

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

Valérie Deckert, Annie Brunet, Frédérique Lantoin, 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, 7 $\beta$ -hydroxycholesterol, 5 $\alpha$ ,6 $\alpha$ -epoxycholesterol, 19-hydroxycholesterol (60  $\mu$ g/mL), and  $\alpha$ -lysophosphatidylcholine (10  $\mu$ g/mL) were compared. Pretreatment of HUVECs with cholesterol, 5 $\alpha$ ,6 $\alpha$ -epoxycholesterol, or 19-hydroxycholesterol did not alter histamine-activated NO production. In contrast, pretreatment with 7-ketocholesterol or 7 $\beta$ -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.<sup>1–3</sup> 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,<sup>1,4</sup> identified as NO.<sup>5,6</sup> Recent studies have demonstrated that alterations in vascular reactivity can be associated with cardiovascular disorders<sup>7–10</sup> and that arteries from hypercholesterolemic and atherosclerotic patients exhibit marked attenuation of endothelium-dependent relaxation.<sup>7,11,12</sup> 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.<sup>13–16</sup> Whereas in some previous studies the inhibition of endothelium-dependent relaxation was attributed to LPC,<sup>14,15,17</sup> other studies did not support this view.<sup>16,18</sup> In particular, no significant relationship between the LPC content of LDLs and their ability to reduce endothelium-dependent relax-

ation was observed in a recent study from our group, despite a potent, concentration-dependent inhibition by pure LPC.<sup>19</sup> 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, 7 $\alpha$ -hydroxycholesterol, and 7 $\beta$ -hydroxycholesterol, unlike unmodified cholesterol, can reduce maximal arterial relaxation, and concordant observations were made by using either oxidized LDL or pure cholesterol derivatives.<sup>19</sup> 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.<sup>20</sup>

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

Received August 27, 1997; revision accepted January 22, 1998.

From the Laboratoire de Pharmacologie, URA CNRS 1482, Université Paris V, Faculté de Médecine Necker, Paris (A.B., F.L., E.M.-van B., M.D.-D., M.-A.D.), and the Laboratoire de Biochimie des Lipoprotéines, INSERM U498, Faculté de Médecine, Dijon (V.D., G.L., S.M., L.L., P.G.), France.

Correspondence to Marie-Aude Devynck, Laboratoire de Pharmacologie, URA CNRS 1482, Université Paris V, Faculté de Médecine Necker, Paris, France.

© 1998 American Heart Association, Inc.

**Selected Abbreviations and Acronyms**

HUVEC = human umbilical vein endothelial cell  
 L-NMMA = *N*<sup>G</sup>-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).<sup>21</sup> The cells were plated into 25-cm<sup>2</sup> flasks (Corning) and then incubated at 37°C in a humidified 5%–95% CO<sub>2</sub>/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 reseeded 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 reseeded into 6-well microplates (Nunc).

**Cells Treatments**

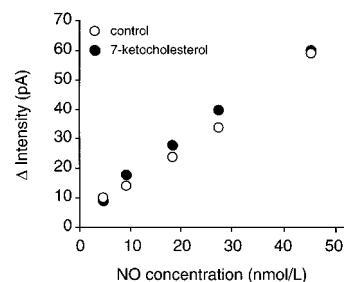
As indicated by the supplier, cholesterol derivatives were ≥98% pure. This high degree of purity was confirmed in our laboratory by capillary gas chromatography analysis. Cholesterol and cholesterol oxides were first dissolved in ethanol, and 1 volume of ethanolic solution was mixed with 150 to 600 volumes 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 MgCl<sub>2</sub>, and 1 mmol/L CaCl<sub>2</sub> 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.<sup>22</sup> The working electrode was a carbon microfiber (8-µm diameter, ≈1 mm 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 (Biologic) as previously described.<sup>22</sup> 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 measurements, 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.<sup>23</sup> 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.<sup>23,24</sup>

In agreement with previous data obtained with the same electrode system,<sup>22</sup> 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



**Figure 1.** Electrochemical detection of NO. Graph shows oxidation current responses to authentic NO added in PBS supplemented (●) or not (○) with 60 µg/mL 7-ketocholesterol.

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.<sup>20,25</sup> 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**

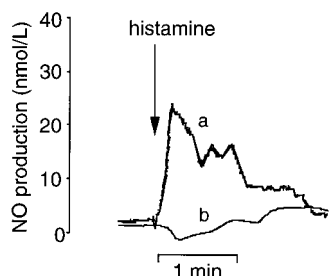
5α,6α-Epoxycholestan-3β-ol (5α,6α-epoxycholesterol), 5-cholestene-3β,7β-diol (7β-hydroxycholesterol), 5-cholestene-3β-ol-7-one (7-ketocholesterol), 5-cholestene-3β,19-diol (19-hydroxycholesterol), cholesterol, LPC, histamine dihydrochloride, EDTA, NO gas, Nafion, BHT, L-arginine, and propidium iodide 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. Tetrakis(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%<sup>23,24</sup>). 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).



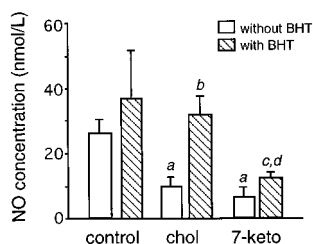
**Figure 2.** Time course of histamine-induced NO production by HUVECs. Cells were preincubated (b) or not (a) with 7-ketocholesterol (60  $\mu\text{mol/L}$ ) for 3 hours, then washed, and stimulated by 10  $\mu\text{mol/l}$  histamine.

The possibility of cholesterol oxidation during incubation in the culture medium had first been checked. The addition of BHT (20  $\mu\text{mol/L}$ ) 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 5 $\alpha$ ,6 $\alpha$ -epoxycholesterol or 19-hydroxycholesterol, the NO production evoked by histamine was not significantly modified. In contrast, another cholesterol derivative oxidized at position 7, 7 $\beta$ -hydroxycholesterol, also significantly inhibited histamine-induced NO release (Table 1). The preincubation of HUVECs with 10  $\mu\text{g/mL}$  LPC added in the culture medium containing 20% serum had no effect on histamine-activated NO production (107 $\pm$ 25% of the control value,  $n=7$ ). However, when preincubated in serum-free PBS, LPC inhibited NO release by 46 $\pm$ 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  $\mu\text{g/mL}$  did not



**Figure 3.** Effect of preincubation with cholesterol or 7-ketocholesterol on histamine-induced release of NO from HUVECs. Bar graph shows amount of NO released from cells stimulated by 10  $\mu\text{mol/L}$  histamine after preincubation for 3 hours with vehicle or with 60  $\mu\text{g/mL}$  cholesterol or 7-ketocholesterol. Compounds were added in the presence (hatched bars) or absence (open bars) of BHT (20  $\mu\text{mol/L}$ ). Each point represents the mean $\pm$ SEM of 4 to 13 experiments. <sup>a</sup> $P<0.01$  vs control without BHT samples; <sup>b</sup> $P<0.01$  vs cholesterol without BHT samples; <sup>c</sup> $P<0.05$  vs control with BHT samples; and <sup>d</sup> $P<0.05$  vs cholesterol with BHT samples.

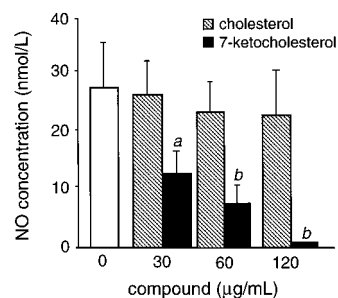
**TABLE 1. Comparative Effects of Cholesterol and Cholesterol Oxides on Histamine-Induced NO Release by HUVECs**

Pretreatment	NO Production, %	n	P
Cholesterol	96 $\pm$ 13	6	NS
7-Ketocholesterol	62 $\pm$ 9	26	<0.001
7 $\beta$ -Hydroxycholesterol	51 $\pm$ 9	9	<0.001
5 $\alpha$ ,6 $\alpha$ -Epoxycholesterol	88 $\pm$ 13	7	NS
19-Hydroxycholesterol	87 $\pm$ 10	7	NS

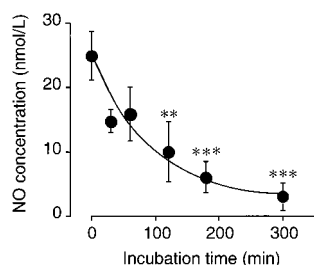
HUVECs were pretreated for 2 hours at 37°C with 60  $\mu\text{g/mL}$  cholesterol or cholesterol oxides. They were then washed and incubated for 5 minutes in PBS before stimulation with 10  $\mu\text{mol/L}$  histamine. Results are expressed as percentage of control values measured in untreated cells mean $\pm$ SEM. Values obtained in pretreated HUVECs were compared with those of untreated cells.

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  $\mu\text{g/mL}$ , 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  $\mu\text{g/mL}$ ) did not modify the NO concentrations measured after histamine stimulation (25.0 $\pm$ 4.0 and 26.0 $\pm$ 5.0 nmol/L with and without cholesterol, respectively). These values did not differ significantly from the NO levels measured with control cells at zero time (24.9 $\pm$ 3.7 nmol/L). In contrast, as shown in Figure 5, NO levels measured after stimulation by 10  $\mu\text{mol/L}$  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  $\mu\text{g/mL}$  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.



**Figure 4.** Concentration-dependent effect of cholesterol and 7-ketocholesterol on histamine-induced release of NO from HUVECs. Graph shows amount of NO released by 10  $\mu\text{mol/L}$  histamine after preincubation of cells for 2 hours at 37°C without (open bars) or with 30, 60, or 120  $\mu\text{g/mL}$  of either 7-ketocholesterol (filled bars) or cholesterol (hatched bars). Each point represents mean $\pm$ SEM of 5 to 13 independent experiments. <sup>a</sup> $P<0.05$  and <sup>b</sup> $P<0.01$  vs control values.



**Figure 5.** Time-dependent inhibition of histamine-induced release of NO from HUVECs pretreated with 7-ketocholesterol. Graph shows release of NO induced by 10  $\mu\text{mol/L}$  histamine after preincubation with 60  $\mu\text{g/mL}$  7-ketocholesterol for 0 to 300 minutes. Each point represents mean  $\pm$  SEM of 4 to 13 independent experiments. \*\* $P < 0.01$  and \*\*\* $P > 0.001$ , respectively vs values at time zero.

### 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 ( $45 \pm 13\%$  versus  $31 \pm 8\%$ , 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  $\mu\text{g/mL}$ ) on the NO response to various histamine concentrations was therefore determined. Histamine from 0.1 to 100  $\mu\text{mol/L}$  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**

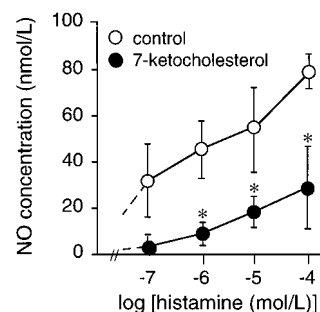
Pretreatment	NO Production, % of Control	
	-1 mmol/L L-Arginine	+1 mmol/L L-Arginine
Controls	100	169 $\pm$ 20*
7-Ketocholesterol	55 $\pm$ 13†	125 $\pm$ 17‡§

HUVECs preincubated for 2 hours at 37°C with or without 60  $\mu\text{g/mL}$  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  $\mu\text{mol/L}$  histamine. Results are expressed as percentage of control values measured in untreated cells (mean  $\pm$  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.



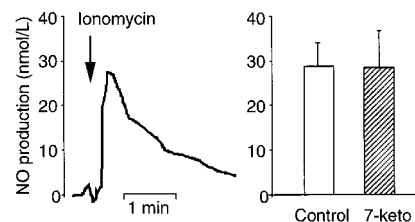
**Figure 6.** Influence of histamine concentrations on 7-ketocholesterol-induced reduction of NO release. Cells were preincubated for 2 hours at 37°C in the presence (●) or absence (○) of 7-ketocholesterol (60  $\mu\text{g/mL}$ ), washed, and then stimulated by histamine concentrations ranging from 0.1 to 100  $\mu\text{mol/L}$ . Each point represents mean  $\pm$  SEM of 3 to 5 independent experiments. \* $P < 0.05$  vs matched control values.

### Effect of 7-Ketocholesterol on Ionomycin-Induced NO Production

To test whether the inhibitory effect described above was also observed in the absence of receptor stimulation, we studied the influence of 7-ketocholesterol on NO production induced by the  $\text{Ca}^{2+}$  ionophore ionomycin. As shown in Figure 7, NO production reached a maximum within 15 seconds after ionomycin addition and returned slowly toward basal levels. Preincubation for 2 hours with 60  $\mu\text{g/mL}$  7-ketocholesterol did not alter the amplitude of ionomycin-activated NO release (Figure 7).

### Effect of 7-Ketocholesterol on Cell Morphology and Viability

As assessed by phase-contrast microscopy, HUVECs pretreated for 120 minutes with 7-ketocholesterol (60  $\mu\text{g/mL}$ ) 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 ( $2.2 \pm 0.2 \times 10^5$  and  $2.0 \pm 0.3 \times 10^5$  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 ( $1.2 \pm 0.5\%$  and  $1.0 \pm 0.4\%$ , respectively). This indicates that the inhibitory effect of 7-ketocholesterol was not due to alterations of cell morphology and viability.



**Figure 7.** Effect of 7-ketocholesterol on ionomycin-induced NO production. Left panel illustrates time course of NO production resulting from addition of 4  $\mu\text{mol/L}$  ionomycin, as indicated by the arrow (trace representative of 5 independent experiments). Right panel shows maximal NO release in control and 7-ketocholesterol-pretreated cells.

## 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 7 $\beta$ -hydroxycholesterol, but not cholesterol, account for the ability of oxidized LDL to reduce the endothelium-dependent relaxation of the isolated rabbit aorta.<sup>19</sup>

It is now well established that oxidized LDL can play a determinant role in cardiovascular disease by inducing alterations in both the morphology<sup>26,27</sup> and reactivity<sup>10,28</sup> 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.<sup>10,28,29</sup> Although oxidized LDLs were shown to enhance contraction<sup>13,30</sup> and to impair the endothelium-dependent relaxation of arteries,<sup>14,16,18,31</sup> 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, 7 $\alpha$ -hydroxycholesterol, and 7 $\beta$ -hydroxycholesterol), can constitute potent inhibitors of the maximal relaxation of rabbit aortic rings.<sup>19</sup> 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,<sup>19</sup> 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 nitrites, 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.<sup>22,23,32-35</sup> Experiments of NO release were conducted on histamine-stimulated HUVECs, an experimental system in which the release of NO has been well documented.<sup>24,25,34,35</sup> The real-time measurement of NO by electrochemical detection revealed for the first time that both 7-ketocholesterol and 7 $\beta$ -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.<sup>20</sup> The inhibition of NO release by 7-ketocholesterol and 7 $\beta$ -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.<sup>19</sup> The inability of 5 $\alpha$ ,6 $\alpha$ -epoxycholesterol and 19-hydroxy-

cholesterol 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,<sup>19</sup> 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.<sup>36,37</sup> 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 H<sub>2</sub>O<sub>2</sub>, has also been suspected in hypercholesterolemic or atherosclerotic animal models,<sup>38-40</sup> and endothelium-dependent vasodilation was reported to be improved by SOD.<sup>41</sup> However, the inability of SOD to antagonize inhibition of endothelium-derived relaxing factor production by cholesterol oxide-containing oxidized LDLs<sup>36,42</sup> and to correct the altered endothelium-dependent relaxation in patients with hypercholesterolemia,<sup>43</sup> 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,<sup>44</sup> and NOS translocates from the membrane to the cytosol by binding of the Ca<sup>2+</sup>-calmodulin complex.<sup>45</sup> Among others, 7-ketocholesterol could have modified the activity of the L-arginine carrier or the Ca<sup>2+</sup>-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 Ca<sup>2+</sup>-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 Ca<sup>2+</sup> concentration due to ionomycin. This suggests that 7-ketocholesterol did not reduce NO release by altering the Ca<sup>2+</sup>-dependent NOS activation steps. Another possible mechanism is a change in the membrane physicochemical properties, similar to that induced by oxidized LDL.<sup>46</sup> Cho-

lesterol oxides have indeed been reported to alter the activity of various membrane-bound enzymes, eg,  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase,  $\text{Ca}^{2+}$ -ATPase, and protein kinase C.<sup>47-49</sup> Membrane-dependent phenomena and particularly acylation processes participate in anchoring NOS in cell membranes<sup>50,51</sup> and thereby in regulating its activity. A characteristic of endothelial NOS is to be targeted to signal-transducing membrane microdomains called plasmalemmal caveolae.<sup>51,52</sup> These cavolae are involved in the storage and processing of various cellular messengers and in initiation of phosphorylation cascades.<sup>53</sup> Membrane cholesterol is essential for their normal function.<sup>54</sup> 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,<sup>55-57</sup> autooxidation of cholesterol in air,<sup>58</sup> or enzymatic transformation of cholesterol,<sup>59</sup> can reduce the production of NO by endothelial cells. Because significant amounts of cholesterol oxides have been found in human hypercholesterolemic plasma,<sup>60,61</sup> in atherosclerotic arteries,<sup>61,62</sup> and in many processed foods,<sup>58,63,64</sup> 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.<sup>31,65</sup> 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.<sup>28</sup>

### Acknowledgments

This work was supported by grants from the Center National de la Recherche Scientifique (Ultimatech No. 95N89/0045), the Ministère de l'Enseignement Supérieur et de la Recherche (ACC SV11 No. 9511021), the Institut National de la Santé et de la Recherche Médicale (INSERM), and the Conseil Régional de Bourgogne. The help of F. Schaller for collecting umbilical cords from the Robert Debré Hospital maternity is gratefully acknowledged.

### References

- Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980;288:373-376.
- Shepherd JT, Katusic ZS. Endothelium-derived vasoactive factors. I: endothelium-dependent relaxation. *Hypertension*. 1991;18(suppl III):III-76-III-85.
- Vanhoutte PM, Lüscher TF, Gräser T. Endothelium-dependent contractions. *Blood Vessels*. 1991;28:74-83.
- Van de Voorde J, Leusen I. Role of the endothelium in the vasodilator response of rat thoracic aorta to histamine. *Eur J Pharmacol*. 1983;87:113-120.
- Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*. 1987;327:524-526.
- Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A*. 1987;84:9265-9269.
- Tanner FC, Boulanger CM, Lüscher TF. Endothelium-derived nitric oxide, endothelin, and platelet vessel wall interaction: alterations in hypercholesterolemia and atherosclerosis. In: Thieme Medical Publishers, ed. *Seminars in Thrombosis and Hemostasis*. New York, NY: Thieme Medical Publishers; 1993;19:167-175.
- Lüscher TF, Boulanger CM, Yang Z, Noll G, Dohi Y. Interactions between endothelium-derived relaxing and contracting factors in health and cardiovascular disease. *Circulation*. 1993;87(suppl V):V-36-V-44.
- Cohen RA. The role of nitric oxide and other endothelium-derived vasoactive substances in vascular disease. *Prog Cardiovasc Dis*. 1995;38:105-128.
- Busse R, Fleming I. Endothelial dysfunction in atherosclerosis. *J Vasc Res*. 1996;33:181-194.
- Creager MA, Cooke JP, Mendelsohn ME, Gallagher SJ, Coleman SM, Loscalzo J, Dzau VJ. Impaired vasodilatation of forearm resistance vessels in hypercholesterolemic humans. *J Clin Invest*. 1990;86:228-234.
- Zeiger AM, Drexler H, Wollschläger H, Just H. Modulation of coronary vasomotor tone in humans: progressive endothelial dysfunction with different early stages of coronary atherosclerosis. *Circulation*. 1991;83:391-401.
- Simon BC, Cunningham LD, Cohen RA. Oxidized low density lipoproteins cause contraction and inhibit endothelium-dependent relaxation in the pig coronary artery. *J Clin Invest*. 1990;86:75-79.
- Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature*. 1990;344:160-162.
- Yokoyama M, Hirata K-I, Miyake R, Akita H, Ishikawa Y, Fukuzaki H. Lysophosphatidylcholine: essential role in the inhibition of endothelium-dependent vasorelaxation by oxidized low density lipoprotein. *Biochem Biophys Res Commun*. 1990;168:301-308.
- Tanner FC, Noll G, Boulanger CM, Lüscher TF. Oxidized low density lipoproteins inhibit relaxations of porcine coronary arteries: role of scavenger receptor and endothelium-derived nitric oxide. *Circulation*. 1991;83:2012-2020.
- Mangin EL, Kugiyama K, Nguy JH, Kerns SA, Henry PD. Effects of lysolipids and oxidatively modified low density lipoprotein on endothelium-dependent relaxation of rabbit aorta. *Circ Res*. 1993;72:161-166.
- Plane F, Bruckdorfer RK, Kerr P, Steuer A, Jacobs M. Oxidative modification of low-density lipoproteins and the inhibition of relaxations mediated by endothelium-derived nitric oxide in rabbit aorta. *Br J Pharmacol*. 1992;105:216-222.
- Deckert V, Persegol L, Viens L, Lizard G, Athias A, Lallemand C, Gambert P, Lagrost L. Inhibitors of arterial relaxation among components of human oxidized low-density lipoproteins: cholesterol derivatives oxidized in position 7 are potent inhibitors of endothelium-dependent relaxation. *Circulation*. 1997;95:723-731.
- Lizard G, Deckert V, Dubrez L, Moisan M, Gambert P, Lagrost L. Induction of apoptosis in endothelial cells treated with cholesterol oxides. *Am J Pathol*. 1996;148:1625-1638.
- Iouzalen L, Devynck MA, David-Dufilho M. Israpidine affects histamine-induced cytosolic  $\text{Ca}^{2+}$  movements in human endothelial cells. *Eur J Pharmacol*. 1995;289:189-195.
- Lantoine F, Trévin S, Bedioui F, Devynck J. Selective and sensitive electrochemical measurement of nitric oxide in aqueous solution: discussion and new results. *J Electroanal Chem*. 1995;392:85-89.
- Bedioui F, Trévin S, Devynck J, Lantoine F, Brunet A, Devynck MA. Elaboration and use of nickel planar macrocyclic complex-based sensors for the direct electrochemical measurement of nitric oxide in biological media. *Biosens Bioelectron*. 1997;12:205-212.
- Privat C, Lantoine F, Bedioui F, Millanvoe E, Devynck J, Devynck MA. Nitric oxide production in endothelial cells: comparison of three methods of quantification. *Life Sci*. 1997;61:1193-1202.
- Yeh CG, Hsi B, Faulk WP. Propidium iodide as a nuclear marker in immunofluorescence. II: use with cellular identification and viability studies. *J Immunol Methods*. 1981;43:269-275.
- Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med*. 1989;320:915-924.
- Berliner JA, Haberland ME. The role of low-density lipoprotein in atherogenesis. *Curr Opin Lipidol*. 1993;4:373-381.
- Cox DA, Cohen ML. Effects of oxidized low-density lipoprotein on vascular contraction and relaxation: clinical and pharmacological implications in atherosclerosis. *Pharmacol Rev*. 1996;48:3-19.
- Meredith IT, Yeung AC, Weidinger FF, Anderson TJ, Uehata A, Ryan TJ, Selwyn AP, Ganz PG. Role of impaired endothelium-dependent vasodilation in ischemic manifestations of coronary artery disease. *Circulation*. 1993;87(suppl V):V-56-V-66.
- Galle J, Bassenge E, Busse R. Oxidized low density lipoproteins potentiate vasoconstrictions to various agonists by direct interactions with vascular smooth muscle. *Circ Res*. 1990;66:1287-1293.

31. Myers PR, Wright TF, Tanner MA, Ostlund RE. The effects of native LDL and oxidized LDL on EDRF bioactivity and nitric oxide production in vascular endothelium. *J Lab Clin Med.* 1994;124:672–683.
32. Malinski T, Taha Z. Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor. *Nature.* 1992;358:676–678.
33. Schüssler O, Lantoiné F, Devynck MA, Glotz D, David-Duflho M. Human immunoglobulins inhibit thrombin-induced  $\text{Ca}^{2+}$  movements and nitric oxide production in endothelial cells. *J Biol Chem.* 1996;271:26473–26476.
34. Iouzalen L, Lantoiné F, Pernollet MG, Millanvoye-Van Brussel E, Devynck M-A, David-Duflho M. SK&F 96365 inhibits intracellular  $\text{Ca}^{2+}$  pumps and raises cytosolic  $\text{Ca}^{2+}$  concentration in human endothelial cells without production of nitric oxide and von Willebrand factor. *Cell Calcium.* 1996;20:501–508.
35. Lantoiné F, Iouzalen L, Millanvoye van Brussel E, Devynck M-A, David-Duflho M. Nitric oxide production in human endothelial cells stimulated by histamine requires  $\text{Ca}^{2+}$  influx. *Biochem J.* 1998;30:695–699.
36. Chin JH, Azhar S, Hoffman BB. Inactivation of endothelial derived relaxing factor by oxidized lipoproteins. *J Clin Invest.* 1992;89:10–18.
37. Galle J, Mülsch A, Busse R, Bassenge E. Effects of native and oxidized low density lipoproteins on formation and inactivation of endothelium-derived relaxing factor. *Arterioscler Thromb.* 1991;11:198–203.
38. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest.* 1993;91:2546–2551.
39. Mügge A, Brandes RP, Böger RH, Dwenger A, Bode-Böger S, Kienke S, Frölich JC, Lichtlen PR. Vascular release of superoxide radicals is enhanced in hypercholesterolemic rabbits. *J Cardiovasc Pharmacol.* 1994;24:994–998.
40. Harrison DG, Ohara Y. Physiologic consequences of increased vascular oxidant stresses in hypercholesterolemia and atherosclerosis: implications for impaired vasomotion. *Am J Cardiol.* 1995;75:75B–81B.
41. Mügge A, Elwell JH, Peterson TE, Hofmeyer TG, Heistad DD, Harrison DG. Chronic treatment with polyethylene glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circ Res.* 1991;69:1293–1300.
42. Ezaki M, Ikeda M, Tomita I, Tomita T. Inhibition of EDRF release by native low-density lipoprotein from cultured porcine endothelial cells through intracellular mechanisms. *J Cardiovasc Pharmacol.* 1994;24:552–558.
43. Garcia CE, Kilcoyne CM, Cardillo C, Cannon RO, Quyyumi AA, Panza JA. Evidence that endothelial dysfunction in patients with hypercholesterolemia is not due to increased extracellular nitric oxide breakdown by superoxide anions. *Am J Cardiol.* 1995;76:1157–1161.
44. Bogle RG, Coade SB, Moncada S, Pearson JD, Mann GE. Bradykinin and ATP stimulate L-arginine uptake and nitric oxide release in vascular endothelial cells. *Biochem Biophys Res Commun.* 1991;180:926–932.
45. Michel JB, Feron O, Sase K, Prabhakar P, Michel T. Cavaeolin versus calmodulin. *J Biol Chem.* 1997;272:25907–25912.
46. Thorin E, Hamilton C, Dominiczak AF, Dominiczak MH, Reid JL. Oxidized-LDL induced changes in membrane physico-chemical properties and  $[\text{Ca}^{2+}]_i$  of bovine aortic endothelial cells: influence of vitamin E. *Atherosclerosis.* 1995;114:185–195.
47. Peng SK, Hill JC, Morin RJ, Taylor BC. Influence of cholesterol oxidation derivatives on membrane bound enzymes in cultured aortic smooth muscle cells. *Proc Soc Exp Biol Med.* 1985;180:126–132.
48. Ramasamy S, Boissonneault GA, Hennig B. Oxysterol-induced endothelial cell dysfunction in culture. *J Am Coll Nutr.* 1992;11:532–538.
49. Moog C, Deloulme JC, Baudier J, Revel MO, Bischoff P, Hietter H, Luu B. Membrane-related oxysterol function: preliminary results on the modification of protein kinase C activity and substrate phosphorylation by 7 $\beta$ ,25-dihydroxycholesterol. *Biochimie.* 1991;73:1321–1326.
50. Hecker M, Mulsch A, Bassenge E, Forstermann U, Busse R. Subcellular localization and characterization of nitric oxide synthase(s) in endothelial cells: physiological implications. *Biochem J.* 1994;299:247–250.
51. Shaul PW, Smart EJ, Robinson LJ, German Z, Yuhanna IS, Ying Y, Anderson RGW, Michel T. Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. *J Biol Chem.* 1996;271:6518–6522.
52. Garcia-Gardena G, Martasek P, Siler Masters BS, Skidd PM, Couet J, Li S, Lisanti MP, Sessa WC. Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. *J Biol Chem.* 1997;272:25437–25440.
53. Anderson RGW. Caveolae: where incoming and outgoing messengers meet. *Proc Natl Acad Sci U S A.* 1993;90:10909–10913.
54. Chang WJ, Rothberg KG, Kamen BA, Anderson RGW. Lowering the cholesterol content of MA104 cells inhibits receptor-mediated transport of folate. *J Cell Biol.* 1992;118:63–69.
55. Bhadra S, Arshad MAQ, Rymaszewski Z, Norman E, Wherley R, Subbiah MTR. Oxidation of cholesterol moiety of low density lipoprotein in the presence of human endothelial cells or  $\text{Cu}^{2+}$  ions: identification of major products and their effects. *Biochem Biophys Res Commun.* 1991;176:431–440.
56. Malavasi B, Rasetti MF, Roma P, Fogliatto R, Allevi P, Catapano AL, Galli G. Evidence for the presence of 7-hydroperoxycholest-5-en-3 $\beta$ -ol in oxidized human LDL. *Chem Phys Lipids.* 1992;62:209–214.
57. Tamasawa N, Takebe K. Analytical examination of oxidized free and esterified 7-ketocholesterol and related oxysterols in human plasma incubated with copper. *Tohoku J Exp Med.* 1992;168:37–45.
58. Smith LL. Cholesterol autooxidation. *Chem Phys Lipids.* 1987;44:87–125.
59. Martin CM, Nicholas HJ. Metabolism of cholesteryl palmitate by rat brain in vitro: formation of cholesterol epoxides and cholestan-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol. *J Lipid Res.* 1973;14:618–624.
60. Gray MF, Lawrie TDV. Isolation and identification of cholesterol  $\alpha$ -oxide and other minor sterols in human serum. *Lipids.* 1971;6:836–843.
61. Carpenter KLH, Taylor SE, Ballantine JA, Fussell B, Halliwell B, Mitchinson MJ. Lipids and oxidised lipids in human atheroma and normal aorta. *Biochim Biophys Acta.* 1993;1167:121–130.
62. Breuer O, Dzeletovic S, Lund E, Diczfalusy U. The oxysterols cholest-5-ene-3 $\beta$ ,4 $\alpha$ -diol, cholest-5-ene-3 $\beta$ ,4 $\beta$ -diol and cholestan-3 $\beta$ ,5 $\alpha$ ,6 $\alpha$ -triol are formed during in vitro oxidation of low density lipoprotein, and are present in human atherosclerotic plaques. *Biochim Biophys Acta.* 1996;1302:145–152.
63. Guardiola F, Codony R, Addis PB, Rafecas M, Boatella J. Biological effects of oxysterols: current status. *Food Chem Toxicol.* 1996;34:193–211.
64. Addis PB. Occurrence of lipid oxidation products in foods. *Food Chem Toxicol.* 1986;24:1021–1030.
65. Fries DM, Penha RG, D'Amico EA, Abdalla DSP, Monteiro HQ. Oxidized low-density lipoprotein stimulates nitric oxide release by rabbit aortic endothelial cells. *Biochem Biophys Res Commun.* 1995;207:231–237.