Lysophosphatidylcholine and Reactive Oxygen Species Mediate the Synergistic Effect of Mildly Oxidized LDL With Serotonin on Vascular Smooth Muscle Cell Proliferation

Takuya Watanabe, MD; Rajbabu Pakala, PhD; Shinji Koba, MD; Takashi Katagiri, MD; Claude R. Benedict, MD, DPhil

Background—Mild oxidation of LDL enhances its atherogenic potential and induces a synergistic interaction with serotonin (5HT) on vascular smooth muscle cell (VSMC) proliferation. Because of its complex chemical nature, the mitogenic components of mildly oxidized LDL (moxLDL) remain unclear.

Methods and Results—We examined both the effects of lysophosphatidylcholine (LPC) and hydrogen peroxide (H2O2), a donor of reactive oxygen species, as major components of moxLDL and their interactions with 5HT on VSMC proliferation. Growth-arrested VSMCs were incubated with different concentrations of moxLDL, LPC, H2O2, or LPC with H2O2 in the absence or presence of 5HT. DNA synthesis in VSMCs was examined by [3H]thymidine incorporation. MoxLDL, LPC, H2O2, and 5HT stimulated DNA synthesis in a dose-dependent manner. MoxLDL had a maximal stimulatory effect at a concentration of 5 µg/mL (211%), LPC at 15 µmol/L (156%), H2O2 at 5 µmol/L (179%), and 5HT at 50 µmol/L (205%). Added together, moxLDL (50 ng/mL) and 5HT (50 µmol/L) synergistically increased DNA synthesis (443%). Coincubation of LPC (1 µmol/L) with H2O2 (0.5 µmol/L) and 5HT (5 µmol/L) resulted in a synergistic increase in DNA synthesis (439%), which was nearly equal to that of moxLDL with 5HT (443%). The combined effects of LPC, H2O2, and 5HT on DNA synthesis were completely reversed by the combined use of an antioxidant, N-acetylcysteine (400 µmol/L) or butylated hydroxytoluene (20 µmol/L), with a 5HT2 receptor antagonist, LY281067 (10 µg/mL).

Conclusions—Our results suggest that both LPC and reactive oxygen species may contribute to the mitogenic effect of moxLDL on VSMCs and its synergistic effect with 5HT. (Circulation. 2001;103:1440-1445.)

Key Words: antioxidants ■ atherosclerosis ■ lipoproteins ■ muscle, smooth ■ platelet-derived factors

Vascular smooth muscle cells (VSMCs) are implicated in the pathogenesis of atherosclerosis and other vascular proliferative disorders, including restenosis after percutaneous angioplasty. Currently, restenosis of the coronary arteries and to a lesser extent the peripheral arteries after angioplasty is a major clinical problem. Hypothetical mechanisms for restenosis after balloon dilation include activation and release of platelet growth factors, intimal migration and proliferation of VSMCs induced by growth factors, and the multiple effects of modified LDL. Several lines of evidence suggest that oxidative modification of LDL enhances its atherogenicity and plays a key role in the development of atherosclerosis. It is also known that the oxidation of LDL ultimately leads to the generation of reactive oxygen species (ROS) and the conversion of phosphatidylcholine (PC) to lysophosphatidylcholine (LPC). Recent evidence indicates that ROS, such as hydrogen peroxide (H2O2), superoxide anion (O2−), and the hydroxy radical (HO2·), stimulate VSMC growth and that LPC possesses mitogenic and chemotactic properties for VSMCs. Serotonin (5HT), which is released from activated platelets, is a potent vasoactive substance and is associated with coronary artery events. Our previous study showed that 5HT is a mitogen for VSMCs, which may contribute to the progression of atherosclerotic lesion or neointimal proliferation after angioplasty. Furthermore, we have also demonstrated that 5HT exerts a synergistic interaction with mildly oxidized LDL (moxLDL) on VSMC proliferation. Because of its complex chemical nature, however, the mechanism by which moxLDL acts synergistically with 5HT in promoting VSMC proliferation is unclear.

In this study, we examined both the effects of LPC and H2O2, a donor of ROS, as major chemical components of moxLDL and their interactions with 5HT on VSMC proliferation. We tested the effect of defatted BSA and catalase on...
moxLDL-induced DNA synthesis to show that LPC and ROS are responsible for the mitogenic effect of moxLDL. Furthermore, we assessed the effects of N-acetylcysteine (NAC) or butylated hydroxytoluene (BHT) and/or LY281067, a 5HT3 receptor antagonist, on DNA synthesis induced by LPC, H2O2, and 5HT.

**Methods**

**Materials**

LDL (human), LPC, PC, H2O2, 5HT, NAC, BHT, BSA, and catalase were purchased from Sigma. LY281067 was donated by Eli Lilly Laboratories. Dulbecco’s modified Eagle’s medium (DMEM), FBS, and PBS were purchased from Gibco BRL. [3 H]Thymidine (specific activity 20 Ci/mol) was obtained from DuPont-NEN.

**LDL Oxidation**

Human LDL was oxidized with CuSO4 at a final concentration of 5 μmol/L at 37°C, and the thiobarbituric acid–reactive substances (TBARS) content was determined every 30 minutes by removing an aliquot as described previously. All the samples were tested for their electrophoretic mobility by 1% agarose gel electrophoresis. The point at which there is no change in the electrophoretic mobility but an increase in TBARS content was selected as moxLDL. The LDL was oxidized at 37°C for 30 hours before EDTA was added to obtain oxLDL. Lipoprotein concentrations are expressed as protein concentrations. Even at a concentration of 10 mg/mL, native LDL (nLDL) showed no development of TBARS, whereas moxLDL showed a slight increase in TBARS formation (2 to 4 nmol/mg protein), with no change in the electrophoretic mobility. In contrast, oxLDL showed a significant increase in TBARS formation (35 nmol/mg protein) and an increase in the electrophoretic mobility.

**Cell Culture**

VSMCs were isolated from the thoracic aortas of male New Zealand White rabbits (body weight ~3 kg, n=95) by the explant method and were cultured in a humidified atmosphere (5% CO2/95% air) at 37°C. After 3 to 4 weeks, the tissue blocks were removed and the migrated VSMCs were cultured, followed by a subculture with trypsinization.

**DNA Synthesis**

DNA synthesis was examined by measurement of [3 H]thymidine incorporation into the cellular DNA.8,9 VSMCs in passage 1 or 2 were seeded in 35-mm-diameter tissue culture plates and grown to semiconfluence in DMEM containing 10% FBS. Then, the medium was replaced with DMEM containing 0.1% FBS and incubated for ~72 hours for growth arrest. After that, the medium was replaced with DMEM containing 500 μg/mL BSA, 10 μg/mL insulin, 20 μg/mL transferrin, 25 mg/mL sodium, and 100 μmol/L pargyline (serum-free medium). At the same, experiments were divided into 3 groups. In group 1, VSMCs were incubated with the indicated concentrations of nLDL, moxLDL, oxLDL, PC, LPC, H2O2, or LPC with H2O2. After 24 hours of incubation, indicated concentrations of 5HT were added, and the cells were incubated for another 24 hours. In group 2, VSMCs were incubated with different concentrations of LPC, H2O2, or LPC with H2O2 in the presence of NAC or BHT for 48 hours. In group 3, VSMCs were incubated with LPC, H2O2, or LPC with H2O2 in the presence of NAC or BHT. After 24 hours of incubation, 5HT was added, and the cells were incubated for another 24 hours. LY281067 was added 4 hours before the addition of 5HT. For all experiments, VSMCs were exposed to [3 H]thymidine at a concentration of 5 μCi/plate for the last 5 hours of the 48-hour incubation period. [3 H]Thymidine incorporation into VSMC DNA was quantified in a liquid scintillation counter. All the experiments were performed in triplicate, and each experiment was repeated a minimum of 3 times.

**Results**

**Effect of PC, LPC, and H2O2 on VSMC DNA Synthesis**

The concentration-dependent effects of PC, LPC, and H2O2 on [3 H]thymidine incorporation into DNA are shown in Figure 1A and 1B. PC at concentrations tested did not have a significant effect on DNA synthesis. By contrast, LPC stimulated DNA synthesis in a dose-dependent manner between 0.5 and 15 μmol/L, with a maximal effect at 15 μmol/L (156±8%, P<0.0001 versus other concentrations of LPC). H2O2 also stimulated DNA synthesis in a dose-dependent manner between 0.1 and 5 μmol/L. Maximal stimulation was observed at 5 μmol/L (177±7%, P<0.0001 versus other
concentrations of H₂O₂. When VSMCs were incubated with >5 μmol/L H₂O₂, there was a significant decrease in the [³H]thymidine incorporation, indicating that higher concentrations of H₂O₂ may be cytotoxic.

Effect of LPC or H₂O₂ With 5HT on VSMC DNA Synthesis

The interaction of LPC or H₂O₂ with 5HT on [³H]thymidine incorporation into DNA is shown in Figure 2A and 2B. 5HT at 5 or 50 μmol/L, induced DNA synthesis 1.8- or 2.1-fold higher than the control value, respectively (P<0.0001). When LPC at concentrations <15 μmol/L was added together with either concentration of 5HT, a synergistic rather than an additive effect was observed on DNA synthesis. The synergistic interaction between LPC and 5HT was prominent when nonmitogenic concentrations of LPC were incubated with either concentration of 5HT. For example, when VSMCs were incubated with 1 μmol/L LPC alone, LPC did not have any significant mitogenic effect. When VSMCs were incubated with 1 μmol/L LPC and 5 μmol/L 5HT, however, there was a significant increase in [³H]thymidine incorporation to 339±12% (P<0.0001), compared with 183±2% with 5 μmol/L 5HT alone. Like LPC, the synergistic interaction between H₂O₂ and 5HT was more significant when nonmitogenic concentrations of H₂O₂ were incubated with either concentration of 5HT. When VSMCs were incubated with 0.5 μmol/L H₂O₂ and 5 μmol/L 5HT, the amount of [³H]thymidine incorporation increased significantly to 304±13% (P<0.0001), compared with 182±3% with 5 μmol/L 5HT alone.

Effect of LPC and H₂O₂ With 5HT on VSMC DNA Synthesis

The interaction between subthreshold concentrations of LPC and H₂O₂ and 5 μmol/L 5HT on DNA synthesis is shown in Figure 3. When VSMCs were incubated with nonmitogenic concentrations of LPC (1 μmol/L) and H₂O₂ (0.5 μmol/L), there was a significant increase in [³H]thymidine incorporation to 262±8% (P<0.0001), suggesting a synergistic interaction between LPC and H₂O₂. When 5 μmol/L 5HT was added to this combination of LPC (1 μmol/L) and H₂O₂ (0.5 μmol/L), there was a further increase in the amount of [³H]thymidine incorporation (439±13%, P<0.0001). Similar synergistic interactions could be observed with all the concentrations tested. This stimulatory effect cannot be explained by simple additive effects of LPC, H₂O₂, and 5HT.

Interaction of LPC and H₂O₂ or Different Forms of LDL With 5HT on VSMC DNA Synthesis

The interaction of different forms of LDL or LPC and H₂O₂ with 5HT on DNA synthesis is shown in Figure 4. When tested alone for their effect on [³H]thymidine incorporation into DNA, oxLDL, moxLDL (both 50 ng/mL), or LPC with H₂O₂ (1 and 0.5 μmol/L, respectively) significantly stimulated [³H]thymidine incorporation (129±10% or 134±15%, both P<0.05; 262±8%, P<0.0001) compared with nLDL, PC, LPC, or H₂O₂. When the same experiment was repeated in the presence of 50 μmol/L 5HT, the amount of [³H]thymidine incorporation in the VSMCs increased significantly. Interestingly, the amount of [³H]thymidine incorporation into the VSMCs was nearly equal when VSMCs were stimulated...
with moxLDL and 5HT or with LPC, H$_2$O$_2$, and 5HT (443±12% versus 438±12%, P=NS).

**Effect of LPC, H$_2$O$_2$, and 5HT on VSMC Number**

To determine whether the induction of DNA synthesis by LPC, H$_2$O$_2$, and 5HT resulted in an increase in cell number, VSMC number was also examined (Figure 5). Because a significant synergistic interaction between LPC and H$_2$O$_2$ was observed at lower concentration of LPC and H$_2$O$_2$, we determined the cell number at 1 μmol/L LPC and/or 0.5 μmol/L H$_2$O$_2$. LPC and H$_2$O$_2$ were without any effect; however, in combinations with 5HT (5 μmol/L), the cell number increased (both P<0.05). When LPC (1 μmol/L) and H$_2$O$_2$ (0.5 μmol/L) were added together, a significant increase in cell number was observed (P=0.01). In particular, coinubcation of LPC (1 μmol/L) with H$_2$O$_2$ (0.5 μmol/L) and 5HT (5 μmol/L) resulted in the greatest increase in cell number (P<0.01).

**Effect of Antioxidants and LY281067 on VSMC DNA Synthesis**

To find out whether antioxidants block the mitogenic effect of LPC and H$_2$O$_2$ and their synergistic interaction, VSMCs were preincubated with the antioxidant NAC (400 μmol/L) or BHT (20 μmol/L) along with LPC (1 μmol/L), H$_2$O$_2$ (0.5 μmol/L), or LPC (1 μmol/L) with H$_2$O$_2$ (0.5 μmol/L). When VSMCs were incubated with antioxidants, they completely blocked the mitogenic effects of LPC and H$_2$O$_2$ and their synergistic interaction (Figure 6). We evaluated the combined effects of the 5HT$_2$ receptor antagonist LY281067 (10 μg/mL) and NAC or BHT on VSMC proliferation induced by LPC (1 μmol/L), H$_2$O$_2$ (0.5 μmol/L), or LPC (1 μmol/L) with H$_2$O$_2$ (0.5 μmol/L) in combination with 5HT (5 μmol/L). When VSMCs were incubated with antioxidants and LY281067, they blocked the mitogenic effect of LPC and/or H$_2$O$_2$ and their synergistic interaction with 5HT without affecting the mitogenic effect of 5HT (Figure 7). Similarly, when VSMCs were incubated with LY281067 and H$_2$O$_2$ with 5HT, LY281067 blocked the mitogenic effect of 5HT and synergistic interaction with LPC and/or H$_2$O$_2$ without affecting the mitogenic effect of LPC and/or H$_2$O$_2$. When VSMCs were incubated with both antioxidants and LY281067 and then stimulated with LPC and/or H$_2$O$_2$ with 5HT, the mitogenic effects and the synergistic interaction of all the 3 mitogens were blocked.

**Effect of Defatted BSA and Catalase on MoxLDL- and 5HT-Induced VSMC DNA Synthesis**

To ensure that LPC and H$_2$O$_2$ are the components responsible for the mitogenic effect of moxLDL and its synergistic...
ROS. Thus, oxLDL may contain only LPC, whereas mox-LDL contains large amounts of LPC but only small amounts of ROS, which are still relatively low. After 24 hours of oxidative stress, LDL and presumably also other ROS, whereas the LPC levels are increased. The increased LDL oxidation is a progressive process leading to the formation of mox-LDL and later to oxLDL. The increased atherogenic effect of mox-LDL or oxLDL is attributed to the chemical changes brought about by the oxidation processes to the components of LDL, namely, generation of ROS and conversion of PC to LPC. During the early stages of oxidation, there is a significant accumulation of peroxides and presumably also other ROS, whereas the LPC levels are still relatively low. After 24 hours of oxidative stress, LDL contains large amounts of LPC but only small amounts of ROS. Thus, oxLDL may contain only LPC, whereas mox-LDL may contain both LPC and ROS. This hypothesis is demonstrated by the facts that defatted BSA could deplete the mitogenic effects of both mox-LDL and oxLDL and that catalase could reverse the mitogenic effect of mox-LDL but not that of oxLDL. Recent studies have shown that a major source of ROS and H2O2 in vascular tissues is NAD(P)H oxidase, and the activity of NAD(P)H oxidase is increased by angiotensin II, thrombin, platelet-derived growth factor, tumor necrosis factor-α, and LPC. Thus, the source of ROS in the present study can be either mox-LDL or LPC-stimulated NAD(P)H oxidase.

Several investigators have reported that G protein–mediated signal transduction is impaired by high levels of LPC. A low concentration of LPC has been shown to stimulate smooth muscle growth factors, such as basic fibroblast growth factor, which may be responsible for VSMC migration and proliferation. Others have reported, however, that LPC itself is a mitogen for VSMCs under serum-free conditions.

**Discussion**

LDL oxidation is a progressive process leading to the formation of mox-LDL and later to oxLDL. The increased atherogenic effect of mox-LDL or oxLDL is attributed to the chemical changes brought about by the oxidation processes to the components of LDL, namely, generation of ROS and conversion of PC to LPC. During the early stages of oxidation, there is a significant accumulation of peroxides and presumably also other ROS, whereas the LPC levels are still relatively low. After 24 hours of oxidative stress, LDL contains large amounts of LPC but only small amounts of ROS. Thus, oxLDL may contain only LPC, whereas mox-LDL may contain both LPC and ROS. This hypothesis is demonstrated by the facts that defatted BSA could deplete the mitogenic effects of both mox-LDL and oxLDL and that catalase could reverse the mitogenic effect of mox-LDL but not that of oxLDL. Recent studies have shown that a major source of ROS and H2O2 in vascular tissues is NAD(P)H oxidase, and the activity of NAD(P)H oxidase is increased by angiotensin II, thrombin, platelet-derived growth factor, tumor necrosis factor-α, and LPC. Thus, the source of ROS in the present study can be either mox-LDL or LPC-stimulated NAD(P)H oxidase.

**Figure 7.** Effect of antioxidants and/or LY281067 on mitogenic interaction among H2O2, LPC, and 5HT. Growth-arrested VSMCs were stimulated with LPC (1 μmol/L) and/or H2O2 (0.5 μmol/L) in presence of NAC (400 μmol/L) or BHT (20 μmol/L). LY281067 (10 μg/mL) was added 4 hours before addition of 5HT. After 24 hours of incubation with LPC and/or H2O2, 5HT (5 μmol/L) was added and incubated for 24 hours, and amount of [3H]thymidine incorporation into DNA was measured. Data are shown as mean±SEM (n=10). Control value (234±15 cpm)=100%. *P<0.0001 vs control; †P<0.0001 vs LPC+5HT; **P<0.0001 vs H2O2+5HT; ***P<0.0001 vs LPC+H2O2+5HT (unpaired t test); ††P<0.0001 vs all others in group of LPC+H2O2+5HT (ANOVA).

**Figure 8.** Effect of defatted BSA and catalase on mox-LDL- and 5HT-induced VSMC proliferation. Growth-arrested VSMCs were incubated with untreated (control), defatted BSA (100 μg/mL)-pretreated or catalase (10 U/mL)-treated mox-LDL or ox-LDL (both 5 μg/mL, showing maximal effect), LPC (15 μmol/L), H2O2 (5 μmol/L), or 5HT (5 μmol/L) for 48 hours. When both mox-LDL or ox-LDL and 5HT were used, VSMCs were incubated with defatted BSA (100 μg/mL)-pretreated or catalase (10 U/mL)-treated mox-LDL or ox-LDL (both 50 ng/mL, showing maximal interaction with 5 μmol/L 5HT). At 24 hours after addition of mox-LDL or ox-LDL, 5HT (5 μmol/L) was added and incubated for 24 hours, and amount of [3H]thymidine incorporation into DNA was measured. Data are shown as mean±SEM (n=10). Control value (240±11 cpm)=100%. *P<0.0001, †P<0.0001, ‡P<0.0005, ††P<0.0001 vs 5HT; **P<0.001, ***P<0.0001 vs all others in group of corresponding controls.
kinases and ERK5. Banes et al showed that 5HT also activates ERK1/2. Therefore, the activation of the ERK1/2 pathway by 5HT and redox-sensitive pathways by LPC and 

\[ \text{H}_2\text{O}_2 \] may explain the synergistic interaction in our studies. Watanabe et al 10 and Cox et al 22 showed that 

\[ \text{H}_2\text{O}_2, \text{LPC, and oxLDL act synergistically with 5HT in vasoconstriction, which may also support our findings.} \]

Our previous studies have shown that the mitogenic effect of 5HT on VSMCs is mediated predominantly by 5HT \(_2\) receptors. 8,9 5HT acting via the 5HT \(_2\) receptor subtype induced a 5-fold increase in the mobilization of intracellular calcium and a 10-fold increase in c-fos mRNA in VSMCs. In the present study, the 5HT \(_2\) receptor antagonist LY281067 reversed only the mitogenic effect of 5HT but not those of LPC and 

\[ \text{H}_2\text{O}_2 \] on VSMCs.

This study suggests that PC, the major component of nLDL, does not have a significant mitogenic effect on VSMCs and that this effect is not enhanced by 5HT. LPC and ROS, the major components of moxLDL, act synergistically in inducing DNA synthesis. The increase in DNA synthesis induced by the combined use of LPC (1 \(\mu\)mol/L) and 

\[ \text{H}_2\text{O}_2 \] (0.5 \(\mu\)mol/L) (262±8% of the control) is similar to that of 

\[ \text{moxLDL} \] (5 \(\mu\)g/ml) (211±25%), which suggests that the mitogenic effect of moxLDL may be due to the combined effects of LPC and ROS, which are present in moxLDL. The oxLDL was less mitogenic than moxLDL because the major component of oxLDL is LPC. Under physiological conditions that include the presence of various antioxidants in the plasma, complete oxidation of LDL may not be feasible, but these conditions are more likely to result in partial oxidation of LDL with production of moxLDL, which is the most atherogenic of the 3 forms of LDL. Thus, moxLDL interacting with other vasoactive substances and growth factors present in the vasculature may play an important role in the development of atherosclerosis and restenosis after restenosis.

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