Synergistic Effect of Urotensin II With Mildly Oxidized LDL on DNA Synthesis in Vascular Smooth Muscle Cells

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

**Background**—The urotensin II (UII) found in coronary atheroma is the most potent vasoconstrictor known to date. Mildly oxidized LDL (moxLDL) contributes to atherogenesis and plaque formation. We assessed the effect of UII and its interaction with moxLDL on vascular smooth muscle cell (VSMC) proliferation.

**Methods and Results**—Growth-arrested VSMCs were incubated in serum-free medium with different concentrations of LDL, moxLDL, oxLDL, hydrogen peroxide, lysophosphatidylcholine, or 4-hydroxy-2-nonenal, with or without UII. [3H]Thymidine incorporation into DNA was measured as an index of VSMC proliferation. UII stimulated [3H]thymidine incorporation in a dose-dependent manner, with a maximal effect at a concentration of 50 nmol/L (161%). Low concentrations of UII potentiated the mitogenic effect of LDL (108% to 242%), oxLDL (129% to 302%), moxLDL (120% to 337%), hydrogen peroxide (177% to 226%), lysophosphatidylcholine (115% to 332%), and 4-hydroxy-2-nonenal (142% to 299%). The synergistic interaction between UII and moxLDL was partially inhibited by anti-Gq/11a antibody, the epidermal growth factor receptor tyrosine kinase inhibitor erbstatin A (10 μmol/L), and the intracellular free radical scavenger N-acetylcysteine (400 μmol/L) and was completely inhibited by the c-Src tyrosine kinase inhibitor radicicol (10 μmol/L), the protein kinase C (PKC) inhibitor Ro31-8220 (0.1 μmol/L), and the mitogen-activated protein kinase (MAPK) kinase inhibitor PD098059 (10 μmol/L).

**Conclusions**—Our results suggest that UII acts synergistically with moxLDL in inducing VSMC proliferation via the c-Src/PKC/MAPK pathway, which may explain the relatively rapid progression of atherosclerosis in patients with hypertension and hypercholesterolemia. *(Circulation. 2001;104:16-18.)*

**Key Words:** atherosclerosis ■ hypertension ■ lipoproteins ■ muscle, smooth ■ urotensins

Urotensin II (UII) is a cyclic 12-amino acid peptide detected in fish spinal cords and plasma (≈30 pmol/L) that was recently cloned from the spinal cord of humans. Human UII is found in vascular and cardiac tissue (including coronary atheroma) and shows a contractile effect on many arteries from nonhuman primates, including coronary, pulmonary and carotid arteries, suggesting a hypertensive response. The potency of the vasoconstriction of UII is an order of magnitude greater than that of endothelin-1, making UII the most potent mammalian mammalian vasokonstractor identified thus far. Recent studies have shown that the vasoconstrictive effect of UII is mediated via GPR14, an orphan G proteincoupled receptor that may couple to the Gq/11α pathway. However, the downstream signaling pathway remains unclear.

Oxidized LDL (oxLDL) is a well-established risk factor for atherosclerosis that stimulates vascular smooth muscle cell (VSMC) differentiation and proliferation. In particular, mildly oxidized LDL (moxLDL) interacting with other vasoactive agents and growth factors present in the vasculature may play an important role in the development of atherosclerosis and hypertension. Recently, we showed that the mitogenic effect of moxLDL is mediated by its oxidative components, such as reactive oxygen species (ROS), lysophosphatidylcholine (LPC), and 4-hydroxy-2-nonenal (HNE).

This study sought to examine the proliferative effect of UII on VSMCs and its interaction with moxLDL and to demonstrate the mechanism responsible for synergistic interaction between these 2 agents in inducing VSMC proliferation.

**Methods**

**Materials**

Human UII, LDL, LPC, HNE, hydrogen peroxide (H2O2), anti-Gq/11α antibody, erbstatin A, radicicol, Ro31-8220, PD098059, and N-acetylcysteine (NAC) were purchased from Sigma. [3H]Thymidine (specific activity, 20 Ci/mol) was obtained from DuPont-NEN.
LDL Oxidation
MoxLDL and oxLDL were prepared as described previously. Lipoprotein concentrations are expressed as protein concentrations. Even at a concentration of 10 mg/mL, native LDL showed no development of thiobarbituric acid–reactive substances (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=35) by the explant method and were cultured in a humidified atmosphere (5% CO₂/95% air) at 37°C. After ~3 weeks, the tissue blocks were removed, and the migrated VSMCs were cultured; this was followed by a subculture with trypsinization. The identity of the VSMCs was confirmed by morphological examination and by staining for α-actin.

DNA Synthesis
DNA synthesis was examined by measuring [³H]thymidine incorporation into the cellular DNA, as described previously. After synchronization or growth arrest of VSMCs, medium was replaced with Dulbecco’s modified Eagle’s medium containing 500 μg/mL BSA, 10 μg/mL insulin, 20 μg/mL transferrin, and 25 ng/mL selenium. VSMCs were incubated with different concentrations of UII and with LDL, moxLDL, oxLDL, H₂O₂, LPC, or HNE for 48 hours. Otherwise, VSMCs were incubated with indicated concentrations of UII and moxLDL and with the epidermal growth factor receptor tyrosine kinase inhibitor erbstatin A, the c-Src tyrosine kinase inhibitor radicicol, the protein kinase C (PKC) inhibitor Ro31-8220, the mitogen-activated protein kinase (MAPK) kinase inhibitor PD098059, or the intracellular free radical scavenger NAC for 48 hours. Anti-Gq/11 antibody was added 1 hour before the addition of UII and moxLDL. VSMCs were exposed to [³H]thymidine at a concentration of 1 μCi/plate for the last 5 hours of the 48 hour incubation period, and [³H]thymidine incorporation into VSMC DNA was measured. All the experiments were performed in quadruplicate, and each experiment was repeated a minimum of 3 times.

Statistical Analysis
All values are expressed as mean±SEM. The data were compared by 2-tailed unpaired Student’s t test between 2 groups and by 1-way ANOVA followed by Bonferroni test when >2 groups were involved. Differences were considered statistically significant at P<0.05.

Results
Effect of UII With LDL, MoxLDL, or OxLDL on VSMC DNA Synthesis
UII significantly increased [³H]thymidine incorporation in a concentration-dependent manner, with a maximal effect at a concentration of 5 μmol/L (161±10%, P<0.0001 versus control; Figure 1A). LDL, moxLDL, and oxLDL had a maximal effect at a concentration of 5 μg/mL (data not shown). When tested in combination, UII (50 nmol/L) significantly increased the mitogenic effect of LDL (500 ng/mL; by 242±23%; P<0.005; Figure 1A). When added together, even nonmitogenic concentrations of UII (10 or 25 nmol/L) acted synergistically with moxLDL (100 ng/mL) or oxLDL (50 ng/mL) in inducing [³H]thymidine incorporation (337±35%, P<0.0001; 302±36%; P<0.001, respectively; Figures 1B and 1C).

Effect of Anti-Gq/11α Antibody, Erbstatin A, Radicicol, Ro31-8220, PD098059, or NAC on Mitogenic Interaction Between UII and MoxLDL
To discover how UII and moxLDL exert their synergistic interaction, we assessed the effects of anti-Gq/11α antibody (2.5 μL/plate of 2 mL), the epidermal growth factor receptor tyrosine kinase inhibitor erbstatin A (10 μmol/L), the c-Src tyrosine kinase inhibitor radicicol (10 μmol/L), the PKC inhibitor Ro31-8220 (0.1 μmol/L), the MAPK kinase inhibitor PD098059 (10 μmol/L), and the intracellular free radical scavenger NAC (400 μmol/L) on the interaction between UII (10 nmol/L) and moxLDL (100 ng/mL) in inducing [³H]thymidine incorporation. Anti-Gq/11α antibody, erbstatin A,
Figure 2. Effect of anti-Gq/11α antibody, erbastin A, radicicol, Ro31-8220, PD098059, or NAC on mitogenic interaction between UII and moxLDL. Growth-arrested VSMCs were stimulated with UII and/or moxLDL in serum-free medium with anti-Gq/11α antibody, erbastin A, radicicol, Ro31-8220, PD098059, or NAC for 48 hours, and amount of [3H]thymidine incorporation was measured. Data are shown as mean±SEM (n=12). Control value (238±11 counts per minute) was 100%. *P<0.0001 vs untreated control; †P<0.0001, ‡P<0.0005, and #P<0.05 vs UII+moxLDL.

Discussion

It is well established that hypertension and hypercholesterolemia enhance the development of atherosclerosis in human and animal studies. Vasoconstrictors such as endothelin-1, angiotensin II, and oxLDL accelerate atherosclerosis by inducing VSMC proliferation. The extent of changes in the LDL particle induced by oxidation (moxLDL to oxLDL) depends on the pro-oxidant conditions and the length of time the particle is exposed to these conditions. Under physiological conditions, which include the presence of various antioxidants in the plasma, complete oxidation of LDL may not be feasible; instead, such conditions are more likely to result in partial oxidation of LDL, with production of moxLDL, which was shown to be the most potent of the 3 forms of LDL. In this study, the combination of UII (10 nmol/L) with moxLDL (100 ng/mL) resulted in the highest induction of VSMC proliferation among cells incubated with UII and LDL or oxLDL (Figure 1) and endothelin-1 or angiotensin II with moxLDL (Table).

The increased atherogenic effect of moxLDL is attributed to the chemical changes brought about by the oxidation processes. During the early stages of oxidation, there is a significant accumulation of peroxidases and other ROS. As oxidation proceeds, phosphatidylcholine is converted to LPC. More or less at the same time, unsaturated aldehydes, such as HNE, are generated by β-scission of alkoyl radicals in the polyunsaturated fatty acids that are present in LDL.

Several studies from our laboratory and others have shown that moxLDL and oxLDL and their oxidative components (ie, ROS, LPC, and HNE) induce VSMC proliferation via the redox-sensitive pathway and the extracellular signal–regulated kinase (ERK) 1/2 MAPK pathway.

Like most vasoactive agents, such as angiotensin II, endothelin-1, and serotonin, UII may also induce VSMC proliferation via the activation of the G protein-coupled receptor or the PKC/c-Src tyrosine kinase/MAPK pathway. In this study, the synergistic interaction between UII and moxLDL was abolished by the PKC inhibitor Ro31-8220, the c-Src tyrosine kinase inhibitor radicicol, and the MAPK kinase inhibitor PD098059, suggesting that the amplification of the PKC/c-Src tyrosine kinase/MAPK pathway may play a key role in inducing synergistic interaction between the two agents. These findings provide an understanding of the potential molecular mechanisms responsible for the long-standing clinical observations that the interaction of risk factors promotes the development of atherosclerosis.

References

Synergistic Effect of Urotensin II With Mildly Oxidized LDL on DNA Synthesis in Vascular Smooth Muscle Cells
Takuya Watanabe, Rajbabu Pakala, Takashi Katagiri and Claude R. Benedict

Circulation. 2001;104:16-18
doi: 10.1161/hc2601.092848
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/104/1/16