

Apoptotic Action of Peroxisome Proliferator-Activated Receptor- γ Activation in Human Non-Small-Cell Lung Cancer Is Mediated via Proline Oxidase-Induced Reactive Oxygen Species Formation

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ABSTRACT

Peroxisome proliferator-activated receptor (PPAR)- γ ligands have been shown to inhibit human lung cancers by inducing apoptosis and differentiation. In the present study, we elucidated the apoptotic mechanism of PPAR γ activation in human lung cancers by using a novel PPAR γ agonist, 1-(trans-methylimino-*N*-oxy)-6-(2-morpholinoethoxy)-3-phenyl-(1*H*-indene-2-carboxylic acid ethyl ester (KR-62980), and rosiglitazone. PPAR γ activation selectively inhibited cell viability of non-small-cell lung cancer with little effect on small-cell lung cancer and normal lung cells. The cell death induced by PPAR γ activation presented apoptotic features of oligonucleosomal DNA fragmentation in A549 human non-small-cell lung cancer cell line. Reactive oxygen species (ROS) production was accompa-

nied by increased expression of proline oxidase (POX), a redox enzyme expressed in mitochondria, upon incubation with the agonists. POX RNA interference treatment blocked PPAR γ -induced ROS formation and cytotoxicity, suggesting that POX plays a functional role in apoptosis through ROS formation. The apoptotic effects by the agonists were antagonized by bisphenol A diglycidyl ether, a PPAR γ antagonist, and by knockdown of PPAR γ expression, indicating the involvement of PPAR γ in these actions. The results of the present study suggest that PPAR γ activation induces apoptotic cell death in non-small-cell lung carcinoma mainly through ROS formation via POX induction.

Lung cancer is the most common cause of cancer death in the world for men and women, and its epidemic is closely associated with smoking. The cancer can be divided into two groups as small-cell lung cancer and non-small-cell lung cancer (NSCLC), of which NSCLC constitutes major populations (Ginsber et al., 1993). NSCLC are subdivided into squamous, adenocarcinoma, and large cell carcinoma phenotypes, and they are frequently associated with Ras mutation. Various approaches for lung cancer treatment, including induction of differentiation and apoptosis, have been attempted,

but conventional chemotherapy and radiotherapy of lung cancer are still of limited effectiveness, necessitating the development of new treatment strategies.

Peroxisome proliferator-activated receptors (PPARs) are members of the family of ligand-activated transcription factors that include receptors for steroids, thyroid hormone, retinoic acid, and vitamin D. Of subtypes of PPAR (PPAR α , PPAR β , and PPAR γ), each encoded by a separate gene, the role of PPAR γ , in particular, in the proliferation and differentiation of various cancers has been extensively investigated. PPAR γ expression has been identified in various cancer cell lines and in human tumors, including breast, colon, gastric, prostate, and lung cancers. Furthermore, various PPAR γ ligands, including thiazolidinediones, exhibited anticancer effects in many types of cancer cells, and their actions were associated with the induction of differentiation and apoptosis. With regard to the role of

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ABBREVIATIONS: NSCLC, non-small-cell lung cancer(s); PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; POX, proline oxidase; KR-62980, 1-(trans-methylimino-*N*-oxy)-6-(2-morpholinoethoxy)-3-phenyl-(1*H*-indene-2-carboxylic acid ethyl ester; BADGE, bisphenol A diglycidyl ether; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; 15d-PGJ2, 15-deoxy- $\Delta^{12,14}$ -prostaglandin-J2; RNAi, RNA interference; DCFH, 2',7'-dichlorodihydrofluorescein; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse transcription-polymerase chain reaction.

PPAR γ in lung cancer, decreased expression of PPAR γ was correlated with poor prognosis (Sasaki et al., 2002), and Tsubouchi et al. (2000) demonstrated that PPAR γ ligands lead to the inhibition of human lung cancer cell growth through induction of apoptosis. Conversely, the growth arrest by troglitazone was associated with antiproliferative effects without significant induction of apoptosis (Keshamouni et al., 2004). PPAR γ ligands have also been found to induce changes associated with differentiation as well as apoptosis in lung cancer cells (Chang and Szabo, 2000; Sasaki et al., 2002).

The mechanism studies involved in the anticancer effects of PPAR γ ligands in lung cancers have been carried out previously. For example, induction of the phosphatase and tensin homolog deleted from chromosome 10 expression (Lee et al., 2006), activation of mitogen-activated protein kinase (Li et al., 2005, 2006), p21 up-regulation (Han et al., 2004), and suppression of prostaglandin E2 receptor (Han and Roman, 2004) have been reported, and their contributions for anticancer effects are likely variable depending on the lung cancer type. Recent investigation in HCT116 colon cancer cells revealed that apoptosis by troglitazone, a well-known PPAR γ ligand, is mediated via ROS formation by proline oxidase (POX), a mitochondrial inner membrane enzyme that catalyzes the first step of proline degradation (Pandhare et al., 2006).

The main objective of this study was to determine whether ROS generation had a role for PPAR γ -induced antiproliferative effects in A549 human lung cancer cells, a representative cell line for non-small-cell lung cancer. For this purpose, rosiglitazone and KR-62980, two structurally unrelated compounds, were chosen as PPAR γ activators. KR-62980 was synthesized in the Korea Research Institute of Chemical Technology (Daejeon, Korea) as a novel PPAR γ agonist. The present study shows that ROS generation via POX up-regulation seems to be responsible for PPAR γ -induced apoptosis of A549 human lung cancer cells. There has been no report so far on the POX-mediated ROS formation as a key apoptotic mechanism by PPAR γ in lung cancer cells, and our results provide further insight into the molecular mechanism underlying PPAR γ -induced growth arrests in human lung cancer cells that may contribute to tumor growth inhibition achieved by PPAR γ activation.

Materials and Methods

Drugs and Chemicals. KR-62980 and rosiglitazone were synthesized in the Korea Research Institute of Chemical Technology with purity of >95%. Bisphenol A diglycidyl ether (BADGE), troglitazone, ciglitazone, agar, crystal violet, and mitochondrial tetrazolium bromide (MTT) were from Sigma Chemical Co. (St. Louis, MO). Prostaglandin J2 (15d-PGJ2) was from Cayman Chemical (Ann Arbor, MI). Stealth Select RNAi oligonucleotide, stealth RNAi negative control duplex, and Lipofectamine 2000 were from the Invitrogen (Carlsbad, CA). 2',7'-Dichlorodihydrofluorescein (DCFH) diacetate was from Invitrogen (Carlsbad, CA). Bongkreikic acid was from A. G. Scientific (San Diego, CA). KR-62980, rosiglitazone, troglitazone, ciglitazone, 15d-PGJ2, BADGE, and bongkreikic acid were dissolved in dimethyl sulfoxide as a 20 mM stock solution, and then they were diluted with the phosphate-buffered saline.

Cell Culture. A549 cells (human lung carcinoma; American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine

serum. NCI-H460 (Korean Cell Line Bank 30177, human large-cell lung cancer; Korean Cell Line Bank, Seoul, Korea), NCI-H596 (Korean Cell Line Bank 90596, human adenosquamous carcinoma), and NCI-H1688 cells (human classic small-cell lung cancer) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. CCD-8Lu (human normal lung cell) cells were cultured in Eagle's minimal essential medium supplemented with 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10% fetal bovine serum. NCI-H1876 (human classic small-cell lung cancer) cells were cultured in 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 0.005 mg/ml insulin, 0.01 mg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β -estradiol, and 10% fetal bovine serum. Cells were grown to confluence at 37°C in 5% CO₂.

Cell Viability Assay. For MTT assay procedure, cells were seeded at 1×10^4 cells/well in 96-well tissue culture plates. After 24 h, the indicated concentrations of the compounds were added, and the cells were incubated for 48 h. MTT solution (20 μ l/well) in 5 mg/ml phosphate-buffered saline was then added, and the cells were incubated for 2 h. The medium was aspirated and replaced with 150 μ l of ethanol/dimethyl sulfoxide solution (1:1) per well. The plates were shaken for 20 min, and the optical density was measured at 570 nm using a microplate spectrometer (Bio-Rad Laboratories, Hercules, CA).

Soft Agar Colony Assay. Effects of KR-62980 and rosiglitazone on A549 proliferation were assessed using an agar cloning technique. An underlay of 0.5% agar in DMEM containing 5% fetal bovine serum was prepared by mixing equal volumes of 1% agar and 2 \times DMEM plus 10% fetal bovine serum. Two milliliters of this mixture was pipetted into the wells of six-well plates, and the mixture was allowed to set. A549 cells were diluted to a final concentration of 2000 cells/ml in a mixture of 0.7% agar and 2 \times DMEM. KR-62980 and rosiglitazone were added from appropriate stock solutions to achieve the indicated final concentrations and a final solvent concentration of 0.1%. Vehicle controls received similar volumes of solvent (dimethyl sulfoxide) alone. Two milliliters of the cell suspension was aliquoted into each well. The agar was allowed to set, and the plates were incubated in a humidified chamber at 37°C for 14 days. Colonies were stained with 0.005% crystal violet, and then they were counted in a blinded manner using a 10 \times objective on a Nikon inverted microscope (Nikon, Tokyo, Japan). Experiments were carried out two times in triplicate. Data are expressed as percentage of control.

DNA Fragmentation Assay. A549 cells were pretreated with 20 μ M BADGE for 1 h, followed by KR-62980 or rosiglitazone treatment for 0 to 48 h. DNA fragmentation was detected by electrophoresis of DNA (15–20 μ g/well) on 1.6% agarose gel, followed by visualization using ethidium bromide staining. Gel pictures were taken by UV transillumination.

Western Blot Analysis. A549 cells were pretreated with 20 μ M BADGE for 1 h, followed by KR-62980 or rosiglitazone treatment for 24 h. After centrifugation of cell lysates at 12,000 rpm, 10 μ g of total protein was loaded into a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Protein bands were visualized using chemiluminescence (Pierce Chemical, Rockford, IL), and they were quantified with UN-SCAN-IT gel 5.1 software (Silk Scientific Inc., Orem, UT). Polyclonal antibodies against Bax, cytochrome *c*, gelsolin, Mad1, and glyceraldehyde-3-phosphate dehydrogenase and monoclonal antibodies against Bcl-2 and E-cadherin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Measurement of Intracellular ROS Production. Intracellular ROS was measured by using conversion of nonfluorescent DCFH diacetate (Invitrogen) into free DCFH. A549 cells were preincubated for 1 h in the absence or presence of 20 μ M BADGE. Thereafter, cells were stimulated with the PPAR γ agonists for 4 h, followed by incubation in the dark for 15 min in 50 mM phosphate buffer, pH 7.4, containing DCFH diacetate. This agent is a nonpolar compound that

readily diffuses into cells, where it is hydrolyzed to the fluorescent polar derivative DCFH and thereby trapped within the cells. The quantity of DCFH fluorescence was measured at an emission wavelength of 545 nm and an excitation wavelength of 485 nm using a fluorescence plate reader (Bio-Tek Instruments, Winooski, VT). Results were expressed as percentage of fluorescence intensity of control (nonstimulated A549 cells).

Measurement of Cytochrome *c* Release. For analysis of cytochrome *c* release, A549 cells were preincubated for 1 h in the absence or presence of 20 μ M BADGE. Thereafter, cells were stimulated with KR-62980 or rosiglitazone for 24 h, trypsinized, washed with phosphate-buffered saline, and resuspended in 30 μ l of phosphate-buffered saline containing 80 mM KCl, 250 mM sucrose, 1 mM dithiothreitol, protease inhibitors, and 500 μ g/ml digitonin. Cells were incubated in the lysis buffer for 8 min at 4°C, and then they were collected at 10,000g for 5 min. The obtained pellet represented the mitochondria-containing nuclear heavy-membrane fraction. The supernatant was resuspended for a further 5 min at 10,000g. The second supernatant, representing the cytosol including the light-membrane fraction, was loaded on a 10% polyacrylamide gel, and cytochrome *c* release was analyzed by immunoblotting.

POX or PPAR γ Knockdown Using Stealth RNAi Oligonucleotide. The POX stealth Select RNAi oligonucleotide (target accessions no. NM 016335) and the PPAR γ stealth Select RNAi oligonucleotide (target accessions nos. NM 138712.1, NM015869.2,

NM138711.1, and NM 005037.3) were synthesized by Invitrogen. The stealth RNAi negative control Duplex (Invitrogen) was used as a control oligonucleotide. Transfection efficiency was monitored using a fluorescent oligonucleotide (BLOCK-iT fluorescent oligonucleotide; Invitrogen), and it was estimated to be 80 to 90%.

The stealth RNAi molecules were transfected into A549 cells using Lipofectamine 2000 following Invitrogen's protocols. The final concentrations of 30 nM PPAR γ stealth Select RNAi oligonucleotide and 100 nM POX stealth Select RNAi oligonucleotide were empirically determined to maximally suppress target RNA expression, and the stealth RNAi oligonucleotide was transfected to the medium 48 h before the treatment of compounds. The ability of the stealth RNAi oligonucleotide to knock down POX and PPAR γ expression was analyzed by Western blot and real-time reverse transcription-polymerase chain reaction (RT-PCR) on whole cell extracts. A549 cells transfected with each RNAi were preincubated for 1 h in the absence or presence of 20 μ M BADGE. Thereafter, cells were stimulated with either KR-62980 or rosiglitazone for 48 h (cell viability and DNA fragmentation) or for 4 h (ROS).

RT-PCR and Real-Time Quantitative RT-PCR Analysis. Total RNA was isolated from compound-treated cells using easy-BLUE Total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea). Reverse transcription of total RNA (1 μ g) was performed using AccuPower RT PreMix (Bioneer Inc., Seoul, Korea). Polymerase chain reaction primers for amplification of POX were designed based

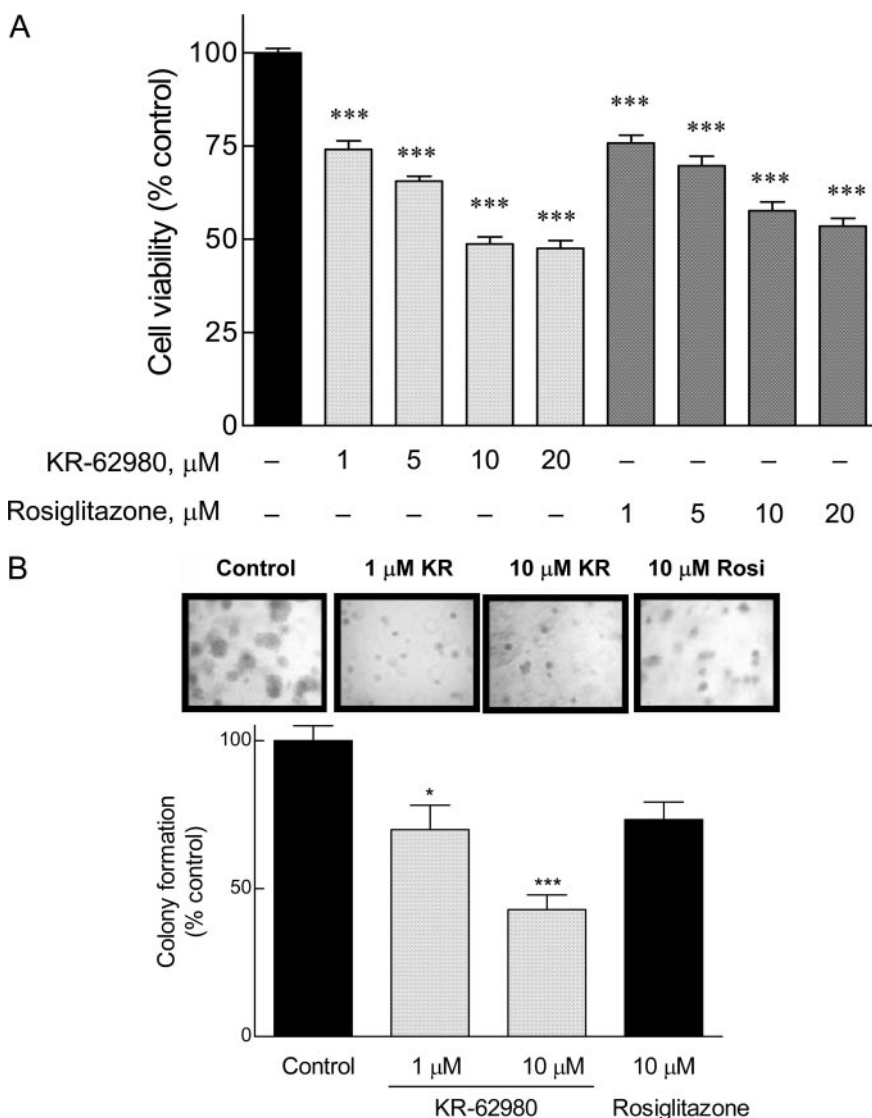


Fig. 1. Inhibitory effects of KR-62980 on cell viability in A549 cells. A, concentration-dependent effects of KR-62980 and rosiglitazone on cell viability of A549 cells. Cells were incubated with the indicated concentrations of KR-62980 or rosiglitazone for 2 days, and then cell viability was determined using MTT assay. Values are means \pm S.E.M. of two experiments each done in quadruplicate. ***, $P < 0.001$ versus control group. B, effects of KR-62980 and rosiglitazone on colony formation of A549 cells. Cells were incubated with the indicated concentrations of KR-62980 (KR) or rosiglitazone (Rosi) for 14 days, and colonies were stained with 0.005% crystal violet. Colonies were counted in a blinded manner using a 10 \times objective on a Nikon inverted microscope. Experiments were carried out two times in triplicate. Data are expressed as percentage of control. *, $P < 0.05$; ***, $P < 0.001$ versus control group.

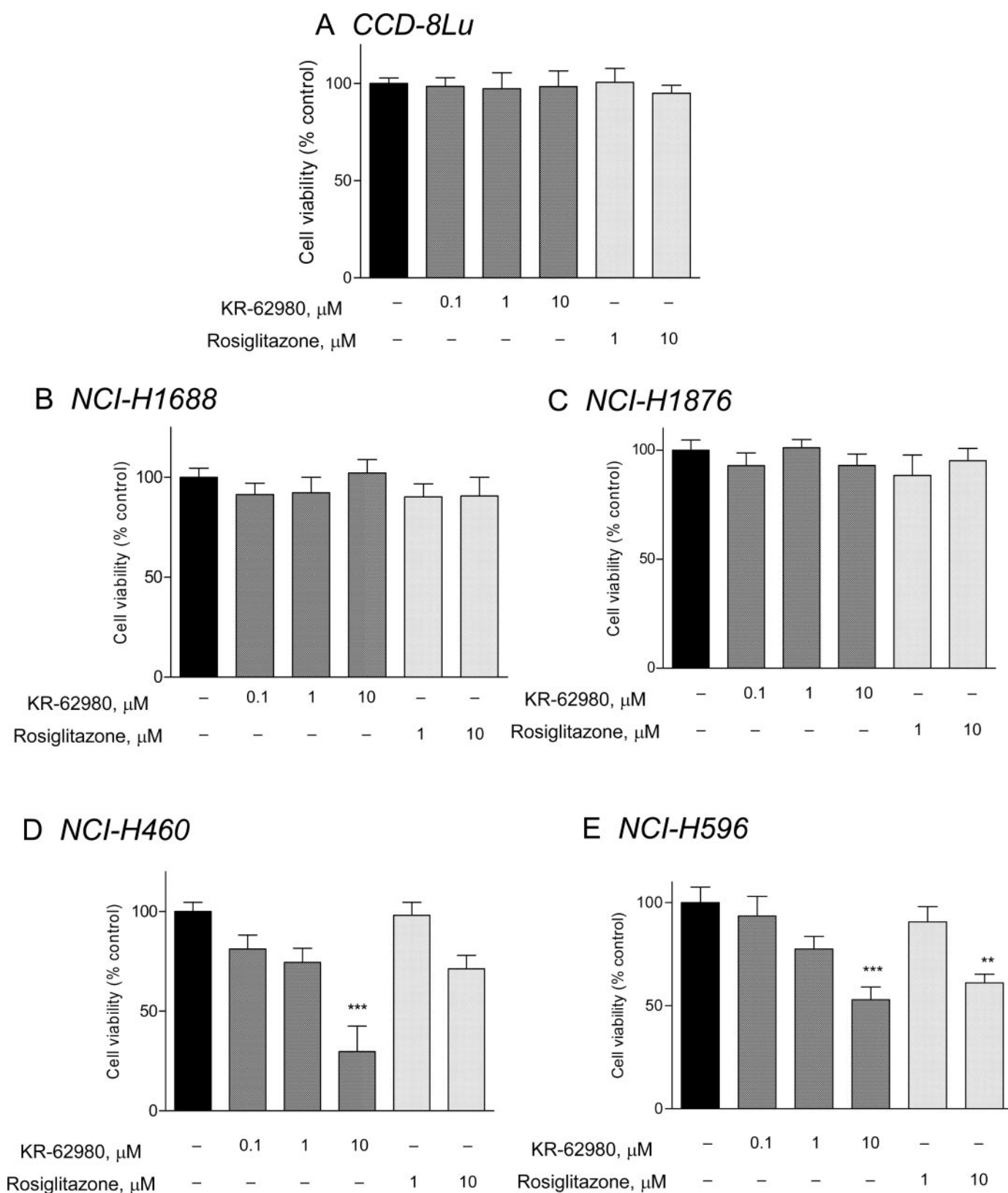


Fig. 2. Effects of KR-62980 on cell viability in CCD-8Lu cells, a normal lung cell line (A); NCI-H1688 (B) and NCI-H1876 (C) cells, the classic small-cell lung cancer cell lines; and NCI-H460 (D) and NCI-H596 cells (E), the non-small-cell lung cancer cell lines. Cells were incubated with the indicated concentrations of KR-62980 or rosiglitazone for 2 days, and then cell viability was determined using MTT assay. Values are means \pm S.E.M. of two experiments each done in quadruplicate. **, $P < 0.01$; ***, $P < 0.001$ versus control group.

on the sequences obtained: sense, 5'-GCC ATT AAG CTC ACA GCA CTG GG-3' and antisense, 5'-CTG ATG GCC GGC TGG AAG TAG-3'. RT-PCR conditions were 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, followed by a 10-min extension reaction at 72°C. Aliquots of the reaction from polymerase chain reaction were checked by melting curve analysis as provided by the Roter-Gene 3000 system (Corbett Research, Sydney, Australia). The instrument settings were as follows: POX denaturing at 95°C for 15 min, with 40 repeated cycles of denaturing at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s; and PPAR γ denaturing at 95°C for 15 min, with 40 repeated cycles of denaturing at 94°C for 30 s, annealing at 62°C for 30 s, and elongation at 72°C for 30 s. Relative abundance of mRNA was calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase.

Statistical Analysis. The results are expressed as means \pm S.E.M. Statistical significance was determined by one-way analysis

of variance followed by a Tukey multiple comparison test. $P < 0.05$ was considered to be statistically significant for all experiments.

Results

Effect of PPAR γ Activation on Cell Viability and Colony Formation in Human Non-Small-Lung Carcinoma. Because the A549 cell line represents the histological type of the majority of lung cancer patients, we first accessed the effects of two PPAR γ agonists on A549 cell viability by MTT assay. KR-62980 decreased A549 cell viability in a concentration-dependent manner, with approximately maximal effects occurring at 10 μ M KR-62980 (~55% inhibition) (Fig. 1A). Likewise, KR-62980 caused a dose-dependent inhibition of A549 colony formation, a measure of transformed growth (Fig. 1B). Exposure to 10 μ M KR-62980 for 14 days

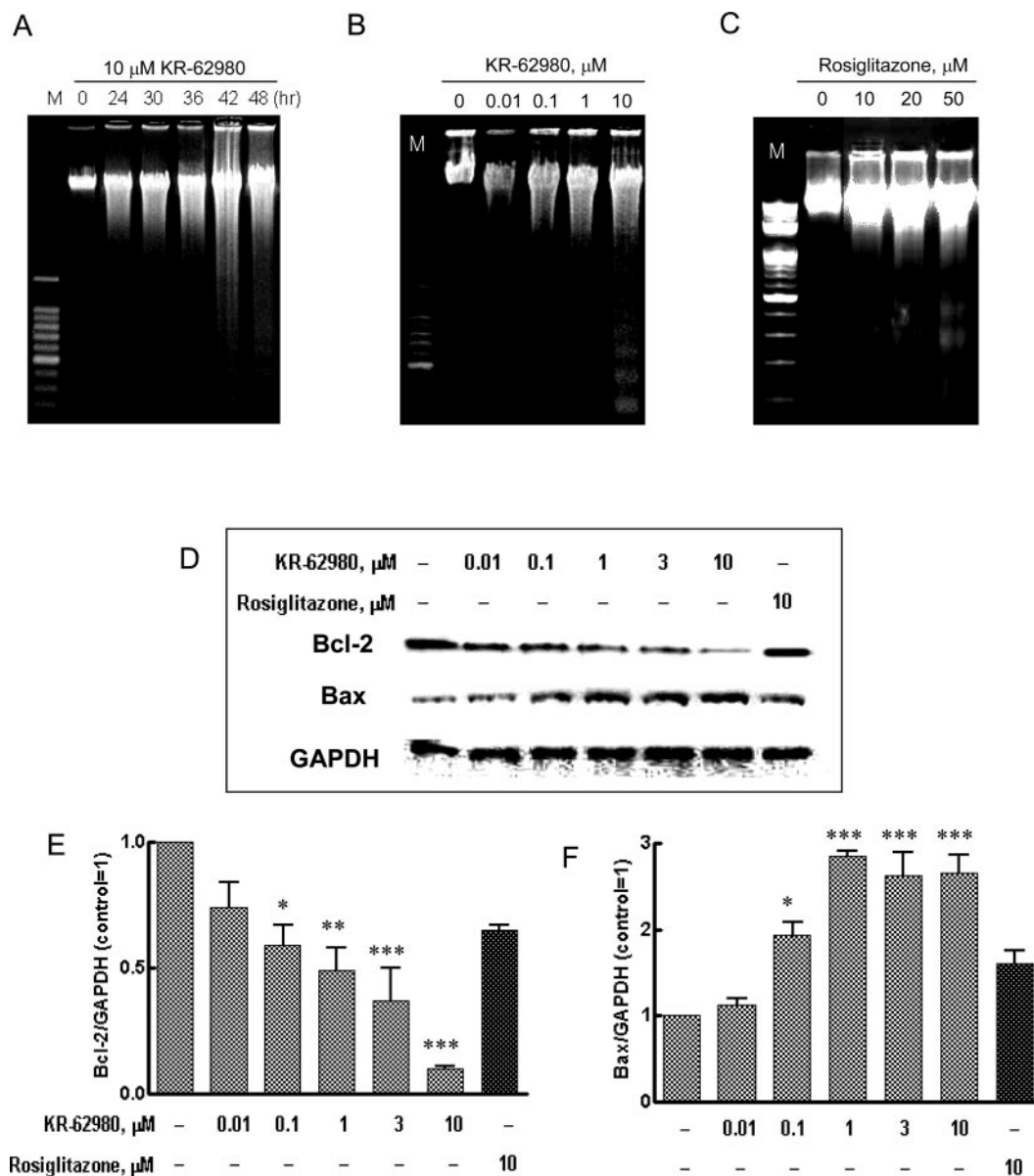


Fig. 3. Apoptotic action of KR-62980 and rosiglitazone in A549 cells. A and B, time- and concentration-dependent effects of 10 μ M KR-62980 on the oligonucleosomal DNA laddering. C, concentration-dependent effects of rosiglitazone on the oligonucleosomal DNA laddering upon 48-h incubation. Each was confirmed with two different preparations. D to F, effects of KR-62980 and rosiglitazone on the expression of Bcl-2 protein and Bax protein in A549 cells. Cells were incubated with the indicated concentrations of KR-62980 or rosiglitazone for 24 h, and protein expression was determined using Western blotting. Values are means \pm S.E.M. of three different preparations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus control group.

caused a reduction in colony formation to $40 \pm 5.7\%$ of control. By comparison, the effects of rosiglitazone were examined, and its effects were weaker than those of KR-62980.

Next, we measured the effects of the agonists on other lung cell lines to investigate whether the suppressive effects of PPAR γ activation are a general phenomena in lung cells. We used CCD-8Lu, a normal lung cell line; NCI-H1876 and NCI-H1688 cell lines, the classic small-cell lung cancer cell lines; and NCI-H596 and NCI-H460, the non-small-cell lung cancer cell lines. PPAR γ activation by the agonists induced cell death in non-small-cancer cell lines to a similar extent to that of the death observed in A549 cells, but it had no effect on both normal lung cells and classic small-cancer cell lines at the concentration range used (Fig. 2), suggesting that PPAR γ action is likely to be restricted to non-small-cell lung cancer cells.

Induction of Apoptotic Cell Death by PPAR γ Activation. To determine the mechanism of PPAR γ -induced reduction in cell viability, cell death caused by KR-62980 was monitored by oligonucleosomal DNA fragmentation assay. KR-62980 induced DNA fragmentation in a time- and con-

centration-dependent manner (Fig. 3, A and B), indicating that decreased cell viability by KR-62980 was due to the apoptotic action. Concomitant with the apoptotic feature by the compound, there was a significant reduction in Bcl-2 protein level by 24-h exposure to the compound, whereas Bax protein expression was markedly and concentration-dependently elevated by 1, 3, and 10 μ M KR-62980 to 2.1 ± 0.2 , 2.6 ± 0.3 , and 2.7 ± 0.3 relative density, respectively (Fig. 3, D–F). Rosiglitazone also induced DNA fragmentation, and it altered Bcl-2 and Bax expression, although the change was not as potent as that with KR-62980 (Fig. 3, C–F).

PPAR γ has been shown to induce differentiation-promoting effects, thereby producing antiproliferative activities. When we measured the level of E-cadherin, p21, gelsolin, and Mad 1, the well-known markers of epithelial differentiation, in A549 cells after agonist treatments, rosiglitazone induced differentiation marker expression with morphological changes to a more differentiated phenotype, whereas the change was not seen by KR-62980 treatment (Fig. 4). This result suggests that the two agonists have overlapping but distinct profiles in A549 cells.

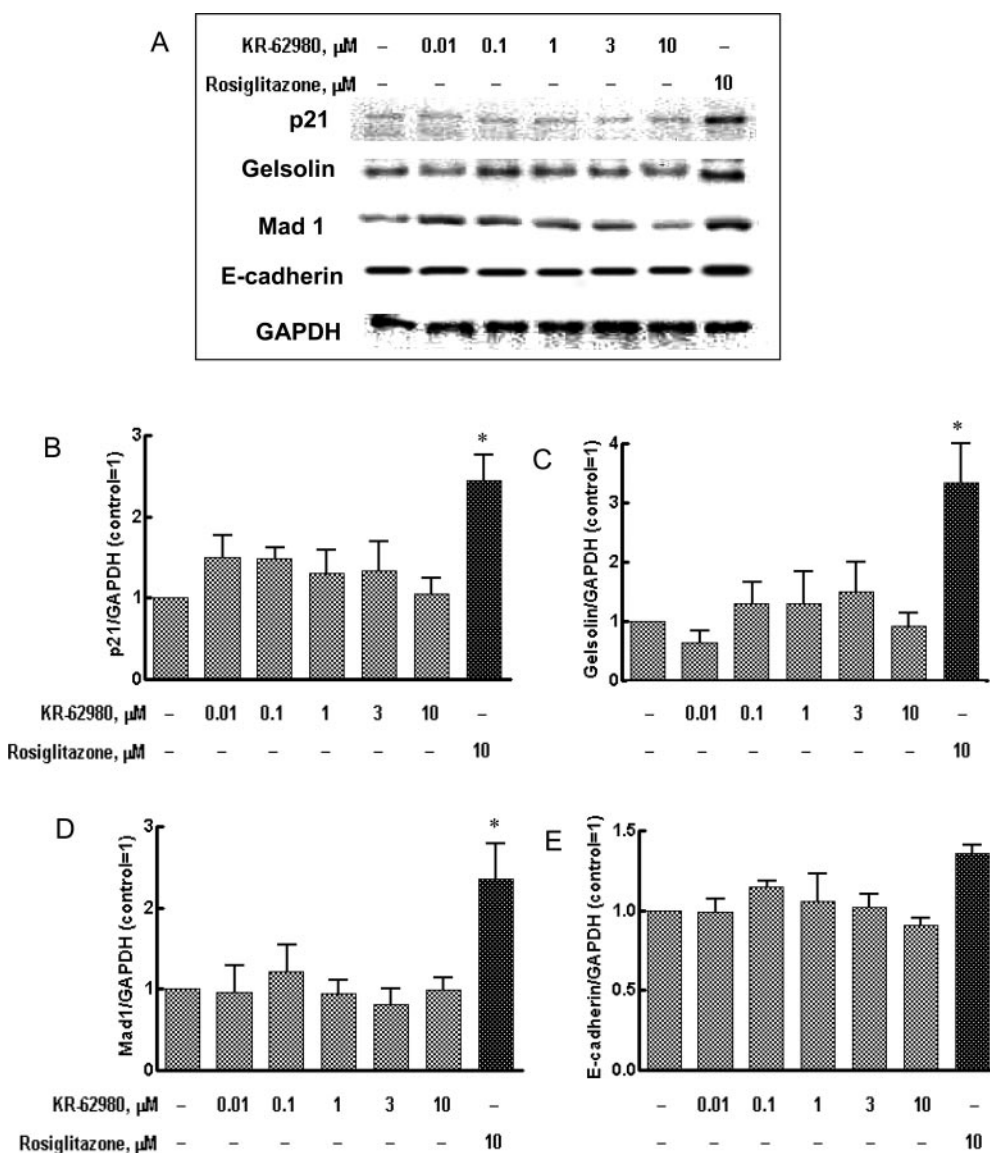


Fig. 4. Effects of KR-62980 and rosiglitazone on the expression of differentiation markers in A549 cells. Cells were incubated with the indicated concentrations of KR-62980 or rosiglitazone for 24 h, and protein expression was determined using Western blotting. Values are means \pm S.E.M. of three different preparations. *, $P < 0.05$ versus control group.

Induction of Intracellular ROS Formation by PPAR γ Activation. To investigate whether ROS generation is involved in the inhibitory effects of KR-62980 on A549 cell viability, ROS production was evaluated in DCFH diacetate-loaded cells by fluorescence plate reader (Fig. 5A). KR-62980 (1–10 μ M) concentration-dependently increased the ROS formation ($211 \pm 16\%$ of control at 10 μ M), but ROS concentration induced by KR-62980 decreased after 4 h (data not shown).

To examine whether ROS is generated from the mitochondrial fraction, we determined cytochrome *c* release to the cytosolic fraction by Western blot. Both agonists (10 μ M) increased cytochrome *c* release to the cytosol (Fig. 5B), suggesting that ROS produced by the agonists originated from mitochondria. Moreover, treatment with bongkreikic acid, an inhibitor of mitochondrial permeability transition and mitochondrial depolarization, abrogated KR-62980-induced cell death in a dose-dependent manner (Fig. 5C), suggesting that mitochondrial ROS is important for the compound-induced apoptosis.

Effect of PPAR γ Activation on POX Expression in A549 Cells. Previous study demonstrated that troglitazone induced ROS formation by POX expression in part via PPAR γ activation in colon cancer cells (Pandhare et al., 2006). Thus, to explore the potential role of POX in ROS production in A549 cells, the mRNA expression of POX was determined after treatment with the agonists by RT-PCR and real-time RT-PCR. As seen in Fig. 6A, 10 μ M KR-62980 increased the POX expression to 5-fold relative to control, whereas less profound effects were detected after rosiglitazone treatment.

To confirm the involvement of POX in the action, we used POX stealth RNAi to knock down the POX expression. The transfection of POX stealth RNAi in A549 cells resulted in the reduction of POX mRNA to 25% of control (Fig. 6B). In accordance with increased POX expression by the agonists, the effects of 10 μ M KR-62980 on ROS formation and cell viability were significantly blocked in POX knockdown cells (Fig. 6, C and D), suggesting that POX induction is important for KR-62980-induced apoptosis of lung cancer cells via ROS generation. Similar results were obtained when rosiglitazone was tested, suggesting a common pathway involved in two agonists action.

We further examined the effects of other PPAR γ agonists on POX expression to check whether POX-induced ROS generation is a universal mechanism for PPAR γ -mediated apoptotic death of A549 cells. As shown in Fig. 7A, well-known PPAR γ agonists troglitazone, ciglitazone, and 15d-PGJ2 (10 μ M) all induced POX mRNA expression, and the extent of POX induction was correlated with ROS level and cell death (Fig. 7, B and C), suggesting that POX-induced ROS formation is critical for the apoptotic cell death induced by PPAR γ activation and that these phenomena may apply to the PPAR γ agonists in general.

Effects of PPAR γ Activation on POX Expression and ROS Formation in Other NSCLC. Because KR-62980 and rosiglitazone induced selective cell death of NSCLC, we next measured the level of POX and ROS in other NSCLC than A549 after the agonist treatments. Similar to A549 cells, the agonists concentration-dependently induced ROS formation in both NSCLC cell lines we used, and an increase in POX

expression was detected at 10 μ M concentrations of either KR-62980 or rosiglitazone (Fig. 8).

Effects of PPAR γ Blockade on the Antiproliferative and Apoptotic Actions by the Agonists. To check whether the effects of KR-62980 were due to the activation of PPAR γ , we first examined the effects of BADGE, a PPAR γ antagonist on cell viability, ROS formation, and POX expression induced by the agonists. All the agonistic effects were significantly, if not completely, blocked in the presence of 20 μ M BADGE (Fig. 9), implying that the effects are mediated through PPAR γ activation. BADGE alone did not influence cell viability, ROS formation, and POX expression.

In parallel studies, RNAi was used to eliminate the endog-

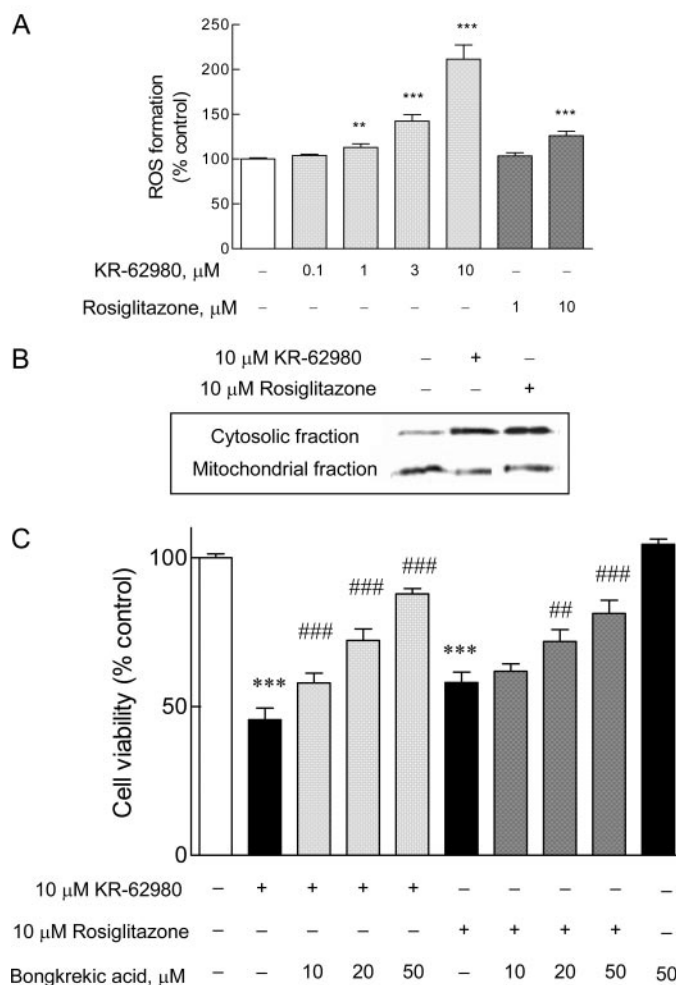


Fig. 5. Effects of KR-62980 and rosiglitazone on the intracellular ROS induction in A549 cells. **A**, intracellular ROS formation was estimated by the oxidation of cell-trapped DCFH diacetate to the fluorescent compound dichlorofluorescein. Cells treated with either KR-62980 or rosiglitazone for 4 h were incubated with 5 μ M DCFH diacetate for 15 min, washed in phosphate-buffered saline, and read by fluorescence plate reader. **B**, effects of KR-62980 and rosiglitazone on cytochrome *c* release in A549 cells. A549 cells were treated with KR-62980 or rosiglitazone (both at 10 μ M) for 24 h, and the amount of cytochrome *c* in cytosolic and mitochondrial fraction was analyzed by immunoblotting. **C**, effect of bongkreikic acid, an inhibitor of mitochondrial permeability transition and mitochondrial depolarization, on KR-62980- and rosiglitazone-induced cell death in A549 cells. A549 cells were pretreated with bongkreikic acid for 1 h, followed by KR-62980 or rosiglitazone (both at 10 μ M) treatment for 48 h. Values are means \pm S.E.M. of two different preparations with quadruplicate experiments. ***, $P < 0.001$ versus control group; ##, $P < 0.01$; ###, $P < 0.001$ versus 10 μ M KR-62980- or rosiglitazone-treated group.

enous expression of PPAR γ . The transfection of stealth RNAi oligonucleotide in A549 cells resulted in the reduction of PPAR γ protein expression to 20% of control (Fig. 10A). In contrast to the antiproliferative and apoptotic effects of KR-62980 in stealth RNAi negative control oligonucleotide-transfected cells, 10 μ M KR-62980 was ineffective on cell viability and DNA fragmentation in PPAR γ knockdown cells (Fig. 10, B and C). These results provide conclusive evidence that PPAR γ activation is required for the antiproliferative and apoptotic effects of KR-62980 on lung cancer cells. The effects of rosiglitazone were also blocked by either BADGE or PPAR γ stealth RNAi.

Discussion

Present study demonstrated that PPAR γ activation by either KR-62980, a novel PPAR γ agonist, or by rosiglitazone induces apoptosis in human non-small-cell lung cancer A549 cells and that POX induction with subsequent production of ROS seems to be a key mechanism responsible for apoptotic actions. In addition, the effects of the PPAR γ agonists are limited to the non-small-cell lung cancer cells, because little effect was seen in small-cell lung cancer cells and in normal lung cells.

PPAR γ is a ligand-activated transcription factor belonging to the nuclear receptor family, and its functions are diverse, including regulation of cell proliferation, differentiation, and apoptosis. Because PPAR γ expression has been found in var-

ious cancer types, such as liposarcoma, human breast cancer, colon cancer, prostate cancer, and lung cancer (Butler et al., 2000; Tsubouchi et al., 2000; Lapillonne et al., 2003), several studies have been carried out to confirm the utility of PPAR γ agonists as anticancer agents. Based on the results, PPAR γ activation by synthetic ligands has been proven as an effective anticancer regimen by induction and maintenance of a more differentiated state and/or by apoptosis. However, the molecular mechanisms underlying these effects remain to be explored.

The present study with two PPAR γ agonists indicates that PPAR γ activation had antiproliferative activities in A549 cells that are known to express wild-type and functional PPAR γ , and its antiproliferative activities were due to apoptosis as shown by oligonucleosomal DNA fragmentation and changes in Bcl-2 and Bax expression. This finding is in agreement with a previous report demonstrating that synthetic and endogenous PPAR γ ligands induce apoptosis of human lung cancers (Tsubouchi et al., 2000; Sasaki et al., 2002; Li et al., 2006). The apoptotic effects seen in our study seem to be mediated, at least in part, by PPAR γ , because BADGE (a PPAR γ antagonist) treatment or PPAR γ knockdown by RNAi reversed growth inhibitory effects induced by the agonists.

One interesting observation in the present study is that two agonists exhibit differential effects on A549 cell differentiation. Despite that KR-62980 is more potent than rosiglitazone in transactivation activities, only rosiglitazone in-

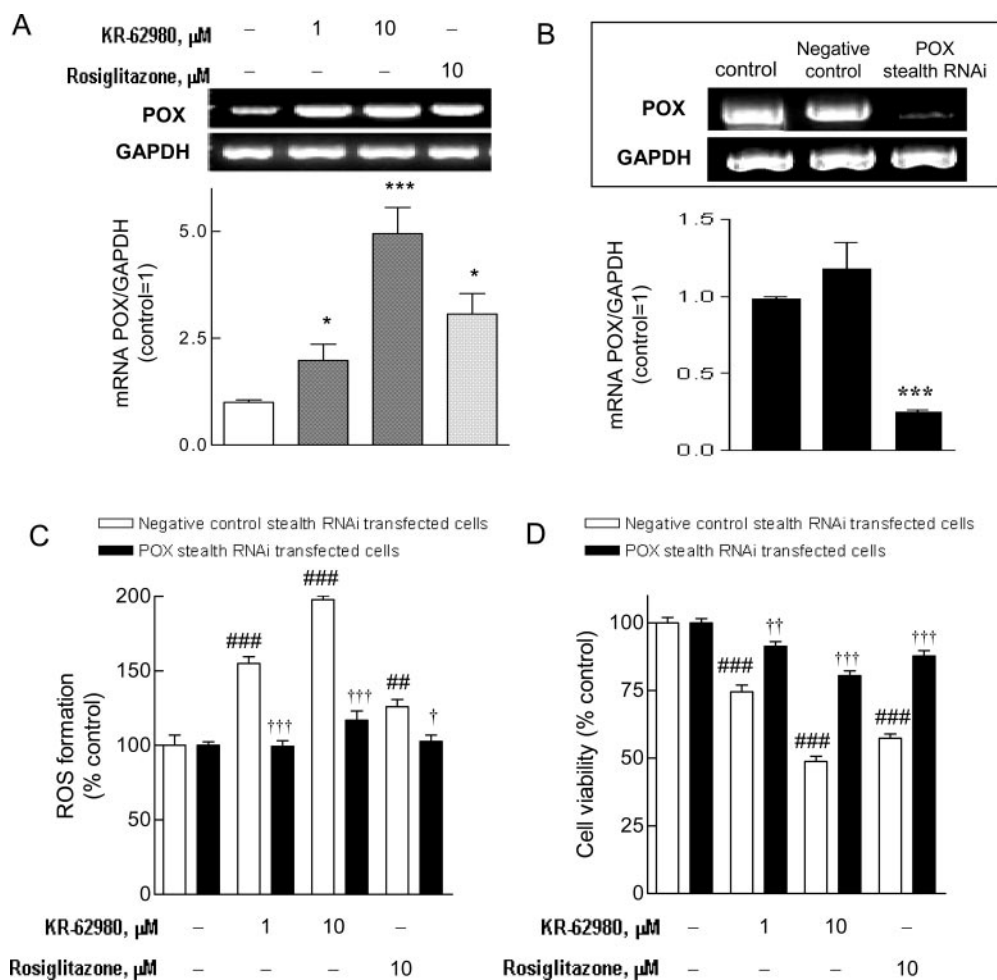


Fig. 6. Effects of KR-62980 and rosiglitazone on POX expression (A) in A549 cells and on ROS formation (C) and cell viability (D) in POX knockdown A549 cells. A, A549 cells were treated with KR-62980 or rosiglitazone treatment for 4 h, and POX expression was determined using RT-PCR (top) or real-time RT-PCR (bottom). *, $P < 0.05$; ***, $P < 0.001$ versus control group. B, after transfection of negative control stealth RNAi or with POX stealth RNAi, POX mRNA expressions were determined using RT-PCR (top) or real-time RT-PCR (bottom). ***, $P < 0.001$ versus negative control stealth RNAi-transfected cells group. C and D, effects of KR-62980 and rosiglitazone on ROS formation (C) and cell viability (D) in POX knock down cells. Cells were treated with KR-62980 or rosiglitazone stimulation for 4 h (C) or 48 h (D) in POX stealth RNAi oligonucleotide-transfected cells or negative control stealth RNAi oligonucleotide-transfected cells, and ROS formation assay or MTT assay was carried out. Values are means \pm S.E.M. of two different preparations with quadruplicate experiments. ##, $P < 0.01$; ###, $P < 0.001$ versus control group; †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$ versus negative control stealth RNAi-transfected cells group.

duced differentiation of A549 cells as indicated by the expression of E-cadherin, p21, Mad 1, and gelsolin, the lung cancer differentiation markers. These results suggest that KR-62980-induced antiproliferative action is not associated with cell differentiation, which may agree with the notion that KR-62980 lacks the capacity for adipocyte differentiation (Kim et al., 2006a). In conjunction with this, the growth inhibitory action of KR-62980 in MCF-7 breast cancer also was not associated with differentiation-promoting effects, whereas rosiglitazone induced terminal differentiation of the cells (Kim et al., 2006b). It can be speculated that this distinct effect may be due to the difference in binding mode to the receptor, which may lead to alternate coactivator and corepressor complex formation.

While elucidating the mechanisms producing apoptotic cell death by PPAR γ activation, we observed that ROS is involved in the growth inhibition of the two agonists. ROS generation seems to be a common cellular mechanism for multiple death pathways (Martindale and Holbrook, 2002), implicated as second messengers in proliferation, gene activation, cell cycle arrest, and apoptosis. In support of ROS involvement, rotenone, a radical scavenger, significantly reversed suppression of cell survival, ROS formation, and DNA fragmentation by the agonists (data not shown), indicating the important role of ROS in PPAR γ action. Because ROS can be derived from either mitochondria or extramitochondrial origin (i.e., NADPH oxidase), we further investigated the possible involvement of mitochondria in ROS production. In parallel with ROS formation, the level of cytochrome *c* in cytosolic fraction was increased, suggesting that mitochondria are a likely source of ROS. Moreover, increased cell death by the agonists was accompanied by loss of mitochondrial membrane potential (data not shown), and it was suppressed by bongkrekic acid, an inhibitor of mitochondrial permeability transition and mitochondrial depolarization. In line with this finding, troglitazone and pioglitazone were noted to inhibit state III respiration in isolated brain mitochondria (Dello Russo et al., 2003), and ciglitazone caused a dose-dependent and rapid loss of mitochondrial membrane potential, and it induced ROS formation in glioma C6 cells (Pérez-Ortiz et al., 2004).

Recent findings provided the evidence that POX is an important source for ROS formation; indeed, troglitazone increased POX expression in a PPAR γ -dependent and -independent manner in colon cancer cells (Pandhare et al., 2006). Likewise, in our studies with A549 cells, five different PPAR γ agonists, including KR-62980 and rosiglitazone, increased POX expression in parallel with ROS formation and cell death. In addition, POX knockdown abolished the ROS generation and growth-inhibitory actions of the agonists in A549 cells, suggesting that POX is a critical player in the PPAR γ -induced growth arrest of the cells. Previous study revealed that POX expression could be directly regulated by PPAR γ activation via transcriptional control (Pandhare et al., 2006), but an additional mechanism for POX regulation involving p53 seems to exist. Based on our results, ROS formation seems to be attained via PPAR γ -mediated POX induction, because both POX and PPAR γ blockades almost prevented the agonist effects. On the contrary, apoptotic effects by the agonists were not abrogated, reflecting that the growth arrest by the agonists may also be mediated partly through

other pathways that are independent of PPAR γ and ROS formation.

We determined the effects of PPAR γ agonists on a variety panel of lung cell lines: CCD-8Lu (a normal lung cell line), NCI-H1876 and NCI-H1688 cells (the classic small-cell lung cancer lines), and NCI-H596 and NCI-H460 (the non-small-cell lung cancer lines). The agonists inhibited cell growth with increased POX and ROS levels of non-small-cell lung cancer cells, including A549 cells, whereas they were incapable of inhibiting growth of either normal lung cells or small-cell lung cancer cells. So far, most studies delving into the

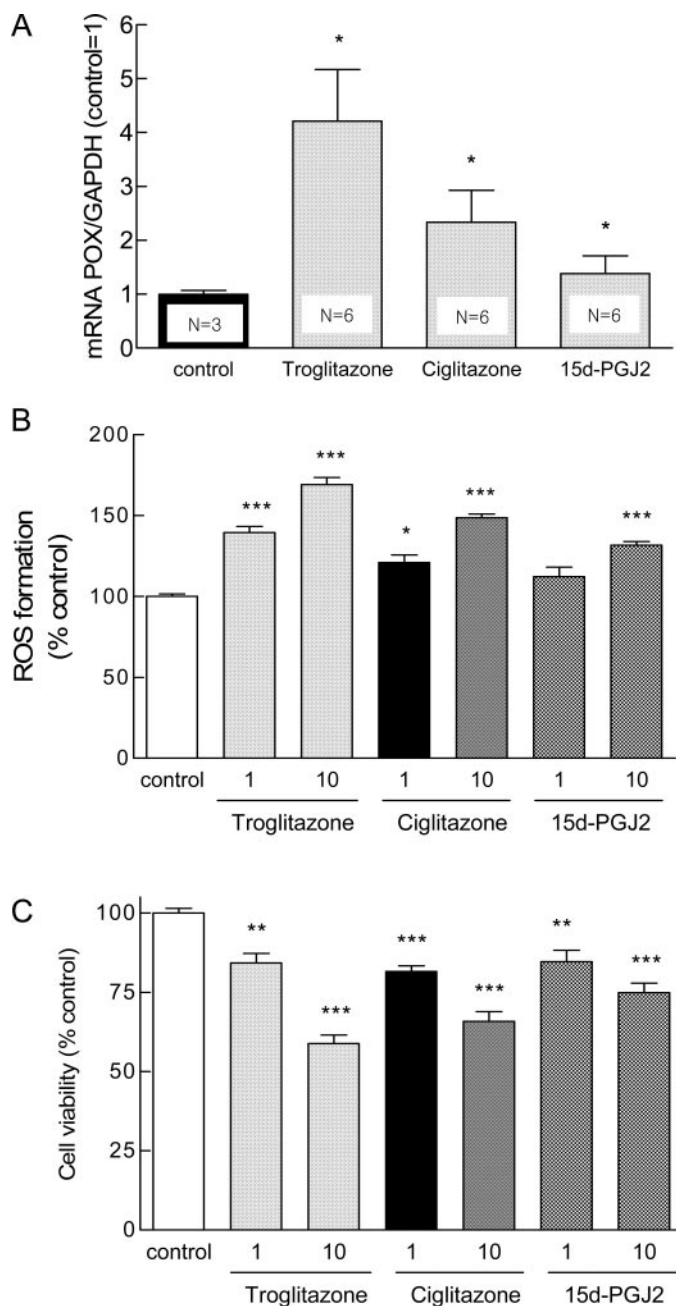


Fig. 7. Effects of troglitazone, ciglitazone, and 15d-PGJ2 on the POX expression (A), ROS formation (B), and cell viability (C) in A549 cells. A549 cells were treated with PPAR γ agonists [10 μ M (A) and 1 or 10 μ M (B and C)] for 4 h (A and B) or 2 days (C), and then POX expression, ROS formation, and cell viability were determined. Values are means \pm S.E.M. of two different preparations with quadruplicate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus control group.

role of PPAR γ in lung cancers have focused on non-small-cell lung cancer types, but the responses of various PPAR γ ligands in different lung cell lines are still controversial (Allred and Kilgore, 2005). In our current study, the two agonists

had no effect on small-cell lung cancer cells, which seems to be contradictory to the previous reports mentioning that growth-inhibitory effects of PPAR γ ligands were similar in human small-cell lung cancer and non-small-cell lung can-

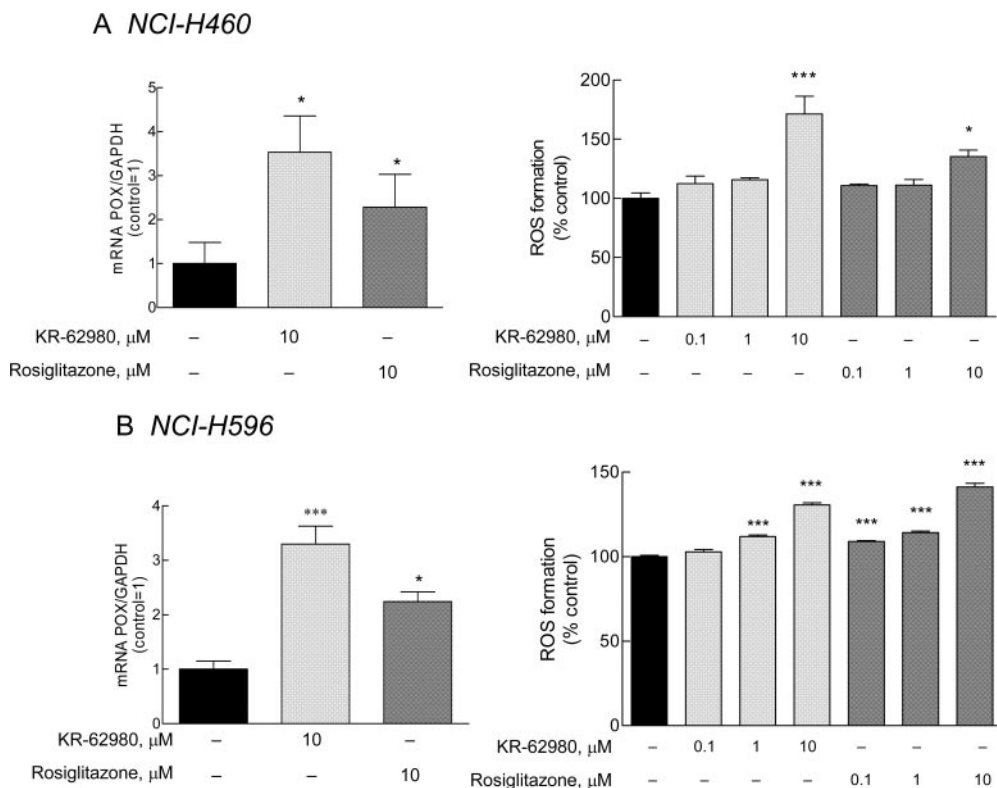


Fig. 8. Effects of KR-62980 and rosiglitazone on the POX expression and ROS formation in NCI-H460 (A) and NCI-H596 (B) cells. Cells were treated with KR-62980 or rosiglitazone for 4 h, and then POX expression and ROS formation were determined. Values are means \pm S.E.M. of two different preparations with duplicate (POX expression) or quadruplicate experiments (ROS formation). *, $P < 0.05$; ***, $P < 0.001$ versus control group.

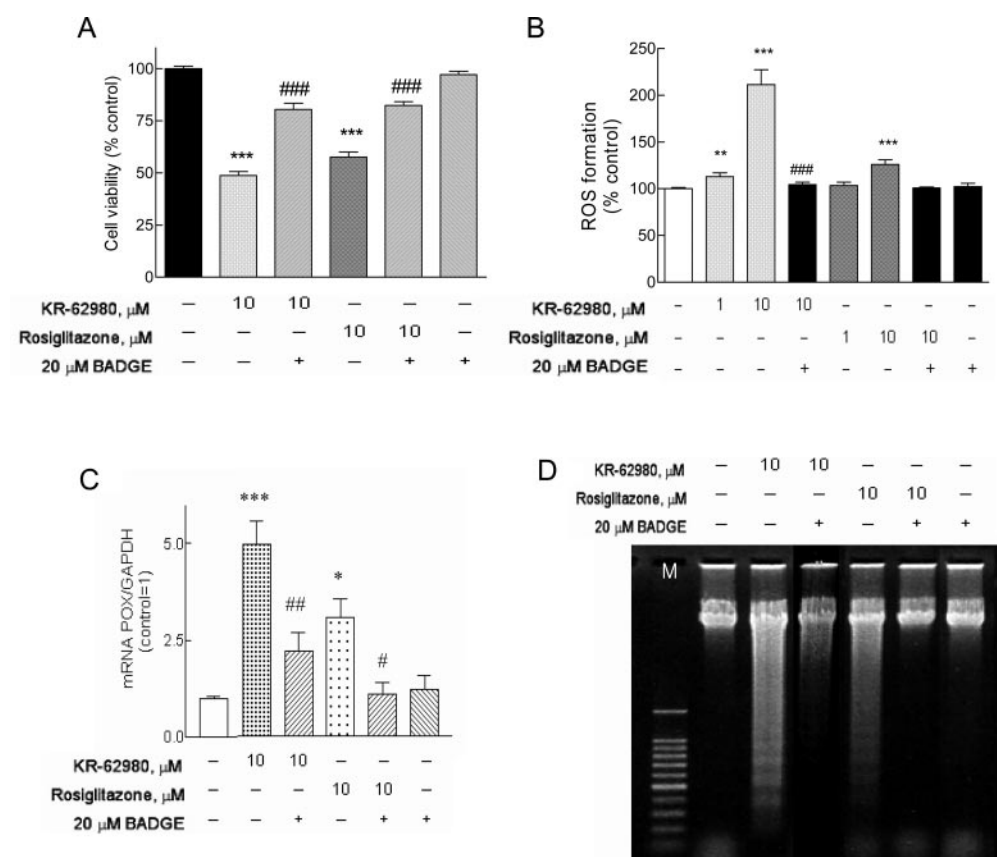


Fig. 9. Effects of BADGE, a PPAR γ antagonist, on KR-62980- and rosiglitazone-induced cell death (A), ROS formation (B), POX expression (C), and DNA fragmentation (D) in A549 cells. A549 cells were pretreated with 20 μ M BADGE for 1 h, followed by KR-62980 or rosiglitazone treatment for 4 h (B and C) or 2 days (A and D), and then cell viability, ROS formation, POX expression, and DNA fragmentation were determined. Values are means \pm S.E.M. of two experiments each done in quadruplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus control group; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ versus 10 μ M KR-62980- or rosiglitazone-treated group. D, M represents the 100-base pair DNA ladder markers. Each was confirmed with two different preparations.

cers (Han et al., 2004; Li et al., 2005). At present, the reason for this discrepancy is unclear, except that different PPAR γ ligands and small-cell lung cancer cells were used between the two studies as speculated. Because it remains unclear whether molecular etiology between two lung cancer cell types is distinct, the different response to the agonists in our system may need further investigation. Alternatively, in accordance with previous observations (Chang and Szabo, 2000; Tsubouchi et al., 2000; Keshamouni et al., 2004), PPAR γ expression was confirmed in all lung cell lines we tested, although the expression was much less in small-cell lung cancer cell types (data not shown). The observation that no apoptosis occurred by the agonists in normal lung cells

may add therapeutic significance to using PPAR γ agonists for lung cancer.

Overall, our results demonstrate that 1) ROS formation by POX up-regulation is important for PPAR γ -induced apoptosis of NSCLC, including A549 cells; 2) PPAR γ activation seems to be selectively effective on NSCLC; and 3) two PPAR γ agonists exhibit differential effects on A549 differentiation. Although several mechanisms for PPAR γ -activated antiproliferative activity in lung cancers have been reported, the involvement of ROS in apoptotic effects of PPAR γ in human non-small-cell lung cancer cells may provide a novel mechanism by which PPAR γ activation attenuates human lung cancer growth.

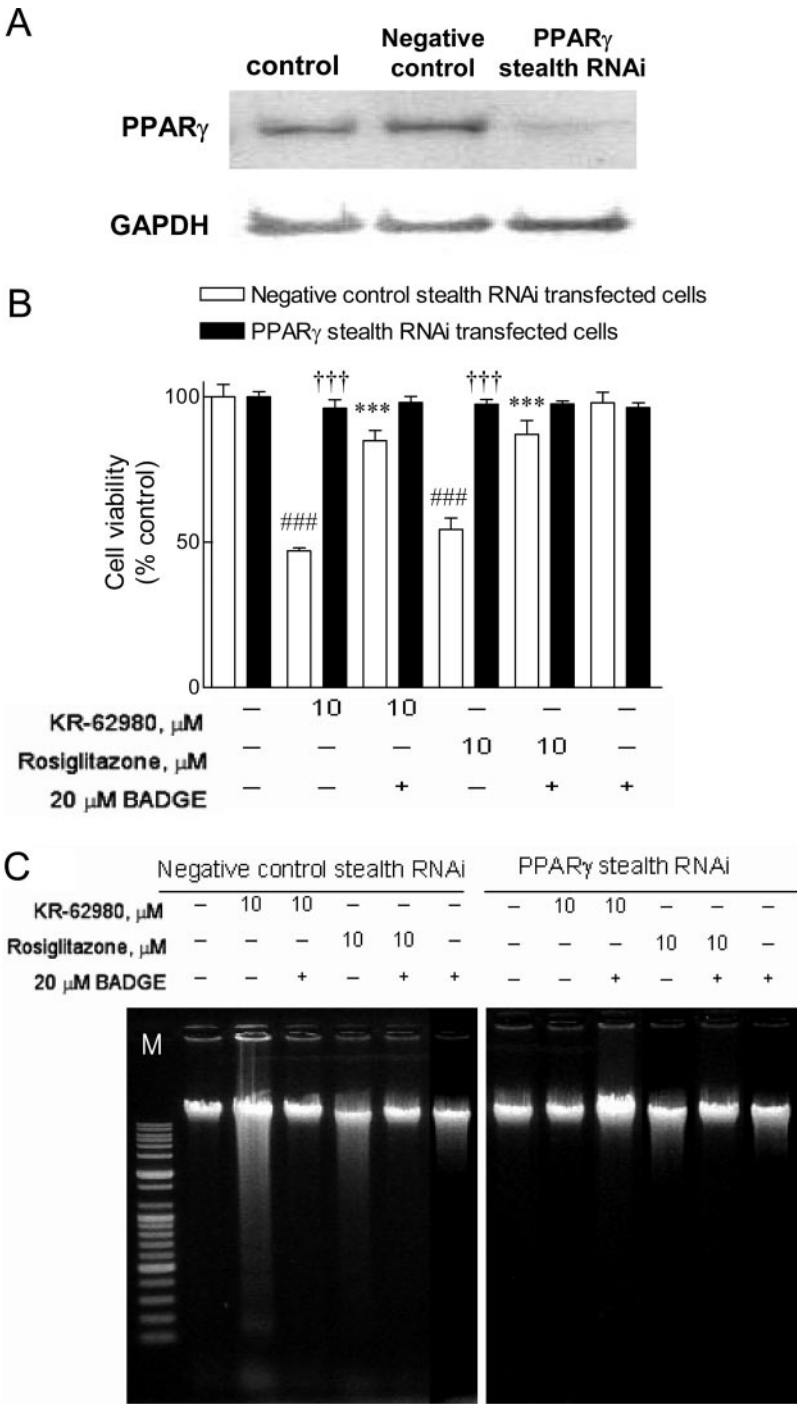


Fig. 10. Effects of KR-62980 and rosiglitazone on cell viability (B) and DNA fragmentation (C) in PPAR γ knockdown A549 cells. A, after transfection of negative control stealth RNAi or PPAR γ stealth RNAi, PPAR γ protein expressions were determined using Western blotting. Cells were pretreated with 20 μ M BADGE for 1 h followed by KR-62980 or rosiglitazone stimulation for 48 h in PPAR γ stealth RNAi oligonucleotide-transfected cells or in negative control stealth RNAi oligonucleotide-transfected cells. Then, DNA fragmentation assay or MTT assay was carried out. Values are means \pm S.E.M. of two different experiments, each done in quadruplicate. ###, $P < 0.001$ versus control group; ***, $P < 0.001$ versus 10 μ M KR-62980- or rosiglitazone-treated group; †††, $P < 0.001$ versus negative control stealth RNAi-transfected cell group.

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