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Incubation of Endothelial Cells in a Superoxide-Generating System: Impaired Low-Density Lipoprotein Receptor-Mediated Endocytosis

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Endothelial cells (EC) of blood vessels are submitted to oxidative stress under various circumstances. These conditions may modify EC functions; therefore, in the present work we have studied the receptor-mediated endocytosis of low-density lipoproteins (LDL) and malondialdehyde-modified LDL by the LDL receptor and the "scavenger" receptor, respectively, in cultured human umbilical vein EC after short (0–120 minutes) incubations in a superoxide anion (O_2^-) generating system. In both receptor-mediated processes, the oxidative stress produces a significant decrease at four different LDL concentrations (5–50 µg/ml) after 120 minutes of oxidation. On the other hand, the fluid-phase endocytosis of sucrose by EC seems to be stimulated by these conditions. Furthermore, incorporation of antioxidant enzymes in the O_2^- -producing system shows that H_2O_2 is an obligatory intermediate in order to produce the effect on the receptor-mediated processes. Hypotheses concerning the mechanisms involved in the modifications of endocytotic processes and their implications in vivo are discussed.

So far, many reports on the interactions between endothelial cells (EC) and low-density lipoproteins (LDL) have been published. *In vivo*, Vasile et al. (1983) have demonstrated a dual possibility for LDL processing in EC of rat aorta: a transcytosis mechanism using plasmalemmal vesicles permits the transport of LDL towards the subendothelial intima, whereas a receptor-dependent endocytotic mechanism is responsible for the uptake of LDL and for its degradation in lysosomes with the subsequent release of cholesterol for EC's membrane synthesis. *In vitro*, Van Hinsbergh and coworkers (1983) have shown that EC exhibit two processes towards LDL: a high-affinity receptor-dependent saturable one and a low-affinity nonsaturable process accounting for LDL concentrations above 50 µg of protein/ml. Furthermore, Smith and Staples (1980, 1982) have shown that LDL are selectively concentrated in the intima of normal arteries, probably via an active transcytosis process. However, under these conditions, a normal functioning of the receptor-dependent mechanism keeps the LDL level below pathological values, such as those found in type II familial hypercholesterolemia where this specific receptor is lacking (Goldstein and Brown, 1974).

On the other hand, the presence of a "scavenger receptor" has been demonstrated on bovine EC (Stein and Stein, 1980) and human umbilical vein EC (Van Hinsbergh et al., 1983). This receptor has been first shown on macrophages (Goldstein et al., 1979) and is able to recognize negatively charged proteins like acetylated LDL but also other modified LDL with increased electrophoretic mobilities: malondialdehyde-treated LDL (MDA-LDL) (Shechter et al., 1981) or LDL

modified by incubation with cultured endothelial cells (Henriksen et al., 1981). However, the mechanisms leading to local intimal cholesterol deposition in atherosclerosis are still not clear.

Due to their critical localization in the vascular wall, EC are highly susceptible to an exposure in an oxidant-rich environment. Indeed, superoxide (O_2^-) is generated as a product of the respiratory burst of neutrophils which have been activated under many physiopathological circumstances involving acute inflammatory responses. Following margination of these activated cells or incubation in an O_2^- -producing system, alteration of EC functions occurs: cell lysis has been observed in long-term incubations (Weiss et al., 1981; Sacks et al., 1978; Shingu et al., 1985), but shorter experiments have revealed the perturbation of cell functions: plasma membrane organization (Freeman et al., 1986) and alteration of the endothelial barrier function (Shasby et al., 1985).

In the present work we study the effects of relatively short incubation times (0–120 minutes) in an O_2^- -generating system on the receptor-mediated endocytotic processes of LDL and MDA-LDL in cultured human umbilical vein EC together with the effects on the pinocytotic uptake of sucrose by these cells.

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MATERIALS AND METHODS

Culture and identification of endothelial cells

Human umbilical vein EC were isolated as described by Jaffe et al. (1973) and cultured on gelatin-coated 60-mm petri dishes (Nunc Products, Gibco Europe, Gent, Belgium) in medium 199 (Flow Laboratories, Brussels) supplemented with 20% newborn calf serum. Cells were grown at 37°C in a humidified atmosphere (5% CO₂). After the first passage, EC from several umbilical cords were pooled and transferred in a 1:2 split ratio in Multidish (Nunc, 24 wells) and used at confluence. Cells exhibited a typical cobblestone pattern as has been observed by phase-contrast microscopy. The presence of Von Willebrand factor-associated antigen was demonstrated by immunofluorescence microscopy. Typical Weibel-Palade bodies were regularly observed by electron microscopy (Weibel and Palade, 1964).

LDL isolation, modification and labeling

Lipoprotein-deficient serum (LPDS) and LDL were prepared from pooled serum using a modification of a vertical rotor gradient ultracentrifugation technique (Poumay and Ronveaux-Dupal, 1985). Modification of native LDL by malondialdehyde was performed according to Haberland et al. (1982). The modification of LDL with malondialdehyde gives a lipoprotein which exhibits a mean relative electrophoretic mobility of 2.06 in agarose gel at pH 8.6 compared with the native LDL from which it is derived.

LDL and MDA-LDL were radiolabeled with Na¹²⁵I (Amersham) by the ICI method of Mac Farlane (1958) as has been described for lipoprotein labeling (Bilheimer et al., 1970). Their specific activities were, respectively, about 150 and 15 cpm/ng. For the measurement of LDL endocytosis, EC were first incubated for 18–24 hours in the medium supplemented with 10% LPDS for maximal expression of LDL receptors.

Oxidative incubations

Endothelial cells were incubated in the oxidizing medium containing xanthine (500 μM). The enzyme xanthine oxidase (Boehringer Mannheim) (10 mU/ml) was added in order to initiate the production of O₂⁻ for periods varying from 0 to 120 minutes at room temperature. This was followed by one rapid wash in normal medium.

Determination of endocytotic processes

These processes were tested during a 150-minute incubation of EC at 37°C in the presence of ¹²⁵I-LDL, ¹²⁵I-MDA-LDL, or ¹⁴C-sucrose.

LDL endocytosis. The washing medium was replaced by the same medium containing ¹²⁵I-LDL (10 μg/ml or otherwise stated). High-affinity binding and internalization were determined after a 2.5-hour incubation at 37°C according to Goldstein and coworkers (1983). In brief, at the end of the incubation, the medium was immediately removed and replaced by ice-cold 50 mM Tris-HCl buffer containing NaCl (150 mM) and 2 mg/ml of bovine serum albumin (BSA). Each EC monolayer was rapidly washed three times

and incubated twice for 10 minutes with this buffer. These incubations were followed by two rapid washes in a similar buffer where BSA was omitted. Each dish then received 500 μl of 10 mM HEPES buffer containing NaCl (50 mM) and 10 mg/ml of heparin (grade I; purchased from Sigma), then the Multidish was placed on a shaker for 60 minutes at 4°C. This buffer was then collected and an aliquot was counted for determination of surface-associated ¹²⁵I-LDL. On the other hand, EC were dissolved in 0.5 N NaOH. One aliquot of the cell suspension was counted to determine the amount of internalized ¹²⁵I-LDL while the cellular protein content was determined by the method of Lowry et al. (1951). Lipoprotein degradation was determined in the incubation medium as 10% trichloroacetic acid-soluble products. Low-affinity binding and internalization were determined in the presence of an excess of unlabeled LDL (500 μg/ml).

MDA-LDL endocytosis. As above, the washing medium was replaced by the same medium containing ¹²⁵I-MDA-LDL (10 μg/ml) and total cell capture was determined after a 2.5-hour incubation at 37°C. At the end of the incubation, EC were washed as described above for LDL endocytosis measurements. EC were then dissolved in 0.5 N NaOH, and one aliquot of the cell suspension was counted to determine the amount of captured ¹²⁵I-MDA-LDL while the cellular protein content was determined as above. Low-affinity capture was determined in the presence of an excess of unlabeled MDA-LDL (500 μg/ml).

Sucrose pinocytosis. Following the oxidative incubation of EC, a complete culture medium containing 1 μCi of ¹⁴C-sucrose/ml was placed in the petri dishes for a further 2.5-hour incubation at 37°C. The cells were subsequently washed seven times with PBS and then collected in 0.25 M sucrose for homogenization with a Dounce homogenizer. The radioactivity was counted in a 500-μl aliquot in 4 ml of Aqualuma in a liquid scintillation counter.

Antioxidant enzymes

Antioxidant enzymes (catalase [CAT], 3,250 U/ml; and superoxide dismutase [SOD], 600 U/ml, purchased from Boehringer Mannheim) were added to the oxidizing media before incubation and before the addition of xanthine oxidase.

Enzymes measurements

Lactate dehydrogenase. EC were first incubated in the oxidative medium for 0, 60, or 120 minutes, after which this medium was replaced by a normal culture medium similar to the medium used for the 2.5-hour incubation of EC and incubated during the same time at 37°C. At the end of this incubation, cell lysis was checked by the measurement of the release of lactate dehydrogenase (LDH) activity in the culture medium compared with the total cellular LDH activity. The LDH activity was determined by a spectroscopic method.

Adenosine 5'-monophosphatase. Following incubation (60 minutes) of EC in the oxidizing medium, their adenosine 5'-monophosphatase (5'-AMPase) activity was measured as described by Beaufay et al. (1974) and compared with the activity of control EC.

TABLE 1. Endocytosis (binding + internalization) of ^{125}I -LDL and ^{125}I -MDA-LDL by two different receptors¹

Incubation medium ($\mu\text{g/ml}$)	High-affinity endocytosis (ng/mg protein)
^{125}I -LDL (10)	$1,100 \pm 27$
^{125}I -LDL (10) unlabeled LDL (500)	72 ± 15
^{125}I -MDA-LDL (10)	$2,472 \pm 351$
^{125}I -MDA-LDL (10) unlabeled MDA-LDL (500)	518 ± 111
^{125}I -MDA-LDL (10) unlabeled native LDL (500)	$2,717 \pm 158$

¹EC were rinsed and incubated in the presence of 10 $\mu\text{g/ml}$ of ^{125}I -LDL or ^{125}I -MDA-LDL with or without an excess of an unlabeled lipoprotein (native LDL or MDA-LDL) at a concentration of 500 $\mu\text{g/ml}$. High-affinity endocytosis of ^{125}I -MDA-LDL was determined after a 2.5-hour incubation at 37°C. Data are presented as means of triplicates \pm SE of representative experiments.

Statistical methods

Differences between groups of cells were compared using the unpaired Student's t-test. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of the incubation in the superoxide-generating medium on endothelial cells lysis

EC were first incubated for 0, 60, or 120 minutes in the oxidative medium. After these preincubations, EC were incubated in a normal medium and the percent of LDH activity released was determined after 2.5, 4, and 24 hours of incubation at 37°C. At the end of the 2.5 hours of incubation, only $2.0\% \pm 1.5\%$, $3.8\% \pm 2.3\%$, and $8.3\% \pm 2.5\%$ ($n=8$) of the cells were lysed in EC cultures, respectively, preincubated for 0, 60, and 120 minutes in the xanthine-xanthine oxidase-containing medium. However, after 4 hours, the percentage of EC lysis increased to $3.8\% \pm 1.0\%$, $9.5\% \pm 1.3\%$, and $14.4\% \pm 3.4\%$ ($n=6$), and a 24-hour incubation resulted in $7.9\% \pm 3.3\%$ ($n=3$), $11.5\% \pm 1.8\%$ ($n=5$), and $17.3\% \pm 3.7\%$ ($n=6$) of EC lysis. At the same time, the cell density was significantly reduced by the oxidative preincubations (not shown).

Effect of the incubation in the superoxide-generating medium on the endocytosis of LDL and malondialdehyde-modified LDL by endothelial cells

The specific affinity of ^{125}I -LDL for the LDL receptor and the affinity of ^{125}I -MDA-LDL for a receptor different from the LDL receptor is shown in Table 1. An excess of native LDL (500 $\mu\text{g/ml}$) competes with the labeled LDL (10 $\mu\text{g/ml}$) for the LDL receptor present in EC plasma membrane, but the same excess of native LDL is unable to compete with the labeled MDA-LDL (10 $\mu\text{g/ml}$) for the "scavenger" receptor, whereas an excess of unlabeled MDA-LDL (500 $\mu\text{g/ml}$) diminishes this process by 80%.

Inhibition of receptor-dependent processes. In the first incubation medium, the oxidation of xanthine by xanthine oxidase produces superoxide anion (O_2^-). Experiments were necessary to exclude an eventual direct effect of xanthine alone, or of the commercial preparation of xanthine oxidase on the studied process.

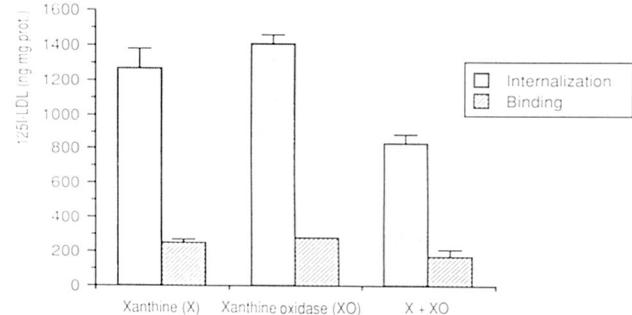


Fig. 1. High-affinity binding and internalization of ^{125}I -LDL in EC after incubation (60 minutes) in the superoxide-generating system (xanthine, 500 μM ; xanthine oxidase, 10 mU/ml) or in presence of xanthine (500 μM) alone or of xanthine oxidase (10 mU/ml) alone. High-affinity binding and internalization of ^{125}I -LDL (10 $\mu\text{g/ml}$) in cells were determined after a 2.5-hour incubation at 37°C. Data are presented as means of triplicates \pm SE.

The results presented in Figure 1 demonstrate that the production of O_2^- in the first incubation medium is required for the modifications observed during the incubation with labeled LDL. In a second group of experiments, we observed the binding and internalization of ^{125}I -LDL by EC and then we measured the endocytosis of ^{125}I -MDA-LDL under the same conditions. The time course of the effects of O_2^- on the processing of both ligands in cultured human umbilical vein EC is shown in Figure 2. Internalization of ^{125}I -LDL is progressively reduced after an increased exposure to the oxidative medium, and the binding process is also markedly modified. Following 120 minutes of oxidative preincubation, there is a highly significant difference ($P < 0.001$) in the binding and internalization. In the same way, the endocytosis of ^{125}I -MDA-LDL is diminished after this treatment. After 120 minutes of oxidative preincubation, the relative inhibition values obtained in these experiments are 73% for binding of LDL, 86% for internalization of LDL, and 72% for MDA-LDL endocytosis, compared with control. In all the experiments we performed, the relative inhibition value varied from about 70% to 85%. The inhibitory potential of O_2^- produced during 120 minutes of incubation of EC has been also tested on the receptor-dependent binding (Fig. 3A) and internalization (Fig. 3B) processes at four ligand concentrations (5, 10, 20, and 50 $\mu\text{g/ml}$ of ^{125}I -LDL). The 80% inhibition observed under these conditions for the internalization process was confirmed. Moreover, a significant effect has been detected for the binding process. In all these experiments, no significant change has been observed in the low affinity processes (not shown).

Furthermore, the inhibitory potential of O_2^- produced during 60 and 120 minutes of incubation of EC has been also tested for the receptor-dependent degradation of ^{125}I -LDL which is also shown to diminish under these conditions. Figure 4 shows the relative values obtained after these oxidative conditions for the binding, the internalization, and the degradation of ^{125}I -LDL in a representative experiment. Under these conditions, three processes were affected, especially the degradation, since it was undetectable after a 120-minute preincubation of cells in the oxidative medium.

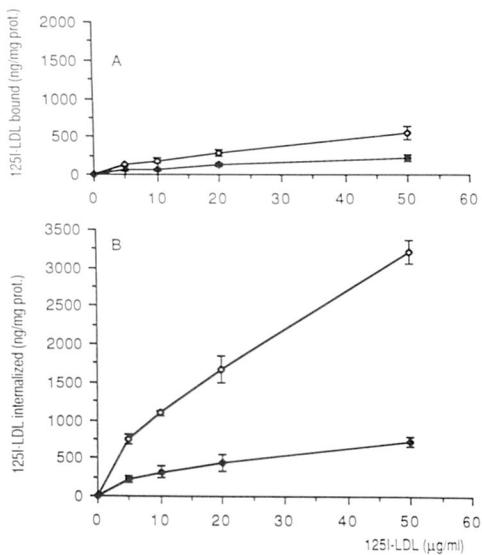


Fig. 2. **A:** Effect of the oxidation time on the high-affinity binding (■—■) and internalization (□—□) of ^{125}I -LDL in EC. **B:** Effect of the oxidation time on the high-affinity endocytosis of ^{125}I -MDA-LDL in EC. Cells were first incubated in the superoxide-generating system (xanthine, 500 μM ; xanthine oxidase, 10 mU/ml). Xanthine oxidase was added to initiate the reaction during the indicated times. Next, high-affinity binding and internalization of ^{125}I -LDL (10 $\mu\text{g}/\text{ml}$) or endocytosis of ^{125}I -MDA-LDL (10 $\mu\text{g}/\text{ml}$) in cells were determined after a 2.5-hour incubation at 37°C. Data are presented as means of triplicates \pm SE obtained in typical experiments.

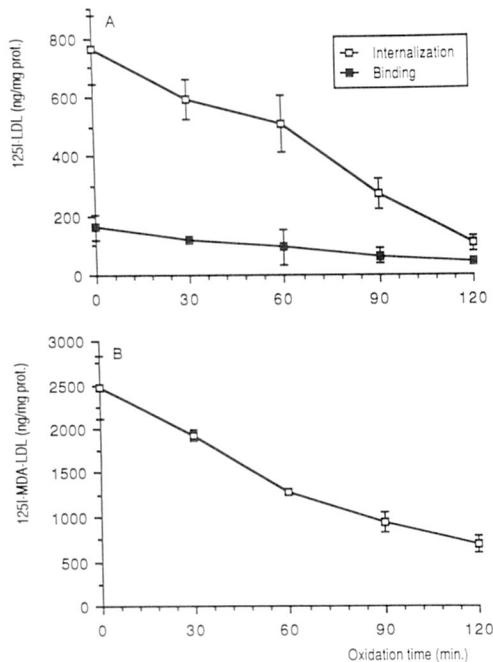


Fig. 3. Typical experiment showing the high-affinity binding (A) and internalization (B) of ^{125}I -LDL as a function of the concentration of ligand in control (□—□) and treated (■—■) cells. EC were first incubated for 120 minutes in the superoxide-generating system (xanthine, 500 μM ; XO, 10 mU/ml). Xanthine oxidase was added to the tests in order to initiate the production of the superoxide anion. High-affinity binding and internalization of ^{125}I -LDL were then determined after a 2.5-hour incubation at 37°C. Data are presented as means of triplicates \pm SE.

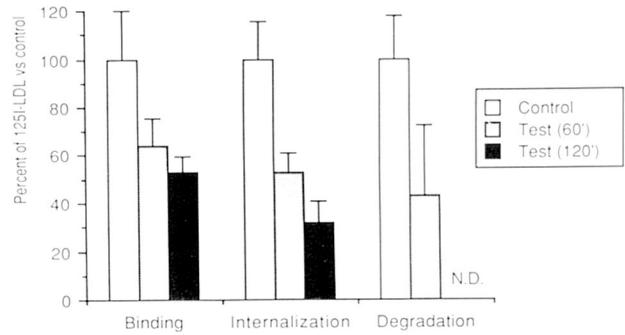


Fig. 4. Effect of 60- and 120-minute oxidative incubations of EC on the receptor-dependent binding, internalization, and degradation of ^{125}I -LDL. Cells were first incubated in the superoxide-generating system (xanthine, 500 μM ; xanthine oxidase, 10 mU/ml). Xanthine oxidase was added to initiate the reaction during the indicated times. Data are presented as means of triplicates \pm SE of the relative values determined in comparison with the values obtained in control EC for the three processes in the same typical experiment. N.D. = not detected.

Measurements of 5'-AMPase activity determined in EC oxidized for 60 minutes revealed that this activity was decreased ($87.4\% \pm 2.8\%$; $n=4$) compared with that of the control (100%).

Are oxygen-derived radicals involved in the inhibition of LDL receptor-dependent internalization? In order to investigate whether the effect observed on the receptor-dependent processes of ^{125}I -LDL was actually the consequence of the O_2^- presence in the preincubation medium, SOD was incorporated into this medium. SOD catalyzes the dismutation of O_2^- into hydrogen peroxide (H_2O_2). In the presence of SOD, the receptor-dependent binding and internalization are not protected against the previously observed inhibitor (Table 2). This allows one to assume an indirect action of the O_2^- production on these mechanisms. On the other hand, Table 2 also shows that if CAT is incorporated into the preincubation medium producing O_2^- , the receptor-dependent processes don't show any inhibitory change under these conditions, revealing that in order to produce this inhibition H_2O_2 is an obligatory intermediate. This protective action of SOD together with the unprotective action of CAT were also proved by studying the endocytosis of ^{125}I -MDA-LDL after preincubation of EC in these media (Table 3).

Effect of the incubation in the superoxide-generating medium on the pinocytotic uptake of sucrose by endothelial cells

In the previous experiments, it was shown that receptor-dependent processes could be altered by oxidative preincubation. Hence, the pinocytosis of ^{14}C -sucrose, a receptor-independent endocytic process, was of interest in EC receiving the same treatment. Results presented in Figure 5 show the stimulation of sucrose uptake during the incubation (2.5 hours) which follows oxidative preincubations (from 0 to 120 minutes). This stimulation seems to be progressive with the duration of the oxidative preincubation for the first 90 minutes, but seems to accelerate during the follow-

TABLE 3. Protective effect of antioxidant enzymes on the receptor-mediated endocytosis of ^{125}I -MDA-LDL¹

	ng of MDA-LDL protein mg of cell protein	Percent of control	P values (vs. control)
Control	1,722 ± 200	(100)	
Xanthine oxidase	482 ± 59	28.0	<0.001
Xanthine oxidase + SOD	448 ± 40	26.0	<0.001
Xanthine oxidase + CAT	1,601 ± 259	93.0	NS

¹Endothelial cells were first incubated for 120 minutes in the presence or absence of xanthine oxidase (10 mU/ml) in a medium containing xanthine (500 μM) and superoxide dismutase (SOD, 600 U/ml) or catalase (CAT, 3,250 U/ml), where indicated. The cells were then rinsed and incubated in the presence of 10 μg/ml of ^{125}I -MDA-LDL. High-affinity endocytosis of ^{125}I -MDA-LDL was determined after a 2.5-hour incubation at 37°C. Data are presented as means of triplicates ± SE.

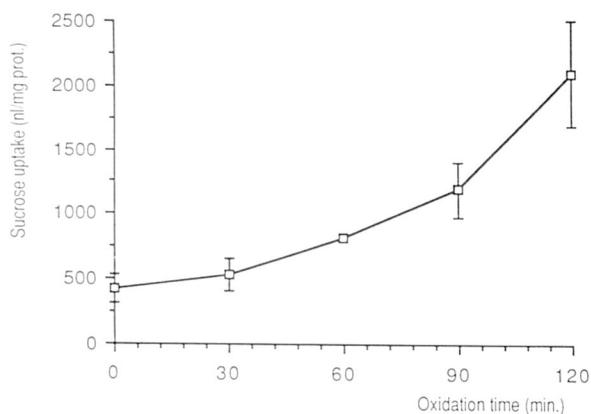


Fig. 5. Effect of the oxidation time on the ^{14}C -sucrose uptake in EC. Cells were first incubated in the superoxide-generating system (xanthine, 500 μM; xanthine oxidase, 10 mU/ml). Xanthine oxidase was added to initiate the reaction during the indicated times. Next, the radioactivity incorporated in EC during a further 2.5-hour incubation at 37°C was measured in order to determine the corresponding volume of medium pinocytosed. Data are presented as means of three experiments ± SE.

ing 30 minutes, as revealed by high uptake measured after 120 minutes of preincubation.

DISCUSSION

Cell lysis and detachment are well documented to be the consequence of incubations of EC in the presence of O_2^- (Weiss et al., 1981; Harlan et al., 1981; Niwa et al., 1982; Shingu et al., 1985). However, these studies showed that a constant production of oxygen-derived radicals for several hours is required to obtain the above effects.

In contrast with these results, shorter incubations in a O_2^- -generating system showed modifications in EC functions without important cell lysis. Studies by Shasby et al. (1985) on the transfer of serum albumin across cultured EC monolayers revealed a reversible oxidant-induced increase of this process. Recently, Freeman et al. (1986) have shown, using spin-labeling of membrane proteins, that the organization of EC membranes was perturbed by O_2^- . As far as we know, the present paper is the first report of an impairment of the receptor-dependent endocytosis of LDL and MDA-LDL in EC following a short incubation with O_2^- , showing that this very important cellular mechanism is sensitive to oxidative conditions *in vitro*. The binding process is affected in a small way after 60 minutes of oxidation, but this process is highly significantly decreased after 120 minutes of oxidation. This could reflect a decrease in the receptor number or accessibil-

ity produced by the oxidative molecular species. Oxidative stress may perturb membranes and proteins (Freeman et al., 1986; Wolff et al., 1986), modulating the recognition of the apolipoprotein B by the LDL receptor. The internalization process is more significantly reduced after 60 minutes than the binding one and is highly significantly decreased after 120 minutes. As a consequence of these inhibited processes, the degradation is reduced by a higher percentage compared with the controls and is not even detectable after 120 minutes of oxidation of EC.

It is relevant to note that some variations were observed in the absolute values obtained in different experiments; however, the relative values of the inhibition were maintained between 40% and 50% after 60 minutes and between 70% and 85% after 120 minutes of oxidation as a result of the good reproducibility obtained within each experiment. Such a considerable variation among experiments on the processing of LDL in human endothelial cells has been previously reported by others (Coetze et al., 1979; Van Hinsbergh et al., 1983) and is probably the consequence of the different origins of EC.

The stimulation of sucrose pinocytotic uptake could be due to an increased invagination of plasma membrane with the subsequent formation of more pinocytotic vesicles in treated EC. However, it has been shown recently that an increased entry of sucrose in isolated hepatocytes could result from the formation of hyperpermeable surface blebs in these cells, leading to a diffusional uptake of the molecule in addition to the pinocytotic uptake (Gordon et al., 1987). The explanation of our results would have to consider this possibility. Note that in contrast with the LDL endocytosis, the reproducibility for the sucrose pinocytosis was very high within different experiments.

But how could the O_2^- -producing system induce the observed changes in the cellular endocytic systems? First, we supposed that membrane lipids oxidation could perturb the organization of EC membranes. Indeed, Freeman et al. (1986) demonstrated an increased mobility of the spin-labeled membrane proteins under these conditions. However this effect is opposed to other reported experiments in which lipid peroxidation of other membranes has been shown to decrease their fluidity (Dobretsov et al., 1977; Ohki et al., 1984). As the cellular metabolism of LDL involves membranes in most of its steps, these changes in EC membrane organization could be related to the effects reported here: not only is the receptor-dependent internalization of LDL decreased, but so is the 5'-AMPase activity.

TABLE 2. Protective effect of antioxidant enzymes on the receptor-mediated processing of ^{125}I -LDL.¹

	High-affinity binding of ^{125}I -LDL				High-affinity internalization of ^{125}I -LDL			
	60 minutes of oxidation		120 minutes of oxidation		60 minutes of oxidation		120 minutes of oxidation	
	ng of LDL protein	mg of cell protein	ng of LDL protein	mg of cell protein	ng of LDL protein	mg of cell protein	Percent of control	P values (vs. control)
Control	164 \pm 42	(100)	138 \pm 15	(100)	762 \pm 117	(100)	684 \pm 71	(100)
Xanthine oxidase	94 \pm 61	57.6	<0.050	49 \pm 16	35.7	<0.001	508 \pm 98	66.7
Xanthine oxidase + SOD	101 \pm 50	61.9	<0.050	41 \pm 6	29.7	<0.001	534 \pm 96	70.1
Xanthine oxidase + CAT	197 \pm 38	120.5	NS	159 \pm 20	115.6	NS	851 \pm 126	111.7
Xanthine oxidase + SOD + CAT	192 \pm 22	117.5	NS	138 \pm 31	100.6	NS	838 \pm 126	109.9

¹Endothelial cells were first incubated for 60 or 120 minutes in the presence or absence of xanthine oxidase (10 mU/ml) in a medium containing xanthine (500 μM) and superoxide dismutase (SOD, 600 U/ml) and/or catalase (CAT, 3250 U/ml), where indicated. The cells were then rinsed and incubated in the presence of 10 $\mu\text{g}/\text{ml}$ of ^{125}I -LDL. High-affinity binding and internalization of ^{125}I -LDL was determined after a 2.5-hour incubation at 37°C. Data are presented as means of triplicates \pm SE.

This enzyme is usually associated with the plasma membrane, and its activity is linked to the membrane's physical properties (Dipple and Houslay, 1978). However, in contrast with our observations, Freeman et al. (1986) demonstrated membrane fluidity changes under such conditions, but reported that SOD was able to slightly protect EC against membrane fluidity changes, whereas CAT had absolutely no effect. This discrepancy with the protection obtained in our system with CAT alone strongly suggests that another mechanism could be involved in the modifications of the receptor-dependent endocytosis reported here. However, EC membrane fluidity is currently under investigation using fluorescence polarization techniques.

Second, we wondered whether the appearance of oxidized derivatives of cholesterol could be responsible for these modifications. Indeed, it is well known, for more than a decade, that these derivatives, for example 25- and 26-hydroxycholesterol, are inhibitors of LDL receptor synthesis and are responsible for a reduced binding of LDL in cultured fibroblasts (Brown and Goldstein, 1975). However, this inhibitory effect is only revealed after longer incubations of cells with these compounds and there is no evidence that they could modify the processing of the scavenger receptor in the same way as that of the LDL receptor.

Third, it has been demonstrated that EC calcium homeostasis is impaired following incubation of these cells with O_2^- (Shasby et al., 1985); a similar effect has been detected after the incubation of P388D₁ cells with H_2O_2 (Hyslop et al., 1986) as well as in hepatocytes subjected to an oxidative stress (Di Monte et al., 1984). It was also shown that oxidized lipids, such as oxidized fatty acids or cholesterol, could increase the permeability of membranes to Ca^{2+} (Serhan et al., 1981; Holmes et al., 1983). The implication of Ca^{2+} in the development of atherosclerosis is crucial for some authors (Holmes and Kummerow, 1985), and the possible modification of Ca^{2+} homeostasis by oxidative conditions could be of great interest and in accordance with our results. It is known that an entry of Ca^{2+} , mediated by an ionophore, is able to impede the LDL processing in cultured fibroblasts (Mazière et al., 1984), and furthermore that such an increase in intracellular Ca^{2+} is also able to stimulate fluid-phase endocytosis (Goldstone et al., 1983). However, the apparent stimulation of fluid-phase endocytosis as determined by sucrose uptake could be, at least in part, the result of an increase in plasma membrane permeability, giving hyperpermeable membranes as in the results of Gordon et al. (1987) and in this way, allowing transmembrane diffusion of small molecules, such as sucrose, to take place. In other words, extracellular sucrose might be capable of entering EC through a diffusional route as well as by pinocytosis. This hypothesis could be checked by using another molecular probe of pinocytotic uptake which enters the cells by pinocytosis only, for example polyvinylpyrrolidone (Gordon et al., 1987).

Lastly, it is difficult to ascertain that the loss in EC plasma membrane integrity, as assessed by the measurement of LDH release in the medium, is produced as a secondary event by modified cellular functions (which act first on the endocytotic processes) or to ascertain that the loss of viability itself produces the modifications observed in endocytosis. The former hy-

pothesis, however, seems to conform more to our results since the processes affected after 120 minutes of oxidation are modified to an extent of about 80%, whereas EC viability was apparently reduced by a maximum of 20%, even 24 hours after the oxidative stress.

In conclusion, all the results presented here indicate that endocytotic processes are affected rapidly in cultured endothelial cells incubated with O_2^- , but it remains to be determined if this is the result of a direct action of oxygen-derived free radicals or if it is the consequence of an important modification in the cell physiology produced by these radicals.

In the present work, as well as in other publications where animal cells have been exposed to O_2^- -generating system, the agent responsible for cellular effects has been identified as H_2O_2 on the basis that addition of SOD didn't protect the cells whereas CAT did (Weiss et al., 1981; Harlan et al., 1984). It is suggested from the above that if an intracellular action is required, H_2O_2 crosses membranes, although O_2^- doesn't. Halliwell and Gutteridge (1986) proposed that in order to cross biological membranes, O_2^- requires anion channels such as those found in erythrocyte membranes only. On the other hand, if an intracellular action is required, H_2O_2 could be cytotoxic because it is able to enter the cells easily. In addition, H_2O_2 is probably not cytotoxic by itself but through its metal-dependent reduction produces the extremely reactive hydroxyl radical (OH^-), a radical considered to be the major cytotoxic agent responsible for membrane lipid peroxidation (Tien et al., 1982).

These results would imply that, *in vivo*, similar modifications could occur locally when EC are subjected to an oxidant-rich environment during margination, for example, of activated leukocytes; this margination is thought to be a possible initial step in the development of cholesterol deposition and atherogenesis (Rogers et al., 1986). Smith and Staples (1980, 1982) have shown that LDL are present in the intima of healthy aortas at concentrations even twice higher than those found in the serum, and Vasile et al. (1983) proved that a transcytosis mechanism is involved in the transport of LDL through EC in the direction of the intima; however, the understanding of the mechanism responsible for the localized abnormal accumulation of cholesterol in atherogenesis is still not known. Territo et al. (1984) have found that monocyte migration across the endothelium can accelerate the transport of LDL into the subendothelial space. All the same, our data would suggest that the decreased receptor-dependent internalization process in EC could lead to a local further increase in the intimal concentration of LDL. Indeed, the normal fate of LDL present in the subendothelial space could be regulated by the abluminal surface of the EC, and by the binding on receptors present on this surface and consequently internalized and degraded by these cells. On the other hand, the results obtained with the measurements of radiolabeled-sucrose following the oxidative preincubation would suggest that an increased transcytotic mechanism resulting from a stimulated fluid-phase pinocytosis in these cells could lead to an increased LDL concentration in the subendothelial space (Holmes and Kummerow, 1985).

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