Original Article

Human Urotensin-II Potentiates the Mitogenic Effect of Mildly Oxidized Low-Density Lipoprotein on Vascular Smooth Muscle Cells: Comparison with Other Vasoactive Agents and Hydrogen Peroxide

Takuya WATANABE¹⁾, Keiko TAKAHASHI¹⁾, Tomoko KANOME¹⁾,
Shigeki HONGO¹⁾, Akira MIYAZAKI¹⁾, Shinji KOBAYASHI²⁾, Takashi KATAGIRI²⁾,
Rajbabu PAKARA³⁾, and Claude R. BENEDICT⁴⁾

Human urotensin-II (U-II) is the most potent vasoactive peptide identified to date, and may be involved in hypertension and atherosclerosis. We investigated the effects of the interactions between U-II or other vasoactive agents and mildly oxidized low-density lipoprotein (mox-LDL) or hydrogen peroxide (H₂O₂) on the induction of vascular smooth muscle cell (VSMC) proliferation. Growth-arrested rabbit VSMCs were incubated with vasoactive agents (U-II, endothelin-1, angiotensin-II, serotonin, or thromboxane-A₂) in the presence or absence of mox-LDL or H₂O₂. [³H]Thymidine incorporation into DNA was measured as an index of VSMC proliferation. On interaction with mox-LDL or H₂O₂, U-II induced the greatest increase in [³H]thymidine incorporation among these vasoactive agents. A low concentration of U-II (10 nmol/l) enhanced the potential mitogenic effect of low concentrations of mox-LDL (120 to 337%) and H₂O₂ (177 to 226%). U-II at 50 nmol/l showed the maximal mitogenic effect (161%), which was abolished by G protein inactivator (GDP-β-S), c-Src tyrosine kinase inhibitor (radicicol), protein kinase C (PKC) inhibitor (Ro31-8220), extracellular signal-regulated kinase (ERK) kinase inhibitor (PD98059), or Rho kinase inhibitor (Y27632). Mox-LDL at 5 μg/ml showed the maximal mitogenic effect (211%), which was inhibited by free radical scavenger (catalase), intracellular and extracellular antioxidants (*N*-acetylcysteine and probucol), nicotinamide adenine dinucleotide phosphate oxidase inhibitor (diphenylene iodonium), or c-Jun N-terminal kinase (JNK) inhibitor (SP600125). These results suggested that U-II acts in synergy with mox-LDL in inducing VSMC DNA synthesis at the highest rate among these vasoactive agents. Activation of the G protein/c-Src/PKC/ERK and Rho kinase pathways by U-II together with the redox-sensitive JNK pathway by mox-LDL may explain the synergistic interaction between these agents. (*Hypertens Res* 2006; 29: 821–831)

Key Words: atherosclerosis, hypertension, lipoproteins, smooth muscle, urotensin-II

From the ¹⁾Department of Biochemistry and ²⁾Third Department of Internal Medicine, Showa University School of Medicine, Tokyo, Japan; ³⁾Cardiovascular Research Institute, Washington Hospital Center, Washington, DC, USA; and ⁴⁾Department of Internal Medicine, University of Texas–Houston Health Science Center, Texas, USA.

This work was presented in part at the 74th Scientific Sessions of the American Heart Association, Anaheim, California, USA, November 11–14, 2001 (*Circulation* 2001; **104** (Suppl): II-65).

Address for Reprints: Takuya Watanabe, M.D., Ph.D., Department of Biochemistry, Showa University School of Medicine, 1–5–8 Hatanodai, Shinagawa-ku, Tokyo 142–8555, Japan. E-mail: watanabemd@med.showa-u.ac.jp

Received February 14, 2006; Accepted in revised form July 4, 2006.

Introduction

Human urotensin-II (U-II) is a cyclic peptide of 11 amino acids, with a molecular weight of ~1,388 (1). U-II has been identified as an endogenous ligand for G-protein coupled receptor 14 (GPR14), which is now known as UT receptor (2). Both U-II and UT receptor are expressed in different tissues, including the cardiovascular, brain, kidney, atherosclerotic plaques in the coronary and carotid arteries, and abdominal aortic aneurysms (1–4). U-II is the most potent vasoconstrictor identified to date, with a potency 1–2 orders of magnitude greater than that of endothelin-1 (ET-1), and U-II has been shown to play an important role in the pathogenesis of pulmonary artery hypertension and remodeling (5–7). The main sources of circulating U-II are thought to be the heart, liver, kidney, vascular endothelium, and lymphocytes (1, 4, 8). Plasma concentrations of U-II average from 2 to 20 pmol/l in healthy volunteers (9), and are increased in hypertension (10), atherosclerosis (11), coronary artery disease (12), congestive heart failure (13), diabetes mellitus (14), renal failure (15), and portal hypertension caused by liver cirrhosis (16). Cheung *et al.* (10) and we (11) reported that U-II is positively correlated with systolic blood pressure and has an etiological role in systemic hypertension and its complications. In addition, increased urinary excretion of U-II has been demonstrated in patients with essential hypertension (17).

Oxidized low-density lipoprotein (ox-LDL) is a well-established risk factor for atherosclerosis, and stimulates the differentiation and proliferation of vascular smooth muscle cells (VSMCs). In particular, mildly oxidized LDL (mox-LDL) interacting with vasoactive agents and growth factors present in the vasculature may play an important role in the development of atherosclerosis, hypertension, and restenosis after angioplasty (18, 19). Recently, we showed that the mitogenic effect of mox-LDL is mediated by its oxidative components, such as reactive oxygen species (ROS), lysophosphatidylcholine (LPC), and 4-hydroxy-2-nonenal (HNE) (19–22). These compounds in combination with U-II synergistically promote the induction of DNA synthesis in VSMCs (23). In recent years, there has been renewal of interest in vasoactive oxidant hydrogen peroxide (H_2O_2) as a risk factor for hypertension and atherosclerosis (24).

In the present study, we investigated the interactions between U-II and mox-LDL in comparison with those between other vasoconstrictor G-protein agonists (ET-1, angiotensin-II [Ang-II], 5-HT, thromboxane- A_2 [TX- A_2]) and mox-LDL or H_2O_2 in inducing VSMC proliferation. In the present study, VSMC proliferation was examined by increases in both DNA synthesis and cell number. To elucidate the underlying mechanisms responsible for the synergistic interactions, we assessed the interactions between mitogenic mediators of mox-LDL (H_2O_2 , LPC, HNE) and U-II, and their intracellular signal transduction pathways in inducing VSMC proliferation.

Methods

Materials

Human U-II, ET-1, Ang-II, 5-HT, LDL, LPC, H_2O_2 , anti-Gq/11 α antibody, GDP- β -S, erbstatin A, radicicol, Ro31-8220, PD98059, SP600125, Y27632, catalase, *N*-acetylcysteine (NAC), probucol, and diphenylene iodonium (DPI) were purchased from Sigma Chemical Co. (St. Louis, USA). HNE was purchased from Cayman Chemical Co. (Ann Arbor, USA). U46619 (TX- A_2 mimic) was a generous gift from Upjohn Co. (Kalamazoo, USA). [3H]Thymidine (specific activity, 20 Ci/mol) was obtained from DuPont-NEN Research Products (Boston, USA).

LDL Oxidation

LDL was oxidized with $CuSO_4$ at a final concentration of 5 μ mol/l at 37°C, and the contents of thiobarbituric acid-reactive substances (TBARS) were determined every 30 min by sampling aliquots as described previously (19, 22). Electrophoretic mobilities of all samples were examined by 1% agarose gel electrophoresis. The conditions that did not alter the electrophoretic mobility but increased TBARS contents were selected for preparation of mox-LDL. To obtain ox-LDL, LDL was oxidized at 37°C for 30 h followed by termination of oxidation with ethylenediamine tetraacetic acid (EDTA). Lipoprotein concentrations are expressed as protein concentrations. Even at a concentration of 10 mg/ml, native LDL showed no development of TBARS, whereas mox-LDL showed a slight increase in TBARS formation (2–4 nmol/mg protein), with no change in the electrophoretic mobility. In contrast, ox-LDL showed a significant increase in TBARS formation (35 nmol/mg protein) and an increase in electrophoretic mobility.

Cell Culture

VSMCs were isolated from the thoracic aortas of male New Zealand White rabbits (body weight ~3 kg, $n=75$) by the explant method and were cultured in a humidified atmosphere (5% CO_2 /95% air) at 37°C (19, 22). After 3–4 weeks, the tissue blocks were removed and the migrated VSMCs were cultured, followed by 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 measurement of [3H]thymidine incorporation into the cellular DNA as described previously (19, 22). VSMCs at passage 1 or 2 were seeded in tissue culture plates 35 mm in diameter and grown to semi-confluence in Dulbecco's modified Eagle's medium (DMEM) con-

taining 10% fetal bovine serum (FBS). Then, the medium was replaced with 2 ml of DMEM containing 0.1% FBS and incubated for ~72 h for growth arrest. Subsequently, the medium was replaced with DMEM containing 500 µg/ml bovine serum albumin, 10 µg/ml bovine insulin, 20 µg/ml human transferrin, and 25 ng/ml selenium (serum-free medium). Three different experiments were then performed, as outlined below.

1) Experiment 1: VSMCs were incubated with the indicated concentrations of U-II with native LDL, mox-LDL, ox-LDL, H₂O₂, LPC, or HNE for 48 h.

2) Experiment 2: VSMCs were incubated with the indicated concentrations of U-II, ET-1, Ang-II, 5-HT, or TX-A₂ with mox-LDL or H₂O₂ for 48 h.

3) Experiment 3: VSMCs were incubated with the indicated concentrations of U-II, mox-LDL, or both along with an anti-Gq/11α antibody (2.5 µl/plate of 2 ml), a G protein inactivator (GDP-β-S, 100 µmol/l), an epidermal growth factor (EGF) receptor tyrosine kinase inhibitor (erbstatin A, 10 µmol/l), a c-Src tyrosine kinase inhibitor (radicolol, 10 µmol/l), a protein kinase C (PKC) inhibitor (Ro31-8220, 0.1 µmol/l), an extracellular signal-regulated kinase (ERK) kinase inhibitor (PD98059, 10 µmol/l), a c-Jun N-terminal kinase (JNK) inhibitor (SP600125, 10 µmol/l), a Rho kinase (ROCK) inhibitor (Y27632, 10 µmol/l), a free radical scavenger (catalase, 10 U/ml), an intracellular antioxidant (NAC, 400 µmol/l), a cholesterol-lowering drug with potent antioxidant properties (probucol, 10 µmol/l), or an nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor (DPI, 1 µmol/l) for 48 h.

To ensure that the blocking activity of the inhibitors was not due to their cytotoxic effect, dose-response studies using inhibitors alone were carried out. From these studies, we selected a concentration that could be attained physiologically if the compound was not cytotoxic or a concentration that was at least 1 log below the cytotoxic concentration. For all 3 experiments, VSMCs were exposed to [³H]thymidine at a concentration of 1 µCi/plate for the last 5 h during the 48 h incubation period and [³H]thymidine incorporation into VSMC DNA was measured with a liquid scintillation counter (Packard Instrument Co., Downers Grove, USA). All of the experiments were performed in quadruplicate, and each experiment was repeated a minimum of 3 times.

Cell Number

Primary VSMCs at passage 1 or 2 were seeded in tissue culture plates 35 mm in diameter at a density of ~100,000 cells/plate in DMEM containing 10% FBS (19, 22). After 96 h, the growth medium was replaced with 2 ml of DMEM containing 0.1% FBS and incubated for ~72 h for growth arrest and synchronization. Then, the medium was replaced with serum-free medium and the indicated concentrations of U-II and mox-LDL and incubated for 48 h. The experiment was terminated by aspiration of the medium, and then plates were

Fig. 1. Effects of U-II on VSMC DNA synthesis. Growth-arrested VSMCs were stimulated with different concentrations of U-II for 48 h, and the amount of [³H]thymidine incorporation into DNA was measured. Data are shown as the mean ± SEM (n = 12). Control value (209 ± 9 cpm) = 100%. *p < 0.0001 vs. the control (U-II, 0 nmol/l).

washed with PBS followed by addition of 1 ml of 2% (w/v) crude pancreatic trypsin in phosphate-buffered saline containing 152 mmol/l EDTA to each dish. The dishes were incubated at room temperature for 5 min. The contents of each dish were diluted to 10 ml with isotone II and the cell number was determined with an electronic cell counter (Coulter Corp., Hialeah, USA). Counting was performed in triplicate in each of the four plates and each experiment was repeated at least three times.

Statistical Analysis

All values are expressed as the means ± SEM. Multiple comparisons were performed by 2-way analysis of variance combined with Bonferroni's post-hoc test to evaluate the synergistic interactions between the two factors. To assess the dose-dependent effect of U-II and the inhibitory effects of several inhibitors, Dunnett's test was used. A value of p < 0.05 was considered statistically significant.

Results

Effects of U-II and Other Vasoactive Agents on VSMC DNA Synthesis

As shown in Fig. 1, U-II significantly increased [³H]thymidine incorporation into VSMC DNA in a concentration-dependent manner with a maximal effect at a concentration of 50 nmol/l (161 ± 10%, p < 0.0001 vs. the control). ET-1, Ang-II, 5-HT, and TX-A₂ also significantly increased [³H]thymidine incorporation in a concentration-dependent manner (data not shown). ET-1 had a maximal effect on [³H]thymidine incorporation at a concentration of 0.1 µmol/l (202 ± 16%, p < 0.0001 vs. the control), Ang-II at 1.75 µmol/l (202 ± 19%, p < 0.0005), 5-HT at 50 µmol/l (205 ± 12%, p < 0.0001), and TX-A₂ at 5 µmol/l (120 ± 19%, p < 0.05).

Fig. 2. Interactions of U-II with mox-LDL, ox-LDL, or their oxidative components in inducing VSMC DNA synthesis. Growth-arrested VSMCs were stimulated with the indicated concentration of native LDL (0.5 μ g/ml), mox-LDL (0.5 μ g/ml), ox-LDL (0.5 μ g/ml), H₂O₂ (5 μ mol/l), LPC (5 μ mol/l), or HNE (1 μ mol/l) with or without U-II (10 nmol/l) for 48 h, and the amount of [³H]thymidine incorporation was measured. Data are shown as the mean $\pm$ SEM (n=12). Control value (223 $\pm$ 12 cpm) = 100%. *p < 0.05, +p < 0.005, #p < 0.0001 vs. the control; **p < 0.05, ++p < 0.005, ##p < 0.0001 vs. the corresponding vasoactive agents without U-II.

Fig. 3. Interactions of mox-LDL with U-II or other vasoactive agents in inducing VSMC DNA synthesis. Growth-arrested VSMCs were stimulated with the indicated concentration of U-II (10 nmol/l), ET-1 (1 nmol/l), Ang-II (0.5 μ mol/l), 5-HT (1 μ mol/l), or TX-A₂ (0.5 μ mol/l) with or without mox-LDL (0.1 μ g/ml) for 48 h, and the amount of [³H]thymidine incorporation was measured. Data are shown as the mean $\pm$ SEM (n=12). Control value (240 $\pm$ 11 cpm) = 100%. *p < 0.05, +p < 0.0005, #p < 0.0001 vs. the control; **p < 0.0001 vs. the corresponding vasoactive agents without mox-LDL.

Fig. 4. Interactions of H_2O_2 with U-II or other vasoactive agents in inducing VSMC DNA synthesis. Growth-arrested VSMCs were stimulated with the indicated concentration of U-II (10 nmol/l), ET-1 (1 nmol/l), Ang-II (0.5 μ mol/l), 5-HT (1 μ mol/l), or TX-A₂ (0.5 μ mol/l) with or without H_2O_2 (1 μ mol/l) for 48 h, and the amount of [3 H]thymidine incorporation was measured. Data are shown as the mean $\pm$ SEM (n = 12). Control value (234 $\pm$ 13 cpm) = 100%. * p < 0.05, + p < 0.0005, # p < 0.0001 vs. the control; ** p < 0.0001 vs. the corresponding vasoactive agents without H_2O_2 .

Fig. 5. Effects of U-II with mox-LDL on VSMC number. Growth-arrested VSMCs were stimulated with U-II (10 nmol/l) and/or mox-LDL (0.1 μ g/ml) in serum-free medium for 48 h, and the cell number was counted. The baseline value of the control is 250,500 $\pm$ 10,500 cells/plate. Data are expressed as the mean $\pm$ SEM (n = 12). * p < 0.05 vs. control; + p < 0.0001 vs. others.

Effects of Native LDL, Mox-LDL, Ox-LDL, and Their Oxidative Components on VSMC DNA Synthesis

According to our preliminary examination, native LDL at all concentrations tested had no significant effect on [3 H]thymidine incorporation. Mox-LDL, ox-LDL, H_2O_2 , LPC, or HNE significantly increased [3 H]thymidine incorporation in a concentration-dependent manner (data not shown). Mox-LDL and ox-LDL showed the maximal effect on [3 H]thymidine

incorporation at a concentration of 5 μ g/ml (211 $\pm$ 25%, 154 $\pm$ 2, p < 0.0001), H_2O_2 at 5 μ mol/l (177 $\pm$ 7%, p < 0.0001), LPC at 15 μ mol/l (156 $\pm$ 8%, p < 0.0001), and HNE at 1 μ mol/l (143 $\pm$ 8%, p < 0.005).

Interactions of Mox-LDL, Ox-LDL, and Their Oxidative Components with U-II in Inducing VSMC DNA Synthesis

Low concentrations of mox-LDL (0.5 μ g/ml), ox-LDL (0.5 μ g/ml), H_2O_2 (5 μ mol/l), LPC (5 μ mol/l), or HNE (1 μ mol/l), but not native LDL (0.5 μ g/ml), added alone induced slight but significant increases in [3 H]thymidine incorporation (Fig. 2). When added concomitantly, U-II even at a non-mitogenic concentration (10 nmol/l) significantly potentiated the effects of native LDL, mox-LDL, ox-LDL, H_2O_2 , LPC, or HNE on [3 H]thymidine incorporation (Fig. 2).

Interactions of Mox-LDL with U-II and Other Vasoactive Agents in Inducing VSMC DNA Synthesis

At low concentrations, mox-LDL (0.1 μ g/ml) or U-II (10 nmol/l) alone did not significantly increase [3 H]thymidine incorporation (Fig. 3). However, ET-1 (1 nmol/l), Ang-II (0.5 μ mol/l), 5-HT (1 μ mol/l), or TX-A₂ (0.5 μ mol/l) alone induced significant increases in [3 H]thymidine incorporation (Fig. 3). When added concomitantly, even the non-mitogenic concentration of mox-LDL acted synergistically with U-II, ET-1, Ang-II, 5-HT, or TX-A₂ in inducing [3 H]thymidine incorporation (all p < 0.0001) (Fig. 3).

Fig. 6. Inhibition of *mox*-LDL- or *U-II*-induced VSMC DNA synthesis by antioxidants or signal transduction pathway inhibitors. Growth-arrested VSMCs were stimulated with *mox*-LDL (5 μ g/ml) or *U-II* (50 nmol/l) in the presence or absence of catalase (10 U/ml), NAC (400 μ mol/l), probucol (10 μ mol/l), DPI (1 μ mol/l), SP600125 (10 μ mol/l), GDP- β -S (100 μ mol/l), radicicol (10 μ mol/l), Ro31-8220 (0.1 μ mol/l), PD98059 (10 μ mol/l), or Y27632 (10 μ mol/l). The amount of [³H]thymidine incorporation was measured. Data are shown as the mean $\pm$ SEM ($n=10$). Control value (242 ± 12 cpm) = 100%. * $p < 0.05$, ⁺ $p < 0.005$ vs. *mox*-LDL + vehicle; # $p < 0.01$ vs. *U-II* + vehicle; ** $p < 0.005$, ++ $p < 0.0001$ vs. the control.

Interactions of H₂O₂ with *U-II* and Other Vasoactive Agents in Inducing VSMC DNA Synthesis

We repeated the experiments to examine whether H₂O₂ (ROS donor) could mimic the *mox*-LDL-potentiated effects of *U-II* and the other vasoactive agents on [³H]thymidine incorporation. As shown in Fig. 4, the combination of a low concentration of H₂O₂ (1 μ mol/l) with *U-II* (10 nmol/l), ET-1 (1 nmol/l), Ang-II (0.5 μ mol/l), 5-HT (1 μ mol/l), or TX-A₂ (0.5 μ mol/l) also exerted synergism in inducing [³H]thymidine incorporation (all $p < 0.0001$) (Fig. 4).

Effects of *U-II* with *Mox*-LDL on VSMC Number

The effects of *U-II* and/or *mox*-LDL on VSMC number are shown in Fig. 5. *U-II* (10 nmol/l) or *mox*-LDL (0.1 μ g/ml) significantly increased the cell number (both $p < 0.05$ vs. the baseline value). When the two were added together, there was an additional increase in cell number compared with the single additions ($p < 0.0001$). The increase in VSMC number paralleled the increase in [³H]thymidine incorporation into DNA described above.

Inhibition of *Mox*-LDL- or *U-II*-Induced VSMC DNA Synthesis by Antioxidants or Signal Transduction Pathway Inhibitors

To determine how *mox*-LDL (5 μ g/ml) exerts its mitogenic

effect, we assessed the effects of the free radical scavenger, catalase, the intracellular antioxidant, NAC, the cholesterol-lowering drug with potent antioxidant properties, probucol, the NADPH oxidase inhibitor, DPI, or the JNK inhibitor, SP600125, on *mox*-LDL-induced [³H]thymidine incorporation. To elucidate the intracellular signal transduction pathway of *U-II* (50 nmol/l), we assessed the effects of the G protein inactivator, GDP- β -S, the c-Src tyrosine kinase inhibitor, radicicol, the PKC inhibitor, Ro31-8220, the ERK inhibitor, PD98059, or the ROCK inhibitor, Y27632, on *U-II*-induced [³H]thymidine incorporation. Although catalase (10 U/ml), NAC (400 μ mol/l), probucol (10 μ mol/l), DPI (1 μ mol/l), or SP600125 (10 μ mol/l) alone had no significant effect on [³H]thymidine incorporation (data not shown), these antioxidants significantly inhibited *mox*-LDL-induced [³H]thymidine incorporation (Fig. 6). Similarly, GDP- β -S (100 μ mol/l), radicicol (10 μ mol/l), Ro31-8220 (0.1 μ mol/l), PD98059 (10 μ mol/l), or Y27632 (10 μ mol/l) alone showed no significant effect on [³H]thymidine incorporation (data not shown), but these inhibitors significantly inhibited *U-II*-induced [³H]thymidine incorporation (Fig. 6).

Inhibition of the Interaction between *U-II* and *Mox*-LDL in Inducing VSMC DNA Synthesis by Signal Transduction Pathway Inhibitors

To determine how *U-II* and *mox*-LDL exert their synergistic interaction, we assessed the effects of anti-Gq/11 α antibody,

Fig. 7. Inhibition of the interaction between U-II and mox-LDL in inducing VSMC DNA synthesis by signal transduction pathway inhibitors. Growth-arrested VSMCs were stimulated with U-II (10 nmol/l), mox-LDL (0.1 μ g/ml), or both in the presence or absence of anti-Gq/11 α antibody (2.5 μ l/plate of 2 ml), erbstatin A (10 μ mol/l), radicicol (10 μ mol/l), Ro31-8220 (0.1 μ mol/l), PD98059 (10 μ mol/l), SP600125 (10 μ mol/l), or Y27632 (10 μ mol/l). The amount of [³H]thymidine incorporation was measured. Data are shown as the mean $\pm$ SEM (n = 12). Control value (236 $\pm$ 12 cpm) = 100%. *p < 0.05, +p < 0.005, #p < 0.0001 vs. mox-LDL + U-II; **p < 0.0001 vs. the control.

Fig. 8. Oxidative components of mox-LDL and ox-LDL. ROS, reactive oxygen species; LPC, lysophosphatidylcholine; HNE, 4-hydroxy-2-nonenal.

an EGF receptor tyrosine kinase inhibitor (erbstatin A), a c-Src tyrosine kinase inhibitor (radicicol), a PKC inhibitor (Ro31-8220), an ERK inhibitor (PD98059), a JNK inhibitor (SP600125), or a ROCK inhibitor (Y27632) on the interaction between U-II and mox-LDL in inducing [³H]thymidine incorporation. Anti-Gq/11 α antibody (2.5 μ l/plate of 2 ml), erbstatin A (10 μ mol/l), radicicol (10 μ mol/l), Ro31-8220 (0.1 μ mol/l), PD98059 (10 μ mol/l), SP600125 (10 μ mol/l), or Y27632 (10 μ mol/l) alone showed no significant effect on [³H]thymidine incorporation (data not shown). As shown in Fig. 7, anti-Gq/11 α antibody, erbstatin A, SP600125, or Y27632 partially inhibited the synergistic interaction between

U-II (10 nmol/l) and mox-LDL (0.1 μ g/ml) ($p < 0.05$, $p < 0.05$, $p < 0.005$, respectively), whereas radicicol, Ro31-8220, or PD98059 completely abolished the synergistic interaction between the two (all $p < 0.0001$).

Discussion

In the present study, when primary rabbit VSMCs were incubated with U-II or other vasoactive agents (ET-1, Ang-II, 5-HT, and TX-A₂) in the presence or absence of mox-LDL or H₂O₂, the combinations of U-II and mox-LDL or U-II and H₂O₂ induced the highest level of DNA synthesis. This is the first evidence of the synergism of these combinations, and the most important finding of this study. The present study also demonstrated that the mechanisms underlying the synergistic interaction between U-II and mox-LDL were the interactions between U-II and the major oxidative components of mox-LDL (LPC, HNE, ROS), as well as multiple intracellular signal transduction pathways involved in the induction of VSMC DNA synthesis. However, further studies are required to demonstrate that these interesting phenomena would occur in primary human VSMCs, which is a limitation of the present study.

Evidence reported by Douglas and colleagues has indicated that U-II is an important contributor to cardiovascular diseases (2, 4–6, 25). U-II is the most potent vasoconstrictor identified to date, with 10-fold and 300-fold greater potency

Fig. 9. Intracellular signal transduction pathways of U-II and *mox*-LDL in VSMC. SR, scavenger receptor; EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; JNK, *c*-Jun N-terminal kinase; ROCK, Rho kinase.

than ET-1 and norepinephrine, respectively (26). U-II causes vasoconstriction in human coronary, pulmonary, mammary, and radial arteries, and saphenous and umbilical veins *in vitro* (26, 27). Infusion of U-II into the forearm brachial artery has been shown to cause vasoconstriction in healthy volunteers (28), and this effect was augmented in patients with essential hypertension (29). Cheung *et al.* (10) showed that plasma concentrations of U-II were elevated in hypertensive patients as compared with normotensive controls and were positively correlated with systolic and diastolic blood pressures (10). Their study showed that plasma concentrations of U-II are positively correlated with body weight and fasting plasma glucose level (10). U-II is known to be present at high levels in the plasma of diabetic patients (14) and to inhibit glucose-induced insulin release from pancreatic β cells, creating a predisposition to diabetes (30). In addition to diabetes, U-II may also be associated with obesity (31). Intracerebroventricular injection of U-II caused an increase in food and water intake in mice (32). U-II has been shown to stimulate hyperlipidemia by increasing plasma concentrations of free fatty acids in coho salmon (33). These findings suggest that U-II may be associated with the pathogenesis of the metabolic syndrome. Further studies using several selective UT receptor antagonists or urotensin converting enzyme inhibitor(s), and UT receptor-derived transgenic animals are required to investigate the precise roles of U-II in pathological conditions such as hypertension, atherosclerosis, and the metabolic syndrome.

Human U-II-like immunoreactivity in the human vasculature was first observed as diffuse staining in cardiac myocytes and intense staining in the macrophage- and VSMC-rich

regions of human coronary atherosclerotic plaque (5). Hassan and colleagues (34) reported that U-II was expressed in endothelial cells, foam cells, and myointimal and medial VSMCs of atherosclerotic human coronary arteries. In contrast, Maguire and Davenport (1) reported the expression of U-II in endothelial cells of the human aorta and epicardial coronary artery as well as in intramyocardial vessels with diameters typical of resistance arteries (60–120 μ m) but not in cardiac myocytes or VSMCs. However, UT receptors are present in VSMCs throughout the human coronary artery tree, from large epicardial to small resistance arteries, and mediate vasoconstriction (27). In atherosclerotic lesions of the human coronary arteries, U-II was detected within the region of infiltrating macrophages of the plaque but not in contractile VSMCs of media or proliferated VSMCs of the thickened intima (1, 3). Double immunostaining with anti-U-II antibody and an anti-CD68 antibody specific for macrophages demonstrated the co-localization of U-II and macrophages, suggesting that U-II may be produced mainly by these cells (3). Boussette and colleagues have recently shown that lymphocytes are by far the largest producers of U-II, whereas monocytes and macrophages are the largest producers of UT receptors, and that there is relatively little expression of UT receptors in foam cells, lymphocytes, or platelets in human blood (4). Recent studies from our laboratory and by other groups have shown that U-II accelerates foam cell formation by up-regulating the expression of acyl-coenzyme A:cholesterol acyltransferase-1 in human monocyte-derived macrophages (35), stimulates cell proliferation and up-regulates the expression of type 1 collagen in human endothelial cells (36,

37), and activates NADPH oxidase and plasminogen activator inhibitor-1 in human VSMCs (38), leading to atherosclerotic plaque formation. The expression of UT receptors is up-regulated by ox-LDL (39) as well as by hypoxia (40) and by the production of interferon- γ from activated T lymphocytes found within the atherosclerotic plaque (41). In the present study, it is possible that mox-LDL may have increased UT receptor expression, thereby contributing to the synergistic interaction between mox-LDL and U-II in inducing VSMC proliferation.

It is well established that ox-LDL, which accumulates in vascular lesions, alters vascular cell function in ways that can be construed as atherogenic. LDL oxidation is a progressive process initially leading to the formation of mox-LDL and later to ox-LDL (22). The extent of oxidation in LDL particles is dependent on the pro-oxidant conditions and the duration of oxidation (22). Because plasma contains various natural antioxidants, extensive oxidation of LDL is unlikely to occur. Instead, LDL oxidation in the plasma may result in production of mox-LDL, which is the most potent of the three forms of LDL (19). The increased mitogenic effect of mox-LDL or ox-LDL is attributable to the chemical changes brought about by oxidation of the components of LDL, *i.e.*, generation of lipid peroxides and ROS and conversion of phosphatidylcholine (PC) to LPC (20). As shown in Fig. 8, during the early stages of oxidation of LDL, there is significant accumulation of peroxides and other ROS, whereas the LPC levels are still relatively low. After 24 h of oxidative stress, LDL contains large amounts of LPC but only small amounts of ROS. More or less at the same time, unsaturated aldehydes, such as HNE, are generated by β -scission of alkoxyl radicals in polyunsaturated fatty acids that are present in LDL. Extensive oxidation is associated with the formation of HNE (42).

Several studies in our laboratory and by other groups have shown that mox-LDL and ox-LDL and their oxidative components, *i.e.*, ROS (H_2O_2), LPC, and HNE, induce VSMC proliferation *via* the NADPH-dependent redox-sensitive JNK pathway as well as the ERK1/2 pathway (42–49). All mitogenic effects of U-II, ET-1, Ang-II, 5-HT, and TX-A₂ on VSMCs are predominantly mediated *via* their specific G protein-coupled receptors (UT, ET_A, AT₁, 5-HT_{2A}, or TP, respectively), c-Src tyrosine kinase, PKC, and the ERK pathway. In addition to the ERK pathway, U-II and other vasoactive agents activate the RhoA/ROCK-dependent pathway (50, 51), ET-1 and TX-A₂ activate the JNK pathway (52–54), and Ang-II activates the JNK pathway and Janus kinase-2 (JAK-2)/signal transducer and activator of transcription-3 (STAT-3) pathway for VSMC proliferation (55, 56). Therefore, it is possible that activation of multiple signal transduction pathways as described above may contribute to the synergistic interaction of mox-LDL or H_2O_2 with vasoactive agents in inducing VSMC proliferation.

In the present study, the synergistic interaction between U-II and mox-LDL was completely abolished by the c-Src-

tyrosine kinase inhibitor (radicolol), the PKC inhibitor (Ro31-8220), or the ERK kinase inhibitor (PD98059), and was partially inhibited by anti-Gq/11 α antibody, the EGF receptor tyrosine kinase inhibitor (erbstatin A), the JNK inhibitor (SP600125), or the ROCK inhibitor (Y27632). This suggests that the c-Src/PKC/ERK pathway may be the common pathway for U-II and mox-LDL, and that the Gq protein, EGF receptor tyrosine kinase, JNK, or Rho A/ROCK pathways may be additional pathways for U-II and/or mox-LDL (Fig. 9). Simultaneous activation of these multiple pathways may explain the underlying mechanism of synergistic interactions between the two agents. However, this has raised several questions. One is whether such a low concentration of U-II (10 nmol/l) or mox-LDL (0.1 μ g/ml) could activate the ERK or JNK pathway. Tamura *et al.* (57) have shown that U-II at 10 nmol/l significantly increases the ERK phosphorylation in VSMCs. We demonstrated that the mitogenic effect of mox-LDL at 0.1 μ g/ml is mediated *via* the redox-sensitive JNK pathway by using the specific inhibitors for JNK (SP600125) or NADPH oxidase (DPI) (125 ± 10 to 97 ± 6 , 95 ± 2 ; each $p < 0.05$) (58). Another is whether the combination of higher concentrations of these two agents could induce more potent interactions. In our previous study (23), the combination of higher concentrations of U-II and mox-LDL attenuated their synergistic interaction. Wang *et al.* (39) have recently shown that increasing concentrations of ox-LDL in combination with U-II down-regulate UT receptors and induce apoptosis in VSMCs.

There is increasing data for clarifying the interaction between classical growth factors and ox-LDL in modulating VSMC growth. Auge *et al.* (59) reported that ox-LDL-induced VSMC proliferation involved the EGF receptor/phosphatidylinositol-3 kinase/Akt and the sphingolipid signaling pathways. Previous studies have shown that platelet-derived growth factor-BB (PDGF-BB), EGF, basic fibroblast growth factor (FGF-2), or insulin-like growth factor (IGF) exerts a synergistic interaction with LDL in inducing VSMC proliferation (60). However, recent studies showed that acidic FGF (FGF-1) interacts with ox-LDL to inhibit VSMC growth by formation of the FGF-1/ox-LDL complex, which has a dramatically decreased ability to bind heparin (61, 62).

In conclusion, the results of the present study suggested that U-II shows synergistic interactions with mox-LDL in inducing VSMC proliferation at the greatest rate among the vasoactive agents. Activation of the Gq protein/tyrosine kinase/PKC/ERK and Rho A/ROCK pathways by U-II together with the redox-sensitive JNK pathway by mox-LDL may explain the synergistic interaction between the two agents. These synergistic interactions of several vasoactive agents, including U-II, with mox-LDL or H_2O_2 may explain the molecular mechanisms underlying the long-standing clinical observation that coexistence of hypertension with oxidative stress or hypercholesterolemia promotes the development of atherosclerosis and restenosis after angioplasty.

References

- Maguire JJ, Davenport AP: Is urotensin-II the new endothelin? *Br J Pharmacol* 2002; **137**: 579–588.
- Douglas SA, Ohlstein EH: Human urotensin-II, the most potent mammalian vasoconstrictor identified to date, as a therapeutic target for the management of cardiovascular disease. *Trends Cardiovasc Med* 2000; **10**: 229–237.
- Maguire JJ, Kuc RE, Wiley KE, Kleinz MJ, Davenport AP: Cellular distribution of immunoreactive urotensin-II in human tissues with evidence of increased expression in atherosclerosis and a greater constrictor response of small compared to large coronary arteries. *Peptides* 2004; **25**: 1767–1774.
- Bousette N, Patel L, Douglas SA, Ohlstein EH, Giaid A: Increased expression of urotensin II and its cognate receptor GPR14 in atherosclerotic lesions of the human aorta. *Atherosclerosis* 2004; **176**: 117–123.
- Ames RS, Sarau HM, Cambers JK, *et al*: Human urotensin II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* 1999; **401**: 282–286.
- MacLean MR, Alexander D, Stirrat A, *et al*: Contractile responses to human urotensin-II in rat and human pulmonary arteries: effect of endothelial factors and chronic hypoxia in the rat. *Br J Pharmacol* 2000; **130**: 201–204.
- Watanabe T, Pakala R, Katagiri T, Benedict CR: Synergistic effect of urotensin II with serotonin on vascular smooth muscle cell proliferation. *J Hypertens* 2001; **19**: 2191–2196.
- Charles CJ, Rademaker MT, Richards AM, Yandle TG: Urotensin II: evidence for cardiac, hepatic and renal production. *Peptides* 2005; **26**: 2211–2214.
- Watanabe T, Kanome T, Miyazaki A, Katagiri T: Human urotensin II linking hypertension and coronary artery disease. *Hypertens Res* 2006; **29**: 375–387.
- Cheung BM, Leung R, Man YB, Wong LY: Plasma concentration of urotensin II is raised in hypertension. *J Hypertens* 2004; **22**: 1341–1344.
- Suguro T, Watanabe T, Kodate S, *et al*: Increased levels of human urotensin II are correlated with carotid atherosclerosis in patient with essential hypertension. *Am J Hypertens* 2006, in press.
- Heringlake M, Kox T, Uzun O, *et al*: The relationship between urotensin II plasma immunoreactivity and left ventricular filling pressures in coronary artery disease. *Regul Pept* 2004; **121**: 129–136.
- Richards AM, Nicholls MG, Lainchbury JG, Fisher S, Yandle TG: Plasma urotensin II in heart failure. *Lancet* 2002; **360**: 545–546.
- Totsune K, Takahashi K, Arihara Z, Sone M, Ito S, Murakami O: Increased plasma urotensin II levels in patients with diabetes mellitus. *Clin Sci (Lond)* 2003; **104**: 1–5.
- Totsune K, Takahashi K, Arihara Z, *et al*: Role of urotensin II in patients on dialysis. *Lancet* 2001; **358**: 810–811.
- Heller J, Schepke M, Neef M, Woitas R, Rabe C, Sauerbruch T: Increased urotensin II plasma levels in patients with cirrhosis and portal hypertension. *J Hepatol* 2002; **37**: 767–772.
- Matsushita M, Shichiri M, Imai T, *et al*: Co-expression of urotensin II and its receptor (GPR14) in human cardiovascular and renal tissues. *J Hypertens* 2001; **19**: 2185–2190.
- Koba S, Pakala R, Watanabe T, Katagiri T, Benedict CR: Vascular smooth muscle proliferation: synergistic interaction between serotonin and low density lipoproteins. *J Am Coll Cardiol* 1999; **34**: 1644–1651.
- Watanabe T, Pakala R, Koba S, Katagiri T, Benedict CR: Lysophosphatidylcholine and reactive oxygen species mediate the synergistic effect of mildly oxidized LDL with serotonin on vascular smooth muscle cell proliferation. *Circulation* 2001; **103**: 1440–1445.
- Stiko A, Regnstrom J, Shah PK, Cercek B, Nilsson J: Active oxygen species and lysophosphatidylcholine are involved in oxidized low density lipoprotein activation of smooth muscle cell DNA synthesis. *Arterioscler Thromb Vasc Biol* 1996; **16**: 194–200.
- Watanabe T, Pakala R, Katagiri T, Benedict CR: Lipid peroxidation product 4-hydroxy-2-nonenal acts synergistically with serotonin in inducing vascular smooth muscle cell proliferation. *Atherosclerosis* 2001; **155**: 37–44.
- Watanabe T, Pakala R, Katagiri T, Benedict CR: Mildly oxidized low-density lipoprotein acts synergistically with angiotensin II in inducing vascular smooth muscle cell proliferation. *J Hypertens* 2001; **19**: 1065–1073.
- Watanabe T, Pakala R, Katagiri T, Benedict CR: Synergistic effect of urotensin II with mildly oxidized LDL on DNA synthesis in vascular smooth muscle cells. *Circulation* 2001; **104**: 16–18.
- Suvorava T, Lauer N, Kumpf S, *et al*: Endogenous vascular hydrogen peroxide regulates arteriolar tension *in vivo*. *Circulation* 2005; **112**: 2487–2495.
- Douglas SA: Human urotensin-II as a novel cardiovascular target: ‘heart’ of the matter or simply a fishy ‘tail’? *Curr Opin Pharmacol* 2003; **3**: 159–167.
- Russell FD, Molenaar P, O’Brien DM: Cardiostimulant effects of urotensin-II in human heart *in vitro*. *Br J Pharmacol* 2001; **132**: 5–9.
- Maguire JJ, Kuc RE, Davenport AP: Orphan-receptor ligand human urotensin II: receptor localization in human tissues and comparison of vasoconstrictor responses with endothelin-1. *Br J Pharmacol* 2000; **131**: 441–446.
- Bohm F, Pernow J: Urotensin II evokes potent vasoconstriction in humans *in vivo*. *Br J Pharmacol* 2002; **135**: 25–27.
- Sondermeijer B, Kompa A, Komesaroff P, Krum H: Effect of exogenous urotensin-II on vascular tone in skin microcirculation of patients with essential hypertension. *Am J Hypertens* 2005; **18**: 1195–1199.
- Silvestre RA, Egido EM, Hernandez R, *et al*: Urotensin-II is present in pancreatic extracts and inhibits insulin release in the perfused rat pancreas. *Eur J Endocrinol* 2004; **151**: 803–809.
- Watanabe T, Kanome T, Suguro T, Miyazaki A: Human urotensin II and metabolic syndrome. *Vascular Disease Prevention* 2006; **3**: 91–98.
- Do-Rego JC, Chatenet D, Orta MH, *et al*: Behavioral effects of urotensin-II centrally administered in mice. *Psychopharmacology (Berl)* 2005; **14**: 1–15.
- Sheridan MA, Plisetskaya EM, Bern HA, Gorbman A: Effects of somatostatin-25 and urotensin II on lipid and car-

- bohydrate metabolism of coho salmon, *Oncorhynchus kisutch*. *Gen Comp Endocrinol* 1987; **66**: 405–414.
34. Hassan GS, Douglas SA, Ohlstein EH, Giaid A: Expression of urotensin-II in human coronary atherosclerosis. *Peptides* 2005; **26**: 2464–2472.
 35. Watanabe T, Suguro T, Kanome T, et al: Human urotensin II accelerates foam cell formation in human monocyte-derived macrophages. *Hypertension* 2005; **46**: 738–744.
 36. Shi L, Ding W, Li D, et al: Proliferation and anti-apoptotic effects of human urotensin II on human endothelial cells. *Atherosclerosis* 2006; **188**: 260–264.
 37. Wang H, Mehta JL, Chen K, Zhang X, Li D: Human urotensin II modulates collagen synthesis and the expression of MMP-1 in human endothelial cells. *J Cardiovasc Pharmacol* 2004; **44**: 577–581.
 38. Djordjevic T, BelAiba RS, Bonello S, Pfeilschifter J, Hess J, Gorlach A: Human urotensin II is a novel activator of NADPH oxidase in human pulmonary artery smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2005; **25**: 519–525.
 39. Wang ZJ, Ding WH, Shi LB, Bu DF, Ren ZW, Tang CS: Effects of oxidized-low density lipoprotein on expression of urotensin II receptor GPR14 in rat aortic smooth muscle cells. *Zhonghua Yi Xue Za Zhi* 2006; **24**: 242–246.
 40. Hongfang J, Bailin C, Bin Z, et al: Effects of hydrogen sulfide on hypoxic pulmonary vascular structural remodeling. *Life Sci* 2006; **78**: 1299–1309.
 41. Birker-Robaczewska M, Boukhadra C, Studer R, Mueller C, Binkert C, Nayler O: The expression of urotensin II receptor (U2R) is up-regulated by interferon- γ . *J Recept Signal Transduct Res* 2003; **23**: 289–305.
 42. Ruef J, Rao GN, Li F, et al: Induction of rat aortic smooth muscle cell growth by the lipid peroxidation product 4-hydroxy-2-nonenal. *Circulation* 1998; **97**: 1071–1078.
 43. Kusuvara M, Chait A, Cader A, Berk BC: Oxidized LDL stimulates mitogen-activated protein kinases in smooth muscle cells and macrophages. *Arterioscler Thromb Vasc Biol* 1997; **17**: 141–148.
 44. Yang CM, Chien CS, Hsiao LD, et al: Mitogenic effect of oxidized low-density lipoprotein on vascular smooth muscle cells mediated by activation of Ras/Raf/MEK/MAPK pathway. *Br J Pharmacol* 2001; **132**: 1531–1541.
 45. Baas AS, Berk BC: Differential activation of mitogen-activated protein kinases by H_2O_2 and O_2^- in vascular smooth muscle cells. *Circ Res* 1995; **77**: 29–36.
 46. Yamakawa T, Eguchi S, Yamakawa Y, et al: Lysophosphatidylcholine stimulates MAP kinase activity in rat vascular smooth muscle cells. *Hypertension* 1998; **31**: 248–253.
 47. Yokoyama M, Inoue N, Kawashima S: Role of the vascular NADH/NADPH oxidase system in atherosclerosis. *Ann NY Acad Sci* 2000; **902**: 241–248.
 48. Fang X, Gibson S, Flowers M, Furui T, Bast RC Jr, Mills GB: Lysophosphatidylcholine stimulates activator protein 1 and the c-jun N-terminal kinase activity. *J Biol Chem* 1997; **272**: 13683–13689.
 49. Watanabe T, Koba S, Katagiri T, Pakala R, Benedict CR: Lysophosphatidylcholine potentiates the mitogenic effect of various vasoactive compounds on rabbit aortic smooth muscle cells. *Jpn Heart J* 2002; **43**: 409–416.
 50. Sauzeau V, Mellionec EL, Bertoglio J, Scalbert E, Pacaud P, Loirand G: Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. *Circ Res* 2001; **88**: 1102–1104.
 51. Seko T, Ito M, Kureishi Y, et al: Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. *Circ Res* 2003; **92**: 411–418.
 52. Fei J, Viedt C, Soto U, Elsing C, Jahn L, Kreuzer J: Endothelin-1 and smooth muscle cells: induction of jun amino-terminal kinase through an oxygen radical-sensitive mechanism. *Arterioscler Thromb Vasc Biol* 2000; **20**: 1244–1249.
 53. Miggin SM, Kinsella BT: Thromboxane A_2 receptor mediated activation of the mitogen activated protein kinase cascades in human uterine smooth muscle cells. *Biochim Biophys Acta* 2001; **1539**: 147–162.
 54. Gao Y, Tang S, Zhou S, Ware A: The thromboxane A_2 receptor activates mitogen-activated protein kinase via protein kinase C-dependent Gi coupling and Src-dependent phosphorylation of the epidermal growth factor receptor. *J Pharmacol Exp Ther* 2001; **296**: 426–433.
 55. Kyaw M, Yoshizumi M, Tsuchiya K, Kirima K, Tamaki T: Antioxidants inhibit JNK and p38 MAPK activation but not ERK 1/2 activation by angiotensin II in rat aortic smooth muscle cells. *Hypertens Res* 2001; **24**: 251–261.
 56. Marrero MB, Schieffer B, Li B, Sun J, Harp JB, Ling BN: Role of Janus kinase/signal transducer and activator of transcription and mitogen-activated protein kinase cascades in angiotensin II- and platelet-derived growth factor-induced vascular smooth muscle cell proliferation. *J Biol Chem* 1997; **272**: 24684–24690.
 57. Tamura K, Okazaki M, Tamura M, Isozumi K, Tasaki H, Nakashima Y: Urotensin II-induced activation of extracellular signal-regulated kinase in cultured vascular smooth muscle cells: involvement of cell adhesion-mediated integrin signaling. *Life Sci* 2003; **72**: 1049–1060.
 58. Watanabe T, Pakala R, Katagiri T, Benedict CR: Lysophosphatidylcholine is a major contributor to the synergistic effect of mildly oxidized low-density lipoprotein with endothelin-1 on vascular smooth muscle cell proliferation. *J Cardiovasc Pharmacol* 2002; **39**: 449–459.
 59. Auge N, Garcia V, Maupas-Schwalm F, Levade T, Salvayre R, Negre-Salvayre A: Oxidized LDL-induced smooth muscle cell proliferation involves the EGF receptor/PI-3 kinase/Akt and the sphingolipid signaling pathways. *Arterioscler Thromb Vasc Biol* 2002; **22**: 1990–1995.
 60. Resink TJ, Bochkov VN, Hahn AW, Philippova MP, Buhler FR, Tkachuk VA: Low- and high-density lipoproteins as mitogenic factors for vascular smooth muscle cells: individual, additive and synergistic effects. *J Vasc Res* 1995; **32**: 328–338.
 61. Ananyeva N, Tjurmin A, Saenko E, Haudenschild C: Low density lipoproteins interact with acidic fibroblast growth factor and modify its function. *Arterioscler Thromb Vasc Biol* 2003; **23**: 601–607.
 62. Zettler ME, Prociuk MA, Austria A, Zhong G, Pierce GN: Oxidized low-density lipoprotein retards the growth of proliferating cells by inhibiting nuclear translocation of cell cycle proteins. *Arterioscler Thromb Vasc Biol* 2004; **24**: 727–732.