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神經鞘氨醇 1-磷酸鹽導致人類前列腺癌 PC-3 細胞株產生細胞自噬現象之研究

Sphingosine 1-phosphate induces autophagy in human prostate cancer PC-3 cells

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摘要

神經鞘氨醇 1-磷酸鹽(sphingosine 1-phosphate, S1P)是一種在血清中含量豐富並且具有生物活性的小型磷脂鹽類，S1P 可以透過五種 G 型蛋白偶合受器，$S1P_{1-5}$，在不同細胞中引發各種不同的反應，包括細胞增生、發炎反應、細胞移行、血管新生以及細胞存活等細胞反應。另外，細胞自噬作用(autophagy)亦是一種常見的細胞反應，可使細胞在處於飢餓或是外在環境壓力下，行使降解反應將一些多餘的或是可以暫時回收利用的蛋白質、胞器以及細胞質消化掉。其消化的方式，是藉由細胞內因應刺激而產生的游離細胞膜將目標物包覆，而後和溶體結合進而達成目標分子消化、回收、再利用的目的，是一種維持細胞內平衡狀態的重要機制。近年來愈來愈多研究指出，細胞自噬作用可能扮演一個能夠壓抑癌症細胞生長的角色，而關於兩者之間的關係至今尚未完全明瞭。本實驗的重點在於 S1P 和細胞自噬之間的關係，並以人類前列腺癌 PC-3 細胞株為實驗對象。關於細胞自噬反應是藉由偵測細胞自噬指標蛋白 microtubule-associated protein light chain 3-II (LC3-II)的表現量，以及在穩定表現 EGFP-LC3 的 PC-3 細胞株中觀察含綠螢光之 LC3-II 集中到細胞自噬體(autophagosome)的現象做為判斷標準。結果顯示，LC3-II 的表現量不論是濃度上或是時間上都和 S1P 有顯著的正相關，而含綠螢光之細胞自噬體的出現亦被證明和濃度梯度有關。此實驗更進一步深入探查反應下游之訊息傳導途徑，包括 Rho 鳥苷三磷酸水解酶(Rho GTPase)相關分子(Rho, Rac and cdc42)、ERK1/2 蛋白激酶、環氧合酶-1 (cyclooxygenase-1)以及環氧合酶-2 (cyclooxygenase-2)等。本實驗結果是第一次發現 S1P 能夠促進前列腺癌 PC-3 細胞株產生細胞自噬。因為細胞自噬與癌症的關係密切，所以進一步深入研究其細節是絕對必要的。

Abstract

Sphingosine 1-phosphate (S1P), a bioactive sphingolipid is abundant in serum. S1P can induce various responses in many cell types, such as cell proliferation, inflammation, migration, angiogenesis and cell survival through a family of S1P-specific G-protein-couple receptors ($S1P_{1-5}$). Autophagy, one of main cellular activities, is a cellular degradation pathway which serves an adaptive role to protect cells under starvation or stress situations. Damaged or superfluous cellular proteins and organelles can be engulfed by autophagosomes, then digested when fused with lysosomes, and recycled back into the cytosol to maintain cellular homeostasis. Recently, scientists have begun to consider autophagy as a tumor-suppressing pathway, but the regulatory mechanisms are still controversial. In addition, the relationship between S1P and autophagy remains unclear. Herein, autophagy was characterized by using Western blotting analysis to detect microtubule-associated protein light chain 3-II (LC3-II, an autophagy marker) formation, and to observe the LC3-II molecules re-localization from cytosol to autophagosomes in EGFP-LC3 stably expressing cells by using fluorescence microscopy. These results suggested that S1P induced autophagy in PC-3 cells, a human prostate cancer cell line, is both time- and concentration-dependent. Furthermore, by using chemical inhibitors, Rho family GTPase (Rho, Rac, and Cdc42)-,

ERK1/2-, COX-1-, and COX-2-dependent pathways were found to be involved in the induction of autophagy. In conclusion, the results demonstrated the molecular mechanisms of S1P-induced autophagy in PC-3 cells during starvation.

Introduction

Sphingosine 1-phosphate

Recently, lipids are being discovered to play increasingly important roles in all areas of cell biology. Sphingomyelin (SM) is a structural component of eukaryotic cell membranes, and it was discovered in some specialized membrane microdomains. Metabolism of SM is necessary to maintain the integrity of plasma membranes (van Blitterswijk *et al.*, 2003). During the breakdown of SM, ceramide and sphingosine are produced in turn, and a subsequent product is sphingosine 1-phosphate (S1P). All of those products have different regulatory functions within cells (Hannun *et al.*, 2001). S1P is a low-molecular-weight sphingolipid which was found to regulate a variety of cellular responses. It was first discovered to be a component of the intracellular second messenger system that is involved in cell proliferation (Zhang *et al.*, 1991). Once S1P is released, it binds to albumin, its carrier within the serum, and then it can reach the entire body through the circulatory system.

There are five S1P receptors ($S1P_{1-5}$), previously called endothelial differentiation gene (Edg) receptors, which are all G-protein-coupled receptors (Graler *et al.*, 1998; Hla *et al.*, 1990; Im *et al.*, 2000; Maclennan *et al.*, 1994; Okazaki *et al.*, 1993; Yamaguchi *et al.*, 1996). The binding between S1P and its receptors leads to activation of different

distinct downstream G-protein-responsive pathways which stimulate Rac, Ras-ERK, PI3K-AKT-Rac, phospholipase C, and Rho, resulting in many events such as changes in motility, cell proliferation, cell migration, cell survival, and stress-fiber formation. Different cells and tissues express various S1P receptors and couple to different G proteins, leading to distinct cellular responses and elaborate signaling pathways (Hla *et al.*, 2001; Pitson *et al.*, 2003; Taha *et al.*, 2004).

S1P is found as a normal component in human serum (0.5 μM), and it is believed that platelets are not only the major S1P generator but are the most abundant reservoir compared to other blood cells (Yatomi *et al.*, 1997). What is more interesting is that over the past 30 years, there have been increasing correlations between the spread of cancer and platelets. Metastatic cancer cells were found to activate platelets, probably through thrombin, ADP (from hypoxic sites within tumors), and other agonists. After platelet activation, the expression of adhesion molecules on platelets is increased; thus cancer cells can carry platelets with them as a helper during metastasis. Once activated, platelets release considerable S1P into the blood, and the local concentration of S1P around cancer cells is much higher than the background. Therefore, attention should be focused on the relationship between S1P and cancer cells (Nash *et al.*, 2002).

Autophagy overview

As a type II programmed cell death, autophagy (from the Greek, “auto” oneself, “phagy” to eat) was discovered more than 40 years ago. Its best-known function of autophagy is to remove damaged or dysfunctional organelles and misfolded proteins within cells. Genes related to autophagy were first identified in yeast, but homologs have been found in all eukaryotes. As an evolutionarily conserved metabolic mechanism, autophagy plays an adaptive role in response to starvation to maintain homeostasis in cells (Mizushima *et al.*, 2008). As a cellular self-digestion mechanism, three major types of autophagy have so far been discovered: microautophagy, chaperone-mediated autophagy, and macroautophagy. Microautophagy and chaperone-mediated autophagy respectively deliver target cargos to lysosomes by lysosomal membrane invagination (De duve *et al.*, 1966) and Hsc70 chaperone complex transfer (Dice, 2007). Herein, this study focus on the well-studied macroautophagy, hereafter referred to as autophagy. Specific or nonspecific cargos are sequestered into a cytosolic double-membrane vesicle and than fused with lysosomes which ultimately leads to the degradation of all of its contents. Abnormalities of autophagy have recently been discovered to cause some severe diseases, such as cancer, neurodegeneration, microbial infection, and aging (Mizushima *et al.*, 2008).

Autophagosome formation

Generally speaking, autophagy can be selective or non-selective. Selective autophagy, such as through the cytoplasm-to-vacuole targeting pathway, pexophagy, and pathogen-containing autophagy, pack suitable cargos into autophagosomes which contain a limited amount of cytosol (Baba et al., 1997; Guan et al., 2001; Ogawa et al., 2005; Sakai et al., 1998; Scott et al., 1997). In contrast, autophagosomes in non-selective autophagy contain considerable cytoplasmic material. The key step in autophagy is the formation of autophagosomes, but the mechanism of determining their loading capacity is unclear at present. Herein, this study focused on investigating non-selective autophagosome formation.

To the present, genetic screening of yeast has identified 31 autophagy-related genes (ATGs) (Klionsky *et al.*, 2003), many of which have known analogues in other eukaryotes. Hereafter, we use the unified yeast nomenclature except for LC3, the mammalian homologue of yeast Atg8. Initially, an expending membrane sac called the isolation membrane or phagophore enwraps a portion of the cytoplasm. The proposed site for autophagosome formation is the phagophore assembly site (PAS) (Kim et al., 2002; Suzuki et al., 2001). Atg9 cycles between the PAS and multiple peripheral sites with the help of many regulatory factors, such as the Atg1 complex, Atg2, Atg18,

Atg23, and Atg27 (Kabeya et al., 2005; Kamada et al., 2000; Legakis et al., 2007; Nair et al., 2005; Reggiori et al., 2004; Tucker et al., 2003; Yen et al., 2007). Potentially, this shuttling might participate in delivering the membrane to the PAS (Reggiori et al., 2004, 2005). In addition to Atg9-related factors, there are still some other important molecules, for example, PI3-K and the Atg12-Atg5-Atg16 complex, which participate in several vesicular trafficking pathways and LC3-II recruitment. Taking all these facts together, autophagosome formation truly is a sophisticated process, after which the fusion of an autophagosome with a lysosome occurs. Finally, lysis of the autophagosome's inner membrane and breakdown of the contents occur within the autolysosome, and the final products are released back into the cytosol by membrane permeases.

LC3 and autophagy

Microtubule-associated protein light chain 3 (MAP-LC3) is a mammalian autophagosomal ortholog of yeast Atg8. Modification of LC3 is essential for autophagosome formation (Kabeya *et al.*, 2000). Once pro-LC3 is produced, it is processed by hAtg4B (a specific cysteine protease for LC3) to form LC3-I (Hemelaar *et al.*, 2003). LC3 is a ubiquitin-like protein (Ichimura *et al.*, 2000), which is activated by an E1-like protein, hAtg7, subsequently transferred to an E2-like enzyme, hAtg3, and finally modified to a lipid-containing form, LC3-II (Tanida *et al.*, 2001, 2002). LC3-II,

which is conjugated with phosphatidylethanolamine (PE), can insert itself into both the outer and inner membranes of autophagosomes, making this protein an autophagosome marker. Following the fusion of autophagosomes and lysosomes, LC3-II in the inner membrane is degraded by lysosomal hydrolases (Kabeya *et al.*, 2000), and those in the outer membrane are released back into the cytosol by the cleavage of hAtg4B (Fig. 1).

Autophagy and cancer

As mentioned above, autophagy holds a pivotal position, because it may both promote and prevent cancer, and its specific role may depend on different cancer types. The first link between autophagy and tumor suppression, discovered in 1999, was that the ATG gene, *beclin 1,* can inhibit tumorigenesis and is expressed at low levels in human breast carcinomas (Liang *et al.*, 1999). Since then, the allelic loss of *beclin 1* has been found with high frequency in human breast, ovarian, prostate, and brain tumors (Aita *et al.*, 1999; Miracco *et al.*, 2007; Paglin *et al.*, 2001), and the incidence of tumor formation is increased in *beclin1*$^{+/-}$ mutant mice (Qu *et al.*, 2003; Yue *et al.*, 2003). However, autophagy can prolong the survival of cells with defects in apoptosis under metabolic stress for weeks (Degenhardt *et al.*, 2006; Karantza-Wadsworth *et al.*, 2007; Lum *et al.*, 2005; Mathew *et al.*, 2007a). Long-term starvation and progressive autophagy gradually causes such cells to shrink, but restoring nutrients can still bring

them back, and the smallest cell is called the minimal cell capable of recovery (MCCR). In addition, cells defective in both apoptosis and autophagy fail to tolerate metabolic stress and thus die by necrosis. In addition, a possible explanation of the pro-survival and tumor-suppressive functions of autophagy is that some types of mismanagement of metabolic stress in autophagy-deficient tumor cells result in genome damage and tumor progression (Jin *et al.*, 2007b; Karantza-Wadsworth *et al.*, 2007; Mathew *et al.*, 2007a, b). Since DNA damage accelerates both cancer and aging, it seems that autophagy might also play a role in lifespan extension other than by protecting cells from environmental stresses. However, relationships between autophagy and cancer are still controversial. Some proper management of metabolic stress through autophagy is essential for tumor cells to repress the accumulation of detrimental mutations (Jin *et al.*, 2007a; Karantza-Wadsworth *et al.*, 2007; Mathew *et al.*, 2007a), but the precise reasons that autophagy-defective cells have more DNA damage is not yet known. Therefore, the best cancer therapy for killing apoptosis-resistant tumor cells is to inhibit autophagy and simultaneously apply metabolic stress through surgery, radiation, and/or chemotherapy; this can lead tumor cells to necrotic cell death. In conclusion, autophagy plays a paradoxical role in tumorigenesis, and more-detailed investigations are required.

Our target cell model is the human prostate cancer PC-3 cell line, which was

derived from epithelium. Prostate cancer is one of the best-known malignancies among men, and is reported to be the most common cancer in American men. In 2007, its estimated occurrence was approximately 220,000 (29% of all cancers in men), with a mortality estimated to be over 27,000 (9% of all male cancer deaths) in the US (American Cancer Society, 2007). Many treatments against it are available nowadays, including active surveillance, a prostatectomy, radiation therapy, and androgen ablation therapy, all of which depend on serum prostate-specific antigen (PSA) levels (Lilja *et al.*, 2008). PSA testing allows physicians to detect prostate tumors when they are still small, low-grade, and localized. Additionally, prostate cancer causes the PSA to be released into the circulatory system, and the level in blood can increase up to 10^5-fold. Although clinically localized prostate cancer is highly curable, the death roll still remains high, due to recurrence of the 'clinically cured' (Denmeade *et al.*, 2002). As we described above, we hypothesized that autophagy might maintain prostate cancer cells which escape from the cancer therapy beneath the detectable level. This could be a major reason for the high recurrence of prostate cancer.

Materials and Methods

Reagents

Sphingosine 1-phosphate (S1P), *Clostridium difficile* toxin B (toxin B), and fatty acid-free bovine serum albumin (faf-BSA) were purchased from Sigma (St. Louis, MO). PD98059 was purchased from Tocris Bioscience (Ellisville, MI). The monoclonal mouse anti-human LC3 antibody was from Abgent (San Diego, CA), and a rabbit polyclonal anti-LC3 antibody was from Novus (Littleton, CO). The goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP antibodies were the products of Santa Cruz (Santa Cruz, CA). SC-560 and NS-398 were purchased from Cayman Chemicals (Ann Arbor, MI). Fluoromount-G was purchased from Electron Microscopy Sciences (Hatfield, PA). RPMI 1640 medium, penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS), and G418 were purchased from Invitrogen (Grand Island, NY).

Cell culture

The human prostate cancer PC-3 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% FBS. Cell density in all the experiments was under precise control within the range of (1~1.5) x 10^5 cells per 9.5 cm² during seeding. Incubation of cell cultures was carried out at 37 °C with 5% CO_2 in a humidified atmosphere. PC-3

cells stably transfected with EGFP-LC3 were previously selected, and kept in complete medium supplemented with 500 μg/ml G418.

Immunoblotting

PC-3 cells were harvested and lysed in RIPA lysis buffer (50 mM Tris (pH 7.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100). Lysates were cleared by centrifugation at 14,000 r.p.m. for 15 min at 4 °C. After centrifugation, the supernatants were boiled with 6x sample buffer in boiling water for 5 min. Cell lysates at 20~30 μl were subjected to 13.5% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (with 6 M urea) with running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) and then electrotransferred with transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) to polyvinylidene fluoride (PVDF) membranes. Membranes were then incubated with 5% nonfat milk in TBSt (10mM Tris, 150mM NaCl, 0.2% Tween-20, pH7.4) for 1.5 h at room temperature. After blocking, membranes were incubated with the anti-human LC3 antibody at a titer of 1:100 (for monoclonal mouse anti-human LC3 antibody from Abgent) or 1:6000 (for rabbit polyclonal anti-LC3 antibody from Novus) overnight at 4 °C. After incubation of the first antibody, it was washed three times for 15 min each with TBSt and then incubated with 1:3000 of the anti-mouse-HRP secondary antibody for 1.5 h at room temperature.

When the last three washes were done, the chemiluminescence reagent was added to the membrane as per the manufacturer's instructions. Luminescence signals were detected by exposure to film in a dark room.

Immunocytochemistry

PC-3 cells stably expressing EGFP-LC3 at a density of (4~4.8) x 10^4 cells per 3.8 cm^2 were seeded on coverslips. After starvation, PC-3 cells stably expressing EGFP-LC3 were pretreated with the indicated inhibitors for 1 h before 5 μM S1P treatment for 8 h. Cells were washed with PBS once and fixed in 3.7% paraformaldehyde at room temperature for 5 min. Soon after another washing, coverslips were mounted with Fluoromount-G, and dried in dark place for 1 day. Fluorescence was detected by a Zeiss Axioskop 2 plus fluorescent microscope (Jena, Germany), and images were analyzed using Axiovision software. Confocal microscopy was performed with a Leica TCS SP5 X Supercontinuum confocal microscope (Wetzlar, Germany).

Statistical analyses

Each x-ray film was scanned, and the bands were measured by TotalLab v2.01. All experiments were done at least three times, and the data are shown as the mean ± SD. Statistical analyses were performed with ANOVA by Statview 5.0, and a p value of $<$

0.05 was considered statistically significant.

Results

S1P induces autophagy in dosage- and time-dependent manners in PC-3 cells

Previous studies in our lab showed that S1P has inhibitory effects on the proliferation of PC-3 cells, a human prostate cancer cell line. The cell cycle was arrested in G_1 phase with S1P treatment, and the death of S1P-treated PC-3 cells might be caused by that they pass through an apoptosis-independent and necrosis-dependent pathway (Liao *et al.*, 2005). In the past few years, autophagy has been demonstrated to be an important non-apoptotic cell death pathway, and its role in cancer chemotherapy is indisputable. Therefore, this study focused on the relationship between S1P and autophagy in PC-3 cells.

To investigate the effect of S1P on autophagy, the PC-3 cells were starved for 14~16 h in serum-free RPMI 1640 medium. The starved cells were then treated with 0.5, 1, 5, or 10 μM S1P for 8 hrs. Treatment with RPMI 1640 medium alone or with methanol/chloroform (M/C) was used as the control (Fig. 2A). After treatment, total cell lysates were harvested, and the Western blotting analysis was utilized as described in "Materials and methods". As shown in figure 2A, S1P caused autophagy in a dose-dependent manner. A 2-fold induction was achieved with 10 μM S1P treatment,

and because we were considering the physiological concentration of S1P in human serum, this study did not use higher concentrations.

To further confirm the effect of S1P on autophagy, the starved PC-3 cells were treated with 5 μM S1P for various times, as indicated in figure 2B. The data showed a time-dependent induction and a peak at 4 or 8 hrs (Fig. 2B), so we used 8 h of treatment with 5 μM S1P as our working concentration hereafter.

S1P induces relocalization of EGFP-LC3 in stably transfected PC-3 cells in a concentration-dependent manner

Since LC3-II, which is conjugated with phosphatidylethanolamine (PE), can be inserted into both the outer and the inner membranes of autophagosomes, we further demonstrated the effect of S1P on autophagy by visualizing the pattern of EGFP-LC3 signals in the stably transfected PC-3 cells. After starvation for 14~16 h in serum-free medium, EGFP-LC3 signals within PC-3 cells formed a punctate pattern, suggesting that transfection of EGFP-LC3 for observing autophagy is workable. Following treatment of EGFP-LC3 PC-3 cells with different concentrations of S1P, we determined that the higher dosage of S1P used, the more-obvious punctate pattern we observed (Fig. 3). In conclusion, our data here further confirm that S1P can induce autophagy in a concentration-dependent manner in the PC-3 cells.

Furthermore, we also observed that cells did not exhibit an autophagy pattern without starvation, but S1P treatment (at a concentration of 5 μM) still caused cells to be rounder than the negative control. Furthermore, we found that the brightness of cells in different treatment groups were all distinct: from low to high brightness, the order was the negative control group, starvation control group, and S1P treatment group (data not shown). These observations suggest that S1P could cause morphological change in PC-3 cells without starvation, and the brightness of EGFP-LC3 PC-3 cells is positive correlated to the autophagy intensity.

S1P-induced autophagy is mediated by *Clostridium difficile* toxin B-, SC-560-, NS-398-, and PD98059-sensitive pathways

It is known that lysophospholipids, especially S1P and LPA, lead to actin reorganization, and the influence of S1P here was thought to be much more potent than that of LPA (Postma *et al.*, 1996). Rho, a ras-related GTP-binding protein, is specifically essential for the coordinated assembly of focal adhesions and stress fibers induced by growth factors (Ridley *et al.*, 1992). In addition, previous studies in our lab suggest that S1P causes significant actin rearrangement and cell rounding in PC-3 cells (Liao *et al.*, 2005). On the other hand, recent research has shown that maturation of autophagosomes into autolysosomes is controlled by MAPK/ERK activation, and sustained activation of the ERK pathway is sufficient to commit cells to autophagy

(Corcelle *et al.*, 2006). Inhibition by ERK1/2 of autophagy-induced breast cancer and neuroblastoma cells leads to downregulation of autophagy (Plowey *et al.*, 2008; Sivaprasad U *et al.*, 2008; Zhu *et al.*, 2007). Furthermore, cyclooxygenase (COX) was lately demonstrated to be a target of anticancer therapy. COX-1 is constitutively expressed in most tissues and plays a housekeeping role in the production of prostaglandins that control normal physiological processes. However, COX-2 is undetectable in most normal tissues, but is induced by various inflammatory and mitogenic stimuli (Meric *et al.*, 2006). COX can convert procarcinogens to carcinogens and thus initiates tumor formation, and overexpression of COX-2 is sufficient to induce tumorigenesis in transgenic mice (Liu *et al.*, 2001). Another study showed that COX-2 plays a key role in early stages of carcinogenesis by promoting the proliferation of tumor cells and their resistance to apoptosis, as well as promoting angiogenesis, tumor cell invasion and establishment of the metastatic process (Rodrigues *et al.*, 2004). Since COX is resistant to apoptosis, it might participate in autophagy pathways, so it would be interesting to determine the relationship between COX and autophagy in PC-3 cells. In conclusion, we took Rho family GTPases (Rho, Rac, and Cdc42), ERK1/2, COX-1, and COX-2 as our suspected downstream signalings.

We pretreated starved PC-3 cells with inhibitors for 1 h before 8 hrs of treatment

with 5 μM S1P. The total cell lysates were harvested and used for the Western blot analysis. *Clostridium difficile* toxin B, PD98059, SC-560, and NS-398, which are respective inhibitors of Rho family GTPases (Rho, Rac, and Cdc42), MEK1/ERK, COX-1, and COX-2, were used to treat PC-3 cells. As shown in Fig. 4, *Clostridium difficile* toxin B and PD98059 had inhibitory effects on S1P-induced autophagy in the PC-3 cells. On the other hand, the data surprisingly suggested that both SC-560 and NS-398 can downregulate S1P-induced autophagy, and all these results above are a brand new discovery of the relationship of autophagy with Rho GTPase, ERK1/2, and COX in the prostate cancer cells (Fig. 4).

As for EGFP-LC3 stably transfected PC-3 cells, after pretreatment with all those inhibitors described above, the punctate pattern of EGFP-LC3 signals induced by S1P treatment was downregulated (Fig. 5A, B, quantified data given in 5C, D), and this was consistent with the Western blotting results shown in Fig. 4. Furthermore, we tested the inhibitors of NFκB (PDTC) and PI-3K (wortmannin), and found no inhibitory effects, so we concluded that they might not participate in this pathway (data not shown).

Discussion

Previous studies showed that during nutrient starvation, overexpression of sphingosine kinase 1 stimulates autophagy in human breast cancer MCF-7 cells (Lavieu *et al.*, 2006). They also proposed that endogenous S1P might cause autophagy but lacked direct evidence to prove this hypothesis. Herein we demonstrate for the first time that exogenous S1P can induce autophagy in prostate cancer PC-3 cells through the $S1P_5$-G_i/G_{12} pathways. Therefore, we speculated that in the study of Lavieu et al., S1P might have acted through an autocrine or paracrine pathway to induce autophagic effects in MCF-7 cells. On the other hand, we also demonstrated that Rho family GTPases (Rho, Rac, and Cdc42), MEK1/ERK, COX-1, and COX-2 are all involved in the downstream signaling of S1P-induced autophagy. Recent studies have shown that S1P upregulates COX-2 through both G_i- and G_{12}-dependent pathways (Ki *et al.*, 2007; Serrano-Sanchez *et al.*, 2008), and Rac and Rho are respectively activated by G_i and G_{12} (Brown *et al.*, 2006). The most interesting and confusing molecule is ERK, as sphingosine kinase 1 can be activated by ERK1/2-mediated phosphorylation (Pitson *et al.*, 2003), while S1P upregulates ERK1/2 through $S1P_{1\sim4}$ but inhibits it through $S1P_5$ (Kluk *et al.*, 2002). Nevertheless, in a previous study, $S1P_5$-mediated repression of ERK1/2 was first induced by serum, and the treatment time of S1P was no more than 60

min (Malek *et al.*, 2001). Therefore, even though S1P did inhibit ERK1/2 through $S1P_5$ within 1 hr, the inhibitory effect might not be sustained for 8 hrs. Accordingly, we propose that S1P indirectly activates ERK1/2, and there might be some other S1P-regulated pathways which activate them within the time period between 1 and 8 h of treatment. Regarding the high S1P concentration (5 and 10 μM) we used, as previously described, even though the physiological concentration of S1P within normal human serum is only 0.5 μM, it is possible to encounter higher concentrations during interactions between cancer cells and platelets, leukocytes, and erythrocytes (Hanel *et al.*, 2007; Jolly *et al.*, 2005; Yatomi *et al.*, 1995).

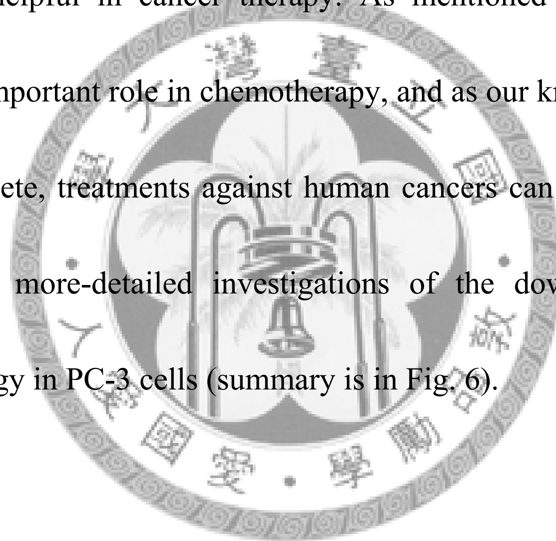
It is worth mentioning that the involvement of Rho GTPase- and COX-related pathways in autophagy have not previously been published. COX-2 inhibitors are thought to act as chemopreventive agents by sensitizing cancer cells to apoptotic signals (Lev-Ari *et al.*, 2008). Therefore, our data suggest that the apoptosis-sensitive effect of COX-2 inhibitors might be caused by an inhibition of autophagy (Boya *et al.*, 2005).

As discussed above, S1P-induced ERK1/2 activation seems to form a positive feedback loop. S1P can upregulate ERK1/2 through most S1P receptors ($S1P_{1\sim4}$), then ERK1/2-mediated phosphorylation activates sphingosine kinase 1, finally sphingosine kinase 1 processes sphingosine to form S1P. As previously described, we observed that

S1P-treated PC-3 cells are much brighter under microscopic view than control cells (data not shown). This was thought to be the result of the S1P-ERK1/2 positive feedback loop, which possibly leads to continuous stimulation of autophagy, so the diffuse pattern of EGFP-LC3 signals becomes more concentrated, and the brightness increases. Moreover, S1P is degraded to hexadecenal and phosphoethanolamine by S1P lyase; these two products are subsequently reused for the biosynthesis of PE (Spiegel *et al.*, 2003), the most important component that enables LC3-II to insert itself into autophagosomes, and this might be one of the reasons why S1P-treated cells are brighter. In addition, the S1P-treated negative control of EGFP-LC3 PC-3 cells displayed a rounder shape compared to non-treated cells, which suggests that PC-3 cells are probably influenced by Rho family GTPases (Rho, Rac, and Cdc42) under normal conditions, but the exact reason is not yet known.

There is no final conclusion about the relationship between autophagy and cancer (Mathew *et al.*, 2007), nor is there one between S1P and cancer (Payne *et al.*, 2002; Sabbadini, 2006; Spiegel *et al.*, 2002). Both autophagy and S1P have tumor-promoting and tumor-suppressing functions, and both of which depend on the situation, such as different cancer cell types, different tumor stages, and various metabolic stresses and environmental stimulants; these are all still controversial issues. At first, previous data

in our lab showed the inhibition of PC-3 cell proliferation by S1P, and we then found the cause of death to be apoptosis-independent and necrosis-dependent (Liao *et al.*, 2005). Herein, we further demonstrate that autophagy is involved in the S1P-induced inhibitory effect on cell proliferation. Although we could not determine which pathway is the major cause of death here and whether other types of cancer cells exhibit this phenomenon or not, we ensured that S1P induces autophagy in PC-3 cells, and this evidence might be helpful in cancer therapy. As mentioned in the "Introduction", autophagy plays an important role in chemotherapy, and as our knowledge of autophagy becomes more complete, treatments against human cancers can be improved. We will put our efforts into more-detailed investigations of the downstream signaling of S1P-induced autophagy in PC-3 cells (summary is in Fig. 6).



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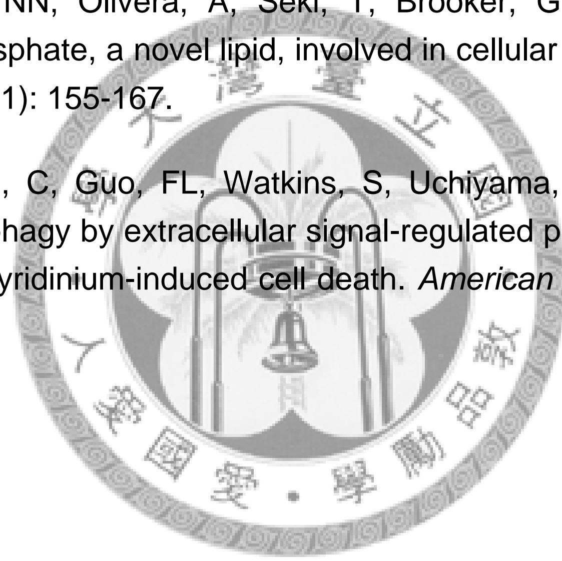


Figures

Figure 1

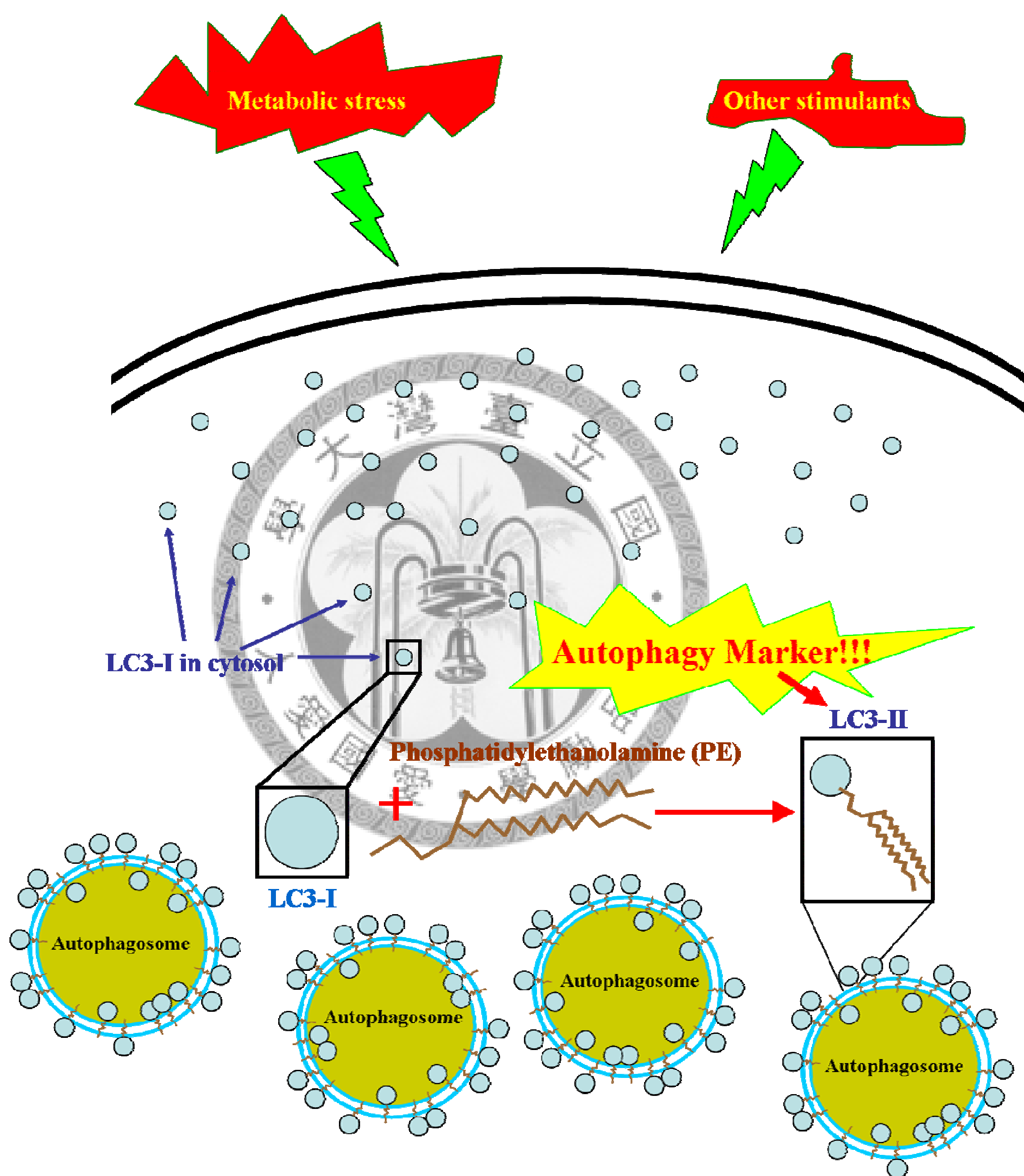
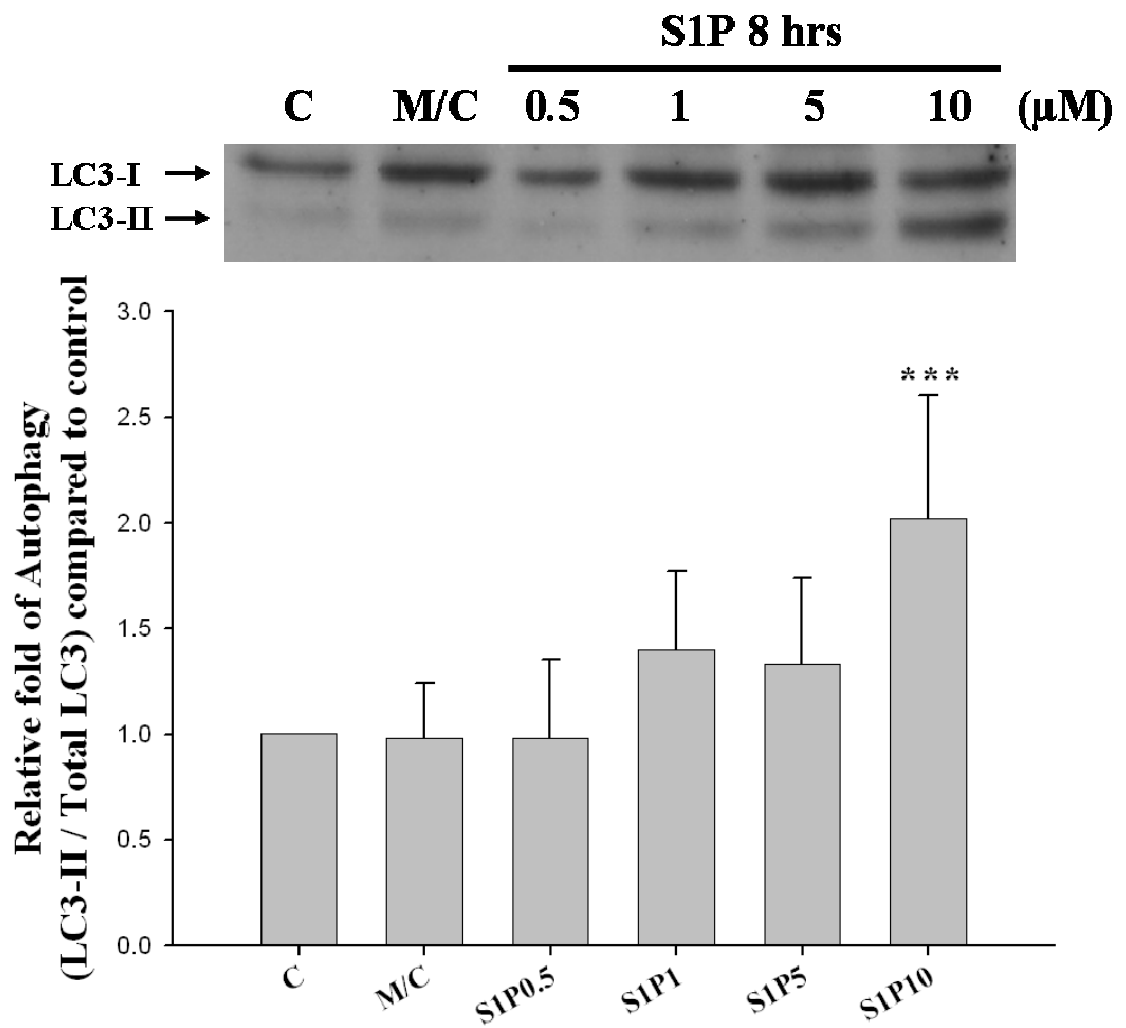


Figure 1. Brief introduction to the transformation from LC3-I to LC3-II, which is an important marker of autophagy. LC3-I is diffuse in the cytosol under normal conditions. When autophagy occurs, LC3-I is processed by hAtg7 and hAtg3 to form LC3-II. LC3-II is an autophagy marker, because it has a phosphatidylethanolamine (PE) domain and thus it can be incorporated into autophagosomes.

Figure 2

A



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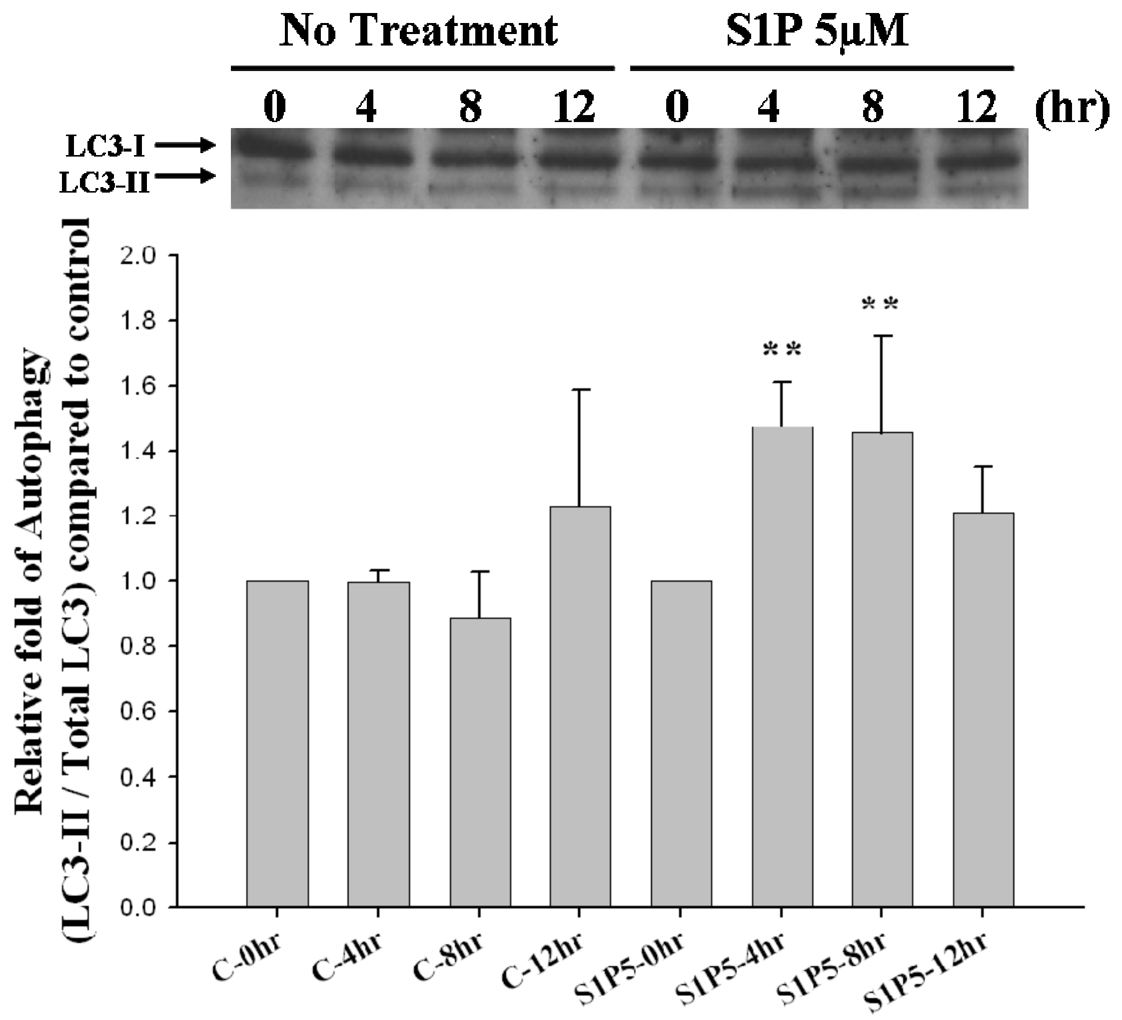


Figure 2. Sphingosine 1-phosphate induces autophagy in dosage- and time-dependent manners in PC-3 cells. (A) After 14~16 h of starvation in RPMI 1640 medium, PC-3 cells were treated with S1P or the control in various concentrations as indicated for 8 h. (B) Starved cells were treated with 5 μM S1P or the control for various times as indicated. After treatment, total cell lysates were collected, and LC3 protein levels were monitored by Western blotting. These results were confirmed by at least three independent experiments, and data from one representative experiment are shown here. Each bar within the diagrams represents the quantified results of a designated band on the x-ray film, and the bar values stand for the relative ratio of LC3-II / total LC3 which are compared to control. Results are shown as the mean value ± SD. ** $p<0.01$, *** $p<0.001$, compared to the control.

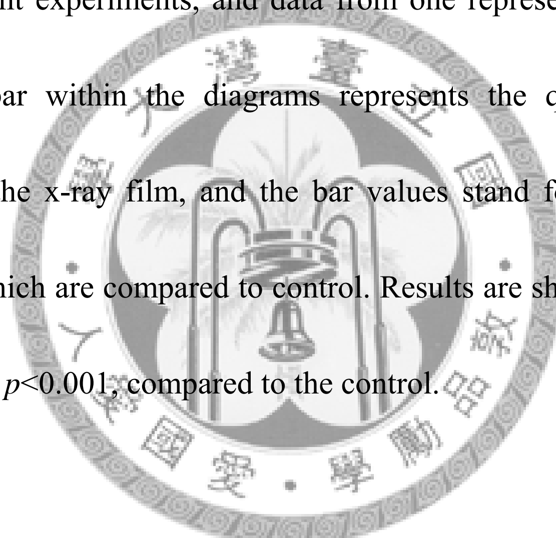


Figure 3

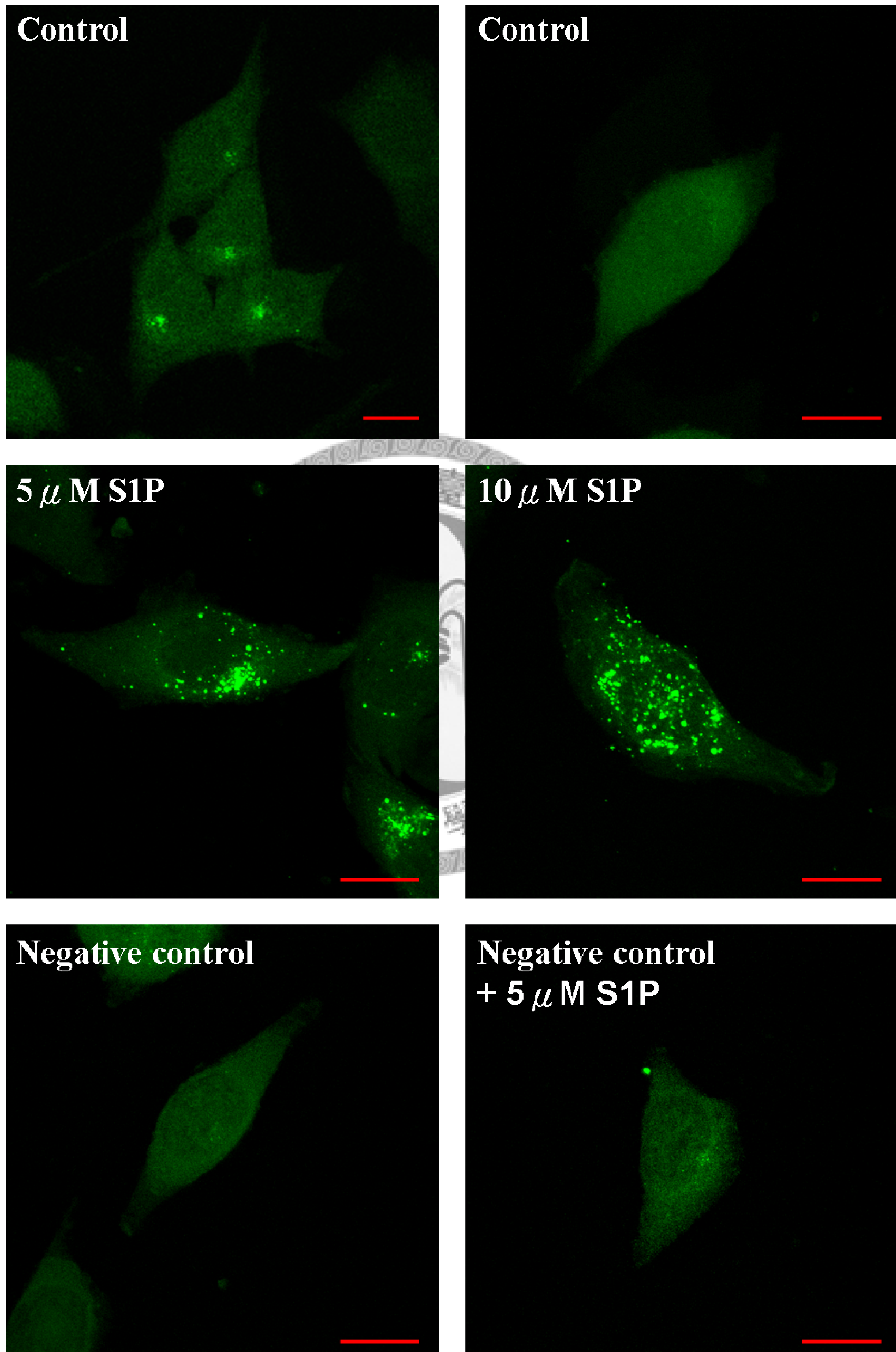


Figure 3. Sphingosine 1-phosphate induces relocalization of EGFP-LC3 in stably transfected PC-3 cells in a concentration-dependent manner. EGFP-LC3 PC-3 cells were treated with S1P or control in various concentrations as indicated for 8 h after 14~16 h of serum-free starvation. After treatment, cells were fixed with 3.7% paraformaldehyde at room temperature for 5 min and analyzed by fluorescence microscopy. The upper left and right panels are different fields photographed from the same coverslip. Representative images shown here were selected from several independent experiments and photographed by confocal microscopy. Control, starvation control; Negative control, cells incubated in complete medium. The red scale bar represents 15 μm.

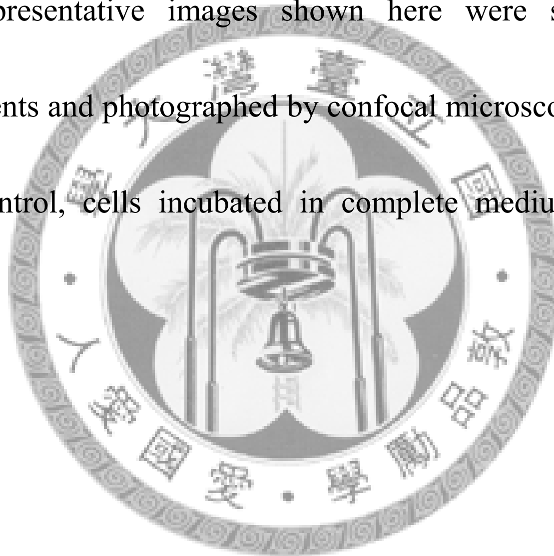


Figure 4

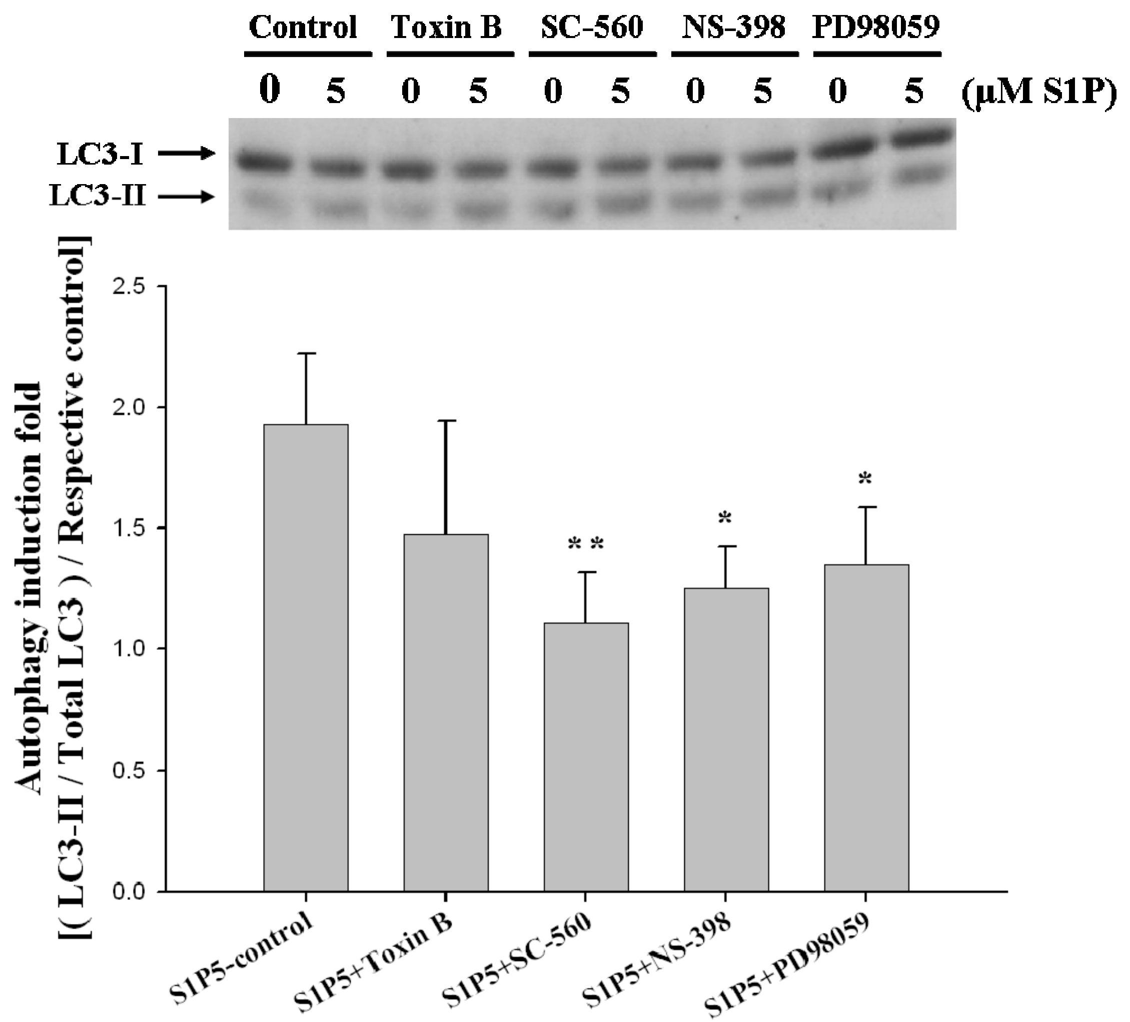


Figure 4. Sphingosine 1-phosphate-induced autophagy is mediated through *Clostridium difficile* toxin B-, SC-560-, NS-398-, and PD98059-sensitive pathways. Starved PC-3 cells were pretreated with *Clostridium difficile* toxin B (0.5 nM), SC-560 (5 μM), NS-398 (10 μM), or PD98059 (50 μM) for 1 h before the 8 h of treatment with 5 μM S1P or the control. After that, cell lysates were collected, and LC3 protein levels were monitored by Western blotting. These results were confirmed by at least three independent experiments, and data from one representative experiment are shown. Each bar within the diagrams represents the quantified results of the indicated band on x-ray film, and the bar values stand for the induction fold calculated from (ratio of LC3-II / total LC3 in S1P-treated group) / (ratio of LC3-II / total LC3 in non-treated group). Results are shown as the mean ± SD. * $p<0.05$, ** $p<0.01$, compared to the control.

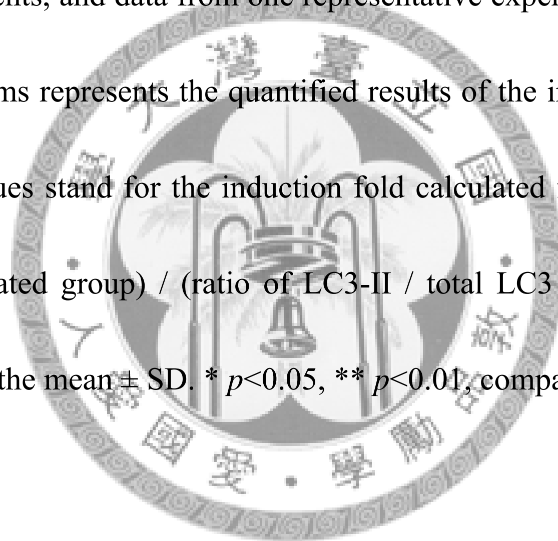
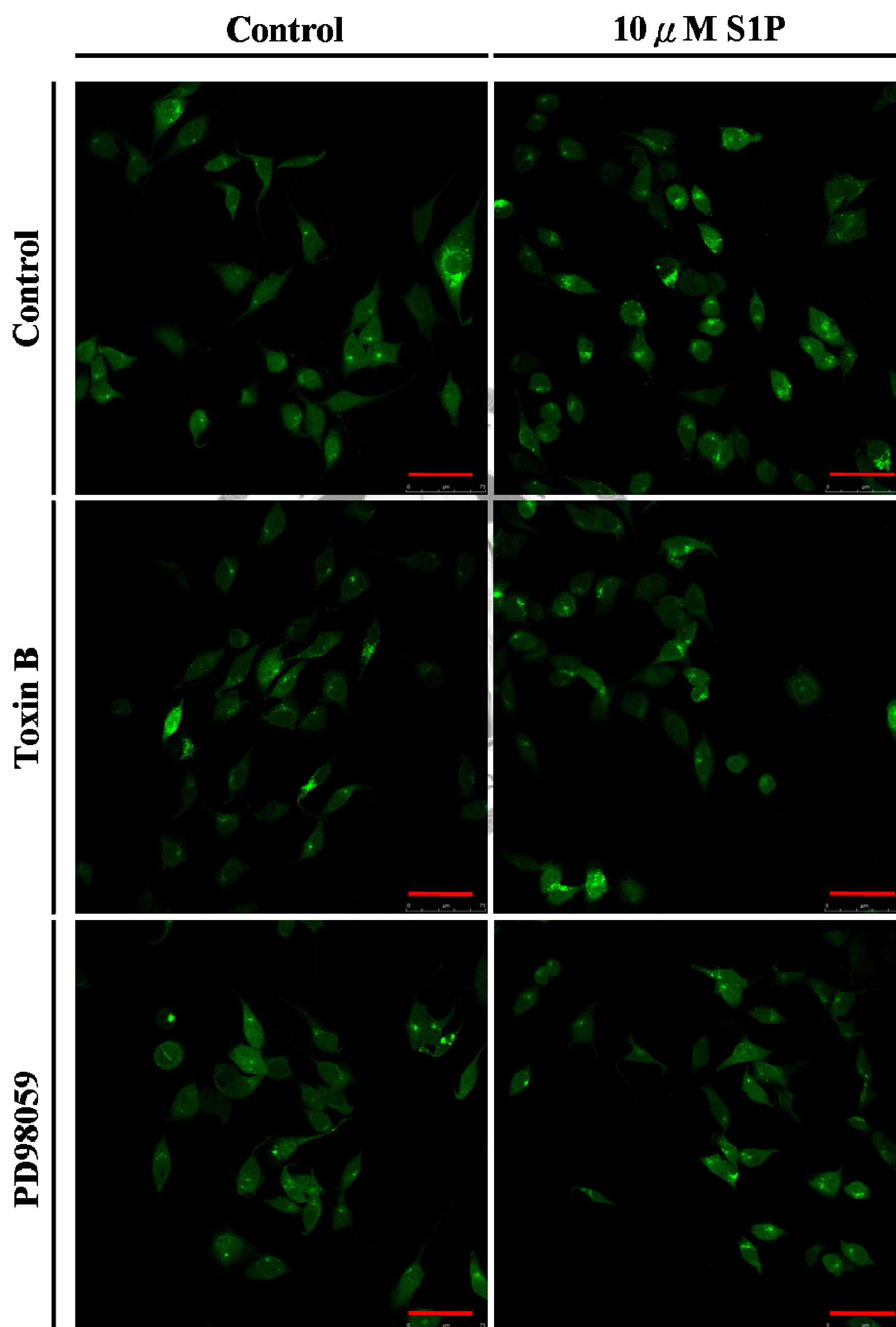


Figure 5

A



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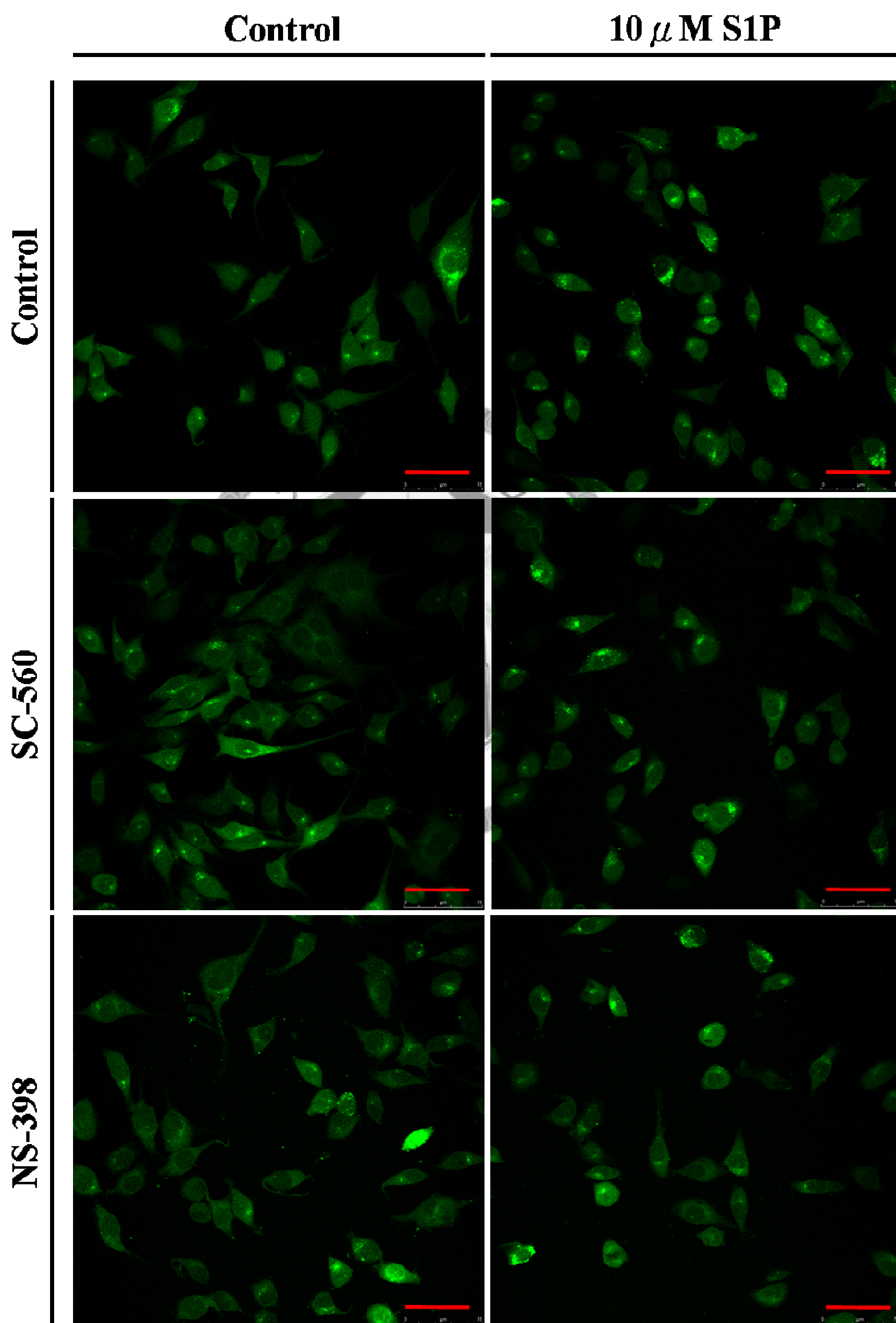
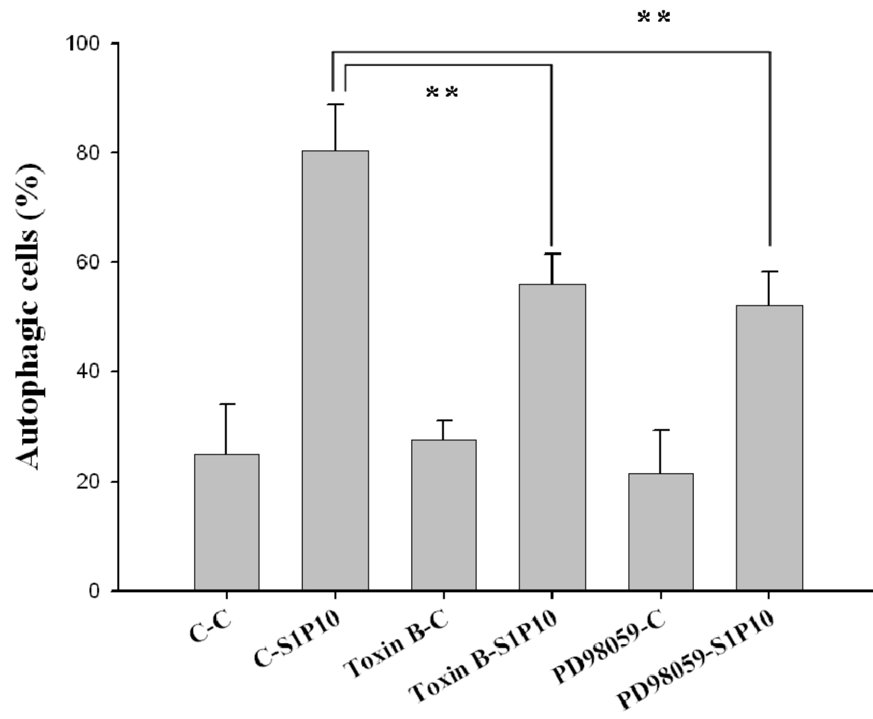


C



D

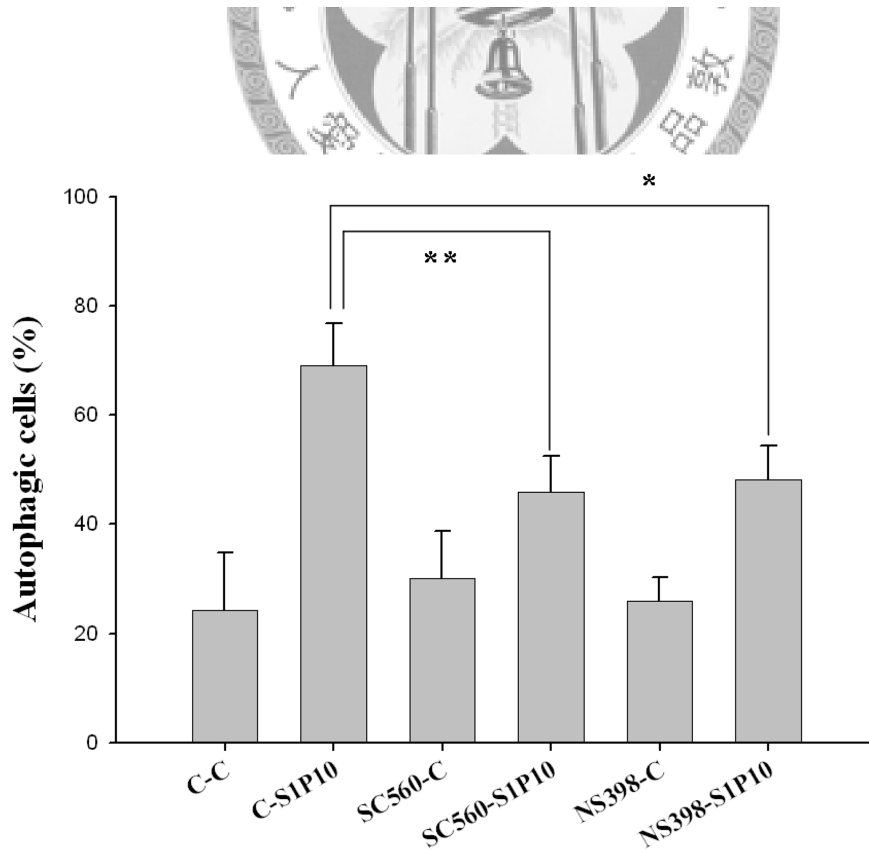


Figure 5. Sphingosine 1-phosphate-induced autophagy is mediated through Rho GTPase-, COX-, and ERK1/2-related pathways in EGFP-LC3 PC-3 cells. Starved EGFP-LC3 stably transfected PC-3 cells on coverslips were pretreated with (A) *Clostridium difficile* toxin B (0.5 nM), PD98059 (50 μM), (B) SC-560 (5 μM), or NS-398 (10 μM) for 1 h before the 8 h of treatment with 5 μM S1P or the control. Then samples were fixed and mounted, the EGFP-LC3 signals were analyzed by fluorescence microscopy, and data of the images were quantified by cell counting (C and D). Representative images shown here were all selected from the same experiment and photographed by confocal microscopy. Each bar within the diagram represents the results of cell counting on images photographed by fluorescence microscopy, and results are shown as the mean ± SD. The red scale bar represents 60 μm. * $p<0.05$, ** $p<0.01$, compared to control of 10 μM S1P.

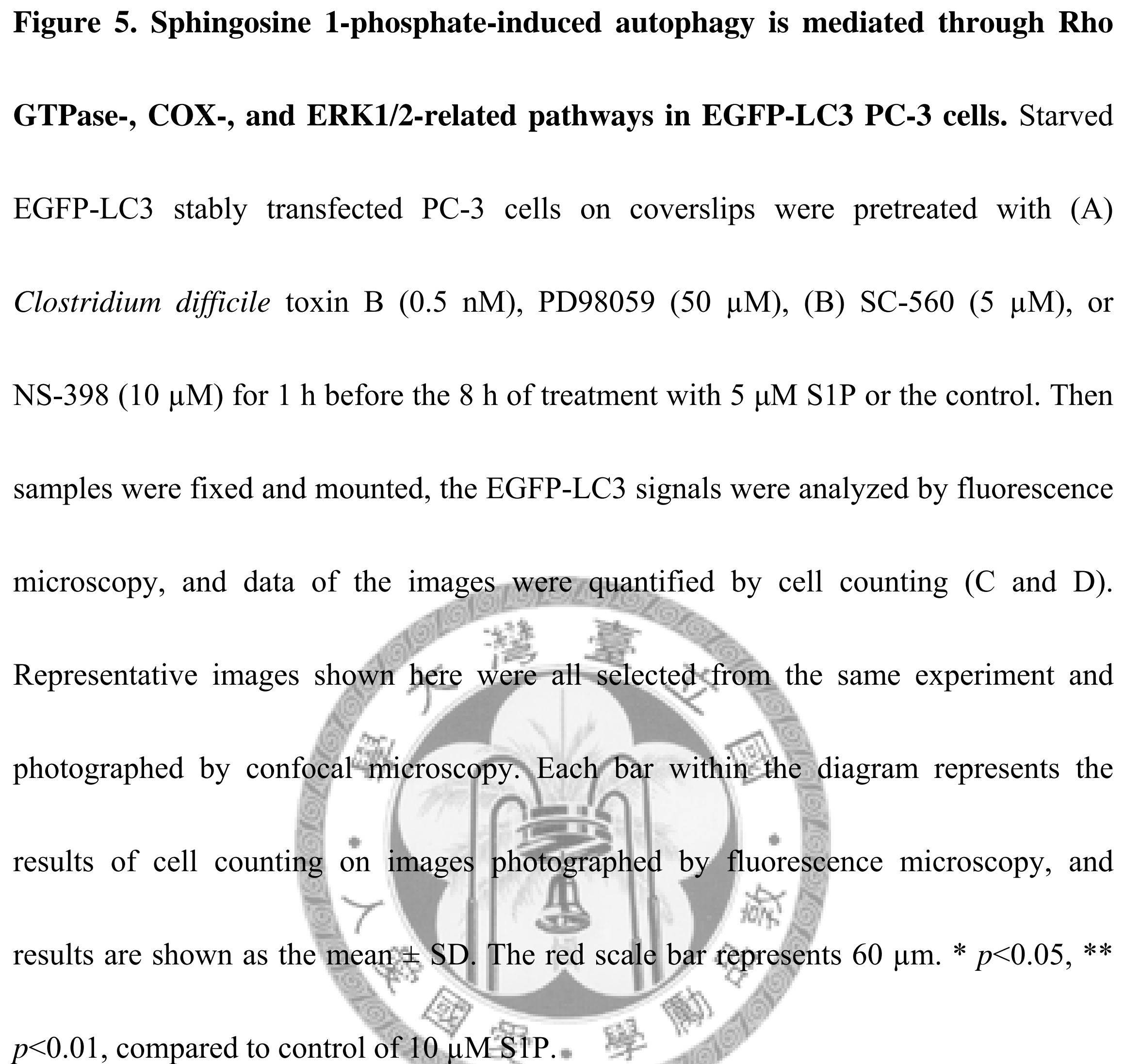


Figure 6

Figure 6. Summary of the proposed mechanisms of sphingosine 1-phosphate-induced autophagy in human prostate cancer PC-3 cells. Taking all our results together, we propose that S1P-induced autophagy is mediated through the $S1P_5$-G_i/G_{12} pathways, and downstream signals include GTPases (Rho, Rac, and Cdc42), ERK1/2, COX-1, and COX-2.