J. Microbiol. Biotechnol. (2017), 27(2), 372–379
https://doi.org/10.4014/jmb.1607.07055

jmb

Effect of Phosphodiesterase in Regulating the Activity of Lysosomes in the HeLa Cell Line

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Received: July 21, 2016
Revised: October 26, 2016
Accepted: October 31, 2016

First published online
November 4, 2016

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pISSN 1017-7825, eISSN 1738-8872

The transport of lysosomal enzymes into the lysosomes depends on the phosphorylation of their chains and the binding of the phosphorylated residues to mannose-6-phosphate receptors. The efficiency of separation depends more on the phosphodiesterases (PDEs) than on the activity of the phosphorylation of mannose residues and can be determined in vitro. PDEs play important roles in regulation of the activation of lysosomes. The expression of proteins was confirmed by western blotting. All PDE4 series protein expression was reduced in high concentrations of rolipram. As a result of observing the fluorescence intensity after rolipram treatment, the lysosomal enzyme was activated at low concentrations and suppressed at high concentrations. High concentrations of rolipram recovered the original function. Antimicrobial activity was not shown in either 10 or 100 μM concentrations of rolipram in treated HeLa cells in vitro. However, the higher anticancer activity at lower rolipram concentration was shown in lysosomal enzyme treated with 10 μM of rolipram. The anticancer activity was confirmed through cathepsin B and D assay. Tranfection allowed examination of the relationship between PDE4 and lysosomal activity in more detail. Protein expression was confirmed to be reduced. Fluorescence intensity showed decreased activity of lysosomes and ROS in cells transfected with the antisense sequences of PDE4 A, B, C, and D. PDE4A showed anticancer activity, whereas lysosome from cells transfected with the antisense sequences of PDE4 B, C, and D had decreased anticancer activity. These results showed the PDE4 A, B, C, and D are conjunctly related with lysosomal activity.

Keywords: Lysosomes, phosphodiesterase 4C, rolipram

Introduction

Lysosomes are membranous organelles that contain enzymes capable of breaking down extracellular and intracellular macromolecules [1]. They are connected with the endocytic network and are implicated in the digestion of macromolecules [2]. Lysosomal enzymes are synthesized in the rough endoplasmic reticulum and are released from the Golgi apparatus in small vesicles that fuse with acidic vesicles called endosomes, becoming full lysosomes [3, 4]. Many soluble lysosomes are synthesized as lysosomal hydrolase precursors that are N-glycosylated in the rough endoplasmic reticulum [5]. Early Golgi areas are recognized and tagged with mannose-6-phosphate (M6P) half in Golgi, where the M6P residues serve as recognition markers for two types of M6P-specific receptors arbitrating the vesicular transport of lysosomal proteins from the Golgi to endosomes [6]. The categorizing and directed transport of newly synthesized proteins to the lysosomes require modification of their *N*-linked oligosaccharides with M6P receptors [7]. The M6P recognition marker is generated in a two-step enzyme reaction [8]. Phosphodiesterases (PDEs) are one of the two enzymes used [9]. The cyclic nucleotide PDEs are made up of a group of enzymes that degrade the phosphodiester bond in the second messenger molecules cAMP and cGMP [10]. Therefore, PDEs are important regulators of signal transduction mediated by these second messenger molecules [11]. The transportation of lysosomal

enzymes into the lysosomes depends on the phosphorylation of their chains and the binding of the phosphorylated residues to the M6P receptors [12, 13]. The efficiency of separation depends more on the phosphotransferase and PDEs than on the activity of the phosphorylation of mannose residues that can be determined in vitro [14]. PDEs play an important role in the regulation of the activation of lysosomes.

In this study, to investigate the relevance of lysosomes and PDE4 changes in the lysosome, processing of rolipram as a PDE4 inhibitor and transfection were observed in HeLa cells. The data demonstrate that lysosomal activity was regulated in cells targeting lysosomal enzymes from the Golgi apparatus associated with the phosphorylation by PDEs.

Materials and Methods

Cell Culture and Total Protein Isolation

HeLa cells (HC18802) were maintained at 37°C under 5% CO_2 with medium. The medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% newborn calf serum (NCS) and 5% penicillin-streptomycin. When the cells grew to about 70%, we treated them with rolipram (1, 2, 5, 10, 20, 50, and 100 μM) and ethanol (99%, 80 μl) for 24 h. The HeLa cells were washed twice with icy Dulbecco's phosphate-buffered saline (DPBS) and treated with a lysis buffer with added phenylmethylsulfony fluoride (PMSF) and protein inhibitor cocktail (PIC). We scraped all cells from the dishes and relevant accumulated E-tubes. After the cells were vortexed for 10 min, they were reacted on ice for 30 min. The mixture was centrifuged at 1,610 ×*g* for 10 min. The separated supernatant was all protein.

Lysosomal Enzymes Isolation

Cells were treated with rolipram (10 and 100 μM) and ethanol (99%, 80 μl) for 24 h. The HeLa cells were rinsed twice with DPBS and treated with a lysis buffer with added PMSF and PIC. The cells were reacted by vortexing for 10 min and then put on the ice for 30 min. The mixture was centrifuged at 1,610 ×*g* for 5 min to remove the cell debris. The supernatant was moved to micro-tubes and ultracentrifuged at 20,000 ×*g* for 30 min to separate the lysosomes and peroxisomes from the supernatant. The lysosomes and peroxisomes were in the pellet and the supernatant was discarded. We mixed the pellets with NP-40 buffer (1 : 1). The cells were reacted by vortexing for 15 min and put on ice for 30 min. The mixture was centrifuged at 13,000 ×*g* for 10 min. The lysosomal enzymes remained in the supernatant.

SDS-PAGE and Western Blot Analysis

After treating with rolipram as described above, samples from the control and experimental groups were examined in 12.5% separating gels. Equal amounts of protein from both the control and experimental groups were suspended in 5× sample buffer and distilled water and boiled for 5 mim, and then subjected to SDS-PAGE. The electrophoresis was carried out for 2.5 h and the separated proteins were transferred to a nitrocellulose membrane. Following blocking with skim milk (1 h), the membrane was blotted with the primary antibody dilution (1:500) overnight. After the membranes were washed with T-TBS buffer, they were blotted with respective secondary antibodies for 1 h at room temperature. After the membrane was washed with T-TBS, the membrane was soaked in Ponceau S solution for staining. The membrane was developed.

Antimicrobial Test

Experiments were conducted to determine the antimicrobial activity of the lysosomal enzymes against *Escherichia coli*. After the *E. coli* reached OD_{600} 0.6–0.7, the cultures were diluted to 10^{-6} cells/ml in sterile water. Lysate (50 μl) containing the lysosome enzyme and *E. coli* culture (50 μl) were spread on a LB plate. The antimicrobial activity of the lysosome enzymes were measured by colony count.

Fluorescence Intensity of Lysosomal and Peroxisomal Activity

To evaluate the activity of cell organelles, Lyso-Tracker and reactive oxygen species (ROS) were used. The HeLa cell were grown in a cover glass-bottom dish and stained with 10 μM DCF-DA in DPBS for 2 h at 37°C. The cells were then washed and stained with 100 μM Lyso-Tracker Red DND 99 in DPBS for 1.5 min at 37°C. The cells were washed with DPBS and observed using a confocal laser-scanning microscope at 543 nm excitation for lysosomes and 488 nm for peroxisomes.

MTT Assay

The MTT assay was performed to evaluate the anticancer capacity of the lysosomal enzyme. After the HeLa cells were cultured in a 96-well plate, the cells were allowed to react. Lysosomal enzyme and medium served as the negative control group, and cells alone served as the positive control for 24 h. After removing the supernatant, each well was washed with DPBS. Then, 100 μl of MTT solution (5 mg/ml in DPBS) was put into the well plate and incubated for 4 h at 37°C. After 4 h reaction, the MTT solution was replaced with dimethyl sulfoxide (200 μl) in each well. After 20 min reaction, the results were measured at 592 nm. All experiments were carried out in triplicates, and cell viability was expressed as a relative percentage of the untreated control cells.

Cathepsin B and D Activity Assay

The HeLa cells were treated with rolipram (10 and 100 μM) and ethanol for 24 h. The cells were washed twice with icy DPBS and then treated with lysis buffer (400 mM sodium-phosphate, 75 mM NaCl, 4 mM EDTA, 0.25% Triton-X 100, and 8 mM DTT, pH 3.5). We raked the cells from the dishes with a scraper and put into E-

Table 1. Antisense oligonucleotides targeted to the PDE4 series for transfection.

Name	Sequence	Property
Missense	5′-GAGGTCTCGAAATCACGAGG-3′	Non-silencing
PDE4A	5′-GGGACGGATCGGGGGTTCCATGG-3′	Silencing
PDE4B	5′-CACACTCCTGCTTTTCTTCATTA-3′	Silencing
PDE4C	5′-TCGCCGACCCCCAGGTTCTC-3′	Silencing
PDE4D	5′-GCGCTGCTGCCCTCTGCCTCCATCC-3′	Silencing

tubes. The cells were reacted by vortexing for 10 min and put on ice for 30 min. The mixture was centrifuged at 1,610 ×*g* for 10 min. This activity was measured using the resulting supernatant. Cathepsin D was confirmed using substrate solution (8% hemoglobin, acetate buffer, pH 3.5). The reaction was stopped with trichloroacetic acid. After addition of Folin-Ciocalteau reagent, the absorbance at 750 nm in ELISA was measured. Cathepsin B activity was measured using z-Arg-Arg-MCA at pH 6.0. Fluorescence was measured with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The cells were treated with lysis buffer (0.05M Tri-HCl, 4 mM NaCl, 1% Triton X-100, pH 7.5) with the previously mentioned method. All experiments were carried out in triplicates.

Transfection of PDE4 Series in HeLa Cell Lines

Transfection of siRNA was performed with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocols. The antisense sequences employed for silencing the PDE4 series were

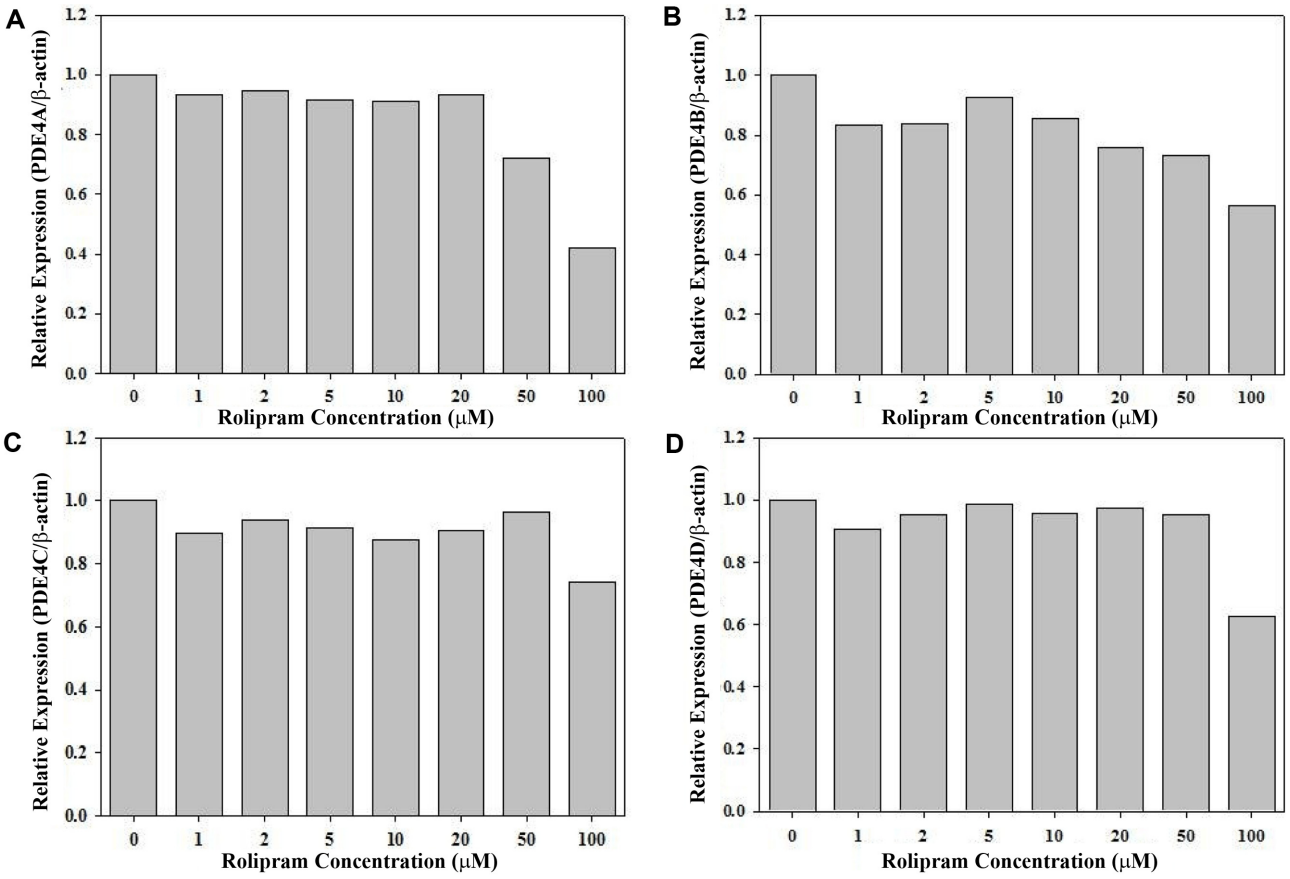

Fig. 1. Relative protein expression of the phosphodiesterase 4 (PDE4) series in rolipram-treated HeLa cells.
After HeLa cell lines were reacted with 0, 1, 2, 5, 10, 20, 50, and 100 μM of rolipram for 24 h, we extracted the total protein. (**A**) PDE4A protein expression was not changed in 0, 1, 2, 5, and 10 μM rolipram, but expression was dramatically decreased at more than 20 μM rolipram. (**B**–**D**) Protein expression of PDE4B, PDE4C, and PDE4D, respectively was not changed in 0, 1, 2, 5, 10, or 20 μM, but protein expression was decreased with more than 50 μM of rolipram.

bought from AccuOligo (Bioneer Co., Korea). The non-silencing antisense (missense) sequence was an unrelated antisense that did not match with any of the target genes. The antisense sequences are shown in Table 1. The HeLa cells were cultured to 70% confluence in DMEM supplemented with 5% NCS and then transfected with antisense sequences of PDE4A, PDE4B, PDE4C, and PDE4D (all PDE4 series antisense, 300 pmol). Transfection was performed in DMEM for 6 h, followed by culturing in DMEM supplemented with 5% NCS for up to 24 h for analysis of lysosomal activity and isolation of protein.

Data Analysis

All data were obtained from three independent samples conducted simultaneously for error analysis, and the results are reported along with the standard deviations. The data was analyzed using Sigma Plot (SPS, USA). A p value < 0.05 was considered significant.

Results and Discussion

Effects of Rolipram, a PDE4 Inhibitor, on the Expression of PDE4A, 4B, 4C, and 4D

We have observed the effect of rolipram in the HeLa cell line. Therefore, after we treated the HeLa cells with rolipram (1, 2, 5, 10, 20, 50, and 100 μM) for 24 h, the total protein was extracted. We then conducted western blotting with the extracted protein. The western blot confirmed PDE4 series expression. Total protein expression of PDE4A showed that treatment with 1, 2, 5, 10, and 20 μM rolipram changed very little. However, PDE4A expression decreased in 50 and 100 μM (Fig. 1A). For expression of PDE4B treated with 1, 2, 5, and 10 μM rolipram, the graph does not seem to show any trend (Fig. 1B). PDE4C expression showed decrease only at 100 μM rolipram (Fig. 1C). Likewise, expression of PDE4D only decreased with 100 μM rolipram (Fig. 1D). Overall, expression of PDE4 protein did not show any trend, until 100 μM rolipram, which consistently decreased the expression.

Lysosomal Confocal Response by Inhibition of PDE4A, 4B, 4C, 4D

Based on the results of western blotting, we showed that the rolipram affects the HeLa cells. Therefore, we wanted to know how damaging rolipram treatment is to cell organelles. We checked the effect of rolipram on cell organelles with a confocal laser-scanning microscope; the green color is ROS and the red color is lysosomes. When cells are exposed to stress such as chemical treatment, cells show oxygen stress. A fluorescent indicator of the oxidative

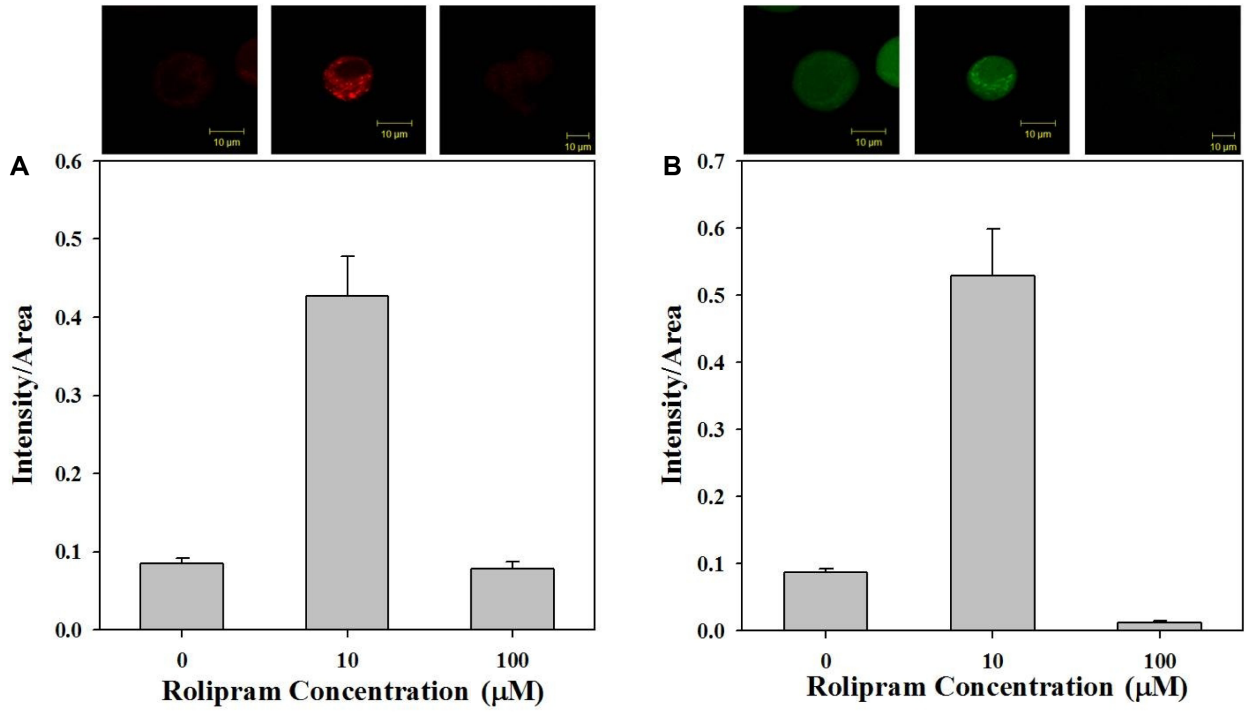

Fig. 2. Confocal microscopy quantification of lysosomal intensity and reactive oxygen species (ROS) production in HeLa cells treated with 10 and 100 μM of rolipram for 24 h.

(**A**) The highest lysosomal intensity was in 10 μM rolipram. (**B**) The highest ROS fluorescence intensity occurred in 10 μM rolipram.

stress in cells is the appearance of ROS. We found that in the PDE4 series, protein expression was not changed by 10 μM rolipram and was reduced by 100 μM rolipram. We looked at the fluorescent intensity at these two concentrations. Fig. 2A shows the highest lysosomal intensity was with 10 μM rolipram. Fig. 2B shows that the highest amount of ROS was with 10 μM rolipram. Lysosomes and ROS had the same response to rolipram treatment. It was found that low-concentration rolipram treatment induced oxidative stress-causing active lysosome. High-concentration rolipram did not disrupt original function.

Evaluation of In Vitro Lysosomal Activity after Rolipram Treatment

We see that lysosomes are activated by rolipram. To investigate the activity of lysosomal enzymes in vitro, we confirmed the antimicrobial and anticancer activities using lysosomal enzymes isolated from rolipram-treated cells. The antimicrobial test used *E. coli*, and the anticancer test used the MTT assay with HeLa cells. We checked the antimicrobial and anticancer activities using cells that had been treated with low-concentration (10 μM) and high-concentration (100 μM) rolipram. It was found that there was no antimicrobial activity with either concentration (Figs. 3A and 3B). The lack of antimicrobial activity might be caused by the ethanol solvent of the solution. Decreased activity of lysosome by ethanol treatment might lead to decreased antimicrobial activity. Treatment with 10 or 100 μM for anticancer activity did not effect the lysosomal enzyme extracted from the treated HeLa cells. The 10 μM rolipram had higher anticancer activity (Figs. 3C and 3D).

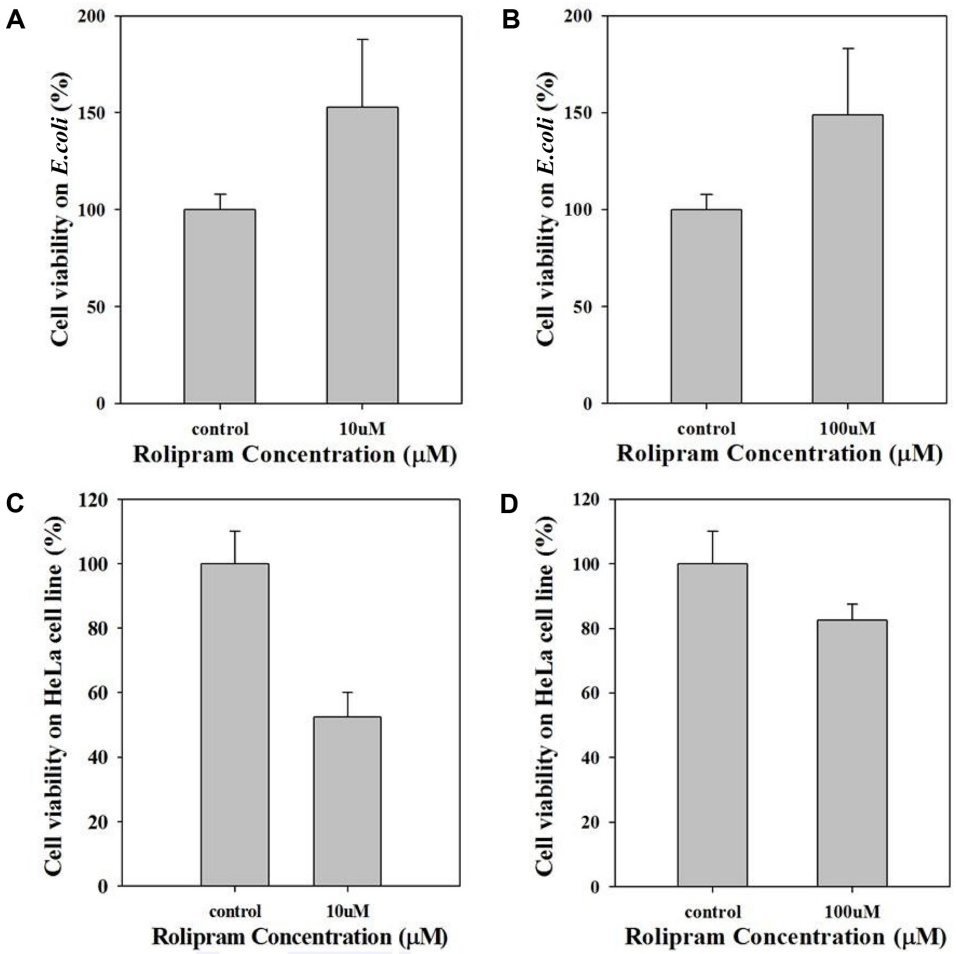

Fig. 3. Antimicrobial and anticancerous activities according to the concentration of rolipram.
(**A**, **B**) Antimicrobial activity test of lysosomal enzyme extracted from the rolipram-treated HeLa cells. (**C**, **D**) Anticancer activity test of lysosomal enzyme extracted from the rolipram-treated HeLa cells. (**A**, **B**) The antimicrobial activity of lysosomal enzyme extracted from the rolipram-treated HeLa cells was not affected. (**C**) The anticancer activity of lysosomal enzyme extracted from the rolipram-treated HeLa cells was not affected.

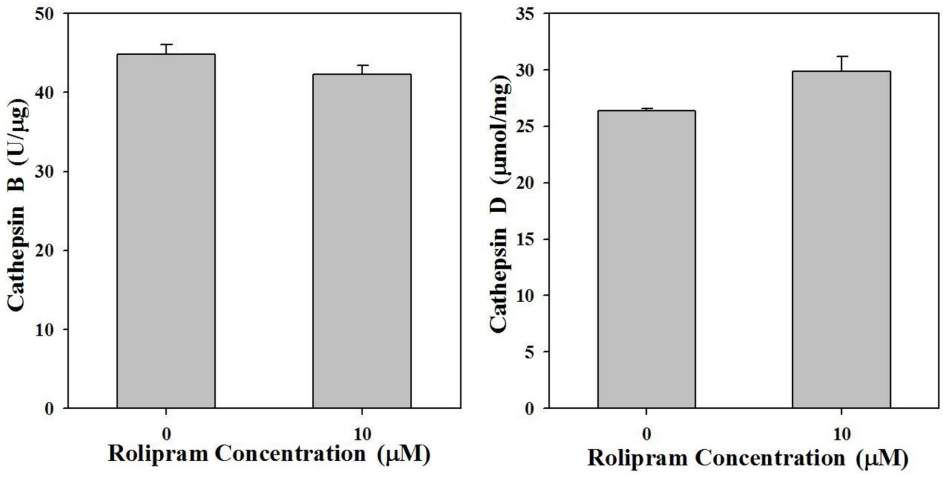

Fig. 4. Analysis of cathepsin B and D activity after 10 μM rolipram treatment.
The activities of cathepsin B (**A**) and cathepsin D (**B**). A decrease in cathepsin B activity and an increase in cathepsin D activity were observed in lysosomes isolated from HeLa cells after treatment with 10 μM rolipram.

To explore the cause of the increased anticancer activity, a cathepsin B and D assay was conducted. Lysosomal cathepsins B and D are major endopeptidases that are important in intracellular protein degradation, antigen processing, and accelerated protein turnover in various pathological conditions [14]. The data showed that the increased levels of cathepsin D (Fig. 4B) and decreased levels of cathepsin B (Fig. 4A) depended on the rolipram. The cathepsin D implied anticancerous activity occurred in the rolipram-treated HeLa cells. Accordingly, it was confirmed that the rolipram caused the anticancer activity.

We carried out transfection in order to examine the relationship between PDE4 and lysosomal activity. The change of lysosomal activity was explained when synthesis of the PDE4 series was repressed, showing the relationship between lysosome and the PDE4 series. The antisense sequences of PDE4 A, B, C, and D were transfected into HeLa cells for inhibition of protein expression. HeLa cells were treated with lipofectamine of the same volume as the negative control and the others with the missense sequence. Negative controls were treated.

First, western blotting was performed to confirm the decrease in the expression of proteins in transfected HeLa cells. Fig. 5 confirms that for PDE4, protein expression was reduced in HeLa cells with the antisense sequence. In order to check the activity of the lysosomes through inhibition of the PDE4 series, the HeLa cells were stained with LysoTracker and ROS staining solution. Expression of the protein was reduced. The ROS amount confirmed that the activity of lysosome increased with rolipram that stressed the cells in the previous experiment. The fact that antisense sequences also stressed the cell was confirmed by ROS. Fig. 6 shows that the control and missense sequence did not show significant differences for activity of lysosomes and ROS. However, cells treated with antisense PDE4 A, B, C, and D showed decreased activity of lysosomes and ROS. Although antisense PDE4A treatment caused lower declines in lysosome activity and ROS, and PDE4B, C, and D antisense

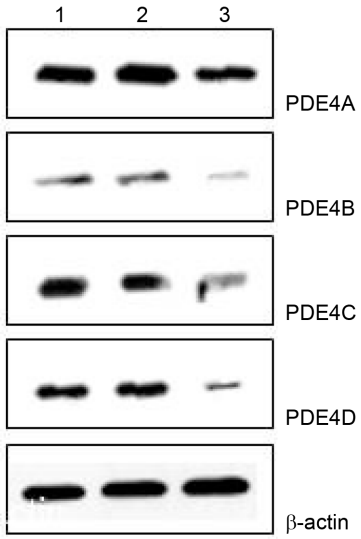

Fig. 5. Western blot assay performed with proteins extracted from the transfected HeLa cells.
HeLa cells treated with lipofectamine (same volume as negative control), and missense sequence. 1 is negative controls, 2 is missense, 3 is treated antisense of the PDE4 series. The molecular mass of each protein is as follows: PDE4A 68 kDa, PDE4B 106 kDa, PDE4C 72 kDa, PDE4D 110 kDa, and β-actin 43 kDa.

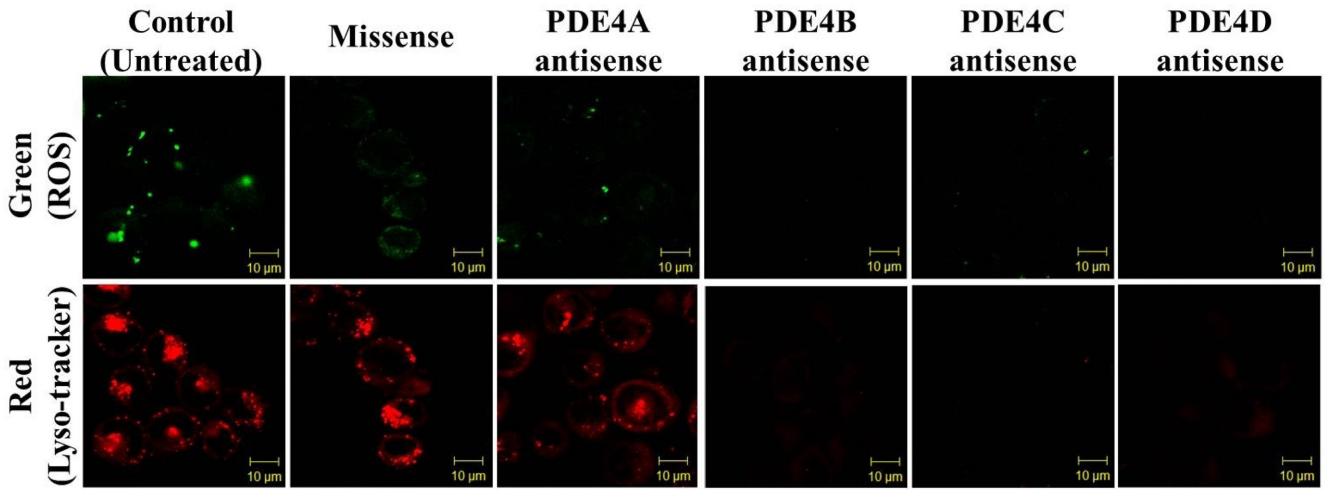

Fig. 6. Lysosomal activity and ROS in HeLa cells transfected with antisense sequences from the PDE4 series.
The control was treated with lipofectamine only. The remaining cells were treated with lipofectamine 2000 and antisense complexes (300 pmol), as described.

treatment showed amazing decreases. Thus, suppression of PDE4, without stressing the cell, also suppressed the activity of the lysosome. Finally, anticancer activity was confirmed via the MTT assay of lysosomes in transfected HeLa. As a result, control, missense, and antisense-treated cells showed anticancer activity. However, lysosmes treated with antisense PDE4B, C, and D had decreased anticancer activity (Fig. 7).

These results show that phosphorylation occurs when lysosomal enzymes are targeted into the lysosome when synthesis of PDE4 is repressed. Therefore, reduced synthesis of PDE inhibited phosphorylation. The results showed the PDE4 A, B, C, and D are conjunctly related to lysosomal activity.

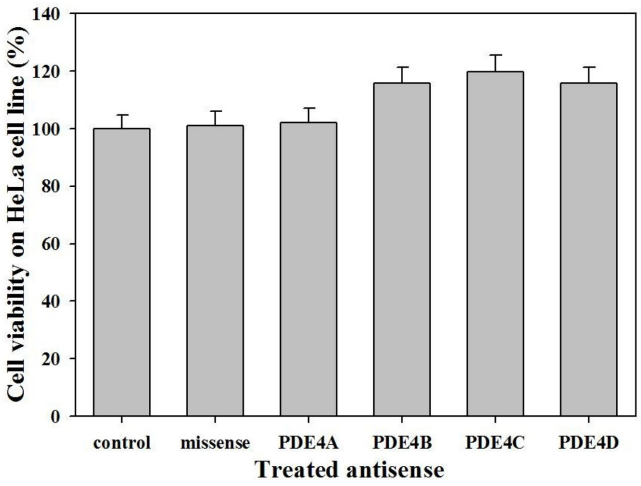

Fig. 7. Anticancer activity of PDE4A, 4B, 4C, and 4D treated with antisense sequences.
Control was treated with lipofectamine only. The remaining HeLa cells were treated with lipofectamine 2000 and the antisense complexes (300 pmol).

In conclusion, lysosomal enzymes are released from the Golgi apparatus in small vesicles, which finally fuse with acidic vesicles called endosomes, becoming full lysosomes. The transportation of lysosomal enzymes into the lysosomes depends on the phosphorylation of their chains and the binding of the phosphorylated residues to M6P receptors [12]. The efficiency of separation depends more on the phosphotransferase and PDEs than on the activity of the phosphorylation of mannose residues [13]. PDEs play important roles in the regulation of activation of lysosomes.

It was observed that lysosomes were inhibited by rolipram. Protein expression of PDE4 showed no effect at up to 50 μM rolipram, but 100 μM rolipram reduced the expression. The fluorescence intensity after rolipram treatment showed that lysosomes were activated at low concentration, and were suppressed at high concentrations.

It was found that treatment of rolipram at low concentration induced oxidative stress and consequently activated lysosomes.

Investigation of the activity of lysosome in vitro showed that there was no antimicrobial activity at either concentration of rolipram (10 and 100 μM) because of the ethanol solvent, which caused reduced antimicrobial activity. Higher anticancer activity was shown for lysosomal enzyme treated with 10 μM rolipram. The anticancer activity was explored with cathepsin B and D assay. These data showed increased

level of cathepsin D and decreased level of cathepsin B depending on the rolipram concentration. The anticancer activity was related to rolipram treatment. Transfection study examined the relationship between PDE4 and lysosomal activity. Protein expression was reduced in HeLa cells with the PDE4 antisense sequence. Fluorescence intensity of cells treated with antisense sequences showed decreased PDE4 A, B, C, and D activity and ROS. Lysosome from cells treated with antisense PDE4B, C, and D showed decreased anticancer activity.

Here, we found a regulation mechanism of lysosomes in cells. These results show that refrained synthesis of PDE inhibited phosphorylation, which decreased lysosomal activity. Moreover, the results showed that PDEA, 4B, 4C, and 4D are related with lysosomal activity. Therefore, the activated lysosome can be used as antimicrobials or anticancer agents without harm to the human body.

Acknowledgments

This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project title: Development of Synthesis Technology of Plant Physiologically Activating Compounds by Genetically Reconstructed Photosynthetic Microorganisms; Project No: PJ01051501)," Rural Development Administration, Republic of Korea. The authors are grateful for their support.

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