Copper and cell-oxidized low-density lipoprotein induces activator protein 1 in fibroblasts, endothelial and smooth muscle cells

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Abstract The effect of cupric ion- or endothelial cell-oxidized low-density lipoproteins (LDL) on transcription factor AP1 activation was investigated by electrophoretic mobility shift assay. Both oxidized LDL induced AP1 activation in fibroblasts, endothelial and smooth muscle cells. This phenomenon was also observed in the presence of cycloheximide. α-Tocopherol, a lipophilic free radical scavenger, and *N*-acetylcysteine, an hydrophilic antioxidant, partially inhibited the stimulatory effect of Cu^{2+}-oxidized LDL. LDL modified by the mixture of the oxygen radicals OH· and $O_2{\cdot}^-$, which generated lipid peroxidation products, also initiated AP1 activation, whereas LDL modified by OH· alone, which did not lead to marked LDL lipid peroxidation, was ineffective. Thus, lipid peroxidation products seem at least partially involved in the activation mechanism. Since AP1 activity is essential for the regulation of genes involved in cell growth and differentiation, our study suggests that the oxidative stress induced by oxidized LDL might be related to the fibroproliferative response observed in the atherosclerotic plaque.

Key words: Oxidized LDL; Oxidative stress; AP1; Fibroblasts; Endothelial cells; Smooth muscle cells; Atherosclerosis

1. Introduction

It is currently believed that oxidized low-density lipoproteins (LDL) play an important role in the generation and progression of the atherosclerotic plaque (review in [1]). Oxidative modification of LDL can be achieved by incubation with cultured cells such as monocytes [2], endothelial [3] or smooth muscle cells [4]. In vitro, transition metals such as copper can also initiate LDL oxidation [5], leading to a cytotoxic form of the particle. The oxidative modification of LDL is accompanied by the appearance of conjugated dienes and lipid peroxidation end-products [6]. A modification of the electrophoretic mobility of the particle was also observed [7].

AP1 (activator protein 1) is a transcriptional activator composed of members of the Jun and Fos families (review in [8]) that mediates the effect of the phorbol ester tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate [9]. AP1 activation can be induced by growth factors, cytokines and neurotransmitters [8]. Furthermore, AP1 binding activity is controlled by the redox state of the cell in that a decrease of the intracellular glutathione level stimulates AP1 activity [10]. It has been also shown that the oxidative stress induced by ultraviolet radiation activates AP1 [11,12]. More recently, Pinkus et al. [13] reported stimulation of AP1 activity by oxygen radicals.

This study was undertaken to investigate whether the oxidative stress induced by copper- or cell-oxidized LDL can activate the transcription factor AP1. It was found that both oxidized LDL stimulated AP1 binding activity in fibroblasts, smooth muscle and endothelial cells. In addition, α-tocopherol significantly reduces the oxidized LDL-induced activation of AP1.

2. Materials and methods

2.1. Materials

Culture medium and foetal calf serum were from Gibco (Grand Island, NY, USA). The double-stranded oligonucleotide containing the AP1 consensus sequence (5′-AGC–TAG–GTG–ACT–CAC–CAA–GCT–TCG–A-3′) was synthesized by Eurogentec (Belgium). [γ-^{32}P]ATP 3000 Ci/mmol was purchased from Amersham. T4 polynucleotide kinase was obtained from Boehringer Mannheim (Meylan, France). *dl*-α-Tocopherol and all other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Cell culture

A7r5 rat smooth muscle cells from The American Type Culture Collection, and MRC5 human fibroblasts from BioMérieux (France) were maintained in Dulbecco's MEM supplemented with 10% foetal calf serum. The murine endothelial cell line UNA was a gift from Pr. J.D. Chapman (The Fox Chase Cancer Center, Philadelphia, PA, USA). This cell line was characterized by the presence of von Willebrand's factor and was maintained in Ham's F10 medium supplemented with 10% foetal calf serum.

2.3. LDL preparation and oxidation

LDL (d 1.024–1.050) was prepared from normal human serum by sequential ultracentrifugation according to Havel et al. [14], and dialysed against 0.005 M Tris, 0.05 M NaCl, 0.02% EDTA, pH 7.4, for conservation. Prior to oxidation, EDTA was removed by dialysis. Oxidation was performed by incubation at 37°C of 1 mg LDL protein/ml with 5.10^{-6} M $CuSO_4$ or of 0.1 mg LDL protein/ml with endothelial UNA cells for 24 h in Ham's F10 medium. Control LDL was incubated at 37°C for 24 h. In some experiments, LDL was modified by the hydroxyl radical (OH·) or by a mixture of OH· and superoxide anion $O_2{\cdot}^-$. The radicals were generated by gamma radiolysis of water using a ^{60}Co irradiator at a radiation dose of 1800 Gy [15]. The degree of LDL oxidation was checked by determination of thiobarbituric-acid-reactive substances (TBARS) according to Yagi [16], by measuring conjugated dienes by absorption at 234 nm, and by determination of the electrophoretic mobility of the LDL particle. Lipid extraction of Cu^{2+}-oxidized LDL was performed according to Bligh and Dyer [17].

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Abbreviations: c-LDL, control low-density lipoprotein; Cu-LDL, Cu^{2+}-oxidized LDL; E-LDL, endothelial cell-oxidized LDL; TBARS, thiobarbituric-acid-reactive substances (lipid peroxidation end-products)

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Table 1
Characterization of control LDL (c-LDL) and copper (Cu-LDL) or endothelial cell-oxidized LDL (E-LDL)

	TBARS (nmol MDA Eq/mg LDL protein)	Conjugated dienes	REM
c-LDL	8 ± 2	144% ± 15	1.0
E-LDL	44 ± 5	323% ± 35	1.6 ± 0.1
Cu-LDL	62 ± 7	455% ± 47	1.9 ± 0.2
OH·-LDL	15 ± 2	194% ± 18	1.2 ± 0.1
$OH\cdot+O_2\cdot^-$-LDL	54 ± 5	396% ± 41	1.7 ± 0.2

After EDTA removal by dialysis, LDL modification was performed by a 24 h incubation with $CuSO_4$ $5\cdot10^{-6}$ M (1 mg LDL protein/ml) or endothelial cells (0.1 mg LDL protein/ml) in Ham's F10 medium at 37°C. Control LDL was incubated in the same medium for 24 h at 37°C. LDL modification by OH· or ($OH\cdot+O_2\cdot^-$) was performed as described [15]. Lipid peroxidation end-products (TBARS) were determined by the technique of Yagi [17], and conjugated dienes by absorption at 234 nm. The modification of the negative net charge of LDL was assessed by agarose gel-electrophoresis in a Ciba Corning system and results expressed in relative electrophoretic mobility (REM). Means ± S.D. ($n = 5$).

2.4. Preparation of nuclear extracts

All experiments were performed on sparse cultures, 2 days after seeding. The cells were preincubated for 24 h in medium devoid of serum and supplemented with 0.1% bovine serum albumin. The cells were then incubated with 0.05 mg control or oxidized LDL protein/ml for 4 h. In some experiments, the lipid extract of Cu^{2+}-oxidized LDL at a concentration equivalent to 0.05 mg LDL protein/ml was introduced in ethanol solution (final concentration 0.3% v/v) during 4 h. *dl*-α-Tocopherol 10^{-5} M was introduced in ethanol solution at the same final concentration 24 h before the addition of modified LDL. Cycloheximide when added was introduced 1 h before the addition of oxidized LDL. Nuclear extracts were then prepared according to Dignam et al. [18]. Cells were resuspended in 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% Nonidet P40 and the protease inhibitors PMSF 0.2 mM, aprotinin 2 mM, antipain, pepstatin, benzamidine and leupeptin 1 μg/ml. After homogenization with a Dounce homogenizer and a 10 min incubation at 4°C, nuclei were collected by centrifugation at $2000\times g$ for 30 min. The pellet was washed with the same buffer without Nonidet P40 and resuspended in 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol and the above cited antiproteases. The nuclear proteins were extracted by incubation at 4°C during 30 min. After centrifugation at $13\,000\times g$ for 15 min, the supernatant was kept at −80°C.

2.5. Electrophoretic mobility shift assay

One strand on the AP1 duplex was ^{32}P-end-labelled with T4 kinase and [γ-^{32}P]ATP and hybridized with the complementary strand. The binding reaction was performed at room temperature for 25 min with 3–5 μg nuclear protein in 20 ml of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 10% glycerol, 0.2% Nonidet P40, 3 μg poly dI-dC and 0.1 pmol of the double-stranded labelled probe. The binding mixture was submitted to non-denaturing polyacrylamide gel electrophoresis. After autoradiography, radioactivity was determined by liquid scintillation counting. Each experiment was repeated 2–4 times.

3. Results

The Cu^{2+}- and endothelial-cell-modified LDL were first characterized for the extent of oxidation by determination of the lipid peroxidation end-product (TBARS) content, the conjugated diene level and the electrophoretic mobility of the particle. With these parameters, it was checked that LDL was more extensively modified by copper ions than by endothelial cells (Table 1). It is of note that, as previously demonstrated [15], the hydroxyl radical alone induced only a very slight increase in LDL lipid peroxidation as compared to copper- or endothelial cell-modified LDL, or to LDL modified by the hydroxyl radical/superoxide mixture.

The effect of oxidized LDL on AP1 binding activity was studied on different cell types of the arterial wall, namely fibroblasts, smooth muscle and endothelial cells. Fig. 1 shows that a 4 h treatment with Cu^{2+}- or cell-oxidized LDL induced a marked stimulation of AP1 activation in fibroblasts, leading to 3.2- and 3.7-fold activation for E-LDL and Cu-LDL, respectively. Control LDL also caused an about 2-fold increase in AP1 activity under the same experimental conditions. Cu^{2+} alone had no significant effect on AP1 binding activity (result not shown).

The activation of AP1 was also observed after treatment of smooth muscle cells with E-LDL and Cu-LDL (Fig. 2), with a more marked effect for Cu-LDL, which initiated an about 6-fold increase in AP1 activity. Finally, in endothelial cells (Fig. 3), similar results were obtained: about a 3- and 4-fold stimulation of AP1 activity was observed for E-LDL and Cu-LDL, respectively. Again, it is of note that control LDL already induced a significant activation of AP1 in both cell types (1.5–2-fold increase in smooth muscle and endothelial cells, depending upon the experimental set). It is of note that the lipid extract also induced a significant stimulation of AP1 binding activity, albeit less marked than that obtained with whole Cu-LDL (about 2.5-fold increase for the lipid extract as

Table 2
Effect of the antioxidants, α-tocopherol and *N*-acetylcysteine on the intracellular TBARS level and AP1 binding activity of endothelial UNA cells

	TBARS (%)	AP1 binding activity (%)
None	100	100
Ethanol 0.5%	98 ± 8	91 ± 7
α-Tocopherol 10^{-5} M	61 ± 5	88 ± 7
N-Acetylcysteine $5\cdot10^{-3}$ M	82 ± 7	91 ± 8
Cu-LDL	147 ± 11	442 ± 35
Cu-LDL + α-tocopherol	62 ± 5	316 ± 29
Cu-LDL + *N*-acetylcysteine	92 ± 9	210 ± 25
Lipid extract of Cu-LDL	n.d.	245 ± 30
Lipid extract of CU-LDL + α-tocopherol	n.d.	168 ± 22

Cells were pretreated for 24 h with the antioxidants before the addition of 0.05 mg/ml Cu-LDL or its lipid extract during 4 h. The AP1 binding activity was determined by electrophoretic mobility shift assay as indicated in the text. The intracellular TBARS was determined by the Yagi method [17] (100%: 1.4 nmol Eq MDA/mg cellular protein). Means ± S.D. ($n = 3$).

compared to 4-fold increase for Cu-LDL, Table 2). On the other hand, the lipid extract of control LDL had the same effect as c-LDL itself (data not shown).

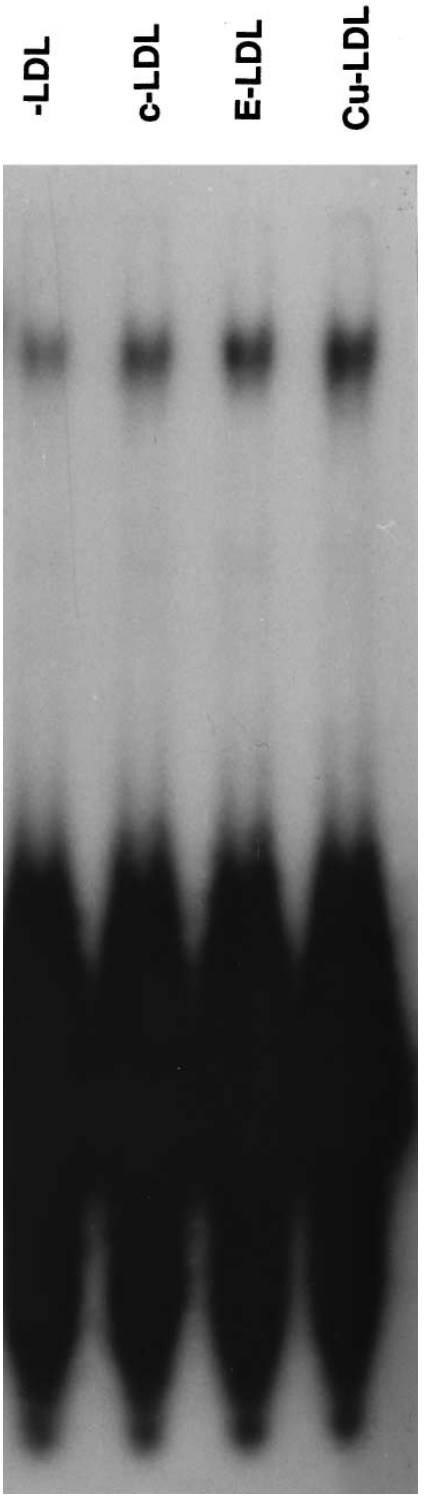

Fig. 1. Autoradiograph showing the activation of AP1 by oxidized LDL in fibroblasts. MRC5 cells were preincubated for 24 h in medium supplemented with bovine serum albumin 0.1% before addition of 0.05 mg/ml control or oxidized LDL for 4 h. Nuclear extracts were then prepared according to Dignam et al. [18] and AP1 binding activity was determined by electrophoretic mobility shift assay. Lane 1: without LDL. Lane 2: c-LDL. Lane 3: E-LDL. Lane 4: Cu-LDL.

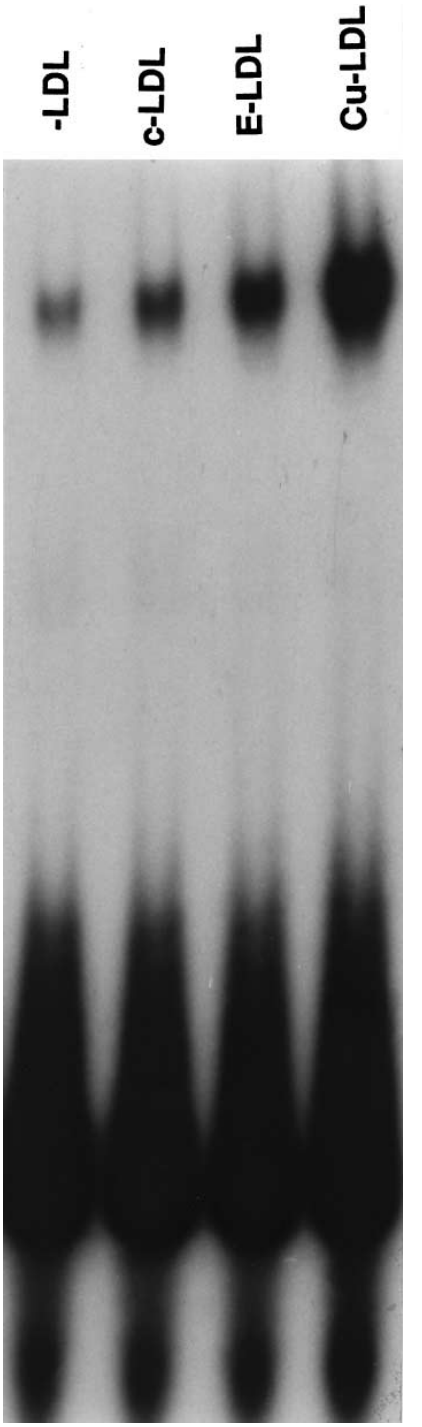

Fig. 2. Autoradiograph showing the activation of AP1 by oxidized LDL in smooth muscle cells. A7r5 cells were preincubated for 24 h in medium supplemented with bovine serum albumin 0.1% before addition of 0.05 mg/ml control or oxidized LDL for 4 h. Nuclear extracts were then prepared according to Dignam et al. [18] and AP1 binding activity was determined by electrophoretic mobility shift assay. Lane 1: without LDL. Lane 2: c-LDL. Lane 3: E-LDL. Lane 4: Cu-LDL.

Since the enhancement of AP1 binding activity might be due to an effect of oxidized LDL on the transcriptional or post-transcriptional regulation of the c-Jun and c-Fos subunits [19], we checked whether it was abolished by cycloheximide. In endothelial cells, it was found that cycloheximide, as

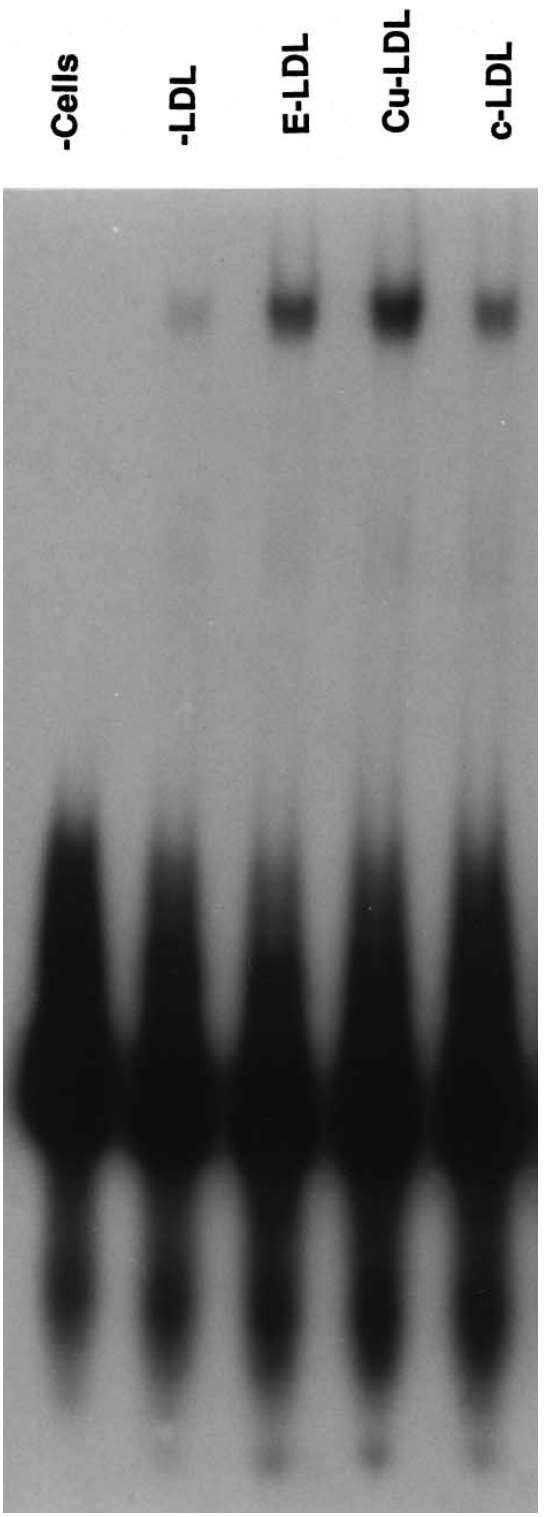

Fig. 3. Autoradiograph showing the activation of AP1 by oxidized LDL in endothelial cells. UNA cells were preincubated for 24 h in medium supplemented with bovine serum albumin 0.1% before addition of 0.05 mg/ml control or oxidized LDL for 4 h. Nuclear extracts were then prepared according to Dignam et al. [18] and AP1 binding activity was determined by electrophoretic mobility shift assay. Lane 1: without cells. Lane 2: without LDL. Lane 3: E-LDL. Lane 4: Cu-LDL. Lane 5: c-LDL.

expected, reduced the AP1 binding activity (Fig. 4). However, the activation of AP1 by Cu-LDL was still observed in the

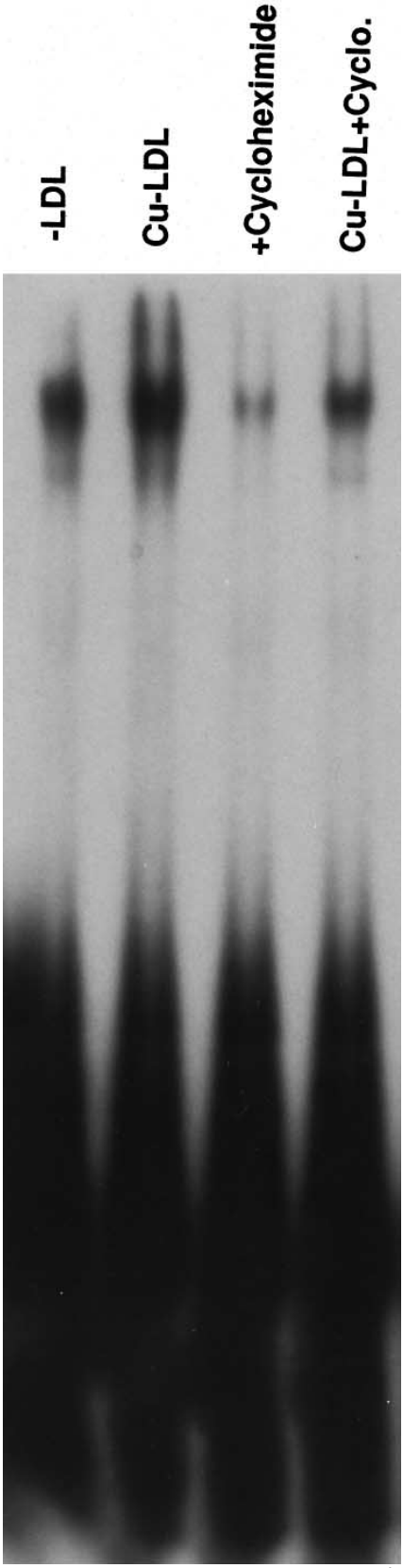

Fig. 4. Autoradiograph showing the effect of cycloheximide on the activation of AP1 by oxidized LDL in endothelial cells. UNA cells were preincubated for 24 h in medium supplemented with bovine serum albumin 0.1% and cycloheximide 10 μg/ml was introduced 1 h before the addition of Cu-LDL 0.05 mg/ml for an additional 4 h incubation. Lane 1: without LDL. Lane 2: Cu-LDL. Lane 3: cycloheximide. Lane 4: Cu-LDL + cycloheximide.

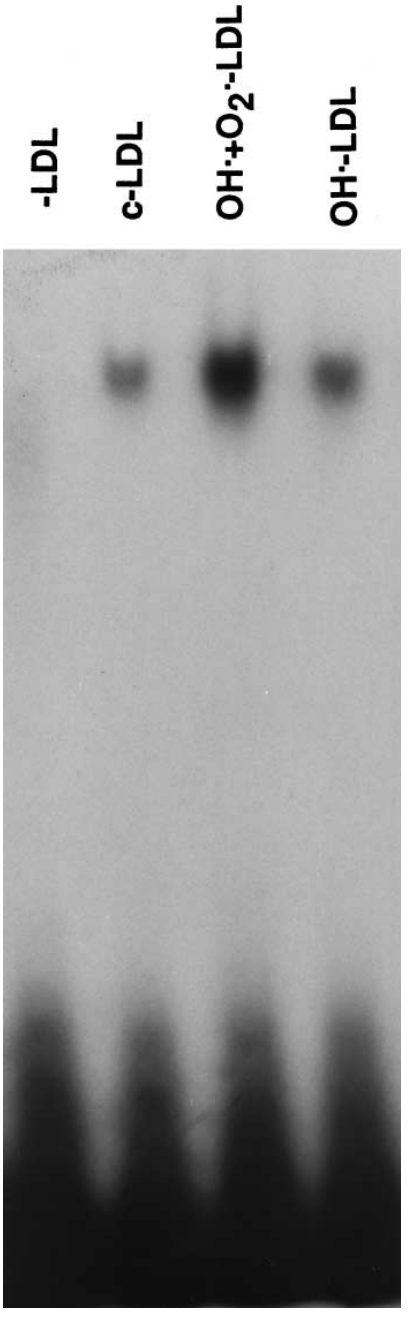

Fig. 5. Autoradiograph showing the effect of OH· and (OH·+O_2·$^-$) modified LDL on AP1 activity. The experimental conditions were the same as described in Fig. 1 legend. Generation of OH· and (OH·+O_2·$^-$) was obtained by gamma radiolysis of water using a ^{60}Co irradiator at a dose of 1800 Gy [15]. Lane 1: without LDL. Lane 2: c-LDL. Lane 3: OH·+O_2·$^-$. Lane 4: OH·-LDL.

presence of cycloheximide, indicating an effect of Cu-LDL on the post-translational regulation of the AP1 subunits.

The next experiment was designed to test the effect of the antioxidants, α-tocopherol and *N*-acetylcysteine, on the intracellular TBARS level and on Cu-LDL-induced AP1 activation in endothelial cells (Table 2). It can be noted that Cu-LDL caused an approximately 1.5-fold increase in intracellular TBARS. Furthermore, the lipophilic antioxidant, α-tocopherol, whether in the presence or absence of Cu-LDL, reduced the TBARS level to approximately 60% of control, whereas the hydrophillic antioxidant, *N*-acetylcysteine had no effect. Concerning the AP1 binding activity, both antioxidants had no significant effect by themselves, but reduced the Cu-LDL-induced AP1 activation, with a more marked effect for *N*-acetylcysteine.

Finally, in order to further investigate the influence of the degree of lipid peroxidation of the LDL particle on AP1 activation, the effect of LDL modified by OH· or by the (OH·+O_2·$^-$) mixture was tested. It was previously demonstrated that only (OH·+O_2·$^-$) modification of LDL is accompanied by a significant increase in the TBARS content of the particle, although both modified LDL were no longer recognized by the Apo B/E receptor of fibroblasts [15]. Fig. 5 shows that the effect of OH·-modified LDL was similar to that of control LDL, whereas (OH·+O_2·$^-$)-modified LDL was much more effective in terms of AP1 activation (Fig. 5).

4. Discussion

Our experiments clearly demonstrate that Cu^{2+}- and cell-oxidized LDL induced AP1 DNA binding activity in fibroblasts, smooth muscle and endothelial cells (Figs. 1–3). This phenomenon was still observed in the presence of cycloheximide (Fig. 4), which indicated that, at least under our experimental conditions, oxidized LDL exerts its effect by a post-translational mechanism on the AP1 c-Fos and c-Jun subunits, possibly via JNKs and FRK kinases [19].

It is of note that the poorly oxidized control LDL already initiated an increase in AP1 activity. This phenomenon might be interpreted by the fact that the AP1 subunits are very sensitive to the redox state of the cell [20], by means of the conserved cysteine residues included in their DNA-binding domains [21,22]. Alternatively, native LDL might act via a signal-transduction pathway: indeed, it has been reported that the LDL particle stimulates the production of diacylglycerol, induces the release of Ca^{2+} from intracellular pools and activates protein kinase C in alveolar type II cells [23] and human fibroblasts [24]. Resink et al. [25] also demonstrated that, in rat smooth muscle cells, native LDL stimulates the accumulation of inositol phosphates.

It can be noted (Table 1) that the extent of AP1 activation is approximately parallel to the degree of LDL oxidative modification in that the extensively oxidized Cu-LDL more efficiently activated AP1 than the cell-oxidized LDL. In addition, we have observed that the lipid extract of Cu-LDL also induces the activation of AP1, which suggests an involvement of lipid peroxidation products in the observed phenomenon. In a recent report, Ares et al. [26] described a stimulating effect of AP1 by Cu-LDL or its lipid extract in human vascular smooth muscle cells, but found no relationship to the extent of LDL peroxidation. These authors mainly ascribed the observed effect to lysophosphatidylcholine arising from LDL phosphatidylcholine partial hydrolysis which accompanies LDL oxidative modification [26]. In our experimental model, lysophosphatidylcholine also induced AP1 activation in the studied cell type (data not shown). However, the partial inhibition by vitamin E of the AP1 activation induced by Cu-LDL or its lipid extract observed in our system suggests that lipid peroxidation products could participate in the observed phenomenon. First, one can suppose that polyunsaturated fatty acid hydroperoxides delivered to cells by oxidized LDL could, in the presence of ferrous ions, or by reacting with intracellular superoxide anion, generate the alkoxyl radical

[27] which might further induce cellular lipid peroxidation. In this hypothesis, vitamin E could exert its protective action by blocking the intracellular lipid peroxidation initiated by the hydroperoxides included in oxidized LDL. This is in accordance with the results presented in Table 2, which show that vitamin E inhibited the increase in intracellular TBARS content induced by Cu-LDL. It is also of interest to note that hydroxy-eicosa-tetraenoic acids derived from arachidonic acid by the lipoxygenase pathway have been demonstrated to activate AP1 [28]. Thus, hydroperoxides contained in Cu-LDL or secondarily generated inside the cells could also act by themselves. Second, among the lipid peroxidation end-products contained in oxidized LDL, aldehydes such as 4-hydroxynonenal are known to strongly react with SH groups and to reduce the cellular glutathione level [29], thus affecting the redox state of the cell, which is known to play a crucial role in the regulation of AP1 activity [20]. Recent studies by Pinkus et al. also confirmed the role of the intracellular glutathione level in AP1 activation [30]. Thus oxidized LDL could also activate AP1 by means of its lipid peroxidation end-products such as 4-hydroxynonenal and other aldehydes inducing a depletion in intracellular glutathione. This hypothesis is supported by the fact that the hydrophilic antioxidant thiol compound *N*-acetylcysteine also reduced, and more efficiently than vitamin E, the activation of AP1 by Cu-LDL (Table 2). In summary, the effects of vitamin E and *N*-acetylcysteine observed under our experimental conditions would be difficult to explain if lysophosphatidylcholine was the only factor involved in oxidized LDL-induced AP1 activation. We therefore suggest that, besides lysophosphatidylcholine, lipid peroxidation intermediary or/and end-products contained in oxidized LDL could also play a role in AP1 activation.

AP1 binding sites are important for the transcriptional regulation of several cytokines involved in inflammation such as tumor necrosis factor alpha [31,32] or interleukin-1 beta [33]. Also, the DNA binding activity of AP1 is essential for the regulation of genes involved in cell growth and differentiation [34]. Furthermore, the pathogenesis of atherosclerosis is closely linked to the mechanism associated with inflammation and fibroproliferative repair [35]. Thus, the observed activation of AP1 in the three cell types present in the artery wall might be related to the essential role of oxidized LDL in the generation and progression of atherosclerosis.

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