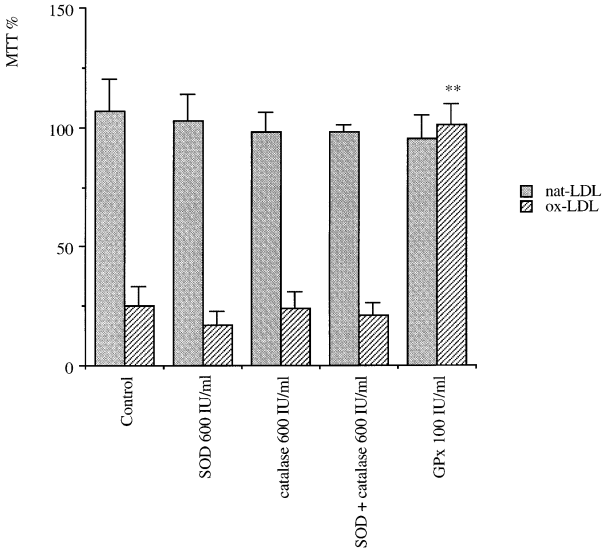


Figure 2. Effect of enzymatic antioxidants superoxide dismutase, catalase and glutathione peroxidase on the cell sensitivity to oxidized LDL. Confluent EA.hy 926 cells in 96-well plates were pre-incubated overnight in the presence of 600 IU/ml of superoxide dismutase, 600 IU/ml catalase, 100 IU/ml glutathione peroxidase, or no addition, and then exposed to 100 μg/ml native LDL (nat-LDL) or oxidized LDL (ox-LDL) in MEM supplemented with 2% (v/v) FCS. After 24 h incubation, the cell viability was assessed by the MTT assay. The results are expressed as a percentage of values for cells incubated in the medium without LDL. Means ± SEM were calculated from three separate experiments, each in triplicate. ** $p < 0.01$ compared to control cells without SOD, catalase and GPx incubated with ox-LDL.

Discussion

We show that EA.hy 926 cells are more sensitive than HUVEC to the cytotoxicity of ox-LDL: at the same concentration of ox-LDL (150 μg/ml), significant cytotoxicity was observed in EA.hy 926 cells but the HUVEC were unaffected. Ox-LDL cytotoxicity, which is dose-dependent, was not apparent on HUVEC up to 200 μg/ml.

Enzyme activities and glutathione levels depend on the growth state of the cells [16, 33] and were therefore determined at cell confluence. They also vary with the number of cell passages [16, 33] but no significant difference resulted from repeated passage of EA.hy 926 cells (data not shown).

The high sensitivity of EA.hy 926 cells was associated with significantly lower SOD, catalase and in particular

GPx activities. The differences in levels of endogenous antioxidant enzymes in the two cell types may account for the higher sensitivity of EA.hy 926 cells to radical effects induced by ox-LDL [11, 13]. GPx counteracts the unsaturated phospholipid peroxidation and eliminates H_2O_2. Indeed, it is the main antioxidant enzyme in endothelial cells [11]. The higher sensitivity to ox-LDL may therefore be attributed primarily to the lower GPx activity. In addition, we show that pre-incubation of EA.hy 926 endothelial cells with GPx for 16 h inhibited the ox-LDL cytotoxic effect concomitant with the enhancement of GPx activity.

The higher glutathione content in EA.hy 926 cells than in HUVEC may be due to the high expression of a rate limiting enzyme activity which acts on the de novo GSH synthesis pathway: γ-glutamyltranspeptidase. High expression of this enzyme has been described in the human lung carcinoma A 549 cells which were fused with HUVEC to form the EA.hy 926 cells [17, 34]. Reduced glutathione (GSH) is the substrate for GPx and is a direct scavenger of free radicals. Oxidized glutathione (GSSG) is regenerated by GR [12]. As GR activity is similar in the two cell types, the GPx activity may be the limiting factor in the glutathione redox cycle of EA.hy 926 cells.

We found that SOD and catalase were ineffective in protecting the cells against ox-LDL cytotoxicity. There are conflicting data concerning cellular uptake of SOD and catalase [35–37]. Nevertheless, our results are in agreement with recent work of Coffey and colleagues, showing that neither SOD conjugated with polyethylene glycol nor catalase protect fibroblasts against ox-LDL injury [38]. As SOD transforms $O_2^{\cdot -}$ into molecular oxygen and hydrogen peroxide, and catalase detoxifies hydrogen peroxide regardless of the cellular GSH [11], the free radical intermediates $O_2^{\cdot -}$ and hydrogen peroxide may not be primarily involved in the ox-LDL cytotoxic process.

Studies on ox-LDL are known to be hindered by difficulties due to the intensity of the peroxidizing stress, which is related to the oxidized lipid fraction [9, 21]. In addition, the chemical composition of ox-LDL is known to vary between the pools of LDL [39]. The use of a different pool of LDL to study the cytoprotective effects of the antioxidant enzymes on EA.hy 926 cells may explain why a stronger cell injury was observed in the experiments presented in figure 2 compared to those presented in figure 1. Although the ox-LDL preparations had reproducible LOOH levels in our study, different hydroperoxides are known to be distributed throughout the triacylglycerol, phospholipid and esterified cholesterol fractions [40].

The GPx-dependent cytoprotection is consistent with the ox-LDL-mediated cell killing involving free radical intermediates originating from the LOOH decomposition in the ox-LDL particle [6, 9]. There is no information concerning cellular GPx penetration in the literature. The GPx enzyme may penetrate cells or may be adsorbed in the outer leaflet of the plasma membrane to antagonize the lipoperoxidation induced by LOOH originating from ox-LDL or those formed from cellular lipids. How toxic LOOH may contact and enter cells is not yet elucidated. Endothelial cells normally express membrane scavenger receptors to take up ox-LDL but there are conflicting data concerning their expression on EA.hy 926 cells [18, 41, 42]. In addition, the receptor-mediated uptake of ox-LDL appears not to be required for cell injury [9]. Ox-LDL may be endocytosed or lipid exchanges between ox-LDL and the plasma membrane may occur [21]. Further studies are thus needed to understand the mechanisms of the ox-LDL-mediated cytotoxic effect.

In conclusion, EA.hy 926 cells had a higher sensitivity to ox-LDL–induced injury than HUVEC and this was associated with weaker enzymatic antioxidant defences. In particular, GPx activity was low, and it is known to detoxify LOOH and thereby prevent their decomposition into peroxyl radical intermediates. Easier to handle than HUVEC, the EA.hy 926 endothelial cells are a useful model of vascular endothelium. However, the differences in levels of endogenous antioxidant enzymes have to be considered when drawing conclusions, which may need to be confirmed using HUVEC.

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