Oxidized LDL activates STAT1 and STAT3 transcription factors: possible involvement of reactive oxygen species

Cécile Mazière*, Gulie Alimardani, Françoise Dantin, Françoise Dubois, Marie-Alix Conte, Jean-Claude Mazière

Laboratoire de Biochimie, CHRU Amiens, Hôpital Nord, 80054 Amiens Cedex 01, France

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Abstract The effect of cupric ion-oxidized low density lipoprotein (Cu-LDL) or endothelial cell-oxidized LDL (E-LDL) on STAT1 and STAT3 (signal transducers and activators of transcription) DNA binding activity was investigated by electrophoretic mobility shift assay in human endothelial cells. Both oxidized LDL enhanced STAT1 and STAT3 binding to their respective consensus binding sites. Furthermore, the activation of STATs was proportional to the oxidation degree of LDL in that the highly oxidized Cu-LDL exhibited a more marked effect than E-LDL. Oxidized LDL induced an intracellular oxidative stress, as shown by the increase in the intracellular level of lipid peroxidation products (thiobarbituric acid-reactive substances) and in the level of reactive oxygen species, measured by the fluorescence of dichlorofluorescein diacetate. The binding activity of STAT1 and STAT3 paralleled these two parameters, which suggests that it is dependent upon the redox state of the cell. The activation of STATs by oxidized LDL was almost completely inhibited by the lipophilic antioxidant vitamin E, and partially antagonized by the hydrophilic thiol-containing compound *N*-acetylcysteine, suggesting that the oxidative stress induced by oxidized LDL is involved in the observed phenomenon. Furthermore, the lipid extract of Cu-LDL also activated STAT1 and STAT3. Since the STAT pathway plays a key role in cytokine and growth factor signal transduction, the activation of STATs by oxidized LDL might be related to their proinflammatory and fibroproliferative effect in the atherosclerotic plaque.

Key words: Signal transducer and activator of transcription; Oxidized low density lipoprotein; Atherosclerosis

1. Introduction

It is recognized that oxidatively modified 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] or endothelial cells [3]. In vitro incubation of the LDL particle with transition metals such as copper can also initiate LDL oxidation [4]. The oxidative modification of LDL involves the generation of lipid peroxidation products such as aldehydes [5] and hydroperoxides [6]. Oxidized LDL induce the secretion of inflammatory cytokines such as interleukin-1 (IL1) [7] and tumor necrosis factor-α [8] by the activated macrophages present in the atherosclerotic lesions, or the secretion of growth factors such as PDGF [9].

Many cytokines and growth factors mediate their effects via the activation of a common signal transduction pathway, the JAK/STAT pathway (review in [10]). The binding of the ligand to its specific membrane receptor results in receptor aggregation, which leads to the catalytic activation of receptor-associated cytoplasmic protein tyrosine kinase termed Janus kinases (JAKs), that mediate ligand-dependent receptor phosphorylation. Receptor phosphotyrosyl residues are in turn recognized by the SH2 domains of the STAT family of cytoplasmic transcription factors [11,12]. The STAT factors are then activated by tyrosine phosphorylation [13,14] followed by dimerization [15], which is a prerequisite for transport to the nucleus, DNA binding and subsequent transcriptional activity [16,17].

This study was undertaken to investigate whether the LDL oxidatively modified by endothelial cells or by copper ions can regulate the DNA binding activity of the transcription factors STAT1 and STAT3. It was found that both oxidized LDL stimulated STAT binding activity in endothelial cells, fibroblasts and T lymphocytes. The degree of STAT activation by oxidized LDL appeared to be proportional to the intracellular oxidative stress, as quantitated by the level of lipid peroxidation products and reactive oxygen species.

2. Materials and methods

2.1. Materials

Ham F10 medium, Dulbecco's minimum essential medium (DMEM) and fetal calf serum were from Gibco (Grand Island, NY, USA). The oligonucleotide probes STAT1 (5′-CAT GTT ATG CAT ATT CCT GTA AGT G-3′) and STAT3 (5′-GAT CCT TCT GGG AAT TCC TAG ATC-3′) consensus binding sites were synthesized by Eurogentec (Belgium). [γ-^{32}P]ATP 7000 Ci/mmol was from ICN (CA, USA). Dichlorofluorescein diacetate was purchased from Molecular Probes. All other chemicals were of Sigma grade.

2.2. Cell culture

The ECV 304 human endothelial cell line was purchased from the European Collection of Cell Cultures. The cells were maintained in Ham's F10 medium supplemented with 10% fetal calf serum, at 37°C under a 5% $C0_2$ humidified atmosphere. All experiments were performed on subconfluent cultures.

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. [18], and dialyzed 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

*Corresponding author.

Abbreviations: LDL, low density lipoprotein; n-LDL, native LDL; c-LDL, control LDL; Cu-LDL, Cu^{2+}-oxidized LDL; E-LDL, endothelial cell-oxidized LDL; STAT, signal transducer and activator of transcription; TBARS, thiobarbituric acid-reactive substances (lipid peroxidation products)

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ECV 304 endothelial cells for 24 h in Ham's F10 medium. Control LDL was incubated at 37°C for 24 h. The degree of LDL oxidation was checked by determination of thiobarbituric acid-reactive substances (TBARS) according to Yagi [19] and by determination of the electrophoretic mobility of the LDL particle. The lipid extracts of control or Cu-LDL were prepared by the method of Bligh and Dyer [20].

2.4. Preparation of nuclear extracts

All experiments were performed on subconfluent cultures, 3 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. Nuclear extracts were then prepared according to Dignam et al. [21]. The 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 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 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

The double stranded oligonucleotides were end-labeled using T4 kinase and [γ-^{32}P]ATP. 7 μg of nuclear extract was incubated with 100 000 dpm of labeled probe (0.5 ng) in the presence of 1 μg of poly(dIdC) at room temperature for 20 min followed by separation of the mixture on a 6% non-denaturing polyacrylamide gel in Tris 50 mM/glycine 0.38 M/EDTA 2 mM buffer at pH 7.5. After autoradiography, the radioactivity was quantified by liquid scintillation counting. Results are representative of at least three independent experiments.

2.6. Quantitation of intracellular oxidation with dichlorofluorescein diacetate

The increase in fluorescence of living cells in the presence of this probe allows the quantitation of reactive oxygen species such as superoxide anion, hydrogen peroxide and the hydroxyl radical [22]. Cells were preincubated in medium devoid of serum supplemented with 0.1% bovine serum albumin for 24 h. After a 4 h incubation with oxidized LDL (0.05 mg LDL protein/ml), the cells were incubated for 30 min with 2×10^{-6} M 2′,7′-dichlorofluorescein diacetate in DMEM without phenol red, washed three times with phosphate buffer saline and solubilized in NaOH 0.1 N. The fluorescence was determined at 503/529 nm, normalized on a protein basis and expressed as a percentage of control.

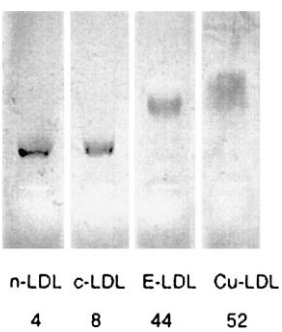

Fig. 1. Characterization of control LDL (c-LDL) and copper- (Cu-LDL) or endothelial cell-oxidized LDL (E-LDL). After EDTA removal by dialysis, LDL modification was performed by a 24 h incubation with $CuSO_4$ 5×10^{-6} M (1 mg LDL protein/ml) or with endothelial cells (0.1 mg LDL protein/ml) in Ham's F10 medium at 37°C. Control LDL was incubated in the same medium for 24 h at 37°C. Lipid peroxidation end products (TBARS) were determined by the technique of Yagi [19] and expressed in nmol malondialdehyde equivalents/mg LDL protein. The modification of the negative net charge of LDL was assessed by agarose gel electrophoresis in a Ciba Corning system. The results shown are from a typical experiment.

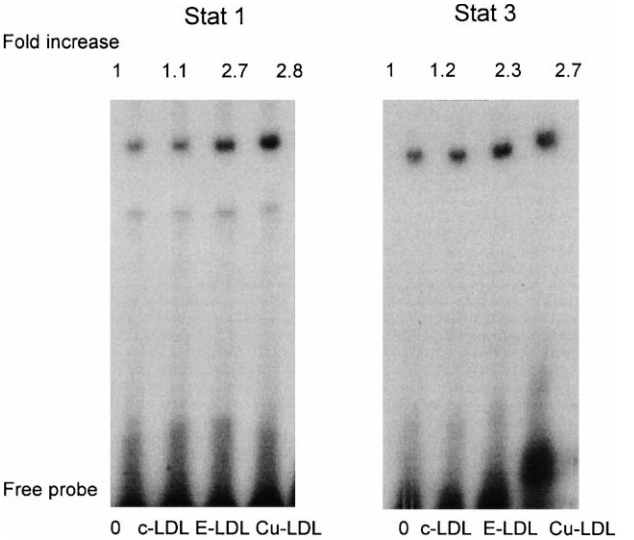

Fig. 2. Autoradiogram showing the activation of STAT1 and STAT3 by oxidized LDL. ECV 304 endothelial cells were preincubated for 24 h in medium supplemented with bovine serum albumin 0.1% before addition of 0.05 mg LDL protein/ml of control or oxidized LDL for 4 h. Nuclear extracts were then prepared and STAT binding activity was determined by electrophoretic mobility shift assay. Left: DNA probe: STAT1 consensus binding site. Right: DNA probe: STAT3 consensus binding site. The autoradiogram shown is from a representative experiment. This experiment was repeated four times with similar results.

2.7. Quantitation of intracellular lipid peroxidation products (TBARS)

After 4 h incubation with oxidized LDL as described above, the cells were harvested and resuspended in H_2O. Intracellular TBARS were determined by the fluorometric method of Yagi [19]. Results, normalized on a protein basis, are expressed as a percentage of control.

3. Results

We first checked the extent of LDL oxidation by copper ions or endothelial cells by measurement of TBARS and by determination of the relative electrophoretic mobility of the LDL particle. Under our experimental conditions, copper ions more extensively modified the LDL particle, in terms of both TBARS and electrophoretic mobility as shown in Fig. 1.

The effect of Cu-LDL and E-LDL was then investigated on STAT DNA binding activity after a 4 h incubation with ECV endothelial cells (Fig. 2). Stimulation of STAT1 and STAT3 activity was noted with both types of oxidized LDL, with a somewhat more marked effect with Cu-LDL, which led to an approximately 3-fold activation of the STAT transcription factors. Control LDL was very poorly effective, in that only 1.1- and 1.2-fold increases were observed for STAT1 and STAT3, respectively.

The time course study indicated that a 30 min incubation with Cu-LDL already induced an enhancement in STAT1 and STAT3 activity, by 1.7- and 1.8-fold respectively (Fig. 3). The STAT1 and STAT3 binding activities increased with the in-

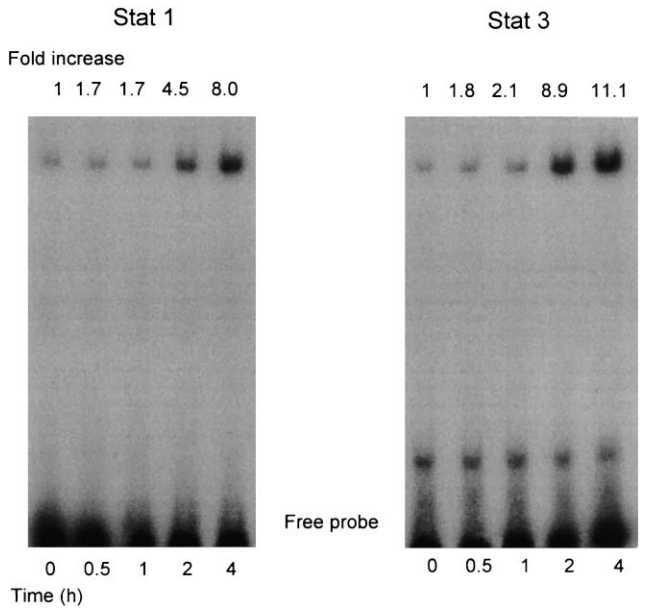

Fig. 3. Time course effect of Cu-LDL on STAT binding activity. ECV 304 endothelial cells were preincubated for 24 h in medium supplemented with bovine serum albumin 0.1% before addition of 0.05 mg Cu-LDL/ml for the indicated time. Left: DNA probe: STAT1 consensus binding site. Right: DNA probe: STAT3 consensus binding site. The autoradiogram shown is from a representative experiment. This experiment was repeated three times with similar results.

cubation time and were 8- and 11-fold increased after 4 h incubation with Cu-LDL for STAT1 and STAT3, respectively. It is of note that in this experiment, the enhancement of STAT activity was the highest ever obtained in our experimental conditions. Indeed, even though the cells were preincubated in medium devoid of serum during 24 h before the addition of oxidized LDL in order to down-regulate the STAT activity, the basal activity of the control was not always low, as can be observed in Figs. 2 and 3. We have noticed in the course of our experiments that the effect of oxidized LDL was more marked when the basal activity was weak, as is the case in Fig. 3.

In order to get insight into the mechanism whereby oxidized LDL stimulate the binding activity of STATs, we measured the intracellular level of lipid peroxidation products (TBARS). It was found (Table 1) that during the incubation with E-LDL or Cu-LDL, the intracellular TBARS increased by 1.3- and 1.4-fold respectively. The effect was somewhat more marked with the extensively oxidized Cu-LDL. Similar results were obtained when the intracellular level of reactive oxygen species was determined by the fluorescence of dichlorofluorescein diacetate. In this case, the values reached were 181% and 234% of control for E-LDL and Cu-LDL respectively.

The next experiment was designed to test the effect of antioxidants (vitamin E and *N*-acetylcysteine) on the activation of STAT1 and STAT3 by oxidized LDL. The results in Table 2 show that the lipophilic antioxidant vitamin E almost completely prevented the activation of STATs by Cu-LDL, while a partial inhibition was observed with *N*-acetylcysteine (about 2-fold reduction as compared to cells treated with Cu-LDL in the absence of antioxidant). Table 2 also shows that the lipid extract of Cu-LDL activated both STAT1 and STAT3, although to a lesser extent than the whole particle.

4. Discussion

In the atheromatous blood vessel, the presence of T lymphocytes suggests that there is an inflammatory reaction during the generation of atherogenesis [23]. Further, during the course of the inflammatory process, numerous cytokines and growth factors are involved in a paracrine or autocrine manner, leading to the settlement of the fibrous plaque [24]. The current studies demonstrate that oxidized LDL activates STAT1 and STAT3, the transcription factors that mediate the effect of cytokines and growth factors. Indeed, different authors have demonstrated the stimulatory effect of oxidized LDL on the production of cytokines or growth factors [7–9], but the action of oxidized LDL on the STAT transduction pathway has not yet been reported. It is of note that in the signalling of IL6-type cytokine receptors, STAT1 and STAT3 are both activated via the common signal transducer gp130, and are able to form homo- and heterodimers [25]. In this case, the kinases JAKs first bind to and phosphorylate the cytoplasmic part of gp130, thereby creating docking sites for STAT1 and STAT3. However, a number of reports demonstrate that STAT activation might occur independently of JAKs, via receptor tyrosine kinases such as EGF, FGF or PDGF receptors [26,27]. In the case of oxidized LDL, whether the activation of JAKs is involved remains to be determined.

Tyrosine phosphorylation is essential for dimerization and DNA binding of STATs. The residues which are involved are Tyr-701 for STAT1 [28] and Tyr-705 for STAT3 [29]. Furthermore, it has also been demonstrated that the activity of the C-terminal transactivation domain of STAT1 and STAT3 is, at least partially, regulated by a serine phosphorylation [30,31]. The effect of oxidized LDL on STAT tyrosine and serine phosphorylation is under investigation.

It is of note that the amino acid sequence surrounding the phosphorylated Ser-727 residue of STAT1 resembles the consensus recognition sites for the MAP kinases [32,33]. Since it has been demonstrated that oxidized LDL stimulate MAP kinase activity in smooth muscle cells and macrophages [34], the hypothesis of an activation of STATs by means of the MAP kinase signalling pathway might be raised.

Table 1
Effect of oxidized LDL on the intracellular level of lipid peroxidation products (TBARS) and of reactive oxygen species

Addition	TBARS (%)	Reactive oxygen species (%)
None	100	100
c-LDL	101 ± 7	99 ± 8
E-LDL	128 ± 8*	181 ± 13**
Cu-LDL	139 ± 10*	234 ± 12***

The cells were preincubated in medium devoid of serum supplemented with 0.1% bovine serum albumin for 24 h. After 4 h incubation with 0.05 mg oxidized LDL protein/ml, the cells were resuspended in H_2O for determination of TBARS by the method of Yagi [19] (100%: 487 pmol eq MDA/mg protein). For the determination of reactive oxygen species, the cells were incubated for 30 min with 2×10^{-6} M 2′,7′-dichorofluorescein diacetate [22] in DMEM without phenol red, washed three times with phosphate buffer saline and solubilized in NaOH 0.1 N. The fluorescence was determined at 503/529 nm, normalized on a protein basis and expressed in percentages of control. Means ± S.D. of six experimental values from three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$ by Student's *t*-test.

Table 2
Effect of antioxidants and of the lipid extract of Cu-LDL on STAT binding activity

Addition	STAT1 binding activity (%)	STAT3 binding activity (%)
None	100	100
Cu-LDL	343 ± 27	326 ± 31
Cu-LDL+α-tocopherol 10^{-5} M	107 ± 15	128 ± 17
Cu-LDL+*N*-acetylcysteine 5×10^{-3} M	188 ± 21	166 ± 18
Lipid extract of Cu-LDL	237 ± 27	178 ± 15

The cells were preincubated with the antioxidants in medium devoid of serum supplemented with 0.1% bovine serum albumin for 24 h. After 4 h incubation with 0.05 mg Cu-LDL protein/ml or the lipid extract of Cu-LDL (equivalent to 0.05 mg Cu-LDL protein/ml), STAT binding activity was determined by electromobility shift assay. Results are from a typical experiment. Means ± S.D. (three distinct nuclear extracts in the same experimental set).

Our studies also demonstrate that oxidized LDL induced an elevation in the level of intracellular lipid oxidation products and of reactive oxygen species (Table 1). The lipid oxidation products measured under our conditions might have two origins, the oxidation products included in the LDL particle itself and those arising from the fatty acids of cellular origin. It can be supposed that the oxidation products delivered to the cell by oxidized LDL might generate reactive oxygen species, which then in turn will attack the fatty acids of cellular origin, thus propagating a chain of lipid peroxidation, and creating oxidative stress. This hypothesis is supported by the report of Rota et al. [35], who demonstrated that, in the presence of cytochrome P450 or Fe^{2+}, superoxide anion and other lipid free radicals could be formed from unsaturated fatty acid hydroperoxides. In any case, the involvement of the oxidized LDL lipids in the observed phenomenon is clearly demonstrated by the activating effect of the Cu-LDL lipid extract (Table 2). Moreover, the preventive action of antioxidants, especially that of the lipophilic compound vitamin E, suggests that further lipid peroxidation steps triggered by the lipid moiety of oxidized LDL and by reactive oxygen species secondarily generated inside the cells are involved in STATs activation.

In conclusion, the current studies suggest that the STAT family of transcription factors are regulated by the redox state of the cell in that they are activated by the oxidative stress induced by oxidized LDL. This effect was also found in other cell types involved in the inflammatory and fibroproliferative process observed in the atherosclerotic lesion, such as T lymphocytes and fibroblasts (data not shown). The stimulation of DNA binding activity of transcription factors by oxidized LDL has already been reported for NF-κB [36,37] and AP1 [38]. In these reports, an involvement of the lipid peroxidation products has also been described [37,38]. Thus, it appears that activation of redox-sensitive transcription factors is a general mechanism whereby oxidized LDL might act on the cellular regulatory network. In this regard, the activation of STATs by oxidized LDL in cells involved in the generation of the atherosclerotic plaque might be interpreted in view of their proinflammatory and fibroproliferative action on the endothelial wall.

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