Inhibition by Cholesterol Oxides of NO Release From Human Vascular Endothelial Cells

Valérie Deckert, Annie Brunet, Frédérique Lantoine, Gérard Lizard, Elisabeth Millanvoye-van Brussel, Serge Monier, Laurent Lagrost, Monique David-Dufilho, Philippe Gambert, Marie-Aude Devynck

Abstract—Recent studies have demonstrated that, unlike cholesterol, cholesterol oxidized at position 7 can reduce the maximal endothelium-dependent relaxation of isolated rabbit aortas (Circulation. 1997;95:723–731). The aim of the current study was to determine whether cholesterol oxides reduce the release of nitric oxide (NO) from human umbilical vein endothelial cells (HUVECs). The amount of NO released by histamine-stimulated HUVECs was determined by differential pulse amperometry using a nickel porphyrin– and Nafion-coated carbon microfiber electrode. The effects of cholesterol (preserved from oxidation by butylated hydroxytoluene), 7-ketocholesterol, 7β-hydroxycholesterol, 5α,6α-epoxycholesterol, 19-hydroxycholesterol (60 µg/mL), and α-lysophosphatidylcholine (10 µg/mL) were compared. Pretreatment of HUVECs with cholesterol, 5α,6α-epoxycholesterol, or 19-hydroxycholesterol did not alter histamine-activated NO production. In contrast, pretreatment with 7-ketocholesterol or 7β-hydroxycholesterol significantly decreased NO release. The inhibitory effect of 7-ketocholesterol was time and dose dependent and was maintained in the presence of l-arginine. In the absence of serum, lysophosphatidylcholine also reduced NO production. In ionomycin-stimulated cells, pretreatment with 7-ketocholesterol did not inhibit NO release. These results demonstrate that cholesterol derivatives oxidized at the 7 position, the main products of low density lipoprotein oxidation, reduce histamine-activated NO release in HUVECs. Such an inhibitory effect of cholesterol oxides may account, at least in part, for the ability of oxidized low density lipoprotein to reduce the endothelium-dependent relaxation of arteries. (Arterioscler Thromb Vasc Biol. 1998;18:1054-1060.)

Key Words: human endothelial cells ■ cholesterol oxides ■ histamine ■ NO production

The endothelium of blood vessels regulates vascular tone by releasing endothelium-derived relaxing and contracting factors.1–3 In particular, it has now been clearly established that the endothelium-dependent relaxation of arteries induced by acetylcholine or histamine involves the release of endothelium-derived relaxing factor,4,5 identified as NO.6,7 Recent studies have demonstrated that alterations in vascular reactivity can be associated with cardiovascular disorders7–10 and that arteries from hypercholesterolemic and atherosclerotic patients exhibit marked attenuation of endothelium-dependent relaxation.7,11,12 Although the impairment of arterial relaxation at an early stage of the atherosclerotic process may constitute a crucial step in disease progression, the related molecular mechanisms remain unclear. Several studies have demonstrated that LDLs, rather than native LDLs, can mimic the impairment of arterial relaxation observed in hyperlipidemia.13–16 Whereas in some previous studies the inhibition of endothelium-dependent relaxation was attributed to LPC,14,15,17 other studies did not support this view.16,18 In particular, no significant relationship between the LPC content of LDLs and their ability to reduce endothelium-dependent relaxation was observed in a recent study from our group, despite a potent, concentration-dependent inhibition by pure LPC.19 The latter observations indicated that LPC may not constitute the major determinant of the ability of oxidized LDL to inhibit arterial relaxation. In fact, complementary investigations revealed that cholesterol derivatives oxidized in position 7, ie, 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol, unlike unmodified cholesterol, can reduce maximal arterial relaxation, and concordant observations were made by using either oxidized LDL, or pure cholesterol derivatives.19 Interestingly, we observed that the early inhibition of arterial relaxation was mediated through a specific effect of cholesterol oxides on vascular endothelial cells, independently of the cytotoxicity of these compounds.20

Since the endothelium-dependent relaxation of arteries is mainly regulated by NO production,3,5,6 we investigated in the current study the effect of cholesterol oxides and LPC on NO release by cultured HUVECs stimulated with histamine. Real-time measurements of NO release were performed by differential amperometry using a selective microelectrode.
**Selected Abbreviations and Acronyms**

HUVEC = human umbilical vein endothelial cell  
L-NMMA = Nω-monomethyl-L-arginine  
LPC = lysophosphatidylcholine  
NO(S) = nitric oxide (synthase)

### Methods

#### Cell Culture

HUVECs were isolated from segments (10 to 30 cm long) of human umbilical cord veins and cultured in medium 199 (40%) and RPMI 1640 (40%) containing FCS (20%), penicillin-streptomycin (100 U/mL–100 μg/mL), L-glutamine (2 mmol/L), and Fungizone (2.5 μg/mL). The cells were plated into 25-cm² flasks (Corning) and then incubated at 37°C in a humidified 5%–95% CO2/air atmosphere. The culture medium was renewed the following day and from then on, every other day. The cells were grown to confluence (4 to 5 days in culture), detached by incubation in PBS containing 0.01% trypsin and 0.005% EDTA for 1 to 2 minutes at room temperature, washed with the culture medium, centrifuged, and resuspended onto tissue-culture dishes (35-mm diameter, Corning). The cells were then allowed to grow to confluence again (3 to 5 days) until needed. For studies of cell morphology and viability, cells were resuspended into 6-well microplates (Nunc).

#### Cells Treatments

As indicated by the supplier, cholesterol derivatives were ≥98% pure. This high degree of purity was confirmed in our laboratory by capillary gas chromatography analysis. Cholesterol and cholesterol oxides were first dissolved in ethanol, and 1 volume of ethanolic solution was mixed with 150 to 600 volumes of culture medium, yielding final concentrations of the added compounds ranging from 30 to 120 μg/mL. Cells were incubated at 37°C with cholesterol or its oxidized derivatives from 30 to 300 minutes. For α-LPC treatment, cells were incubated for 15 minutes at 37°C with 10 μg/mL LPC added either directly to the culture medium or in PBS. At the end of the incubation period, cells were washed with PBS. They were then incubated for 5 minutes in PBS buffer complemented with 5 mmol/L glucose, 0.5 mmol/L MgCl₂, and 1 mmol/L CaCl₂ until measurement of NO production was performed. When specified, the antioxidant BHT was added to the culture medium at a final concentration of 20 μmol/L.

#### Measurement of NO Production

NO release was monitored with an NO-selective microprobe. The working electrode was a carbon microfiber (8-μm long), coated with tetrakis(3-methoxy-4-hydroxyphenyl) nickel(II) porphyrin and Nafion films. Differential pulse amperometry was performed with a 3-electrode potentiostatic EMS-100 system (Bio logic) as previously described. The probe was set 10 to 15 μm above the cell surface by using a micromanipulator (Biologic Instruments) attached to an inverted microscope (Zeiss). All apparatus was enclosed in a Faraday’s chamber. During NO measurement, cells were maintained at 37°C. Each culture dish received a unique dose of histamine (final concentration ranging from 0.1 to 100 μmol/L).

Internal calibration of the electrochemical sensor was performed for each experiment by adding NO standard solutions, as previously described. The sensitivity of electrodes varied from 0.7 to 2.7 nmol/L NO per pA (mean±SD, 1.8±0.4 nmol/L NO per pA, n=6) with a detection limit varying from 4 to 6 nmol/L NO. The identification of NO as the molecule responsible for the amperometric signal induced by histamine was assessed by inhibition of NO synthase by 1 mmol/L L-NMMA, as previously published.

In agreement with previous data obtained with the same electrode system, NO oxidation at 37°C in PBS gave a dose-dependent, linear current response over the 5 to 50 nmol/L concentration range (Figure 1). Similar electrochemical responses to NO were observed in the absence or presence of 60 μg/mL 7-ketocholesterol, indicating the absence of a direct interaction between 7-ketocholesterol and NO (Figure 1).

#### Determination of Cell Viability

Cell viability was determined in confluent cells cultured in 6-well microplates after the dead cells had been stained with propidium iodide. A stock solution of propidium iodide was prepared in PBS buffer at a final concentration of 100 μg/mL and kept in the dark at room temperature. Propidium iodide was used at a final concentration of 4 μg/mL on confluent cells. The fluorescence intensity (maximum excitation wavelength, 540 nm; maximum emission wavelength, 625 nm) was immediately evaluated by an image analysis system (Biocom). The morphological aspect of 300 cells was observed under an inverted phase-contrast microscope IX70 (Olympus).

#### Chemical Compounds

5α,6α-Epoxycholestan-3β-ol (5α,6α-epoxycholesterol), 5-cholestene-3β,7β-diol (7β-hydroxycholesterol), 5-cholestene-3β-ol-7-one (7-ketocholesterol), 5-cholestone-3β,19-diol (19-hydroxycholesterol), cholesterol, LPC, histamine dihydrochloride, EDTA, NO gas, Nafion, BHT, L-arginine, and propidium iodide were purchased from Sigma-Aldrich Chimie. Medium 199 was purchased from Eurobio. RPMI 1640 medium, FCS, L-glutamine, penicillin-streptomycin, Fungizone, and PBS were obtained from Gibco-BRL. Tetraakis(3-methoxy-4-hydroxyphenyl) nickel(II) porphyrin was obtained from Interchim. L-NMMA was purchased from Alexis Corp.

#### Statistical Analysis

Results are expressed as mean±SEM. Multiple comparisons, dose responses, and time-dependent effects were examined by 1-way ANOVA and post hoc Fisher’s test. Comparisons of dose-response effects between 2 different groups were assessed by 2-way ANOVA.

#### Results

**Effect of Cholesterol, Cholesterol Oxides, and LPC on Histamine-Activated NO Production**

Figure 2 shows a typical amperogram obtained with HUVECs stimulated with 10 μmol/L histamine. The rise in oxidation current produced by control cells was due to NO release, as indicated by its marked decrease when the cells were preincubated for 5 minutes with 1 mmol/L L-NMMA (NO release decreased by >90%). It reached a concentration of 26.1±4.2 nmol/L (n=12). The preincubation of cells for 3 hours at 37°C in the presence of 60 μg/mL cholesterol markedly decreased the histamine-induced production of NO (by 61.5±9.4%, n=8) (Figure 3). A similar incubation with 60 μg/mL 7-ketocholesterol also decreased the histamine-induced production of NO (by 75.2±9.2%, n=13).
The possibility of cholesterol oxidation during incubation in the culture medium had first been checked. The addition of BHT (20 μmol/L) had no significant effect on the production of NO induced by histamine either in control cells or in 7-ketocholesterol–treated cells, but it totally prevented the inhibitory effect of cholesterol (Figure 3). These observations indicate that oxidation of cholesterol probably occurred during the incubation period. In subsequent experiments, BHT was systematically added to the incubation medium to avoid oxidation of cholesterol.

When cells were pretreated with 5α,6α-epoxycholesterol or 19-hydroxycholesterol, the NO production evoked by histamine was not significantly modified. In contrast, another cholesterol derivative oxidized at position 7, 7β-hydroxycholesterol, also significantly inhibited histamine-induced NO release (Table 1). The preincubation of HUVECs with 10 μg/mL LPC added in the culture medium containing 20% serum had no effect on histamine-activated NO production (107±25% of the control value, n=7). However, when preincubated in serum-free PBS, LPC inhibited NO release by 46±17% (P=0.03).

### Concentration- and Time-Dependent Effects of 7-Ketocholesterol and Cholesterol on Histamine-Induced Release of NO From HUVECs

As shown in Figure 4, pretreatment for 2 hours with cholesterol concentrations ranging from 30 to 120 μg/mL did not modify NO release from histamine-stimulated HUVECs. On the contrary, similar pretreatment with 7-ketocholesterol dose-dependently decreased NO release (F=4.97, P=0.003). At a dose of 120 μg/mL, 7-ketocholesterol completely suppressed histamine-induced NO production (Figure 4). Under these conditions, the dose–responses curves of 7-ketocholesterol and cholesterol significantly differed from each other (F=3.0, P=0.02).

Increasing the incubation time to 300 minutes with cholesterol (60 μg/mL) did not modify the NO concentrations measured after histamine stimulation (25.0±4.0 and 26.0±5.0 nmol/mL with and without cholesterol, respectively). These values did not differ significantly from the NO levels measured with control cells at zero time (24.9±3.7 nmol/mL). In contrast, as shown in Figure 5, NO levels measured after stimulation by 10 μmol/L histamine gradually decreased with the length of incubation time with 7-ketocholesterol (from 30 to 300 minutes; F=6.3, P=0.002). The decrease in NO concentration became statistically significant after 120 minutes of preincubation with 60 μg/mL 7-ketocholesterol, and NO production was virtually abolished after 300 minutes of pretreatment. These results indicate that the long-lasting presence of low 7-ketocholesterol concentrations could lead to significant reductions in endothelial NO release.

### Table 1. Comparative Effects of Cholesterol and Cholesterol Oxides on Histamine-Induced NO Release by HUVECs

<table>
<thead>
<tr>
<th>Pretreatment</th>
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<tr>
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<td>6</td>
<td>NS</td>
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<tr>
<td>7-Ketocholesterol</td>
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<td>26</td>
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</tr>
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HUVECs were pretreated for 2 hours at 37°C with 60 μg/mL cholesterol or cholesterol oxides. They were then washed and incubated for 5 minutes in PBS before stimulation with 10 μmol/L histamine. Results are expressed as percentage of control values measured in untreated cells mean±SEM. Values obtained in pre-treated HUVECs were compared with those of untreated cells.

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Influence of L-Arginine and Histamine Concentrations on 7-Ketocholesterol–Induced Decrease in NO Production

To investigate whether the inhibition of NO release could be due to an effect of 7-ketocholesterol on L-arginine availability, similar experiments were repeated after L-arginine supplementation. Addition of 1 mmol/L L-arginine increased NO production in both control and 7-ketocholesterol–treated cells (Table 2). However, the NO oxidation current in L-arginine–supplemented cells remained reduced in cells pretreated with 7-ketocholesterol, and the inhibitory effect of 7-ketocholesterol was similar in the absence and presence of L-arginine (45±13% versus 31±8%, respectively).

Another possibility was that 7-ketocholesterol could modify the efficacy of histamine to stimulate NO production. The influence of a given 7-ketocholesterol concentration (60 μg/mL) on the NO response to various histamine concentrations was therefore determined. Histamine from 0.1 to 100 μmol/L induced concentration-dependent increases in the amount of NO released (F=5.3, P=0.006; Figure 6). Irrespective of the histamine concentration studied, NO production was similarly lowered by 7-ketocholesterol (F=6.4, P<0.001).

Table 2. Influence of L-Arginine Supplementation on the Inhibitory Effect of 7-Ketocholesterol on Histamine-Stimulated NO Release

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<th>NO Production, % of Control</th>
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<tr>
<td></td>
<td>Pretreatment</td>
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<tr>
<td></td>
<td>L-arginine supplementation</td>
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<tr>
<td>Controls</td>
<td>100</td>
</tr>
<tr>
<td>7-Ketocholesterol</td>
<td>55±13†</td>
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</table>

HUVECs preincubated for 2 hours at 37°C with or without 60 μg/mL 7-ketocholesterol in culture medium were washed and then incubated for 5 minutes in the presence or absence of mmol/L L-arginine in PBS before stimulation with 10 μmol/L histamine. Results are expressed as percentage of control values measured in untreated cells (mean±SEM). Data are from 9 independent experiments.

*P<0.01 and †P<0.05, respectively, when compared with NO release by untreated cells.

‡P<0.05 when compared with NO release by 7-ketocholesterol–treated cells.

§P<0.01 when compared with NO release by L-arginine–treated cells.

Effect of 7-Ketocholesterol on Cell Morphology and Viability

As assessed by phase-contrast microscopy, HUVECs preincubated for 120 minutes with 7-ketocholesterol (60 μg/mL) presented typical endothelial cell shapes and intercellular connections (results not shown). The total number of cells per microwell did not differ significantly with and without pretreatment with 7-ketocholesterol (2.2±0.2×10⁴ and 2.0±0.3×10⁴ cells, respectively). In addition, detection of dead cells with fluorescence microscopy after propidium iodide staining revealed that the relative proportions of dead cells with and without 7-ketocholesterol treatment were low and remarkably similar (1.2±0.5% and 1.0±0.4%, respectively). This indicates that the inhibitory effect of 7-ketocholesterol was not due to alterations of cell morphology and viability.

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Discussion

This study demonstrates that cholesterol derivatives oxidized at position 7 and LPC, but not unmodified cholesterol or other cholesterol oxides, can significantly reduce the histamine-induced release of NO from HUVECs. These observations extend our recent data revealing that 7-ketocholesterol and 7β-hydroxycholesterol, but not cholesterol, account for the ability of oxidized LDL to reduce the endothelium-dependent relaxation of the isolated rabbit aorta.19

It is now well established that oxidized LDL can play a determinant role in cardiovascular disease by inducing alterations in both the morphology and reactivity of blood vessels. In particular, recent studies indicated that oxidized LDL might contribute to the occurrence of vasospasms that have been observed in hypercholesterolemic and atherosclerotic patients.10,28,29 Although oxidized LDLs were shown to have been observed in hypercholesterolemic and atherosclerotic patients.10,28,29 Although oxidized LDLs were shown to enhance contraction and to impair the endothelium-dependent relaxation of arteries,14,16,18,31 the precise mechanism accounting for their vascular effects has not been clearly identified. We recently demonstrated that cholesterol oxides, and in particular the cholesterol derivatives oxidized at position 7 (ie, 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol), can constitute potent inhibitors of the maximal relaxation of rabbit aortic rings.19 Interestingly, concordant observations were made by using either oxidized LDL or pure cholesterol derivatives. Whereas the inhibition of arterial relaxation by cholesterol oxides was proven to be an endothelium-dependent process because they do not alter the endothelium-independent relaxation to sodium nitroprusside,19 it remained to be established whether they decrease the release of NO. This radical is a reactive molecule with a short half-life in vivo. Its production by endothelial cells has been mainly assessed in earlier studies by bioassays, chemiluminescence of its reaction product with ozone, quantification of nitrates, or electron paramagnetic resonance spectroscopy based on the reaction of NO with spin-trap molecules. These experimental methods are either indirect, or NO consuming with possible drifts in NO production, or related to total NO production and not its real concentration. To directly evaluate the concentrations of NO actually released by endothelial cells, we used a nickel porphyrin–and Nafion-coated carbon microfiber electrode that allows detection of low NO levels.22,23,32–35 Experiments of NO release were conducted on histamine-stimulated HUVECs, an experimental system in which the release of NO has been well documented.24,25,34,35 The real-time measurement of NO by electrochemical detection revealed for the first time that both 7-ketocholesterol and 7β-hydroxycholesterol directly reduced the amounts of NO released by stimulated HUVECs. With the relatively short incubation periods used in the current study, no cytotoxicity was observed, indicating that the reduction of NO release cannot be explained by the cytotoxic, apoptotic properties of 7-ketocholesterol on endothelial cells that has been observed after much longer incubation periods.36 The inhibition of NO release by 7-ketocholesterol and 7β-hydroxycholesterol in HUVECs suggests that a similar mechanism may account for the rapid inhibition of endothelium-dependent relaxation of rabbit aortas by cholesterol derivatives oxidized at position 7.19 The inability of 5α,6α-epoxycholesterol and 19-hydroxycholesterol to alter NO release also agrees with their lack of effects on endothelium-dependent arterial relaxation. Although cholesterol itself could also significantly decrease NO production, its inhibitory effect was abolished by addition of BHT, suggesting that in the absence of an antioxidant, uncontrolled oxidation of cholesterol probably occurred in the culture medium and resulted in artifactual generation of NO production inhibitors.

In agreement with the inhibitory effect of LPC on endothelium-dependent arterial relaxation previously reported,19 we observed that LPC significantly reduced NO release. This observation was not made when LPC was added in the presence of serum-containing culture medium, in which LPC-albumin or LPC-lipoprotein complexes are likely to be formed. This raises the possibility that LPC sequestration by albumin or lipoproteins reduces its ability to inhibit NO release by endothelial cells.

The mechanisms by which oxidized LDL modify NO synthesis or degradation remain unclear. Direct inactivation of NO by oxidized LDLs has been proposed as a possible mechanism of their inhibitory effect on endothelium-dependent relaxation.36,37 This is unlikely to account for the reduction of NO release by cholesterol derivatives oxidized at position 7 observed in the current study, as indicated by the unchanged electrode response to authentic NO solutions in the presence of 7-ketocholesterol. In addition, all experiments were performed on washed cells after removal of the cholesterol derivatives or LPC. An increased breakdown of NO by oxygen-containing free radicals, such as superoxide anion, hydroxyl radical, or H₂O₂, has also been suspected in hypercholesterolemic or atherosclerotic animal models.38–40 and endothelium-dependent vasodilation was reported to be improved by SOD.41 However, the inability of SOD to antagonize inhibition of endothelium-derived relaxing factor production by cholesterol oxide–containing oxidized LDLs41,42 and to correct the altered endothelium-dependent relaxation in patients with hypercholesterolemia.43 did not support the concept of enhanced inactivation of NO by anion superoxide in 7-ketocholesterol–treated cells.

The current results raise the possibility of inhibition of NO synthesis by cholesterol derivatives oxidized at position 7. During activation of endothelial cells by agonists, the uptake of l-arginine, the substrate of NOS, increases,44 and NOS translocates from the membrane to the cytosol by binding of the Ca²⁺-calmodulin complex.45 Among others, 7-ketocholesterol could have modified the activity of the l-arginine carrier or the Ca²⁺-dependent mechanism of NOS translocation. We observed that l-arginine supplementation did not modify the relative 7-ketocholesterol–induced reduction of NO release, indicating that this inhibition was independent of substrate availability. Concerning possible alterations of the Ca²⁺-dependent NOS activation, we observed that 7-ketocholesterol inhibited the NO production associated with receptor activation but not that resulting from the increase in cytosolic Ca²⁺ concentration due to ionomycin. This suggests that 7-ketocholesterol did not reduce NO release by altering the Ca²⁺-dependent NOS activation steps. Another possible mechanism is a change in the membrane physicochemical properties, similar to that induced by oxidized LDL.46 Cho-
Lesterol oxides have indeed been reported to alter the activity of various membrane-bound enzymes, eg, Na^+, K^+-ATPase, Ca^{2+}-ATPase, and protein kinase C. Membrane-dependent phenomena and particularly acylation processes participate in anchoring NOS in cell membranes and thereby in regulating its activity. A characteristic of endothelial NOS is to be targeted to signal-transducing membrane microdomains called plasmalemmal caveolae. These caveolae are involved in the storage and processing of various cellular messengers and in initiation of phosphorylation cascades. Membrane cholesterol is essential for their normal function. Oxidation of cholesterol at position 7 may therefore alter endothelial NO production through direct effects on caveolae.

In conclusion, cholesterol derivatives oxidized at position 7, which include a wide family of molecules resulting from either oxidation of LDL or oxidation of cholesterol in air, or enzymatic transformation of cholesterol, can reduce the production of NO by endothelial cells. Because significant amounts of cholesterol oxides have been found in human hypercholesterolemic plasma, and in many processed foods, the observations of the current study could be of pathophysiological relevance. Whereas the observed reduction of NO release by cholesterol derivatives oxidized at position 7 is likely to influence vascular tone, the inhibition of vasorelaxation by oxidized LDL was not necessarily associated with concomitant decreases in NO production in previous studies. Contrasting observations made with pure cholesterol oxides and with oxidized LDL may indicate a high complexity of interference by oxidized LDL with the endothelium-dependent relaxation that probably combines acute and chronic effects.

Acknowledgments

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