BIOCHEMICAL PHARMACOLOGY 72 (2006) 530–540

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Differential anti-proliferative actions of peroxisome proliferator-activated receptor-γ agonists in MCF-7 breast cancer cells

Ki Young Kim, Sung Soo Kim, Hyae Gyeong Cheon *

Bioorganic Science Division, Korea Research Institute of Chemical Technology, P.O. Box 107, Yuseong-gu, Daejeon, 305-600, Republic of Korea

ARTICLE INFO

Article history:
Received 20 March 2006
Accepted 16 May 2006

Keywords:
KR-62980
Rosiglitazone
Apoptosis
Differentiation
PPARγ
PTEN
Breast cancer

ABSTRACT

Peroxisome proliferator-activated receptor-γ (PPARγ) activation has been a new approach to cancer therapy. In the present study, we investigated the effects of two structurally different PPARγ agonists, rosiglitazone and KR-62980 on MCF-7 breast cancer cells. Both agonists inhibited the cell proliferation and colony formation via apoptosis. PTEN expression was increased with decreased Akt phosphorylation by the agonists, whereas agonists actions were abolished in PTEN knockdown cells, indicating the critical role of PTEN in the anti-proliferative effects of PPARγ activation. Rosiglitazone induced the MCF-7 cell differentiation but KR-62980 did not alter the differentiation pattern with little effects on the lipid accumulation and the expression of lipogenesis markers. These results suggest that PPARγ activation may result in the inhibition of cell proliferation and/or induction of cell differentiation depending on the type of PPARγ agonists, and that KR-62980 may be useful in breast cancer therapy by inducing apoptosis.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) have recently been identified as the transcription factors belonging to the nuclear receptor gene superfamily [1]. Three types of the PPARs have been described in human, rodents, and amphibians: α, β (also called δ) and γ [2,3]. Numerous studies have observed that PPARγ is critical in a variety of biological processes, including adipogenesis, glucose metabolism and inflammation [4], and indeed some PPARγ agonists are clinically used as anti-diabetic agents. In addition, previous studies have shown that PPARγ functions as an important cellular regulator by inhibiting growth and/or inducing differentiation of adipocytes, monocytes, and cancer cells [5–7].

It has been shown that PPARγ ligands attenuate cell growth in carcinomas of various organs including breast, prostate, lung, colon, stomach, bladder, and pancreas [7–9]. Among various tissue cancers, the breast cancers have significant lipogenic capacity, and the modulation of fat metabolism has been associated with alteration of cancer cell growth and apoptosis. PPARγ expression was confirmed in human breast cancer cell lines as well as primary and metastatic breast adenocarcinomas, and PPARγ activation caused inhibition of proliferation, changes in epithelial gene expression associated with a more differentiated, less malignant state and extensive lipid accumulation in cultured breast cancer cells [10,11].

As one of the downstream signaling pathway of PPARγ activation, PTEN (the phosphatase and tensin homolog deleted from chromosome 10), a tumor suppressor gene has been investigated. PTEN has both protein phosphatase (phosphoserine/threonine and phospho-tyrosine) and phosphoinositide 3-phosphatase activities [12], and primarily serves to remove phosphate groups from key intracellular phosphoinositide signaling molecules, thus regulating cell migration, spreading,

* *Corresponding author*. Tel.: +82 42 860 7542; fax: +82 42 861 0770.
E-mail address: hgcheon@pado.krict.re.kr (H.G. Cheon).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.05.009

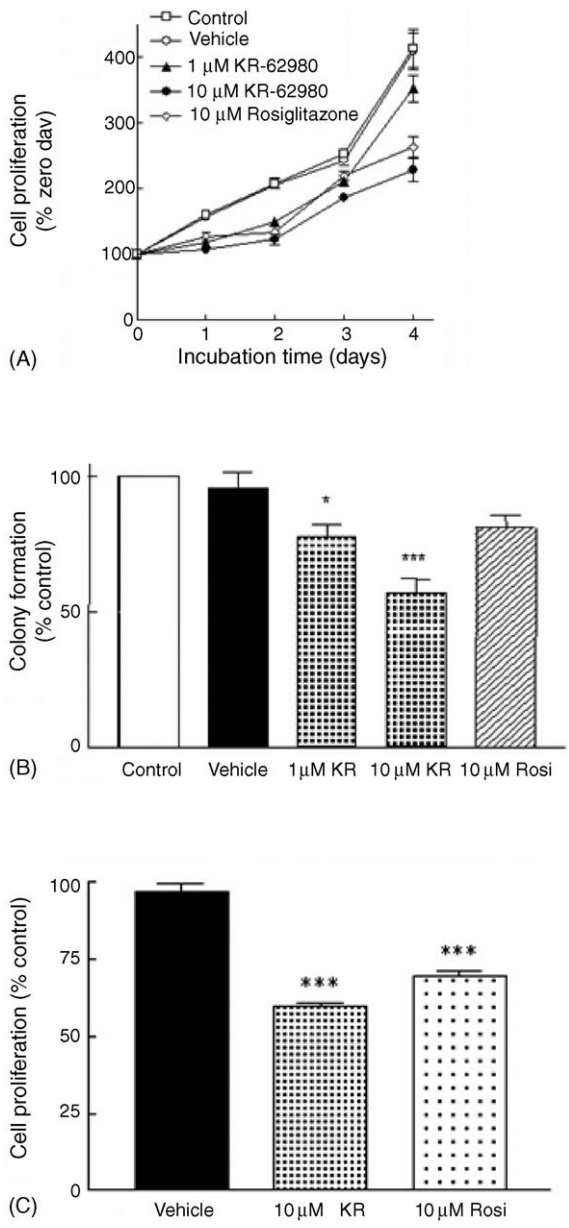

Fig. 1 – Effects of KR-62980 and rosiglitazone on cell proliferation and colony formation in MCF-7 and MDA-MB-231 cells. (A) Dose–response effect of KR-62980 on cell proliferation of MCF-7 cells. Cells were incubated with KR-62980 or rosiglitazone for 4 days, and cell proliferation was determined by MTT assay. Values are means ± S.E.M. of two different experiments each done with quadruplicate. (B) Effects of KR-62980 and rosiglitazone on colony formation of MCF-7 cells. Cells were incubated with KR-62980 or rosiglitazone for 14 days and colonies were stained with 0.005% crystal violet. Colonies were counted in a blinded manner using a ×10 objective on a Nikon inverted microscope. Experiments were carried out two times in triplicate. Data are expressed as percent of control. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. vehicle group. (C) Effects of KR-62980 and rosiglitazone on cell proliferation in MDA-MB-231 cells. Cells (1×10^3 cells/well) were seeded in 96-well plates and incubated with the indicated concentrations of KR-62980 or rosiglitazone for 4 days, and cell proliferation was determined using MTT assay. Values are means ± S.E.M. of two different preparations with quadruplicate experiments. $^{***}P < 0.001$ vs. vehicle group.

and growth signals by limiting activity of the phosphoinositide-3-kinase (PI3-K) pathway [13,14]. Previous study has indicated that PPARγ activation induced PTEN expression leading to anti-proliferative activity in cancer cells [15]. Furthermore, the promoter region of PTEN contains the PPAR responsive element (PPRE) sequence, which indicates that PTEN may be directly regulated by PPARγ in a transcriptional level. Recently, downstream effects of PTEN have been disclosed showing that the Bcl-2 family proteins are regulated [16].

KR-62980, 1-(trans-methylimino-*N*-oxy)-3-phenyl-6-(3-phenylpropoxy)-1H-indene-2-carboxylic acid ethyl ester was synthesized in the Korea Research Institute of Chemical Technology as a novel PPARγ agonist. The compound was initially identified from the chemical library of 70,000 structurally diverse synthetic compounds to discover novel anti-diabetic agents with improvement of side effect profiles of rosiglitazone. In contrast to rosiglitazone, previous observation with KR-62980 showed that the compound had little adipogenic effects on 3T3/L1 preadipocytes and C3H10T1/2 pluripotent stem cells (unpublished results). Because the adipogenic potential of two structurally different PPARγ agonists, rosiglitazone and KR-62980 was different, we examined and compared the effects of two PPARγ agonists on MCF-7 cells, of which activities are related with their lipogenic potentials.

2. Materials and methods

2.1. Cell culture

MCF-7 (ATCC HTB-22, human estrogen receptor-positive breast cancer cell) and MDA-MB-231 (ATCC HTB-26, human estrogen receptor-negative breast cancer cell) cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10 μg/ml insulin and 10% fetal bovine serum. Cells were grown to confluence at 37 °C in 5% CO_2.

2.2. Cell proliferation assay

For mitochondrial tetrazolium assay (MTT) procedure, MCF-7 and MDA-MB-231 cells were seeded at 1×10^3 cells/well in 96-well tissue culture plates. After 24 h, the indicated concentrations of compounds were added for 4 days, and then 20 μl/well of MTT solution (5 mg/ml phosphate buffered saline) was added and incubated for 2 h. The medium was aspirated and replaced with 150 μl/well of ethanol/dimethyl sulfoxide solution (1:1). The plates were shaken for 20 min and the OD was measured at 570–630 nm using microplate spectrometer (Bio-Rad Laboratories, Hercules, CA).

2.3. Soft agar colony assay

Effects of KR-62980 and rosiglitazone on the MCF-7 cell 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× DMEM plus 10% fetal bovine serum. About 2 ml of this mixture were pipetted into the wells of six-well plates and allowed to set. MCF-7 cells, 70–100% confluent, were trypsinized, and the cells were resuspended in growth medium and counted with a hemocytometer. The cells were then diluted to a final concentration of 2000 cells/ml in a mixture of 0.7% agar and 2× DMEM. KR-62980 and rosiglitazone were added from appropriate stock solutions to achieve the indicated final concentrations and a final DMSO concentration of 0.1%. Vehicle controls received similar volume of solvent alone. Two millilitre of the cell suspension were 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. Colonies were counted in a blinded manner using a 10× objective on a Nikon inverted microscope.

2.4. *DNA fragmentation assay*

MCF-7 and MDA-MB-231 cells were seeded in 100 mm tissue culture plates. After 24 h, the indicated concentrations of KR-62980 and rosiglitazone were added for 3 days. Using the Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN), cells were lysed in lysis buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate, and 0.5 mg/ml proteinase K). Digestion was continued for 20 min at 37 °C, followed by addition of RNase A to 0.1 mg/ml and running dye (10 mM EDTA, 0.25% bromophenol blue, 50%

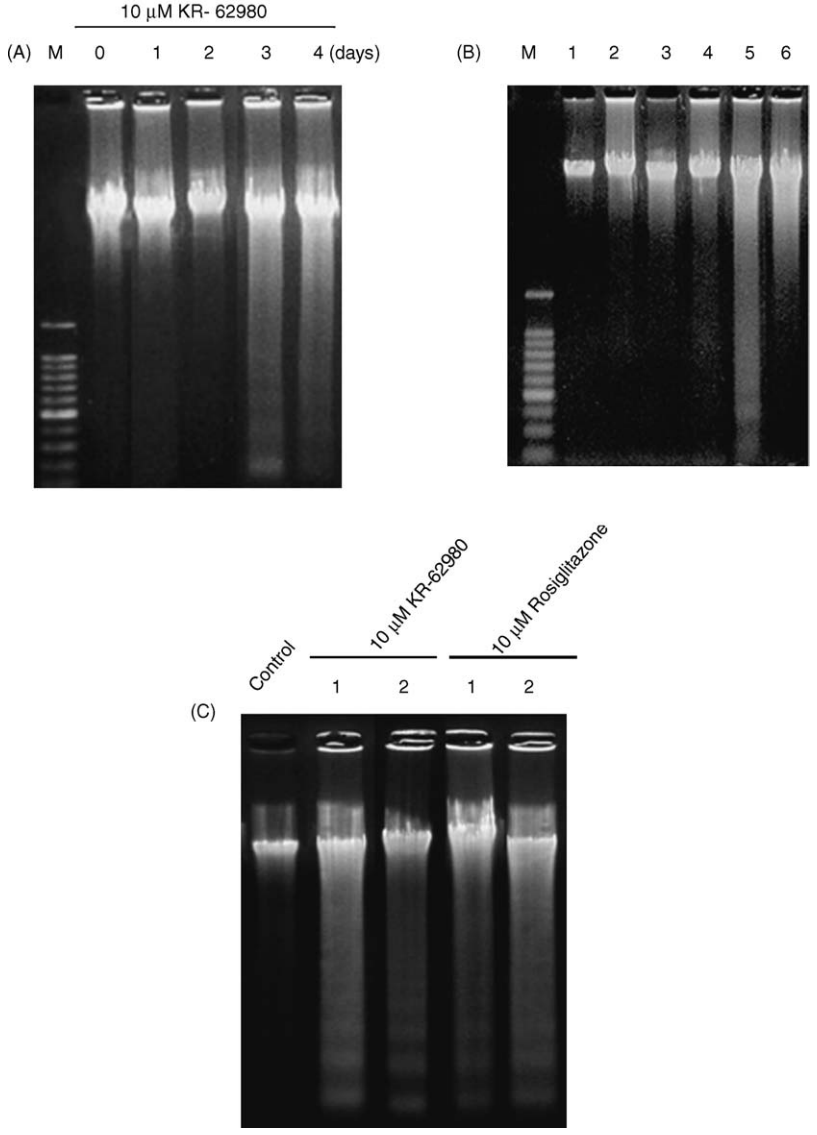

Fig. 2 – Effects of KR-62980 and rosiglitazone on the oligonucleosomal DNA fragmentation in MCF-7 and MDA-MB-231 cells. (A) Time–response effect of KR-62980 on the oligonucleosomal DNA laddering in MCF-7 cells. (B) Dose–response effect of KR-62980 on the oligonucleosomal DNA laddering in MCF-7 cells. Lane 1 represents control; lane 2, 0.01 μM KR-62980; lane 3, 0.1 μM KR-62980; lane 4, 1 μM KR-62980; lane 5, 10 μM KR-62980; lane 6, 10 μM rosiglitazone. (C) Effects of KR-62980 and rosiglitazone on the oligonucleosomal DNA fragmentation in MDA-MB-231 cells. Cells were treated with the indicated concentrations of KR-62980 or rosiglitazone for 3 days. M represents the 100 bp (base pair) DNA ladder markers. Each was confirmed with two different preparations.

glycerol). Equivalent amounts of DNA (15–20 μg) were loaded into wells of 1.6% agarose gel and electrophoresed in 0.5 × TAE buffer (40 mM Tris–acetate, 1 mM EDTA). DNA was visualized by ethidium bromide staining. Gel pictures were taken by UV transillumination.

2.5. Western blot analysis

Cells treated as described above were stimulated with KR-62980 and rosiglitazone for 24 h (PTEN, phospho-PTEN, Akt and phospho-Akt) or 48 h (Bcl-2, Bax, E-cadherin, β-casein, caveolin-1 and caveolin-2). Following centrifugation of cell lysates at 12,000 rpm, 10 μg of total protein was loaded into an 8% sodium dodecyl sulfate–polyacrylamide gel, and transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Protein bands were visualized using chemiluminescence (Pierce, Rockford, IL) and quantified with UN-SCAN-IT gel 5.1 software (Silk Scientific, Inc., Orem, UT). The results were expressed as a relative density. Polyclonal antibodies against Bax, β-casein, caveolin-1 and caveolin-2, and monoclonal antibodies against Bcl-2 and E-cadherin were from the Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against PTEN, phospho-PTEN (Ser380/Thr382/383), Akt and phospho-Akt (Ser473) were from the Cell signaling Technology (Beverly, MA).

2.6. PTEN or PPARγ knockdown using Stealth™ RNAi oligonucleotide

The PTEN stealth™ Select RNAi oligonucleotide (Target Accessions nos. NM016335) and the PPARγ stealth™ Select RNAi oligonucleotide (Target Accessions nos. NM1387121, NM0158692, NM1387111, NM0050373) were synthesized by Invitrogen (Carsbad, CA). The stealth™ RNAi negative control Duplex (Invitrogen) was used as a control oligonucleotide. Transfection efficiency was monitored using a fluorescent oligonucleotide (BLOCK-iT Fluorescent oligo, Invitrogen) and estimated to be 80–90%.

The stealth™ RNAi molecules were transfected into MCF-7 cells using Lipofectamine 2000 following Invitrogen's protocols. The final concentrations of 100 nM PTEN stealth ™ Select RNAi oligonucleotide and 50 nM PPARγ 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 prior to the treatment of compounds. The ability of the stealth™ RNAi oligonucleotide to knockdown PTEN and PPARγ expression was analyzed by Western blot and real-time reverse transcription-polymerase chain reaction (RT-PCR) on whole cell extracts.

2.7. RT-PCR analysis

Total RNA was isolated from compounds-treated cells using easy-BLUE Total RNA extraction Kit (iNtRON Inc., Kyungki-Do, Korea). Reverse transcription of total RNA (1 μg) was performed using AccuPower RT PreMix (Bioneer Inc., Daejeon, Korea). PCR primers for amplification of PTEN were designed based on the sequences obtained; sense, 5′-AGG GAC CGA CTG TAA TG-3′; antisense, 5′-AGT GCC ACG GGT CTG TAA TC-3′. RT-PCR conditions were 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, followed by a 10 min extension reaction at 72 °C. PCR primers for amplification of PPARγ were designed based on the sequences obtained; sense, 5′-CCC TGG CAA AGC ATT TGT AT-3′; antisense, 5′-GAA ACT GGC ACC CTT GAA AA-3′. RT-PCR conditions were 30 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s, followed by a 10 min extension reaction at 72 °C. PCR primers for amplification of aP2, SREBP-1c, and fatty acid synthase (FAS) were designed based on the sequences obtained; aP2 sense, 5′-TGG AAG CTT GTC TCC AGT GA-3′; aP2 antisense, 5′-GCT CTT CAC CTT CCT GTC GT-3′: SREBP-1c sense, 5′-GCC ATG GAT TGC ACT TT-3′; SREBP-1c antisense, 5′-CAA GAG AGG AGC TCA ATG-3′: FAS sense, 5′-GAA ACT GCA GGA GCT GTC-3′; FAS antisense, 5′-CAC GGA GTT GAG GCG CAT-3′. Aliquots of the PCR reaction were checked by melting curve analysis as provided by the Roter-Gene 3000 system (Corbett research, Australia). The instrument settings were: PTEN – denaturing at 95 °C for 15 min; with 40 repeated cycles of denaturing at 94 °C for 30 s, annealing at 64 °C for 30 s, and elongation at 72 °C for 30 s, PPARγ – denaturing at 95 °C for 15 min; with 40 repeated cycles

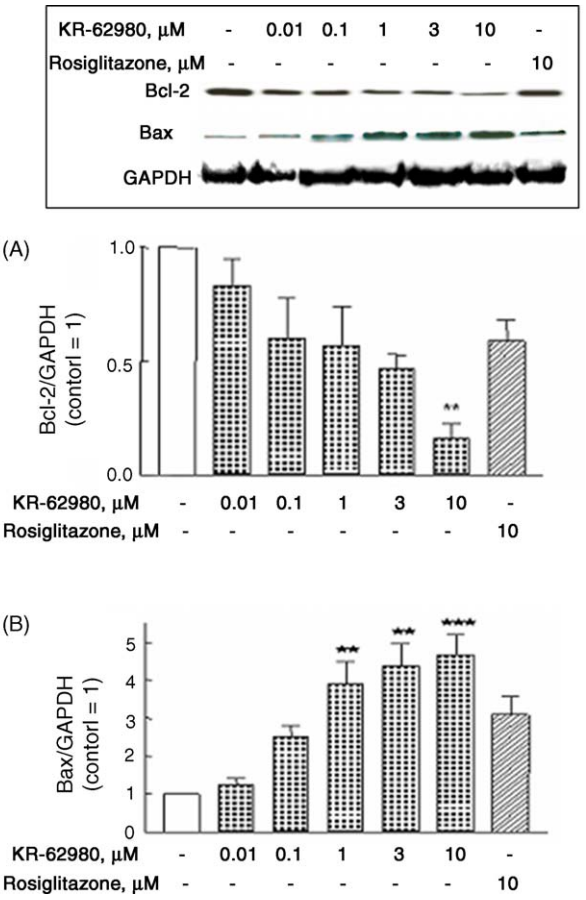

Fig. 3 – Effects of KR-62980 and rosiglitazone on the expressions of Bcl-2 protein (A) and Bax protein (B) in MCF-7 cells. Cells were incubated with the indicated concentrations of KR-62980 or rosiglitazone for 2 days, and protein expression was determined using western blotting. Values are means ± S.E.M. of three different preparations. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group.

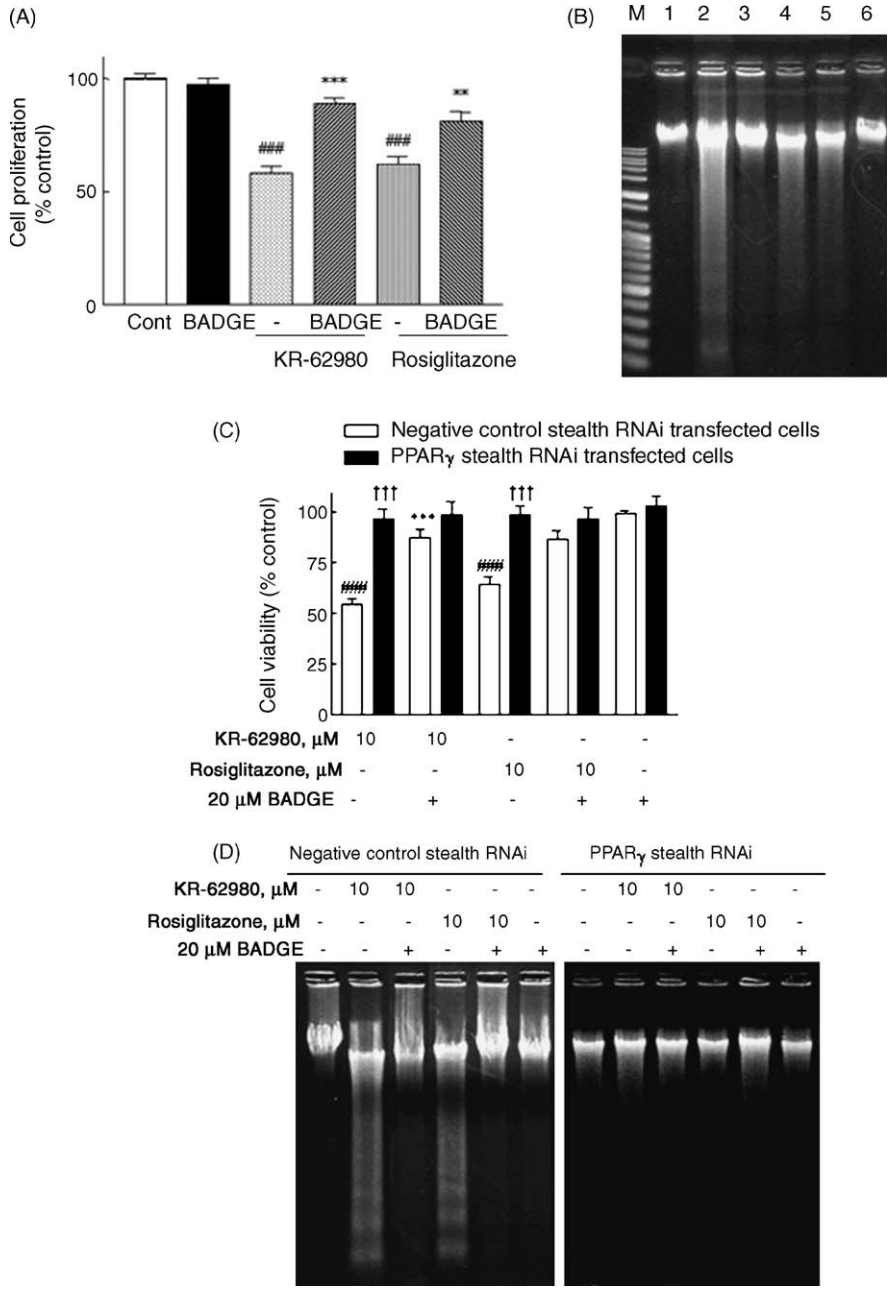

Fig. 4 – Effect of BADGE, a PPARγ antagonist, on KR-62980-suppressed cell proliferation and -induced DNA fragmentation in MCF-7 cells. (A) Inhibition of the anti-proliferative effects of KR-62980 and rosiglitazone by a PPARγ antagonist, BADGE in MCF-7 cells. Cells (1×10^3 cells/well) were seeded in 96-well plates, treated with BADGE (20 μM) for 1 h, and incubated with 10 μM KR-62980 or rosiglitazone for 4 days, and cell proliferation was determined using MTT assay. Values are means ± S.E.M. of two different preparations with quadruplicate experiments. $^{###}P < 0.001$ vs. control group; $^{**}P < 0.01$, $^{***}P < 0.001$ vs. each drug treatment group. (B) Inhibition of the apoptotic effects of KR-62980 and rosiglitazone by a PPARγ antagonist, BADGE in MCF-7 cells. Cells were treated with BADGE (20 μM) for 1 h, and incubated with the indicated concentrations of KR-62980 or rosiglitazone for 3 days. Lane 1, control; lane 2, 10 μM KR-62980; lane 3, 20 μM BADGE plus 10 μM KR-62980; lane 4, 10 μM rosiglitazone; lane 5, 20 μM BADGE plus 10 μM rosiglitazone; lane 6, 20 μM BADGE. (C&D) Effects of KR-62980 and rosiglitazone on cell proliferation (C) and DNA fragmentation (D) in PPARγ knockdown MCF-7 cells. BADGE was pretreated for 1 h followed by KR-62980 stimulation for 3 days (D) or 4 days (C) in PPARγ stealth™ RNAi oligonucleotide transfected cells or negative control stealth™ RNAi oligonucleotide transfected cells, and DNA fragmentation assay or MTT assay was carried out. Values are means ± S.E.M. of two different preparations with quadruplicate experiments. $^{###}P < 0.001$ vs. control group; $^{***}P < 0.001$ vs. 10 μM KR-62980 treated group; $^{†††}P < 0.001$ vs. negative control stealth RNAi transfected group.

of denaturing at 94 °C for 30 s, annealing at 62 °C for 30 s, and elongation at 72 °C for 30 s, aP2 – denaturing at 95 °C for 15 min; with 50 repeated cycles of denaturing at 94 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s, and SREBP-1c and FAS – denaturing at 95 °C for 15 min; with 50 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. Relative abundance of mRNA was calculated after normalization to GAPDH.

2.8. Measurement of lipid accumulation

Cells were incubated with the indicated concentrations of KR-62980 and rosiglitazone for 3 days and lipid accumulation was determined using Oil Red O staining. A stock solution of Oil Red O (0.5 g in 100 ml isopropanol) was prepared and passed through a 0.2 μm filter. To prepare the working solution, 6 ml of the stock solution was mixed with 4 ml of distilled water, left for 1 h at room temperature, and filtered through a 0.2 μm filter prior to use. Cells in 6-well plates were fixed with 10% formalin in phosphate buffered saline for 1 h at 4 °C and stained with 500 μl of the Oil Red O working solution per well for 1 h at room temperature. The wells were rinsed three times with 700 μl of distilled water. Oil Red O-stained cells were then examined with a Nikon microscope.

2.9. Drugs

KR-62980 and rosiglitazone were synthesized in the Korea Research Institute of Chemical Technology (Daejeon, Korea). Bisphenol A diglycidyl ether (BADGE), agar, crystal violet and MTT were from the Sigma Chemical Co. The PTEN and PPARγ stealth™ Select RNAi oligonucleotide, stealth™ RNAi negative control duplex and Lipofectamine 2000 were from the Invitrogen. KR-62980, rosiglitazone and BADGE were dissolved in dimethyl sulfoxide as a 20 mM stock solution and then diluted with the phosphate buffered saline.

2.10. Statistical analysis

The results are expressed as means ± S.E.M. The comparison of changes in cell proliferation between control group and drug-treated groups was analyzed by repeated measures analysis of variance, and followed by Tukey's multiple comparison tests as a post hoc comparison. Student's *t*-test was used for analyzing values between vehicle and PPARγ agonist-treated groups. $P < 0.05$ was considered to be significant.

3. Results

3.1. Inhibitory effects of KR-62980 and rosiglitazone on the proliferation of MCF-7 and MDA-MB-231 cells

Both KR-62980 and rosiglitazone caused inhibition of MCF-7 cell proliferation in a time- and concentration-dependent manner (Fig. 1A). When MCF-7 cells were incubated in the medium for 4 days, cell proliferation increased to 419 ± 29%. KR-62980 (10 μM) significantly suppressed the cell proliferation in MCF-7, estrogen receptor-positive breast cancer cells, and MDA-MB-231 cells, estrogen receptor-negative breast cancer cells (Fig. 1A and C). KR-62980 also inhibited the MCF-7 colony formation concentration-dependently (Fig. 1B). When MCF-7 cells were incubated in the medium containing 10 μM KR-62980 for 14 days, colony formation decreased to 57 ± 5% of control. Rosiglitazone also suppressed the cell proliferation and colony formation but to a lesser degree than KR-62980.

3.2. Effects of KR-62980 and rosiglitazone on apoptotic cell death

Exposure of MCF-7 cells to KR-62980 (10 μM) induced prominent oligonucleosomal DNA fragmentation in a concentration and time-dependent manner, whereas rosiglitazone (10 μM) showed a lesser degree of DNA fragmentation than KR-62980 (Fig. 2A and B). Similarly, KR-62980 (10 μM) and rosiglitazone treatment of MDA-MB231 cells also resulted in the DNA fragmentation, indicating the apoptotic actions of the compounds (Fig. 2C). The LDH assay further supported the anti-proliferative effects of the compounds are mediated by apoptosis (results not shown).

Bcl-2 protein was present at a relatively high level in the control samples and normalized as the relative density of 1.0. KR-62980 (10 μM) caused a significant decrease in Bcl-2 protein expression by 0.16 ± 0.07 relative density (16% of the control value) (Fig. 3A). On the other hand, Bax protein expression was low level in the control MCF-7 cells (expressed as relative density 1.0), which was markedly and concentration-dependently elevated by application of 1, 3 and 10 μM KR-62980 to 3.90 ± 0.59, 4.38 ± 0.58 and 4.67 ± 0.53 relative density, respectively (Fig. 3B).

3.3. Involvement of PPARγ in the KR-62980 and rosiglitazone-induced anti-proliferative and apoptotic actions

To examine whether the effect of KR-62980 is mediated by PPARγ, the PPARγ pathway had been inactivated by treatment

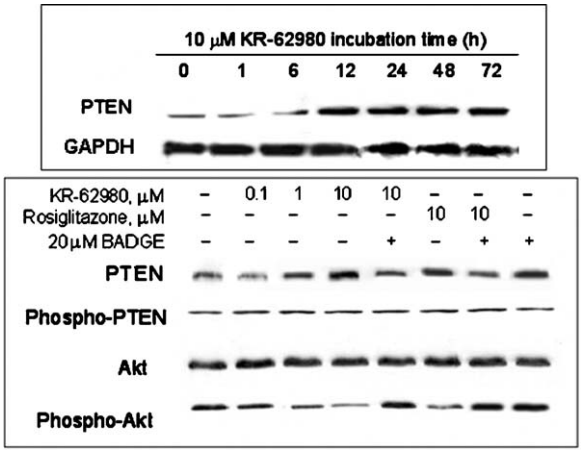

Fig. 5 – Effects of KR-62980 and rosiglitazone on the expressions of PTEN and Akt phosphorylation in MCF-7 cells. Cells were incubated with 10 μM KR-62980 for the indicated times or with the indicated concentrations of KR-62980 or rosiglitazone for 24 h and protein expression was determined using western blotting. Each was confirmed with three different preparations.

with BADGE (20 μM) for 1 h, and then KR-62980 (10 μM) was added to the cells. BADGE decreased both KR-62980 and rosiglitazone-induced cell death and DNA fragmentation (Fig. 4A and B). BADGE alone induced no effect on cell proliferation and DNA fragmentation at a concentration of 20 μM.

The transfection of stealth™ RNAi oligonucleotide in MCF-7 cells resulted in the reduction of PPARγ protein expression to 20% of control (data not shown). In contrast to the anti-proliferative and apoptotic effects of KR-62980 in stealth™ RNAi negative control oligonucleotide transfected cells, KR-62980 (10 μM) was ineffective on cell viability and DNA fragmentation in PPARγ knockdown cells (Fig. 4C and D). These results provide conclusive evidence that PPARγ activation is required for the anti-proliferative and apoptotic effects of KR-62980 on breast cancer cells.

3.4. *Effects of KR-62980 and rosiglitazone on PTEN expression and Akt phosphorylation*

KR-62980 time-dependently increased the PTEN level, showing a maximum effect at 24 h. The PTEN expression level increased with increasing concentrations of KR-62980 and rosiglitazone, which was reversed by BADGE (20 μM). Correlating with decreased PTEN expression, KR-62980 (0.1 ~ 10 μM) and rosiglitazone suppressed the Akt phosphorylation, with no change in the Akt expression (Fig. 5).

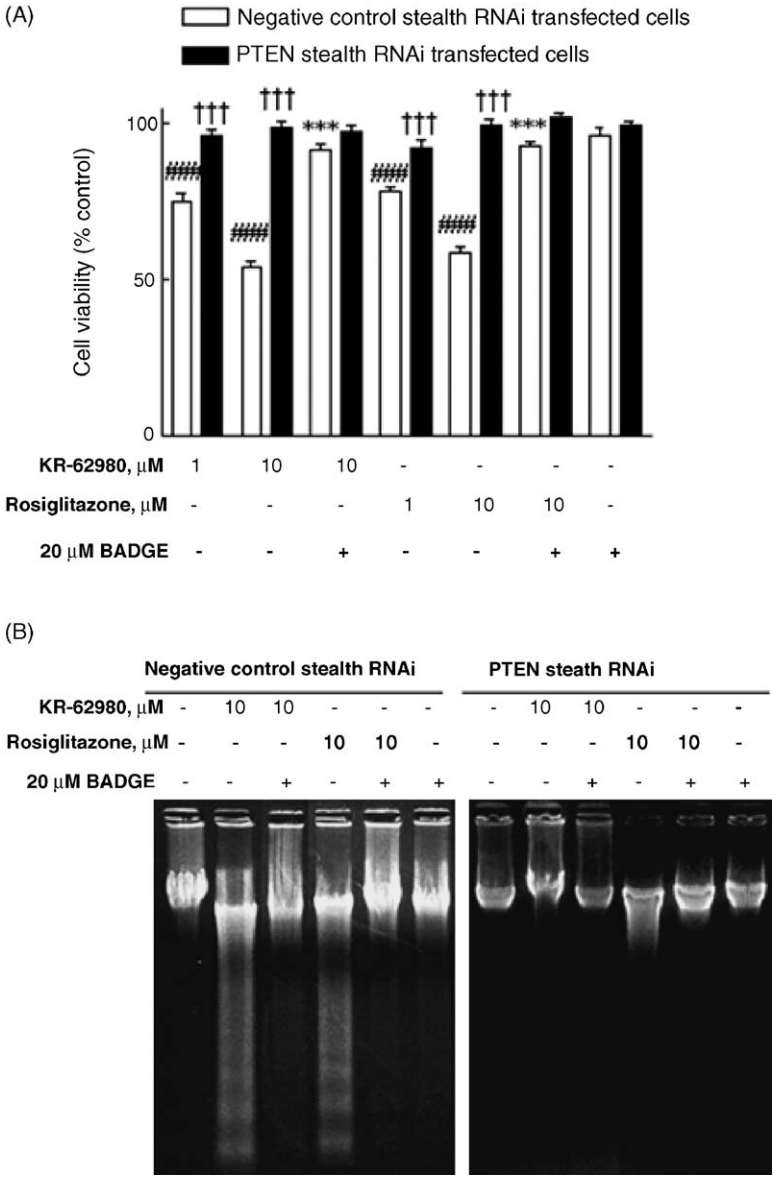

Fig. 6 – Effects of KR-62980 and rosiglitazone on cell proliferation (A) and DNA fragmentation (B) in PTEN-knockdown MCF-7 cells. BADGE was pretreated for 1 h followed by KR-62980 or rosiglitazone stimulation for 4 days (A) or 3 days (B) in PTEN stealth™ RNAi oligonucleotide transfected cells or negative control stealth™ RNAi oligonucleotide transfected cells, and DNA fragmentation assay or MTT assay was carried out. Values are means ± S.E.M. of two different preparations with quadruplicate experiments. ###P < 0.001 vs. control group; ***P < 0.001 vs. 10 μM KR-62980 or rosiglitazone treated group; †††P < 0.001 vs. negative control stealth™ RNAi transfected group.

We examined the effects of PTEN knockdown on the KR-62980 and rosiglitazone actions to elucidate the importance of PTEN. The transfection of PTEN stealth™ RNAi oligonucleotide in MCF-7 cells resulted in the reduction of PTEN protein expression to 45% of control (data not shown). In contrast to the anti-proliferative and apoptotic effects of KR-62980 in stealth™ RNAi negative control oligonucleotide transfected cells, KR-62980 (10 μM) was ineffective on cell proliferation and DNA fragmentation in PTEN knockdown cells (Fig. 6A and B). These results provide important evidence that PTEN activation is required for the anti-proliferative and apoptotic effects of KR-62980 and rosiglitazone on breast cancer cells.

3.5. *Effects of KR-62980 and rosiglitazone on the MCF-7 cell differentiation*

PPARγ activation induced cells to a more differentiated, less malignant state and caused extensive lipid accumulation in cultured breast cancer cells [10,11]. Thus, we examined whether addition of KR-62980 and rosiglitazone in MCF-7 cells also induces differentiation using Oil Red O staining. Untreated MCF-7 cells had little lipid accumulation as measured by Oil Red O staining (Fig. 7). Rosiglitazone treatment (1 and 10 μM, 3 days) strongly induced lipid accumulation whereas little accumulation of lipids was observed by KR-62980.

To further check the effects of KR-62980 and rosiglitazone on cell lipogenesis, the well-known breast cancer lipogenesis markers, aP2, SREBP-1c and FAS were measured after KR-62980 and rosiglitazone treatment in MCF-7 cells. Rosiglitazone induced mRNA aP2, SREBP-1c and FAS level, but KR-62980 did not induce their change (Fig. 8).

When we measured the level of the well-known differentiation marker expressions such as E-cadherin, β-casein, caveolin-1 and -2 in MCF-7 cells after agonists treatment, rosiglitazone significantly induced differentiation marker expression, whereas the expression was not changed by KR-62980 (Fig. 9).

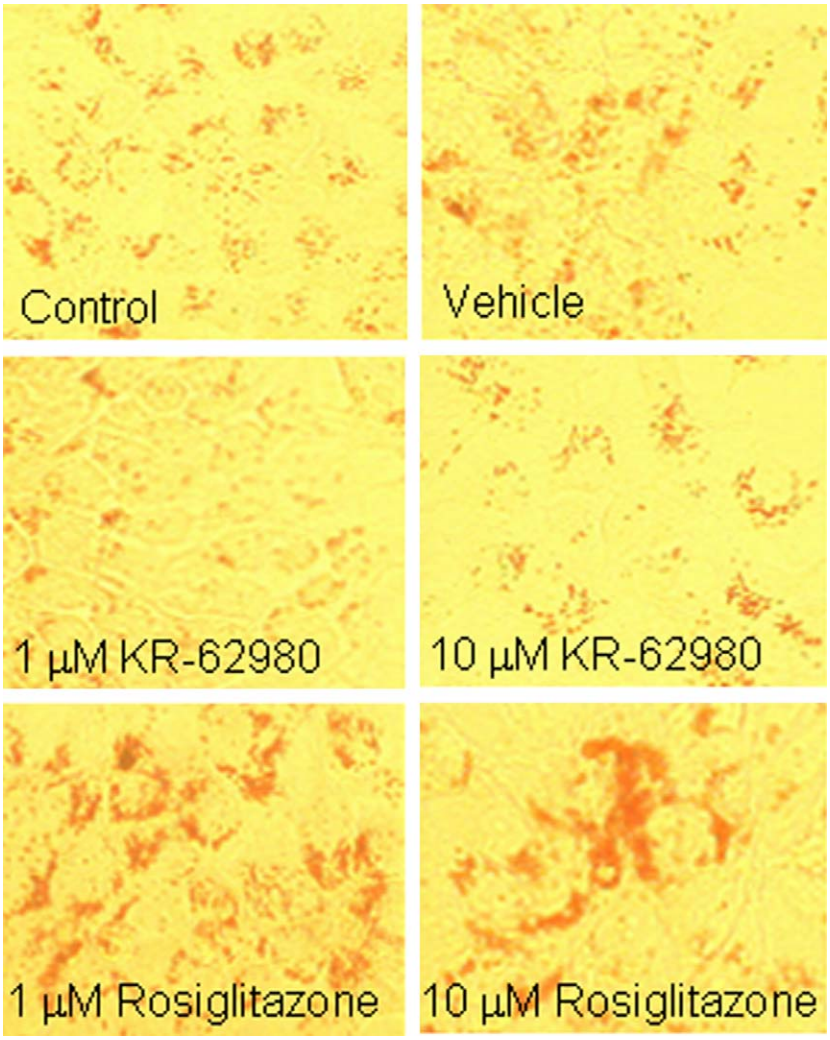

Fig. 7 – Effects of KR-62980 and rosiglitazone on the lipid accumulation in MCF-7 cells. Cells were incubated with the indicated concentrations of KR-62980 or rosiglitazone for 3 days and lipid accumulation was determined using Oil Red O staining. Each was confirmed with three different preparations.

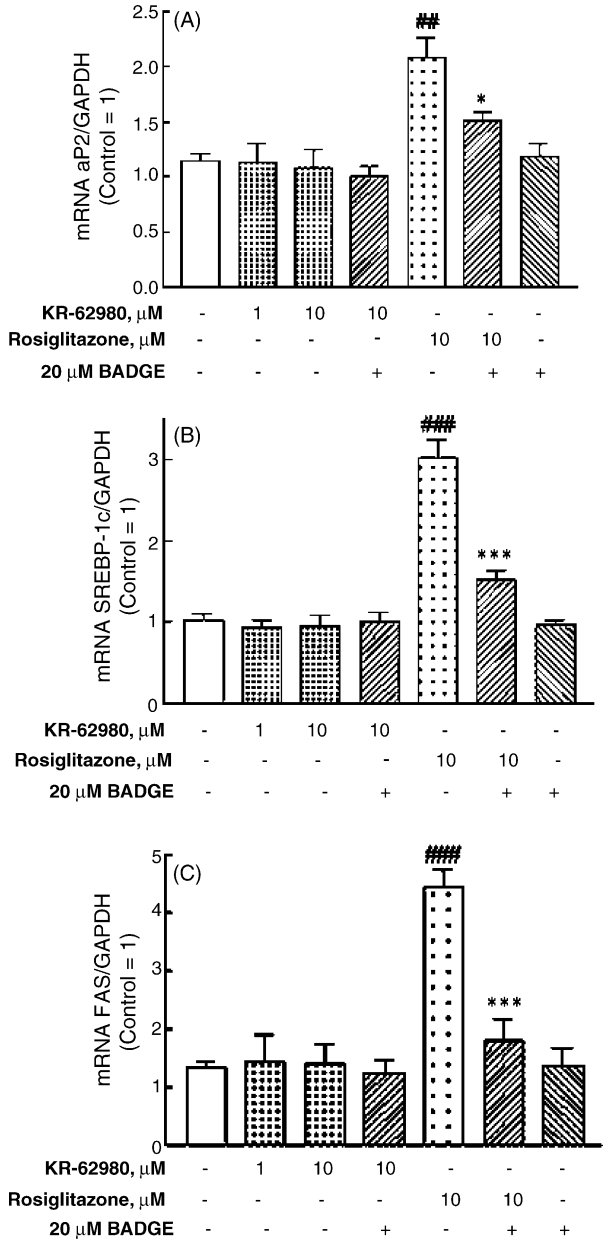

Fig. 8 – Effects of KR-62980 and rosiglitazone on the aP2, SREBP-1c, and FAS expression in MCF-7 cells. BADGE was pretreated for 1 h followed by KR-62980 or rosiglitazone stimulation for 2 days, and real-time RT-PCR was carried out. Values are means ± S.E.M. of quadruplicate experiments. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. control group; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. 10 μM rosiglitazone group.

4. Discussion

The present study shows that rosiglitazone and KR-62980, a novel PPARγ agonist, strongly inhibited cell proliferation in human breast cancer cells, MCF-7, human estrogen receptor-positive cell line, and MDA-MB-231, human estrogen receptor-negative cell line, by pro-apoptotic mechanism. The apoptotic action by PPARγ activation is likely due to the up-regulation of PTEN expression, down-regulation of Akt phosphorylation following Bax and Bcl-2 alteration. The effects of two agonists appear to be different in that rosiglitazone induced both inhibition of cell proliferation and promotion of cell differentiation whereas KR-62980 resulted in only inhibition of cell proliferation. These actions are likely due to the difference in the adipogenic activity, possibly due to the different binding mode to the receptor, thereby different recruitments of coactivators and/or corepressors.

Recently, the role of PPARγ in tumors has been widely studied, and PPARγ agonists have been shown to have direct effects on tumor cells including breast, lung, prostate, colon and gastric cancer cells. For example, the PPARγ ligand 15-deoxy-Δ-12,14-prostaglandin J2 inhibits cell proliferation and induces apoptosis of MCG-803 human gastric cancer cell line in a dose-dependent fashion [17] and the synthetic PPARγ ligands such as thiazolidinediones have potent anti-tumor effects against human prostate cancer both in vitro and in vivo [18]. In the present study, we examined the effects of two different PPARγ agonists, rosiglitazone and KR-62980 on MCF-7 cells. The potency of these compounds on PPARγ was determined as EC_{50} values of 250 nM for rosiglitazone, and 15 nM for KR-62980 (data not shown), suggesting that KR-62980 is more potent. Consistent with their potencies on PPARγ, KR-62980 more strongly suppressed cell proliferation and colony formation in MCF-7 cells and MDA-MB-231 cells than rosiglitazone. This anti-proliferative activity by KR-62980 was accompanied by oligonucleosomal DNA fragmentation with up-regulation of Bax expression and down-regulation of Bcl-2 expression.

Tumor suppressor protein PTEN is known to negatively regulate several cellular functions, including cell migration and survival from apoptosis by suppression of PI-3K and Akt activation via dephosphorylating the phosphatidylinositol (3,4,5) triphosphate to phosphatidylinositol (4,5) diphosphate, an inactive state [12,19]. Weng and coworkers [20] have shown that PTEN suppresses breast cancer growth by phosphatase activity-dependent G1 arrest followed by cell death. Moreover, the regulation of PTEN expression by PPARγ has been reported in several cancer cells and inflammatory cells, and its regulation is likely at the transcriptional manner based on the presence of PPRE in the promoter region of PTEN. In our results, KR-62980 time-dependently and concentration-dependently increased the PTEN expression and suppressed the Akt phosphorylation, which was reversed by BADGE (20 μM). Furthermore, in contrast to the anti-proliferative and apoptotic effects of KR-62980 in stealth™ RNAi negative control oligonucleotide transfected cells, KR-62980 (10 μM) was ineffective on cell viability and DNA fragmentation in PTEN knockdown MCF-7 cells. These results provide conclusive evidence that PTEN activation with reduced Akt phosphorylation is required for the anti-proliferative and apoptotic effects of KR-62980 and rosiglitazone on breast cancer cells.

Two cellular pathways, namely differentiation and apoptosis are main focuses in developing anti-cancer therapies. It was reported previously that thiazolidinediones-induced growth inhibition in tumor cells may be associated with cell cycle arrest, differentiation, and apoptosis [7,18]. In mammary carcinogenesis studies, high fat consumption was related with the incidence of breast cancer and conversely inhibition of fat metabolism in breast cancer cells may result in the inhibition

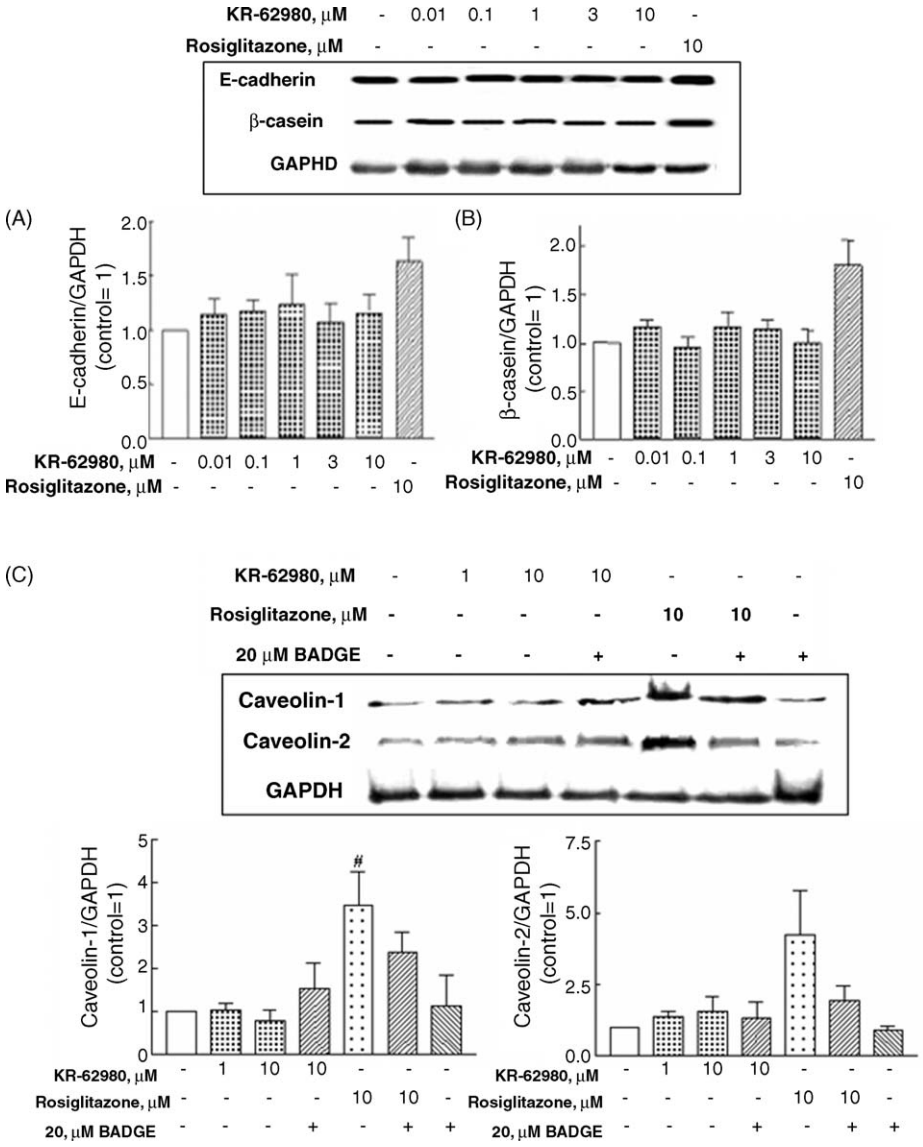

Fig. 9 – Effects of KR-62980 and rosiglitazone on the expressions of E-cadherin (A), β-casein (B), caveolin-1 and -2 (C) in MCF-7 cells. Cells were incubated with the indicated concentrations of KR-62980 or rosiglitazone for 2 days, and protein expression was determined using western blotting. Values are means ± S.E.M. of three different preparations. $^{\#}P < 0.05$ vs. control group.

of growth and apoptosis [21,22]. In addition, PPARγ ligands transcriptionally upregulated the caveolin-1 and -2 expressions in MCF-7 breast carcinoma and HT-29 colon adenocarcinoma cells [23]. The expression of caveolin-1 and -2, 22–24 kDa integral membrane proteins, is increased during differentiation of preadipocytes to adipocytes [24], consistent with Spiegelman and coworkers' results [25] that PPARγ is strongly linked with lipid metabolism. The present study showed that KR-62980 induced no lipid accumulation whereas rosiglitazone strongly increased lipid accumulation in MCF-7 cells with the induction of well-known breast lipogenic markers mRNA levels such as aP2, SREBP-1c and fatty acid synthase. Moreover, the breast cancer differentiation markers including E-cadherin, β-casein, caveolin-1 and -2 were induced only by rosiglitazone. Interestingly, PPAR binding protein (PBP) and PPAR interacting protein (PRIP), the transcriptional coactivators involved in PPARγ transcriptional activity have been suggested as important players in mammary epithelial differentiation with high expression in breast cancers [26]. The differential effects of KR-62980 and rosiglitazone on MCF-7 cell differentiation may be due to the differences in the assembly of the coactivators, particularly PBP and PRIP. In fact, TRAP220, a coactivator involved in adipocyte differentiation was markedly less recruited by KR-62980 than by rosiglitazone.

In summary, our data demonstrated that KR-62980 strongly inhibited cell proliferation and colony formation in human breast cancer cells by means of pro-apoptotic processes including Bcl-2 and Bax regulation via activation of PPARγ, subsequently up-regulation of PTEN expression and down-regulation of Akt phosphorylation. On the other hand, rosiglitazone, a well-known PPARγ agonist, exhibited

differentiation promoting effects as well as anti-proliferative effects on MCF-7 cells. These results suggest that PPARγ activation has different effects on cancer cells depending on the type of the agonists, and that anti-cancer effects of KR-62980 may be primarily due to the apoptotic effects, whereas rosiglitazone may induce anti-cancer effects by both anti-proliferation and pro-differentiation actions. Thus, the activity profile of KR-62980 seems to be different from that of rosiglitazone in that KR-62980 was incapable of accumulating lipid and inducing cell differentiation.

Acknowledgments

This research was supported by grant from the Center for Biological Modulators of the 21st Century Frontier R&D Program, The Ministry of Science and Technology, Korea.

REFERENCES

[1] Calnek DS, Mazzella L, Roser S, Roman J, Hart CM. Peroxisome proliferator-activated receptor gamma ligands increase release of nitric oxide from endothelial cells. Arterioscler Thromb Vasc 2003;23:52–7.
[2] Kliewer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, et al. Differential expression and activation of a family of murine peroxisome-proliferator-activated receptors. Proc Natl Acad Sci USA 1994;91:7355–9.
[3] Niho N, Takahashi M, Shoji Y, Takeuchi Y, Matsubara S, Sugimura T, et al. Dose-dependent suppression of hyperlipidemia and intestinal polyp formation in Min mice by pioglitazone, a PPAR gamma ligand. Cancer Sci 2003;94:960–4.
[4] Rosen ED, Spiegelman BM. PPARgamma: a nuclear regulator of metabolism, differentiation, and cell growth. J Biol Chem 2001;276:37731–4.
[5] Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev 1994;8:1224–34.
[6] Tontonoz P, Singer S, Forman BM, Sarraf P, Fletcher JA, Fletcher CD, et al. Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor gamma and the retinoid X receptor. Proc Natl Acad Sci USA 1997;94:237–41.
[7] Elstner E, Linker-Israeli M, Umiel T, Le J, Grillier I, Said J, et al. Combination of a potent 20-epi-vitamin D3 analogue (KH1060) with 9-*cis*-retinoic acid irreversibly inhibits clonal growth, decreases bcl-2 expression, and induces apoptosis in HL-60 leukemic cells. Cancer Res 1996;56:3570–6.
[8] Motomura W, Nagamine M, Tanno S, Sawamukai M, Takahashi N, Kohgo Y, et al. Inhibition of cell invasion and morphological change by troglitazone in human pancreatic cancer cells. J Gastroenterol 2004;39:461–8.
[9] Han S, Roman J. Suppression of prostaglandin E2 receptor subtype EP2 by PPARgamma ligands inhibits human lung carcinoma cell growth. Biochem Biophys Res Commun 2004;314:1093–9.
[10] Kilgore MW, Tate PL, Pai S, Sengoku E, Price TM. MCF-7 and T47D human breast cancer cells contain a functional peroxisomal response. Mol Cell Endocrinol 1997;129:229–35.
[11] Mueller E, Sarraf P, Tontonoz P, Evans RM, Martin KJ, Zhang M, et al. Terminal differentiation of human breast cancer through PPAR gamma. Mol Cell 1998;1:465–70.
[12] Ning K, Pei L, Liao M, Liu B, Zhang Y, Jiang W, et al. Dual neuroprotective signaling mediated by downregulating two distinct phosphatase activities of PTEN. J Neurosci 2004;24:4052–60.
[13] Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell 1998;95:29–39.
[14] Tamura M, Gu J, Takino T, Yamada KM. Tumor suppressor PTEN inhibition of cell invasion, migration, and growth: differential involvement of focal adhesion kinase and p130Cas. Cancer Res 1999;59:442–9.
[15] Patel L, Pass I, Coxon P, Downes CP, Smith SA, Macphee CH. Tumor suppressor and anti-inflammatory actions of PPARγ agonists are mediated via upregulation of PTEN. Curr Biol 2001;11:764–8.
[16] Gratton JP, Morales-Ruiz M, Kureishi Y, Fulton D, Walsh K, Sessa WC. Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. J Biol Chem 2001;276:30359–65.
[17] Chen YX, Zhong XY, Qin YF, Bing W, He LZ. 15d-PGJ2 inhibits cell growth and induces apoptosis of MCG-803 human gastric cancer cell line. World J Gastroenterol 2003;9:2149–53.
[18] Kubota T, Koshizuka K, Willamson EA, Asou H, Said JW, Holden S, et al. Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. Cancer Res 1999;58:3344–52.
[19] van Golen CM, Schwab TS, Ignatoski KM, Ethier SP, Feldman EL. PTEN/MMAC1 overexpression decreases insulin-like growth factor-I-mediated protection from apoptosis in neuroblastoma cells. Cell Growth Differ 2001;12:371–8.
[20] Weng LP, Smith WM, Dahia PL, Ziebold U, Gil E, Lees JA, et al. PTEN suppresses breast cancer cell growth by phosphatase activity-dependent G1 arrest followed by cell death. Cancer Res 1999;59:5808–14.
[21] Lu J, Jiang C, Fontane S, Thompson HJ. Ras may mediate mammary cancer promotion by high fat. Nutr Cancer 1995;23:283–90.
[22] Pizer ES, Jackisch C, Wood FD, Pasternack GR, Davidson NE, Kuhajda FP. Inhibition of fatty acid synthesis induces programmed cell death in human breast cancer cells. Cancer Res 1996;56:2745–7.
[23] Burgermeister E, Tencer L, Liscovitch M. Peroxisome proliferator-activated receptor-gamma upregulates caveolin-1 and caveolin-2 expression in human carcinoma cells. Oncogene 2003;22:3888–900.
[24] Scherer PE, Lewis RY, Volonte D, Engelman JA, Galbiati F, Couet J, et al. Cell-type and tissue-specific expression of caveolin-2. Caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo. J Biol Chem 1997;272:29337–46.
[25] Spiegelman BM, Hu E, Kim JB, Brun R. PPAR gamma and the control of adipogenesis. Biochimie 1997;79:111–2.
[26] Zhu Y, Qi C, Jain S, LeBeau MM, Espinosa R, Atkins GB, et al. Amplification and overexpression of peroxisome proliferator-activated receptor binding protein (PBP/PPARBP) gene in breast cancer. Proc Natl Acad Sci USA 1999;96:10848–53.