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Title

Sphingosine 1-phosphate has anti-apoptotic effect on liver sinusoidal endothelial cells and proliferative effect on hepatocytes in a paracrine manner in human.

Running title

S1P function in liver sinusoidal endothelial cells

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Abstract

Aim: Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite released from erythrocytes and platelets, and is a potent stimulus for endothelial cell proliferation. However, the role of S1P on human liver sinusoidal endothelial cells (LSECs) remains unclear. The proliferation and inhibition of apoptosis in LSECs are involved in the promotion of liver regeneration and the suppression of liver injury after liver resection and transplantation. The aim of this study is to investigate the role of S1P on human LSECs and the interaction between S1P and LSECs in hepatocyte proliferation *in vitro*.

Methods: Immortalized human LSECs were used. LSECs were cultured with S1P, and the cell proliferation, anti-apoptosis, signal transductions, and production of cytokines and growth factors were subsequently examined. To investigate the interaction between S1P and LSECs in hepatocyte proliferation, primary human hepatocytes were cultured with the supernatants of LSECs with and without S1P. DNA synthesis and signal transductions in hepatocytes were examined.

Results: S1P induced LSEC proliferation through activation of Akt and extracellular signal-related kinase pathways and suppressed LSEC apoptosis by affecting the expression levels of Bcl-2, Bax, and cleaved caspase-3. S1P promoted interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) production in LSECs. The supernatants of LSECs cultured with S1P enhanced hepatocyte DNA synthesis more strongly than the supernatants of LSECs cultured without S1P through activation of the signal transducer and activator of transcription-3 pathway.

Conclusions: S1P has proliferative and anti-apoptotic effects and promotes the production of IL-6 and VEGF in human LSECs, thereby promoting hepatocyte proliferation.

Key words

Sphingosine 1-phosphate; Liver non-parenchymal cell; Hepatocytes; Liver regeneration.

INTRODUCTION

Liver is a heterogeneous tissue composed of approximately 60-70% parenchymal hepatocytes and 30-40% non-parenchymal cells including liver sinusoidal endothelial cells (LSECs), hepatic stellate cells, Kupffer cells, and biliary epithelial cells.¹ LSECs are the first cells to contact the blood flowing into the sinusoids, and they act as the primary barrier between blood flow and parenchymal hepatocytes.² Previous studies revealed that the protection of LSECs against apoptosis prevents hepatocyte death and liver injury in hepatic ischemia/reperfusion injury, which is a common pathological process after liver surgery and transplantation.³⁻⁵ In addition, LSECs are known to produce pro-inflammatory cytokines and growth factors including hepatocyte growth factor (HGF) and interleukin (IL)-6, which are essential in hepatocyte proliferation.⁶⁻⁸ The proliferation and apoptosis resistance of LSECs and the development of secretory function in LSECs can contribute to the treatment of various liver diseases, such as hepatitis and liver failure, after hepatectomy and liver transplantation. Previously, we demonstrated that human platelets promoted the proliferation of human LSECs and human hepatocytes and that sphingosine 1-phosphate (S1P) released from platelets induces the production of IL-6 by human LSECs.⁹ However, there is currently no therapeutic strategy to promote the proliferation and prevent the apoptosis of LSECs or to induce liver regeneration through activation of LSECs.

S1P is a bioactive lipid mediator abundantly stored in erythrocytes and also released from activated platelets, and it exerts a variety of effects, including proliferation, apoptosis inhibition, and migration, in many types of cells.¹⁰⁻¹² Although previous reports demonstrated that S1P stimulates

proliferation and migration and prevents the apoptosis of human vascular endothelial cells, there are few reports regarding the role of S1P in human LSECs.^{13,14}

The aim of this study is to investigate the role of S1P on human LSECs *in vitro* and to discuss the possibility of liver regeneration therapy with S1P.

METHODS

Reagents and Antibodies

S1P, staurosporine (STS), phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, mitogen-activated protein kinase (MAPK) inhibitor PD98059, and nitric oxide synthase (NOS) inhibitor N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma Aldrich (St. Louis, MO, USA). STS is a reagent widely used to promote intracellular stress-induced apoptosis in various cell lines.¹⁵ Anti-human IL-6 receptor MAB227 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). All biochemicals were obtained commercially. S1P was dissolved in methanol as a 1 mM stock solution, which was simply diluted in the medium without any vehicle.

Cell culture

TMNK-1 is a human liver sinusoidal endothelial cell line established by transfection with simian virus 40 large T antigen and human telomerase reverse transcriptase, and was obtained from the JCRB Cell Bank (Osaka, Japan).¹⁶ TMNK-1 cells were cultured in Dulbecco's modified Eagle's

medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 1% penicillin and streptomycin (Invitrogen, Grand Island, NY, USA). The primary human hepatocytes purchased from XenoTech, LLC (Lenexa, KS, USA) were cultured in Williams' medium E (Sigma, St Louis, MO, USA) supplemented with 200 μ M L-glutamine (Invitrogen), 10% FBS, 1 μ M dexamethasone (Sigma), 1 \times ITS liquid media supplement (Sigma), and 1% penicillin and streptomycin. All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Cell viability and anti-apoptosis assay

A total of 5×10^3 TMNK-1 cells in 100 μ L of DMEM supplemented with 10% FBS were seeded in 96-well plates. Following overnight culture, the medium was changed to serum-free medium and different concentrations of S1P were then added to each well. In anti-apoptosis assays, 200 nM STS was added to each well 24 h after the addition of S1P. Cell counts were evaluated using the Cell Counting Kit-8 (CCK-8) (Dojin, Kumamoto, Japan) according to the manufacturer's instructions 48 h after the addition of S1P.

Apoptosis assay

A total of 1×10^5 TMNK-1 cells in 500 μ L of DMEM supplemented with 10% FBS were seeded in a Lab-Tek II Chamber Slide (Nalge Nunc International, Tokyo, Japan). Following overnight culture, the medium was replaced with serum-free medium with or without 5 μ M S1P, the cells

were cultured for an additional 24 h, and 200 nM STS was then added to each well. The effect of S1P on the apoptosis of TMNK-1 cells was evaluated with a DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer's instructions 6 h after the addition of STS.

Western blot analysis

Cells were pre-cultured in 6-well plates and the medium was changed to serum-free medium (2 mL/well) 2 h before the addition of S1P or TMNK-1 cell supernatant. Cell lysates were subjected to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore). All antibodies were purchased from Cell Signaling Technology. The bands for Bcl-2, Bax, cleaved caspase-3, and β -actin were quantified by an Image Quant LAS 4000 mini with Image Quant TL 7.0 software (GE Healthcare Life Sciences).

Assay for cytokines and growth factors

TMNK-1 cells were pre-cultured to confluence in 6-well plates. The medium was replaced with the serum-free medium (2 ml/well), and the cells were cultured for an additional 2 h. The cells were then incubated with or without 2 μ M S1P at 37°C for 0, 6, or 24 h, and the supernatants were sampled and centrifuged at 1000 g for 15 min. Then, the amounts of IL-6, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-1, and HGF in the supernatants were measured. IL-6 was measured using enzyme-linked immunosorbent assay (ELISA) kits from

Biolegend according to the manufacturer's protocols. VEGF, IGF-1, and HGF were measured using ELISA kits from R & D Systems according to the manufacturer's protocols.

DNA synthesis assay

The effect of the interaction between S1P and human LSECs on hepatocyte DNA synthesis was measured using the Cell Proliferation ELISA 5-bromo-2'-deoxyuridine (BrdU) kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Statistical analysis

All data are expressed as the mean \pm standard deviation. Statistical analyses were performed with the Mann–Whitney U-test and one-way analysis of variance, and significant differences were examined by the Bonferroni–Dunn multiple comparisons post hoc test. In all cases, a p value <0.05 was considered significant.

RESULTS

Proliferative and anti-apoptotic effect of S1P on human LSECs

To investigate the proliferative effect of S1P on human LSECs, TMNK-1 cells were incubated with 0.5, 2, or 5 μ M S1P for 48 h, and the CCK-8 was performed. In the anti-apoptosis assay, 200 nM STS was added to each well 24 h after the addition of S1P in addition to the procedure described for the above experiment. TMNK-1 cell proliferation increased in a

concentration-dependent manner upon 48 h of treatment with S1P, cell viability decreased with STS treatment, and the addition of S1P to the culture substantially increased cell viability in a concentration-dependent manner (Fig. 1a). For the histological assessment of the anti-apoptotic effect of S1P, the TUNEL assay was performed. The ratio of TUNEL-positive nuclei to total nuclei was significantly reduced with S1P treatment (Fig. 1b, 1c).

Effect of S1P on signal transduction in human LSECs

We analyzed the signals in TMNK-1 cells activated by S1P using western blotting. TMNK-1 cells were pre-cultured, and 5 μ M S1P was added to each well. The levels of phosphorylation of protein kinase B (Akt), extracellular signal-related kinase 1 and 2 (ERK1/2), signal transducer and activator of transcription-3 (STAT3), and endothelial NOS (eNOS) were analyzed in TMNK-1 cells stimulated by S1P for up to 240 min. The Akt pathway was activated within 60 min, and the ERK1/2 pathway was slightly activated within 5 min after the addition of S1P, whereas the STAT3 and eNOS pathways were not activated (Fig. 1d).

Mechanisms underlying the anti-apoptotic effects of S1P on human LSECs

To investigate the roles of the PI3K/Akt, MAPK/ERK, and eNOS pathways in the anti-apoptotic effect of S1P on human LSECs, TMNK-1 cells were pretreated with the PI3K inhibitor LY294002 (10 μ M), the MAPK inhibitor PD98059 (10 μ M), or the eNOS inhibitor L-NAME (1 mM) for 2 h. The cells were cultured with or without 5 μ M S1P for an additional 24 h and 200 nM STS was then

added to each well. The CCK-8 was performed 24 h after the addition of STS. The anti-apoptotic effect of S1P on TMNK-1 cells was blocked with the inhibition of PI3K by LY294002, whereas the inhibition of MAPK by PD98059 decreased the TMNK-1 cell viability, but did not abolish the anti-apoptotic effect of S1P (Fig. 2a). L-NAME had no effect on the TMNK-1 cell viability with or without S1P treatment (data not shown).

Effect of S1P on apoptosis-related signal transduction in human LSECs

To investigate the underlying mechanisms of the anti-apoptotic effect of S1P on human LSECs, TMNK-1 cells were pre-cultured in the serum-free medium with or without 10 μ M LY294002 for 2 h. The cells were cultured with or without 5 μ M S1P for an additional 24 h, and 200 nM STS was then added to each well. The expression levels of apoptosis-related proteins in the caspase-dependent pathway were measured by western blotting 6 h after the addition of STS. The expression of the anti-apoptotic protein Bcl-2 increased, whereas the expression of the pro-apoptotic protein Bax decreased in TMNK-1 cells treated with S1P, and these effects of S1P were blocked by pretreatment with 10 μ M LY294002 (Fig. 2b-2d). The expression of the anti-apoptotic protein Bcl-xL was not stimulated by S1P (data not shown). Cleavage of caspase-3 was inhibited in TMNK-1 cells upon treatment with S1P, and densitometric analysis of cleaved caspase-3 normalized to β -actin showed a significant difference (Fig. 2e, 2f).

Effect of S1P on the production of cytokines and growth factors in human LSECs

ELISA experiments with the supernatants of TMNK-1 cells revealed that S1P induced the production of both IL-6 and VEGF in TMNK-1 cells at 6 and 24 h (IL-6) or 24 h (VEGF) after the addition of S1P (Fig. 3a, 3b). The phosphorylation of STAT3 and VEGF receptor in TMNK-1 cells was not detected by western blot analysis, indicating that IL-6 and VEGF in the supernatants did not stimulate TMNK-1 cells in an autocrine manner (data not shown). There was no significant difference in the production of IGF-1, and HGF was not detected in the supernatants of TMNK-1 cells with or without S1P (data not shown).

Effect of interaction between S1P and human LSECs on human hepatocyte proliferation

The supernatants of TMNK-1 cells cultured in the serum-free Williams' medium E with or without 5 μ M S1P were sampled 24 h after the addition of S1P. Primary human hepatocytes were pre-cultured in the serum-containing Williams' medium E, and the supernatants or S1P were added to each well. After incubation for 24 or 48 h, the hepatocyte DNA synthesis was measured using the Cell Proliferation ELISA BrdU kit. At 24 h after the addition of the supernatants from TMNK-1 cells, hepatocyte DNA synthesis was enhanced independently of S1P; however, at 48 h, the supernatants cultured with S1P enhanced the hepatocyte DNA synthesis more strongly than the supernatants cultured without S1P (Fig. 4a). S1P had no direct effect on human hepatocyte DNA synthesis (Fig. 4b).

Effects of interaction between S1P and human LSECs on signal transduction in human hepatocytes

We analyzed the signals in hepatocytes activated by the interaction between S1P and TMNK-1 cells using western blotting. Primary human hepatocytes were pre-cultured and the supernatants of TMNK-1 cells were harvested as described above. The medium was changed to the serum-free Williams' medium E and the hepatocytes were cultured for 2 h. The harvested supernatants of TMNK-1 cells were then added to each well. In addition, to investigate the direct effect of S1P on the signal transduction in human hepatocytes, S1P was also added to another wells. The hepatocytes were harvested at 0, 5, 15, 30, 60, and 120 min after the addition of the supernatants or S1P. STAT3 was phosphorylated by the supernatants of TMNK-1 cells cultured without S1P and was strongly activated by the supernatants of cultures treated with S1P, whereas the pathway was not activated by S1P itself. The ERK1/2 pathway was activated by S1P as well as by the supernatants of TMNK-1 cells cultured with or without S1P, whereas the Akt pathway was not activated (Fig. 4c).

Effect of IL-6 produced by human LSECs treated with S1P on human hepatocyte proliferation

We inhibited the effect of the IL-6 produced by TMNK-1 cells cultured with S1P by employing the anti-human IL-6 receptor antibody MAB227. The hepatocytes were pre-cultured as described above and incubated with or without 10 µg/ml MAB227 for 2 h. The supernatants of TMNK-1

cells were harvested as described above and were added to each well. The hepatocyte DNA synthesis was enhanced by the addition of the supernatants and was reduced to control levels by blocking the IL-6 receptors of the hepatocytes (Fig. 4d).

DISCUSSION

This study shows that S1P can have proliferative and anti-apoptotic effects on human LSECs and that S1P promotes the production of IL-6 and VEGF by human LSECs. The promotion of IL-6 production from LSECs by S1P in this action can induce the proliferation of human hepatocytes. Because S1P has been reported to promote the proliferation of human vascular endothelial cells and to protect them against apoptosis, we hypothesize that S1P may have a similar ability in human LSECs.^{13,14} In the present study, although S1P has no direct effect on the proliferation of human hepatocytes, S1P promotes the production of IL-6 by human LSECs, which leads to the proliferation of human hepatocytes.

The reconstruction of sinusoids appears to play an important role in the supply of blood flow to the newly replicating hepatocytes during liver regeneration, and the synchronized replication of hepatocytes and LSECs is a crucial requirement for proper liver regeneration.¹⁷⁻¹⁹ The Akt signaling pathway plays a crucial role in mediating the proliferative and pro-secretion signals in LSECs.⁶ The ERK signaling pathway is a central element in transducing mitogenic signals in endothelial cells.²⁰ However, the regulatory mechanisms of LSEC proliferation have been poorly understood. In the present study, we demonstrate that S1P promotes the proliferation of human

LSECs through the activation of both Akt and ERK1/2 pathways. It has been reported that VEGF is an important stimulator of LSEC proliferation after hepatectomy *in vivo*.²¹ In the present study, although S1P promoted the production of VEGF in human LSECs, VEGF itself did not induce the phosphorylation of VEGF receptor in human LSECs in an autocrine manner. These results indicate that S1P has a direct effect in promoting the proliferation of human LSECs. S1P exerts its physiological effects through activation of cell surface G protein-coupled receptors, that is, S1P1-5. S1P receptors are widely expressed throughout the body and regulate important physiological actions, including cardiac function, immunity, angiogenesis, and vascular permeability.²²⁻²⁴ The S1P 1-3 receptors have a widespread distribution in various tissues including liver.^{25,26} Although we tried to investigate the involvement of the S1P receptors in the anti-apoptotic effect of S1P on LSECs, reproducible results of the experiments with the S1P receptor antagonists including VPC23019 and JTE013 were not obtained. Since one of the limitations of the present study is that we showed the results of the effects of S1P only at 48 h after addition of S1P, it is possible to confirm the involvement of the S1P receptors at an earlier time. The mechanisms underlying the direct effect of S1P on human LSECs remain unclear and deserve further study.

LSECs are the initial target tissue of liver injury induced by ischemia/reperfusion after liver resection and transplantation, and the injury of LSECs in this process is caused by apoptotic cell death.^{3,4} Bcl-2 is an integral inner mitochondrial membrane protein, and its overexpression inhibits apoptosis.²⁷ The pro-apoptotic protein Bax induces cell death via the regulation of mitochondrial membrane permeability.²⁸ Caspase-3 is a critical mediator in the execution of mitochondrial-related

apoptosis, and its cleavage is the final step in the intrinsic apoptotic pathway, which is typically triggered upon accumulation and/or activation of pro-apoptotic proteins.^{29,30} It has been reported that S1P suppressed the cleavage of caspase-3 and protected against mitochondria-mediated apoptosis in several cell types, including human umbilical vein endothelial cells, keratinocytes, and hamster lung fibroblasts.^{31,32} We demonstrate that S1P prevents the apoptosis of human LSECs by affecting the expression of both Bcl-2 and Bax and by inhibiting the cleavage of caspase-3 in human LSECs. It has been reported that the PI3K/Akt and ERK pathways are involved in the inhibition of apoptosis in various cell types, including human endothelial cells, hepatic stellate cells, and hepatocytes.³³⁻³⁵ In addition, the eNOS pathway was also reported to regulate the apoptosis of various types of endothelial cells, including LSECs.³⁶⁻³⁸ In the present study, with the induction of apoptosis by STS, the inhibition of PI3K by LY294002 abolished the anti-apoptotic effect of S1P on human LSECs by affecting the expression of both Bcl-2 and Bax, whereas the inhibition of MAPK by PD98059 or eNOS by L-NAME did not. These results indicate that PI3K/Akt pathway is centrally involved in the anti-apoptotic effect of S1P. This discrepancy may be due to differences in the cell types and apoptosis models used in these studies.

Liver regeneration is a physiopathological phenomenon influenced by a variety of growth factors, cytokines, and cell-cell interactions.³⁹ Clinically, many therapeutic strategies for liver regeneration depend on the ability of the liver to self-replicate, and new therapeutic options are urgently needed. Previous reports showed that the STAT3 pathway, which is activated by cytokines, including IL-6, can play a crucial role in the promotion of liver regeneration after

hepatectomy and the protection of liver injury after ischemia/reperfusion.^{40,41} In the present study, we demonstrate that S1P promotes the production of both IL-6 and VEGF in human LSECs and that the interaction between S1P and human LSECs enhances DNA synthesis in human hepatocytes through the activation of the STAT3 pathway, whereas the inhibition of the IL-6 receptor abolished the interaction. These results suggest that S1P promotes the production of IL-6 by human LSECs, and this effect enhances the human hepatocyte proliferation mainly through the activation of the STAT3 pathway in a paracrine manner. It was reported that S1P protects the apoptosis of human hepatocytes induced by Fas-Ligand through the activation of Akt pathway.⁴² In the present study, S1P has no direct effect on hepatocyte DNA synthesis and Akt activation, whereas the ERK pathway was activated by S1P. The reasons for this discrepancy are unclear, but one possibility is that activation of the PI3K/Akt pathway in hepatocytes can play a critical role in protecting against the injury of hepatocytes.⁴² Further studies are required to define the direct effect of S1P on human hepatocytes.

In conclusion, our results indicate that S1P promotes the viability and the secretory function of LSECs, which may be a novel therapeutic option for promoting liver regeneration and protecting against liver injury. Further investigations both *in vitro* and *in vivo* must be performed to explore these interesting possibilities.

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FIGURE LEGENDS

Figure 1 S1P promotes proliferation and reduces apoptosis in human LSECs. (a) Proliferative and anti-apoptotic effects of S1P on TMNK-1 cells were evaluated using the Cell Counting Kit-8 (CCK-8) 48h after treatment with different doses of S1P. Staurosporine (STS) was added 24 h after addition of S1P in the anti-apoptosis assay. Data are expressed as the mean \pm SD. n = 4. * p < 0.05, ** p < 0.01 versus the 0 μ M S1P, STS (-) group. † p < 0.05, †† p < 0.01 versus the 0 μ M S1P, STS (+) group. (b) Apoptosis of TMNK-1 cells was evaluated by a TUNEL assay. Scale bars are 50 μ m. (c) The TUNEL index was calculated as the ratio of TUNEL-positive nuclei to total nuclei. Data are expressed as the mean \pm SD. n = 6. * p < 0.01 versus the S1P (-) group. (d) Signal transduction in TMNK-1 cells was examined by western blotting.

Figure 2 Mechanisms underlying the anti-apoptotic effect of S1P on human LSECs. (a) The phosphatidylinositol 3-kinase inhibitor LY294002 and the mitogen-activated protein kinase inhibitor PD98059 were used. TMNK-1 cells were pretreated with LY294002 or PD98059 for 2 h and cultured with S1P for an additional 24 h. Staurosporine was then added to each well and the CCK-8 assay was performed 24 h after addition of staurosporine. Data are expressed as the mean \pm SD. n = 4. * p < 0.01. (b) Expression of apoptosis-related proteins including Bcl-2 and Bax was examined by western blotting. Each protein was represented by three samples. Quantification of Bcl-2 (c) and Bax (d) levels normalized to β -actin. Data are expressed as the mean \pm SD. n = 3. * p < 0.01. (e) Cleavage of caspase-3 in TMNK-1 cells was detected by western blotting. Each protein

was represented by three samples. (f) Quantification of cleaved caspase-3 levels normalized to β -actin. Data are expressed as the mean \pm SD. $n = 6$. * $p < 0.05$ versus the S1P (-) group.

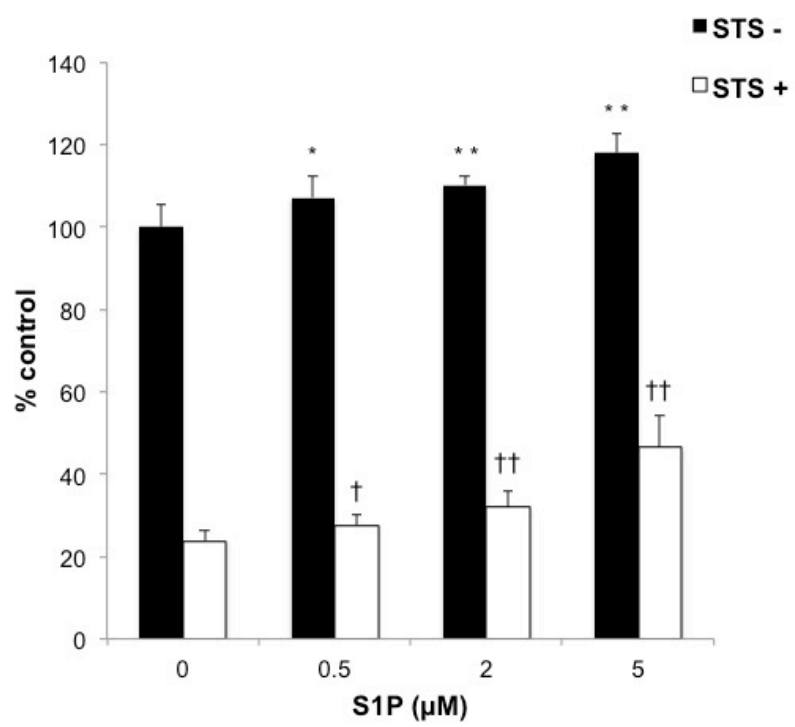
Figure 3 S1P induces the production of cytokines and growth factors in human LSECs. The amounts of IL-6 (a) and VEGF (b) in the supernatants of TMNK-1 cells were measured at 0, 6, and 24 h after treatment with S1P, using ELISA kits. Data are expressed as the mean \pm SD. $n = 4$. * $p < 0.05$, ** $p < 0.01$ versus the S1P (-) group.

Figure 4 Interaction between S1P and human LSECs promotes the proliferation of human hepatocytes. (a) Cultured hepatocytes were divided into three groups: the control group (cultured without the supernatants), the S1P (-) supernatant group (cultured with the S1P (-) supernatants), and the S1P (+) supernatant group (cultured with the S1P (+) supernatants). After 24 and 48 h of incubation, hepatocyte DNA synthesis was measured by the Cell Proliferation ELISA BrdU kit. Data are expressed as the mean \pm SD. $n = 6$. * $p < 0.05$. * * $p < 0.01$. (b) Hepatocyte DNA synthesis at 24 and 48 h after addition of S1P was measured by the Cell Proliferation ELISA BrdU kit. Data are expressed as the mean \pm SD. $n = 6$. (c) Cultured hepatocytes were divided into three groups: the S1P (-) supernatant group, the S1P (+) supernatant group, and the S1P group (cultured with S1P). Signal transduction in human hepatocytes was examined by western blotting. (d) Anti-human IL-6 receptor MAB227 was applied, and hepatocyte DNA synthesis was measured with the BrdU kit. Data are expressed as the mean \pm SD. $n = 6$. * $p < 0.01$ versus the control group.

FIGURES

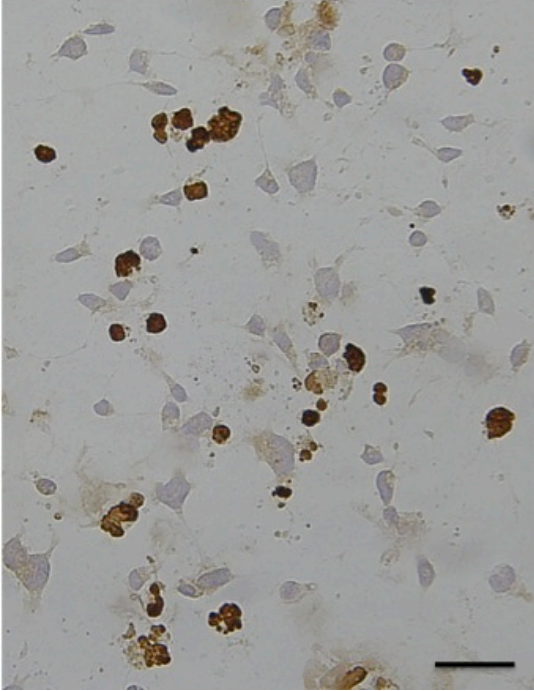
Fig. 1

(a)



(b)

S1P (-)



S1P (+)

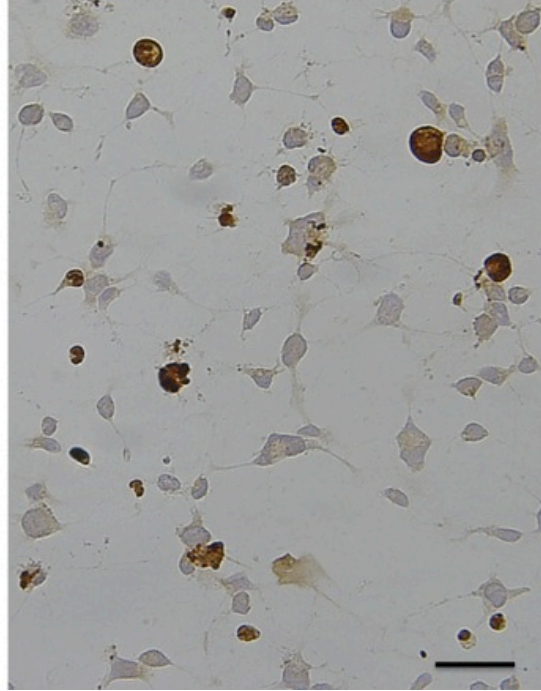
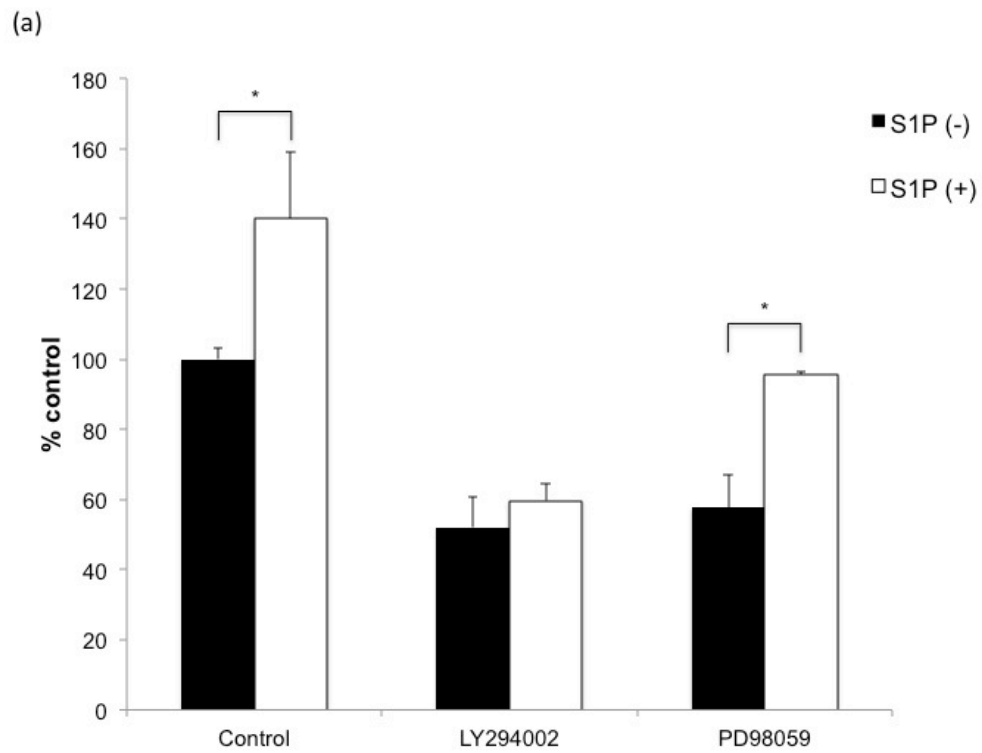
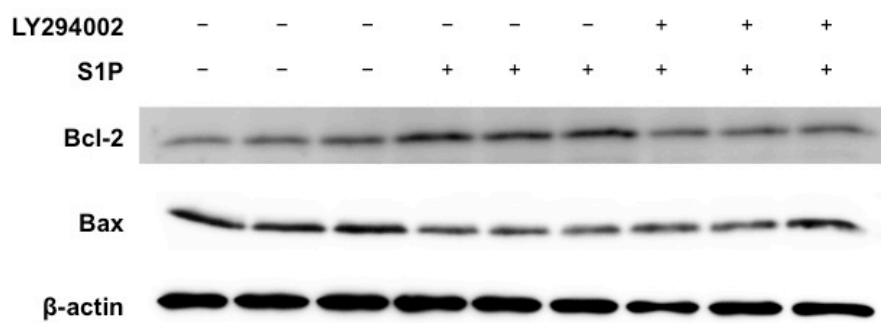


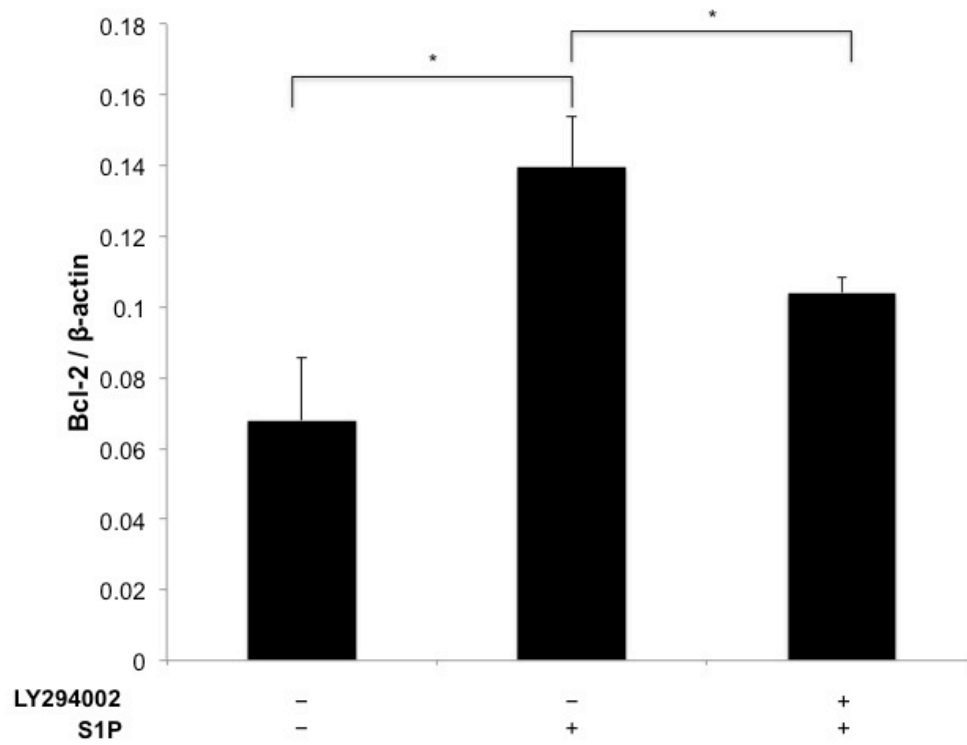
Fig. 2



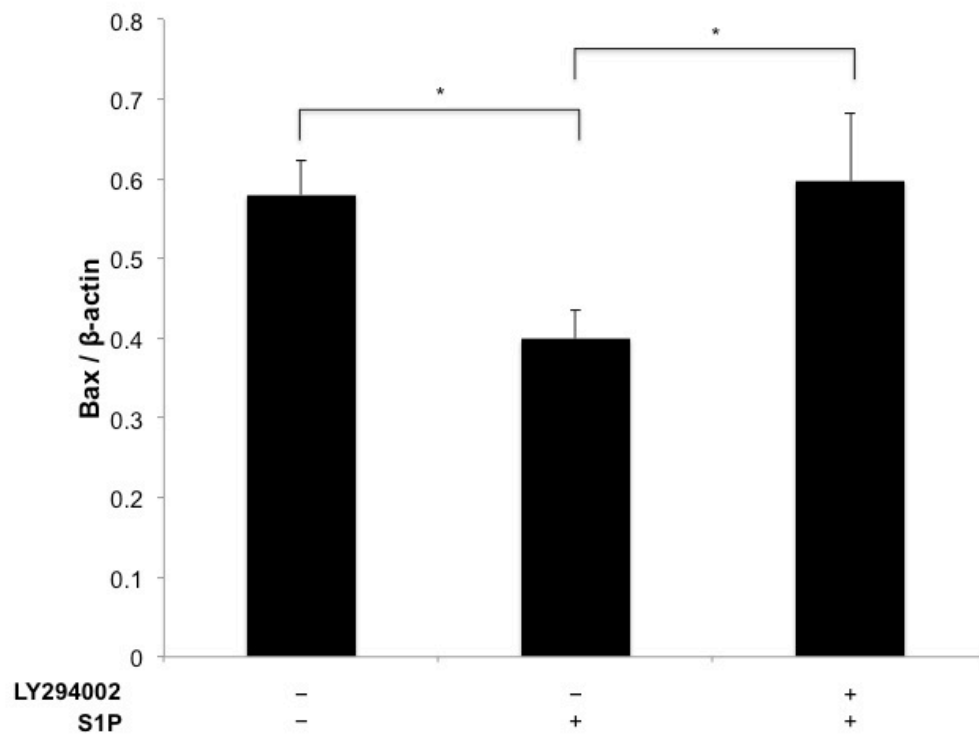
(b)



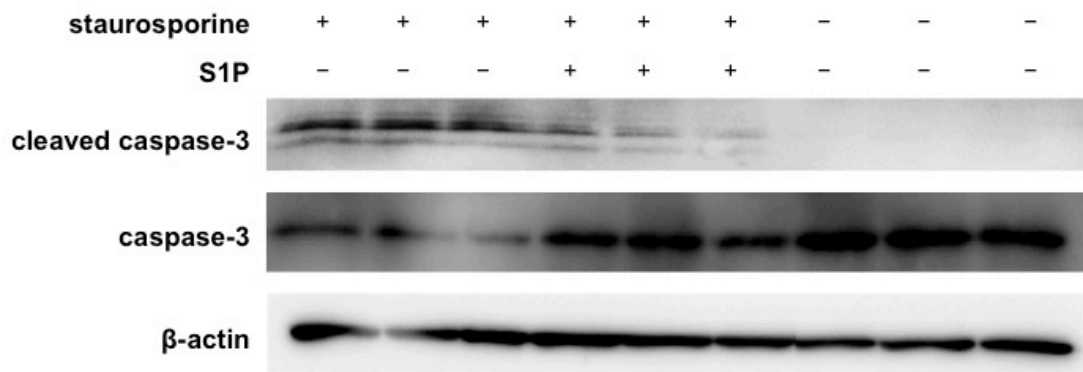
(c)



(d)



(e)



(f)

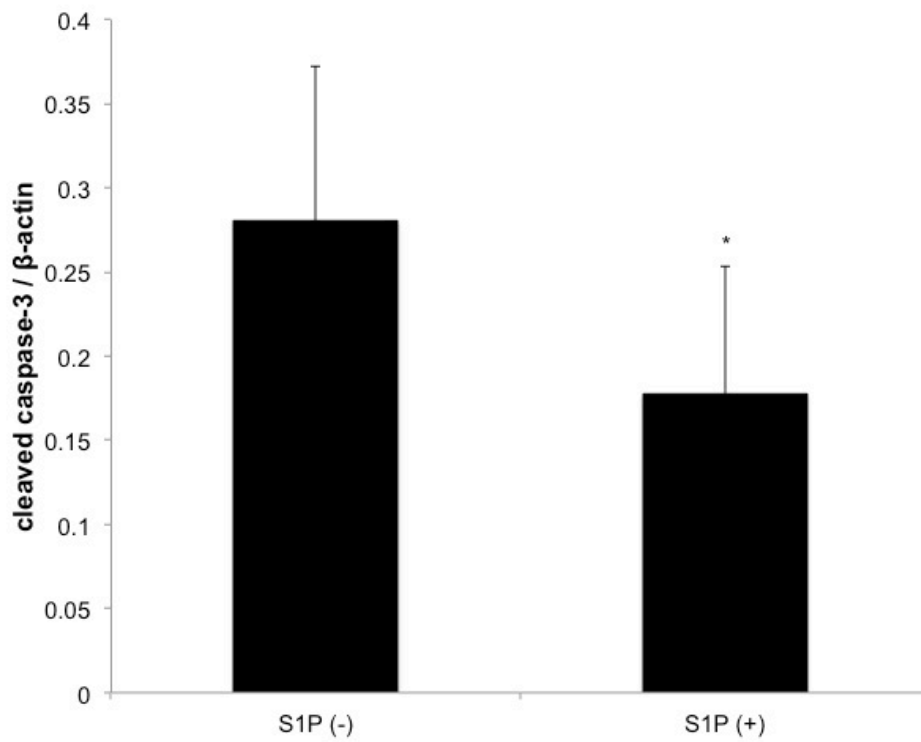


Fig. 3

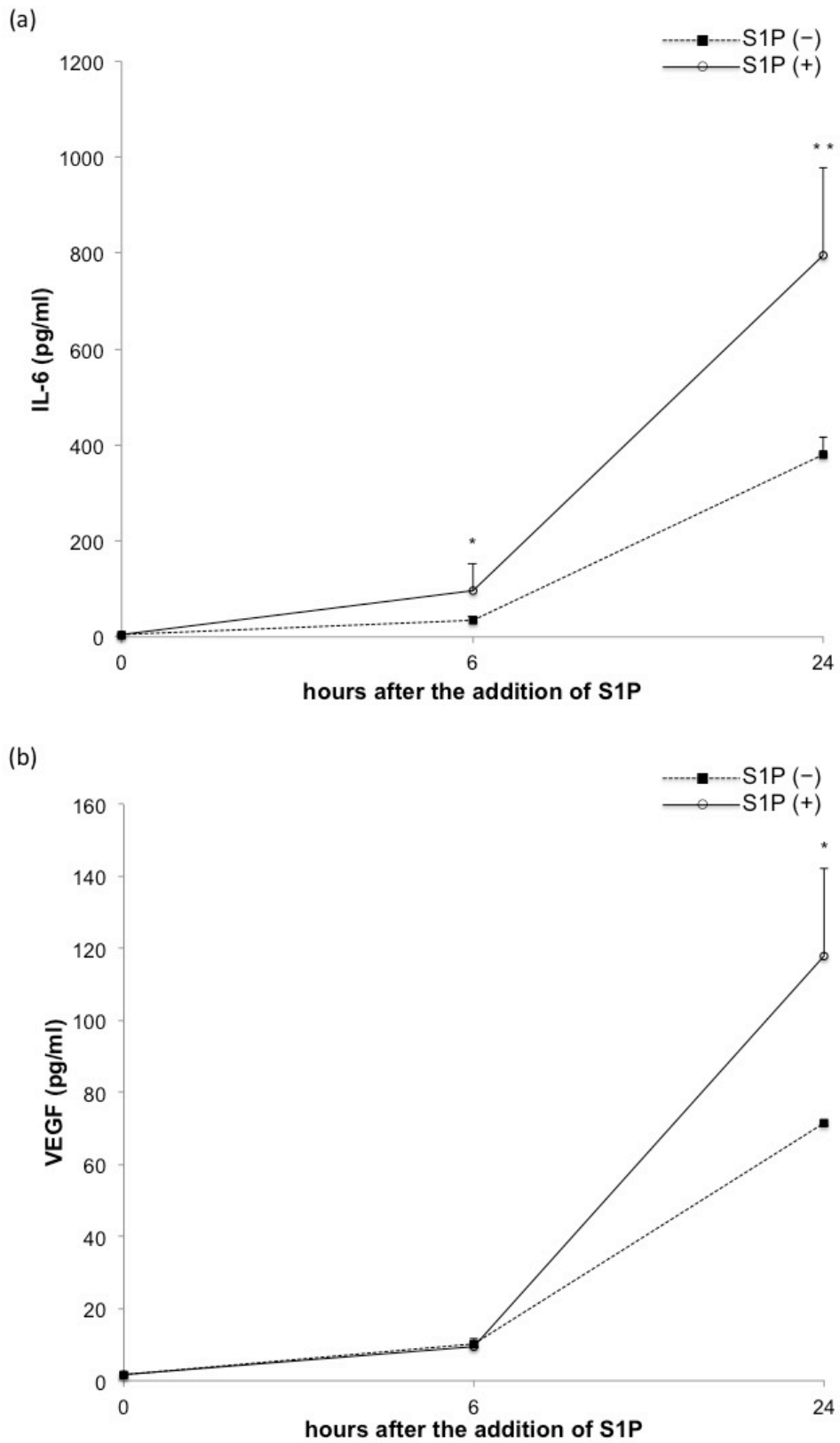
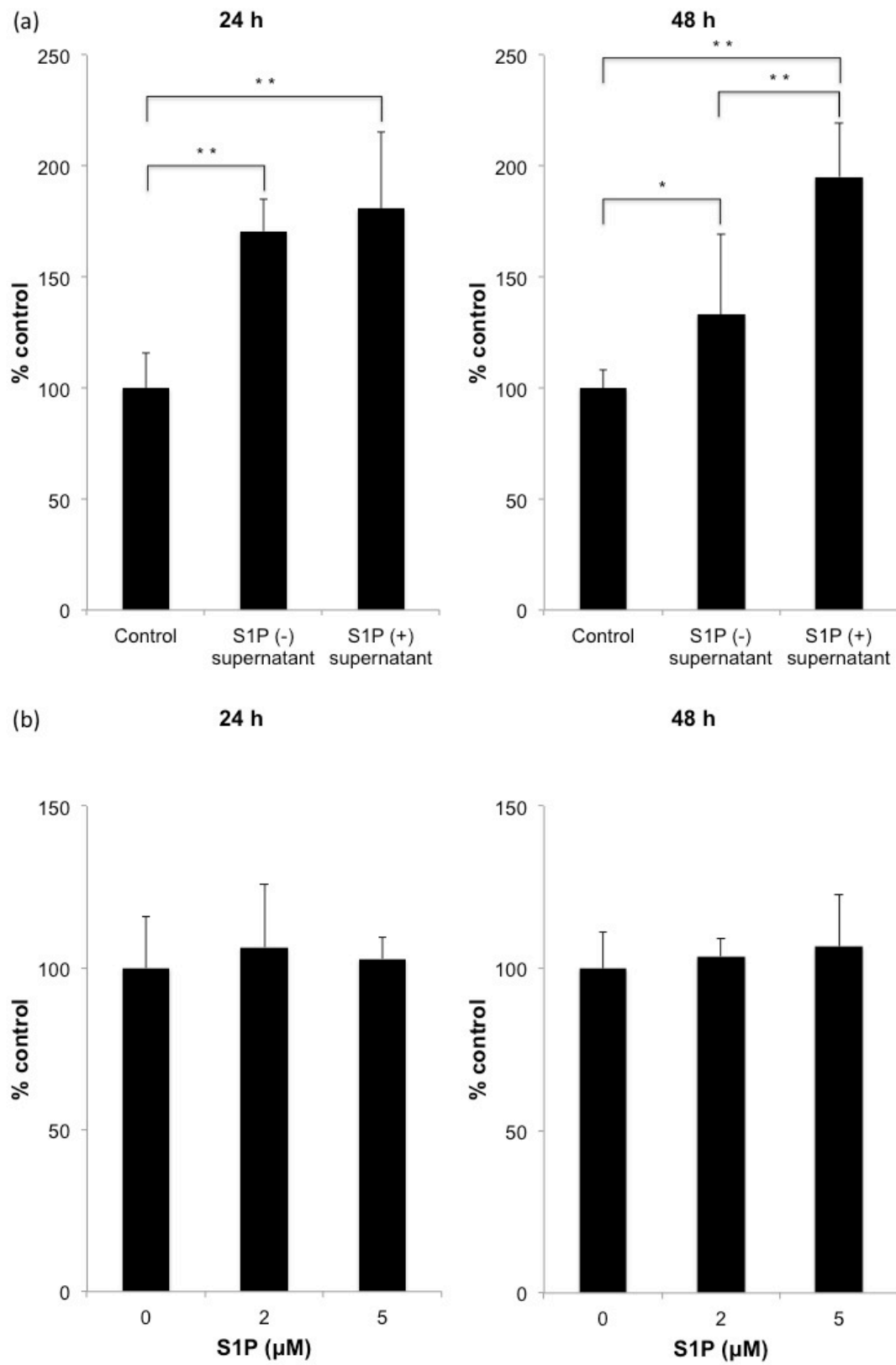
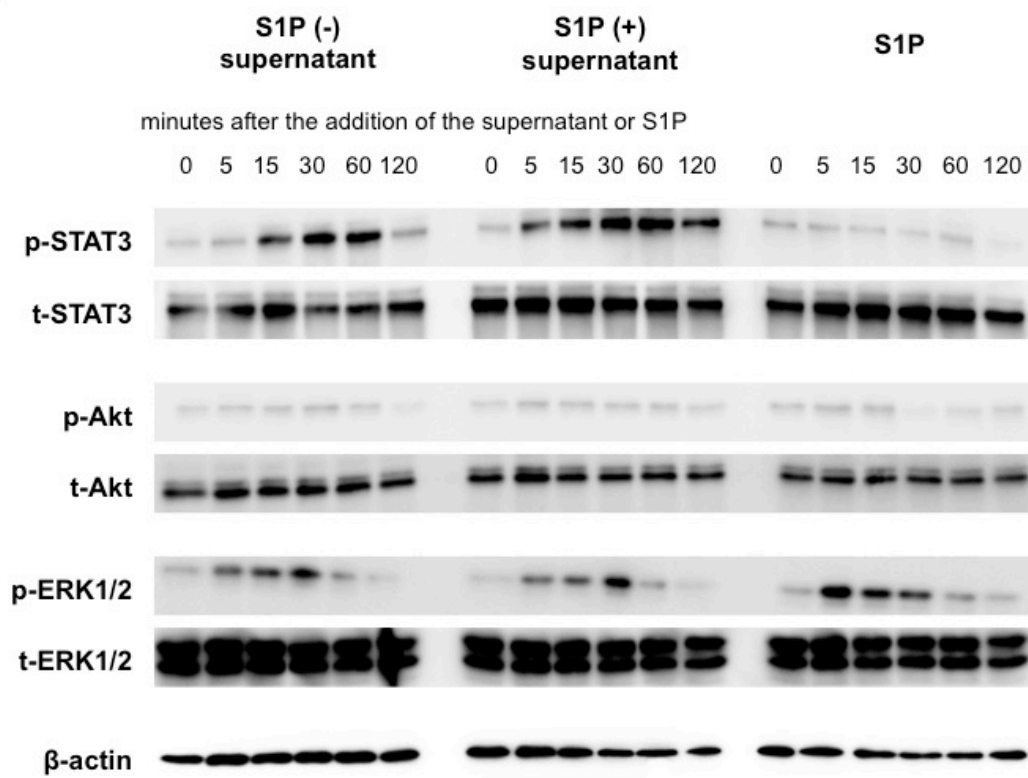


Fig. 4



(c)



(d)

