Human atherosclerotic plaque lipid extract impairs the antioxidant defense capacity of monocytes

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ABSTRACT

Oxidative stress, induced by reactive oxygen species (ROS), is implicated in the pathogenesis of plaque formation and instability. During this ongoing oxidative process, cells in the vasculature are exposed to the atherogenicity of the plaque; previous studies have suggested that the arterial plaque, apart from being a consequence of the development of atherosclerosis, is also a cause of its progression.
Objective: In this study, we challenged this idea by investigating the effect of carotid plaque lipid extract on the human monocyte antioxidant system.
Methods and Results: Exposure of monocytes to carotid plaque lipid extract (LE) for up to 72 h resulted in a significant increase in the ROS level (170%), with a simultaneous rise of 177% in glutathione oxidation. Experiments revealed a significant decrease, in the intracellular antioxidant enzyme activity of CAT, GPx and TRxR, (by 17, 33 and 43%, respectively). Although the activity of these enzymes subsequently returned to those of the controls, the levels of ROS did not decrease but rather continued increasing with extended LE exposure. Intriguingly, intracellular SOD activity rose significantly and remained high (176%), implying that endogenously produced H_2O_2, and not $O_2^-<$ is the factor that promotes the oxidative stress resulting from the presence of LE.
Conclusion: Lipids from the atherosclerotic plaque may contribute to the progression of atherogenic conditions in adjacent regions by weakening the cellular antioxidant system and promoting oxidative stress, mainly through H_2O_2 production.

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1. Introduction

Accumulating evidence indicates that oxidative stress, induced by reactive oxygen species (ROS), is implicated in the pathogenesis of plaque formation and instability [1,2]. Oxidized low density lipoprotein (LDL) and its lipid components induce atherogenic events, such as injury to vascular cells, an increase in interactions between inflammatory and endothelial cells and the induction of vascular smooth muscle cell proliferation. Previous studies have suggested that arterial plaques, apart from being a consequence of the development of the atherosclerosis disease, are also a cause of its progression. A few studies have challenged this idea, e.g., sonicated carotid and coronary plaques were shown to reduce the activity of an antiatherogenic enzyme, paraoxonase I, in vitro [3]. A lipid fraction of human plaques was found to cause LDL oxidation, elevate the oxidative state of mouse macrophages and de-crease the ability of high density lipoprotein (HDL) to cause cholesterol efflux from macrophages, in a dose response manner [3]. In addition, a lipid extract (LE) of atherosclerotic plaque, derived from patients that underwent endarterectomy, was found to mediate cytokine up-regulation in monocytes, causing inflammation [4]. These data imply that plaque lipids may enhance plaque formation and that during this oxidative ongoing progression, cells in the vasculature are widely exposed to the atherogenicity of the plaque.

Studies investigating the role of antioxidant enzymes in the protection of cells from atherogenicity have demonstrated that an increase in the activity of either Cu/Zn-superoxide dismutase (SOD) or catalase reduces cell-mediated LDL oxidation and oxLDL-induced apoptosis [5]. Overexpression of catalase significantly decreased atherosclerotic lesions and the level of oxidized lipids in the arterial wall of apolipoprotein E-deficient (ApoE−/−) mice [6]. Overexpression of GPx4 in ApoE−/− mice inhibited the development of atherosclerosis in association with a decrease in the level of oxidized lipids in the aorta [7], while thioredoxin (TRX) expression in symptomatic unstable coronary plaques was shown to increase and be positively correlated with intraplaque hemorrhage and thrombus formation [8].

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1.1. Aim

The purpose of the current study was, therefore, to investigate the effect of a human carotid plaque lipid extract (LE) on the cellular expression and activity of antioxidant enzymes and on the oxidative state of monocytes. Our experiments demonstrated that LE impairs antioxidant enzyme activity and expression to an extent that lessened their ability to combat the increasing levels of ROS in the cells.

2. Materials and Methods

2.1. Materials

Human carotid plaques were obtained from patients that underwent routine endarterectomy surgery under local anesthesia in the Department of Vascular and Transplantation Surgery at Rambam Medical Center and Mount Carmel Hospital in Haifa, Israel. The research was approved by the Helsinki Committee and by the patients themselves. Immediately upon removal from the patients, the complete atherosclerotic plaques (which included the common internal and external carotid sections) were frozen and kept at $-70\ °C$. Thereafter, they were ground to a powder in liquid nitrogen and pooled. Ethyl acetate (EtAc) was used to extract a lipid fraction, termed LE, which was used at the indicated concentrations.

All chemicals, unless noted otherwise, were of analytical grade and were obtained from Sigma–Aldrich (Rehovot, Israel).

2.2. Cell culture

THP-1 monocytes–THP-1, human leukemic monocytes were grown in RPMI 1640 medium supplemented with 5% FCS, 2 mM L-glutamine solution, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin (Biological Industries, Israel).

All cells were maintained in a standard culture incubator with humidified air containing 5% CO_2 at 37 °C.

Cell treatments – Cells were plated in 6-well culture plates at a concentration, which would give 1×10^6 cells ml^{-1} at the end of the experiment. Cells were treated with 0.5 mg/ml LE or with 200 µM H_2O_2 (positive control). Negative controls were treated with dimethylsulfoxide (DMSO): ethanol (0.096%) only, which is the same concentration as in the LE treatment. Following 4, 24, 48 or 72 h of incubation, the cell number was determined using the fluorescence activated cell sorter (FACS) (Becton Dickinson) at the excitation and emission wavelengths of 488 and 530 nm, respectively. In addition, mRNA expression, protein activities and the glutathione oxidation assay were performed on these cells.

2.3. Intracellular ROS production

Intracellular ROS production was estimated according to the oxidation of the fluorescence probe, dichlorodihydrofluorescein diacetate (DCFDA). The formation of dichlorodihydrofluorescein (DCF) was measured at the excitation and emission wavelengths of 488 and 530 nm, respectively, by means of the FACS (Becton Dickinson) [9].

2.4. Enzyme activity determination

Catalase (Cat) activity was quantified based on the rate at which one catalase unit degrades one micromole of H_2O_2 min^{-1} at 240 nm. Relative superoxide dismutase (SOD) activity was determined according to percent inhibition of the reduction rate of the water-soluble tetrazolium salt WST-1 to WST-1 formazan at 450 nm, in the presence of xanthine and xanthine oxidase, using the SOD cellular assay kit (Stressgen Cat #900–157), according to the manufacturer's instructions. The glutathione-(GSH) peroxidase (GPx) activity was quantified based on the rate of NADPH consumption at 340 nm in the presence of 0.5 mM hydrogen peroxide, as described elsewhere [10]. Thioredoxin reductase (TRxR) activity was quantified using the auranofin (20 nM)- inhibitable proportion of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reduction at 412 nm, as has been described previously [11].

2.5. Intracellular GSH and glutathione disulfide (GSSG) assay

At the end of the experiment 2×10^6 cells were harvested (200 g for 5 min at 4 °C) and washed with cold phosphate buffered saline (PBS). Cell pellets were lyzed with cold extraction buffer (0.1 M PBS with 5 mM ethylenediaminetetraacetate (EDTA), 0.1% triton X-100, 0.6% sulfosalicylic acid, $pH = 7.5$) following 2 min of sonication on ice and two freeze–thaw cycles. Cell lysates were centrifuged (200g for 5 min at 4 °C), and the supernatants were immediately removed and stored at $-80\ °C$ until use.

Total cellular GSH concentrations and GSSG/GSH ratios were quantified using liquid chromatography mass spectrometry (LCMS) and a standard curve based on GSH and GSSG commercial samples. LCMS analysis was performed in the positive ion mode with 6540 UHD accurate mass (Q-TOF) (Agilent technologies, USA) coupled to a Waters HPLC system (Ireland). GSH and GSSG were separated on a reverse-phase column (C18 XTerra; 3.5 µm; 4.6×20 mm, 0.5 ml/min flow rate) at room temperature, using 98% H_2O: 2% acetonitrile + 0.1% acetic acid. Mass spectrometry gas flow was set at 9 l/min, nebulizer gas at 40 PSI, sheath gas temperature at 350 °C and sheath gas flow at 11 l/min. The GSH spectrum was identified at the retention time of 0.9 min with a mass-to-charge (m/z) ratio of 308.0908. The GSSG spectrum was observed at the retention time of 1.2 min with an m/z ratio of 613.1592.

2.6. mRNA expression

The expression of mRNA was analyzed by the real time polymerase chain reaction (PCR). Total RNA was extracted from cell pellets with Trireagent (Sigma, Israel). Each cDNA sample was produced from 1 µg total RNA, which was incubated at 74 °C for 10 min with 100 pmols of oligo dT17 primer. This mixture was added to a final volume of 25 µl solution, containing 2 mM deoxynucleotides (dNTP) (Larova, Germany), 30 U RNasin, 10 U avian myeloblastosis virus-reverse transcriptase (AMV-RT) and the AMV-RT buffer (Promega, Israel), followed by incubation at 42 °C for 2 h, and 52 °C for an additional 1 h. The cDNA of the mRNA transcripts was amplified in a mixture containing Absolute Blue QPCR SYBER Green ROX Mix (ABgene, UK) and specific primers by the spectrofluorometric thermal cycler (Rotor-Gene^{TM} 6000, Corbett Research, Australia), according to the protocol supplied by the manufacturer.

The primers used were: catalase (forward) 5′ AGGCCAGTCCTGA-CAAAATG 3′, (reverse) 5′ GAATCTCCGCACTTCTCCAG 3′; Mn SOD (forward) 5′ GCACTAGCAGCATGTTGAGC 3′, (reverse) 3′ GCGTTGAT GTGAGGTTCCAG 5′; GPx (forward) 5′ AATTCCCTCAAGTACGTCCG 3′, (reverse) 5′ CTCGATGTCAATGGTCTGGAA 3′; TRxR (forward) 5′ ATT-GCCACTGGTGAAAGACC 3′, (reverse) 5′ CGACATAGGATGCTCCAACA 3′. The annealing temperature of all primers was 60 °C, with the exception of GPx, which was 57 °C.

2.7. Statistical analysis

Results from three separate experiments were reported as mean ± SE and considered to be statistically significant if $p < 0.05$.
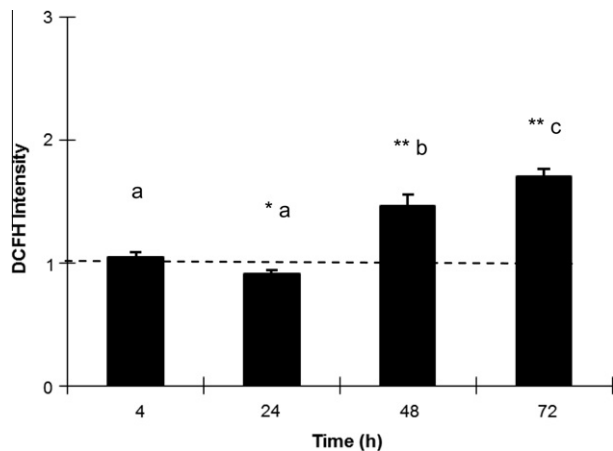
Fig. 1. Plaque lipid extract (LE) increases ROS level in monocytes. THP-1 monocytes were treated with LE–0.5 mg/ml or DMSO–0.275%. After 4, 24, 48 and 72 h of treatment, cells were harvested and counted by flow cytometry (solid line). ROS production was determined according to the oxidation of DCFH (bars). Results: mean ± SE. $^*p < 0.05$, $^{**}p < 0.01$ significant difference from the negative controls (dotted line). $n = 9$, from three independent experiments.

The Student's *t*-Test was employed when comparing the means of two groups. Analysis of variance (ANOVA) was used when more than two groups were compared.

3. Results

3.1. LE elevates ROS production in monocytes

The effect of LE on ROS production and antioxidant enzyme expression and activity was assessed in monocytes, which are the most prevalent leukocyte in the plaque and are also key players in atherogenesis [12,13]. THP-1 human monocytes were treated with 0.5 mg/ml LE or DMSO: EtOH (1:1) (0.096%, negative control) for 4, 24, 48 and 72 h. A significant increase of 47% and 70% ($p < 0.005$) in cellular ROS levels was detected at 48 and 72 h after LE treatment, respectively (Fig. 1). The subsequent count of viable cells revealed no significant change in cell number–up to 48 h after treatment. Following 72 h LE treatment, a slight decrease (by 16%, $p < 0.01$) in the cell number was observed.

3.2. LE reduces CAT activity while enhancing SOD

The effect of LE on CAT and SOD enzyme activities and mRNA expression in the monocytes was assessed after 4, 24, 48 and 72 h of treatment with LE. CAT activity was significantly reduced by 17% (±0.051, $p < 0.01$) after 4 h, as assayed by the rate of NADPH consumption (described in Materials and Methods), and continued to remain at a significantly low level, relative to the control, during the 72 h LE treatment (Fig. 2A). This reduction was accompanied by a significant decrease in cellular CAT expression, (by 12% ± 0.071) following 4 h of LE treatment, which returned to control levels over the course of incubation. In contrast, cellular SOD activity (quantified by the production of WST-1 formazan) and mRNA expression increased after 24 h of LE treatment (by 1.25 ± 0.07 and 1.45 ± 0.07, respectively), relatively to those of the control (Fig. 2B). The elevation in mRNA diminished, while the activity continued to mount significantly, reaching 1.77 ± 0.07 ($p < 0.001$) following 72 h of LE treatment.

3.3. LE affects GPx and TRxR enzyme activities and expression

The exposure of monocytes to LE (as above) significantly reduced GPx enzyme activity after 4 and 24 h of LE treatment by 0.77 ± 0.02 and 0.67 ± 0.06, respectively (Fig. 3A). A concomitant, but not significant, reduction in mRNA levels was also detected during this time period. Such reductions in GPx activity and expression diminished following 48–72 h of treatment.

The activity of the TRxR enzyme, which can potentially reduce oxidized TRx back to its monomer form, was also reduced by 0.57 ± 0.05 and 0.57 ± 0.09, $p < 0.05$, respectively, 4 and 24 h after cells were incubated in the presence of LE (Fig. 3B). The TRxR expression, 4 h after LE treatment, however, did not differ from that of the control level, although it increased significantly by 1.58 ± 0.017, $p < 0.01$, following 24 h of treatment.

3.4. LE increase glutathione oxidation

Intracellular glutathione levels of THP-1 cells were measured to obtain an indication of the cellular response resulting from the oxidative state, which had been induced by exposure to LE. Cell incubation with LE brought about a significant increase in GSH oxidation levels (1.93 ± 0.07-fold that of the control, as determined
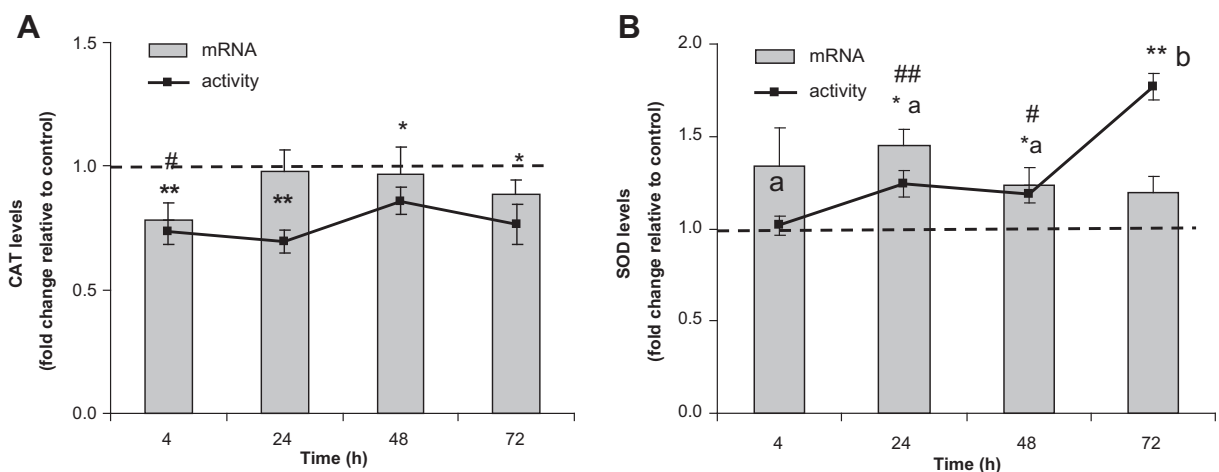

Fig. 2. Plaque lipid extract (LE) affects CAT and SOD enzyme activities and expression. THP-1 monocytes were treated with LE–0.5 mg/ml or DMSO–0.275%. After 4, 24, 48 and 72 h of treatment, mRNA transcription was determined. Each gene was normalized to the housekeeping gene, β-actin. Antioxidant enzyme activity was measured for each enzyme (A- CAT, B- SOD) as described in the Materials and Methods section. Results: mean ± SE. $^*p < 0.05$, $^{**}p < 0.01$ significant difference of enzyme activity from the negative controls (dotted line), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ significant difference of enzyme expression from the negative controls (dotted line). $n = 9$–12 from three independent experiments.
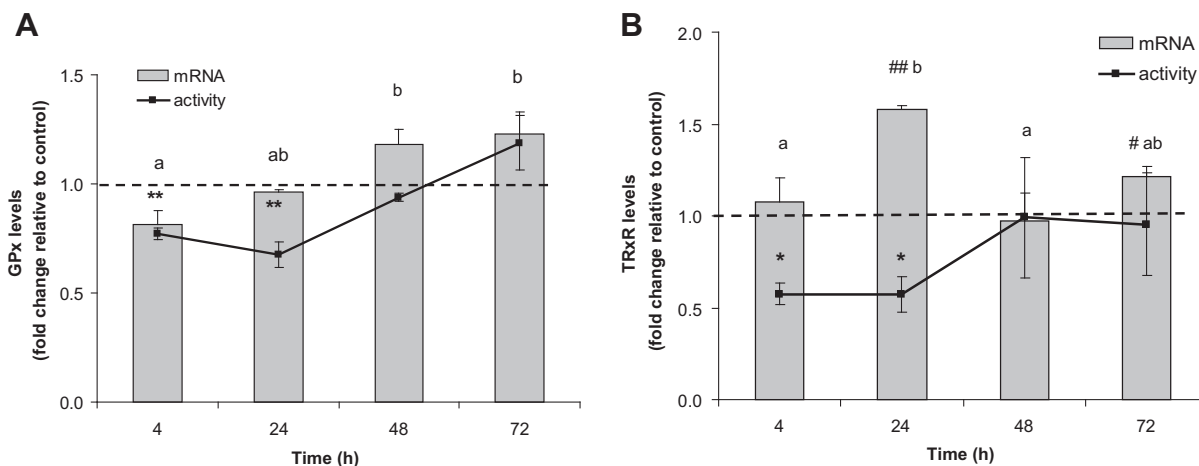
Fig. 3. Plaque lipid extract (LE) affects GPx and TRxR enzyme activities and expression. THP-1 monocytes were treated with LE–0.5 mg/ml or DMSO–0.275%. After 4, 24, 48 and 72 h of treatment, mRNA transcription was determined. Each gene was normalized to the housekeeping gene, β-actin. Antioxidant enzyme activity was measured for each enzyme (A- GPx, B- TRxR), as described in the Materials and Methods section. Results: mean ± SE. *–$p < 0.05$, **–$p < 0.01$ significant difference of enzyme activity from the negative controls (dotted line), #$p < 0.05$, ##$p < 0.01$ significant difference of enzyme expression from the negative controls (dotted line). $n = 9$–12 from three independent experiments.

by the formation of GSSG) 24 h after LE treatment (Fig. 4). This elevation in the cellular GSSG/GSH oxidation ratio became enhanced, increasing by 2.37 ± 0.198 and 1.87 ± 0.11, respectively, during the 48–72 h exposure to LE. However, no change in the GSH levels was detected after 72 h incubation.

4. Discussion

In the present study, the effect of the atherosclerotic plaque lipid moiety on the intracellular oxidative state and on the expression and activity of cellular antioxidant enzymes was investigated. The presence of plaque and its effect within the artery on the adjacent tissue and cells is difficult to assess, as these tissues are not accessible for research. Therefore, the atherogenic effect of plaque on the antioxidant system was studied in vitro using monocytes (THP-1). Exposure of these cells to the plaque lipid moiety during an extended incubation of 72 h revealed changes in antioxidant enzyme expression and activity and characterized the oxidative state variation.

Our results demonstrated that LE promotes cellular oxidative stress as exhibited by the production of ROS, which play a role in

Fig. 4. Plaque lipid extract (LE) increases GSH oxidation. THP-1 monocytes were treated with LE–0.5 mg/ml or DMSO–0.275%. After 4, 24, 48 and 72 h of treatment, cells were harvested. Cellular GSSG/GSH ratio was quantified using LC/MS. Results: mean ± SE. *–$p < 0.05$, **–$p < 0.01$ signicant difference from the negative controls (dotted line). $n = 8$ from two independent experiments.

reducing endogenous antioxidant properties, thereby exacerbating oxidative status. Mammalian cells contain several peroxide scavengers, including catalase, glutathione peroxidases (GPxs) and peroxiredoxins [reviewed in [14]]. In this study, incubation of human monocytes with LE resulted in a significant reduction in intracellular CAT, GPx and TRxR activity during the first 24 h of incubation. Although these reductions subsequently diminished during the 72 h LE treatment, returning to control levels, ROS levels did not decrease and continued to increase up to 70% more than that of the controls. SOD enzymes and catalase act in concert to detoxify O_2^- and H_2O_2 (see Review of Yu BP. [15]); i.e., SOD enzymes convert O_2^- to H_2O_2, while catalase destroys H_2O_2 by converting it to water. During the exposure of monocytes to LE, intracellular SOD activity rose significantly 24 h after treatment and remained high (up to 76% more than that of the control) at 72 h. These results are in agreement with previous findings [6], which showed that overexpression of catalase alone, or of Cu/Zn-SOD and catalase in combination, reduced the level of plasma and aortic F2-isoprostane and retarded the development of atherosclerosis in ApoE–/– mice, in contrary to the effect obtained from the overexpression of Cu/Zn-SOD alone. This implies that endogenously produced H_2O_2, but not O_2^-, is a factor that promotes the formation of oxidative stress in monocytes incubated in the presence of LE. Nevertheless, the rise in SOD activity alone did not combat cellular ROS production.

Glutathione peroxidase (GPx) is a selenium-containing antioxidant enzyme that effectively reduces hydrogen peroxide and lipid peroxides to water and lipid alcohols, respectively, and in turn, oxidizes glutathione (GSH) to glutathione disulfide (GSSG). The decrease in GPx activity, observed in our experiments, may inhibit the detoxification of hydrogen peroxide and lipid peroxides. These peroxides can be converted to hydroxyl radicals and lipid peroxyl radicals, respectively, by transition metals (e.g. Fe_2^-), subsequently contributing to the elevation in cellular ROS levels, at least during the first 24 h of LE treatment. The rise in ROS levels was correlated with a significant increase in the GSSG/GSH levels.

Thioredoxin reductase is another antioxidant enzyme that participates in thiol-dependent cellular reductive processes. The enzyme regenerates reduced thioredoxin, which serves as a reducing equivalent, and may also directly reduce lipid hydroperoxides. In the presence of LE, the activity of the TRxR enzyme was decreased. This may affect the TRxR ability to catalyze the glutathione-dependent disulfide reduction, contributing to the increase in the ratio of GSSG/GSH. The reduction in the enzyme activity
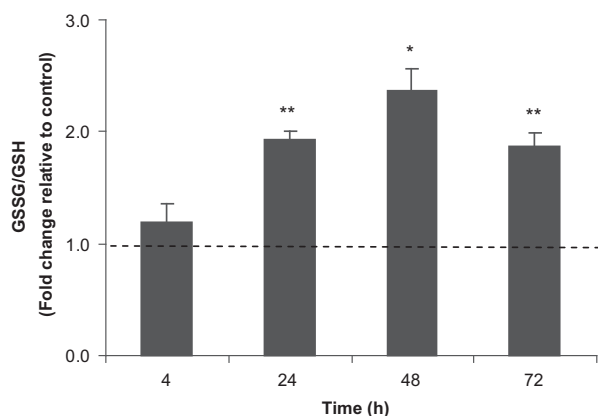
may be explained by post-translational modification, which may occur during exposure to atherosclerotic lipids. However, this remains to be investigated.

Human atherosclerotic plaque contains oxidized lipids, proteins, hydroperoxides [16], cholesterol, oxidized cholesterol products [3] and oxidized and aggregated LDL. Oxidized lipids induce various atherogenic events, including the recruitment of inflammatory cells to the intima and induction of vascular cell death [5]. In a previous study in our laboratory [4], LE was found to promote inflammation by elevating the expression of the pro-inflammatory factors, including interleukin (IL)-1β and tumor necrosis factor (TNF)-α. Such results, concurrent with the present data, support the idea that the plaque lipid content has an atherogenic effect on adjacent cells and tissues.

Acknowledgments

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