Enhancement of Sphingosine 1-Phosphate-Induced Phospholipase C Activation During $G_0$-$G_1$ Transition in Rat Hepatocytes

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Abstract. We previously reported that sphingosine 1-phosphate (S1P) induces inhibition of adenylyl cyclase and activation of phospholipase C via independent G protein-coupled receptors in adult rat hepatocytes. Although S1P activation of phospholipase C and subsequent increase of intracellular $Ca^{2+}$ concentration were enhanced during the primary culture of hepatocytes, S1P inhibition of adenylyl cyclase remained unchanged. Here, we addressed whether enhancement of S1P-induced actions is dependent on change of status from the differentiated ($G_0$) phase to proliferating ($G_1/S$) phase in hepatocytes. By employing cell-density-dependency of the transition ($G_0$-$G_1$) of hepatocytes in primary culture in vitro, it was found that the enhancement of phospholipase C activation by S1P was dependent on cell density and correlated to the $G_0$-$G_1$ transition. The correlation was further confirmed in vivo by 70% hepatectomy as a proliferating hepatocytes model. Northern blot analysis suggested an enhanced expression of S1P receptor in proliferating hepatocytes.

Keywords: hepatocyte, sphingosine 1-phosphate, phospholipase C, G protein-coupled receptor, cell cycle

Introduction

Sphingosine 1-phosphate (S1P), a bio-active sphingolipid metabolite, is highly abundant in platelets and released from activated platelets (1) and has been shown to be involved in a variety of cellular functions, including cell growth, differentiation, and programmed cell death (2, 3). S1P acts as a first messenger through the G protein-coupled receptors, termed S1P$_1$, S1P$_2$, S1P$_3$, S1P$_4$, and S1P$_5$ (formerly known as Edg1, Edg5, Edg3, Edg6, and Edg8, respectively) (4 – 9), and also as a second messenger in certain cell types (10). G protein-couplings and intracellular signaling of each plasma membrane receptor have been investigated by using recombinant DNAs (2, 11, 12), in particular those for S1P$_1$ (4, 13, 14) and S1P$_2$ (6, 15). S1P$_1$ predominantly couples to both G$_i$ and Ras-Raf-MAP (mitogen activated protein) kinase pathways and also induces morphogenetic differentiation in a C3 exoenzyme-sensitive manner (4, 13). S1P$_2$ increases intracellular $Ca^{2+}$ concentration predominantly via the G$_q/11$ protein phospholipase C pathway and activates MAP kinases in a pertussis toxin-insensitive manner (6, 16). Furthermore, S1P$_2$ induces cell rounding and neurite retraction presumably via G$_{12/13}$ proteins (15).

We have earlier investigated the action and signaling mechanisms of S1P in rat hepatocytes (17). S1P triggers at least two independent signaling cascades, that is, one that inhibits adenylyl cyclase and one that activates phospholipase C and subsequent signaling cascades (17). S1P inhibition of adenylyl cyclase activity was blocked by a treatment with pertussis toxin (17), but S1P activation of phospholipase C and subsequent $Ca^{2+}$ increase were not sensitive to the toxin treatment (17). Based on these results, we suggested that two signaling pathways are mediated by two separate G protein-coupled S1P receptors (17). We also found that S1P activation of phospholipase C and subsequent cascades were enhanced during the primary culture of hepatocytes, but S1P inhibition of adenylyl cyclase remained...
unchanged (17). The precise mechanism and physiological significance of the enhancement of S1P-induced actions during the primary culture, however, have not been characterized. In the present study, using an in vitro primary culture system and an in vivo hepatectomy model of liver regeneration, we investigated whether the enhancement of S1P-induced actions during the primary culture was dependent on status change of hepatocytes during progress from the differentiated (G0) phase to proliferating (G1/S) phase. Additionally, we analyzed the expression levels of five S1P receptors in rat hepatocytes.

Materials and Methods

Materials

Fura 2-acetoxyethyl ester and N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) were purchased from Dojindo (Tokyo), and collagenase (type I) was from Wako (Tokyo). The sources of all other reagents were the same as described previously (17).

Isolation of hepatocytes and tissue culture

Hepatocytes were isolated from the liver of Wistar-derived Donryu strain male rats (200 – 250 g) that had been fed ad libitum as described previously (17). Freshly prepared hepatocytes were suspended at a density of 1.92 × 10⁶ cells/ml in the William’s E medium (pH 7.4) containing 5% fetal calf serum, 1 nM insulin, 1 nM dexamethasone, and 100 units/ml penicillin G, and 100 µg/ml streptomycin sulfate; and they were maintained at a density of 4 × 10⁶ cells/cm² or the indicated cell-densities at 37°C under 5% CO₂ and 95% air for 24 h, unless indicated otherwise.

Liver regeneration (70% hepatectomy)

For inducing liver regeneration, we performed two-thirds partial hepatectomy in rats as described by Higgins and Anderson (18). Briefly, in a rat anesthetized with Nembutal, two-thirds of the liver was externalized through a small midabdominal incision, and the externalized portion of the liver was then resected. At 48 h after the hepatectomy, hepatocytes were prepared by the same method as described above.

Inositol phosphate production

Labeling of hepatocytes with myo-[2-³H]inositol (NEN, Boston, MA, USA; 100 µCi/ml) was carried out at 37°C for 90 min in TCM 199 medium (containing 0.1% BSA) (Nissui Seiyaku, Tokyo) for cultured cells or in Krebs-Ringer-Hepes medium for fresh cells. These ³H-labeled cells were washed three times with Krebs-Ringer-HEPES medium and incubated with 15 mM LiCl for 15 min before the start of incubation with receptor agonists at 37°C in the same medium. The reaction was terminated by adding 5% trichloroacetic acid, which was then removed by shaking with water-saturated ether. Inositol phosphates in the supernatant was separated through a Dowex 1-X8 column and the radioactivity of IP₁ and IP₃ was counted as described previously (19).

DNA synthesis

DNA synthesis was examined by measuring [methyl-³H]thymidine (1 µCi/ml, NEN) incorporated into hepatocytes for 4 h in the culture. In the case of Fig. 4B, hepatocytes were cultured for 4 h with [³H]thymidine, and radioactivity of trichloroacetic acid-precipitable materials in the cells was determined as DNA synthesis (20). In the case of Fig. 2A, 20 ng/ml of epidermal growth factor (EGF) was added at 3, 24, or 48 h of culture. After an additional 21 h of culture, cells were labeled with [³H]thymidine (1 µCi/ml, NEN) for 4 h.

Northern blotting

Total RNA was isolated from freshly isolated hepatocytes or indicated time-cultured hepatocytes according to the manufacturer’s instructions for TRIZOL Reagent (Life Technologies, Inc., Langley, OK, USA), and Northern blotting was performed as previously described (21). Briefly, 20 µg of total RNA was electrophoresed through a 1% agarose gel containing 3.7% formaldehyde and 20 mM morpholinepropane sulfonic acid (Mops) buffer and then blotted onto a nylon membrane (Hybond-N) with 20 × SSC. The probes were labeled with [α-³²P]-ATP by random oligonucleotide priming, and hybridization was carried out at 65°C. Following hybridization, the blots were washed with 0.2 × SSC and 0.1% SDS at 65°C. To normalize the amounts of RNA present in the blots, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used (21).

Real-time PCR using TaqMan method

Total RNA from each sample was isolated as described above, and the preparation was treated with DNase I (Promega, Madison, WI, USA) to remove possible traces of genomic DNA contaminating the RNA preparations. After the DNase I treatment, 5 µg of the total RNA was reverse-transcribed using random priming and Multiscribe reverse transcriptase, according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA, USA). Each reverse-transcribed sample was sequentially diluted (n = 5) and used for real-time PCR. To evaluate the expression level of the S1P₁ and S1P₂ genes, real-time PCR was performed using the TaqMan method with a Sequence
Enhancement was cell-density-dependent: the response in freshly-isolated hepatocytes. The degree of enhancement was suppressed in the high-cell-density culture of hepatocytes, however, significant increase in EGF-induced DNA synthesis was not observed until the cells were cultured for 48 h before the growth factor was added (Fig. 2A). Thus, the transition of G₀ phase to G₁ phase or the EGF-responsive cell phase seems to occur in a cell-density-dependent manner. Under the same culture conditions, the S1P-induced activation of phospholipase C was enhanced by 24-h culture at the low cell density, but at the high cell density, it took 48 h to become highly responsive to S1P (Fig. 2B). Thus, there is a strong correlation between the enhancement of the S1P response and the G₀-G₁ transition of cell cycle.

S1P-induced phosphorylase activation was also examined. Culture-induced enhancement of S1P-induced phosphorylase activation was clearly observed in hepatocytes cultured at the low cell density, but the enhancement was suppressed in the high-cell-density culture (16 × 10⁴ cells/cm²) (data not shown).

Enhancement of phospholipase C activation by S1P during primary culture of hepatocytes was dependent on cell density and correlated to the G₀-G₁ transition

In primary culture of rat adult hepatocytes, many metabolic functions as well as cell growth are regulated by cell density (23–25). Growth-related functions, such as DNA synthesis, are stimulated by low cell density (23–25), whereas functions related to hepatocyte-specific characters such as induction of tyrosine aminotransferase are stimulated by high cell density (23–25). We have earlier shown that S1P-induced activation of phospholipase C and its cascade reactions were enhanced when hepatocytes were cultured at low cell density (4 × 10⁴ cells/cm²) (17). In Fig. 1, we examined the effect of cell density on the S1P-induced inositol phosphate production as an activity of phospholipase C. As shown in Fig. 1 (A and B), consistent with the previous report (17), the S1P-induced response in 24-h-cultured hepatocytes was enhanced at low cell density (4 × 10⁴ cells/cm²), compared to the response in freshly-isolated hepatocytes. The degree of enhancement was cell-density-dependent: the response to S1P was weakened by increasing cell density up to 16 × 10⁴ cells/cm² (Fig. 1B). In contrast, LPA-induced responses were not changed during the primary culture (Fig. 1: A and B).

Under low-cell-density culture, DNA synthesis was robust when EGF was added to the culture medium at 24 or 48 h after seeding of the cells (Fig. 2A). Under the high-cell-density culture of hepatocytes, however, significant increase in EGF-induced DNA synthesis was not observed until the cells were cultured for 48 h before the growth factor was added (Fig. 2A). Thus, the transition of G₀ phase to G₁ phase or the EGF-responsive cell phase seems to occur in a cell-density-dependent manner. Under the same culture conditions, the S1P-induced activation of phospholipase C was enhanced by 24-h culture at the low cell density, but at the high cell density, it took 48 h to become highly responsive to S1P (Fig. 2B). Thus, there is a strong correlation between the enhancement of the S1P response and the G₀-G₁ transition of cell cycle.

Results

Effects of S1P and LPA on inositol phosphate production in fresh hepatocytes (A) and 24-h-cultured hepatocytes (B). In A, dispersed hepatocytes that had been incubated with 100 μCi/ml of [³H]inositol were further incubated for 1 min with vehicle, 10 μM S1P, or 10 μM LPA. In B, 24-h-cultured hepatocytes at the indicated cell-densities were used for measurement of inositol phosphate production as in fresh hepatocytes. Results are expressed as percentage of the respective control values. Values are means ± S.E.M. of 3 separate experiments.

Data presentation

All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as representative or means ± S.E.M. of at least three separate experiments unless otherwise stated. Results were analyzed by Students’ t-test.

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Enhancement of S1P-induced activation was further confirmed in vivo by 70% hepatectomy as a proliferating hepatocytes model

The correlation between the enhancement and the cell cycle transition observed in vitro in a primary culture model was further examined with in vivo proliferating hepatocytes induced by conducting hepatectomy. Since two-thirds hepatectomy is a well-known model for inducing liver regeneration, phospholipase C activation by S1P was measured in hepatocytes freshly prepared from normal (Fig. 3A) and partial hepatectomized rats (Fig. 3B). The production of IP$_2$ and IP$_3$ induced by S1P was clearly increased in the hepatocytes prepared from partial hepatectomized rats, compared with those from normal rats. Abilities of DNA synthesis in hepatocytes from normal, sham-operated, and partial hepatectomized rats were also tested by measuring $[^3]$H]thymidine incorporation. As shown in Fig. 4A, the hepatocytes isolated from partial hepatectomized rats showed extremely robust activity of DNA synthesis, confirming the well-known finding that the hepatocytes are in the growth phase. Thus, it is obvious that the enhancement of S1P-induced phospholipase C activation was pararell to the cell cycle of hepatocytes transiting from the differentiated (G$_0$) phase to proliferating or dedifferentiated (G$_1$/S) phase in vivo.

In contrast to the enhancement of S1P-induced activation of phospholipase C in the hepatocytes from partial hepatectomized rats, lysophosphatidic acid (LPA) or phenylephrine, which is Ca$^{2+}$-mobilizing agonists in hepatocytes, induced rather weak responses in the hepatocytes isolated from partial hepatectomized rats, compared with those from normal rats (Fig. 4B). On the other hand, NaF-induced activation of phospholipase C was not changed by hepatectomy (Fig. 4B), thus indicating that the enhancement of the response is a phenotype specific to S1P.

Expression of S1P$_2$ was up-regulated in proliferating hepatocytes

Finally, we studied expression levels of five S1P receptors by Northern blotting and real time PCR. As shown in Fig. 5, two S1P receptors, S1P$_1$ and S1P$_2$, were found to be expressed in rat hepatocytes, whereas the rest of the three receptors were not detectable in freshly-isolated as well as 24-h-cultured hepatocytes. S1P$_1$ receptor was strongly expressed in freshly-isolated hepatocytes and 3-h-cultured hepatocytes, but markedly decreased in 24 h- and 48 h-cultured hepatocytes. In
Fig. 4. Effects of partial hepatectomy on DNA synthesis (A) and phospholipase C activation (B). In A, hepatocytes isolated from normal rats (open), sham-operated rats (hatched), or partial hepatectomized rats at 48 h after the operation (closed) were cultured at a low cell density of $4 \times 10^4$ cells/cm² and simultaneously labeled with 1 $\mu$Ci/ml of $[^3]$H]thymidine for 4 h. Values are means ± S.E.M. of 3 separate experiments. In B, hepatocytes isolated from normal rats (open), sham-operated rats (hatched), or partial hepatectomized rats at 48 h after the operation (closed) were labeled with 100 $\mu$Ci/ml $[^3]$H]inositol and stimulated with 10 $\mu$M S1P, 10 $\mu$M LPA, 100 $\mu$M phenylephrine, or 10 mM NaF. Inositol phosphate production was normalized to the respective control values. Values are means ± S.E.M. of 3 separate experiments.

Fig. 5. Northern blot analysis of S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅ mRNAs in rat hepatocytes (A) and real time PCR of S1P₁ and S1P₂ (B). A: Total RNA was extracted from fresh hepatocytes or hepatocytes that had been cultured at the cell density of $4 \times 10^4$ cells/cm² for 3, 24, or 48 h. Twenty micrograms of total RNA was separated by electrophoresis on agarose gels and transferred onto nylon filters. The filters were hybridized with $^{32}$P-labeled S1P₁, S1P₂, S1P₃, S1P₄, or S1P₅ cDNA. GADPH mRNA is shown as an index of the amount of RNA in each lane. Similar experimental results were obtained twice. B: S1P₁ and S1P₂ mRNA expressions were also measured using real-time PCR by the TaqMan method. Five micrograms of total RNA from fresh hepatocytes or from hepatocytes cultured at the cell density of $4 \times 10^4$ cells/cm² for 3, 24, or 48 h was reverse-transcribed and sequentially diluted. Each sample was used for the TaqMan analysis. Expression levels are presented as relative ratio of these genes to GADPH gene. The results represent the mean ± S.D. from a representative result. Another similar experiment gave a similar result.
contrast, however, S1P₂ receptor was weakly expressed in freshly-isolated hepatocytes, but up-regulated in the primary cultured hepatocytes, even in the 3-h-cultured ones. Since S1P₂ has been suggested to couple to the G12/13 proteins-phospholipase C pathway and G38 proteins-Rho pathway (6, 15, 16), increased expression of S1P₂ might likely be responsible for the enhancement of S1P-induced phospholipase C activation in proliferating hepatocytes.

Discussion

In our previous study, we demonstrated that the S1P-induced Ca²⁺ response and phosphorylase activation were enhanced in parallel with an enhancement of the lipid-induced phospholipase C activation during primary culture of rat adult hepatocytes (17). In the present study, a strong correlation between the enhancement of S1P activation of phospholipase C and the cell cycle transition (G0-G1) was shown by two systems: an in vitro primary culture system and an in vivo model of liver regeneration. Furthermore, up-regulated expression of S1P₂ in proliferating hepatocytes was found and suggested to be a cause of the enhancement.

Among five S1P receptors, expressions of S1P₁ and S1P₂ were detected in rat hepatocytes. Considering G protein coupling properties of S1P₁ and S1P₂ receptors, S1P₁ may mediate the inhibition of adenylyl cyclase and S1P₂ may modulate phospholipase C activity in rat hepatocytes. Enhanced expression of S1P₂ in the primary cultured hepatocytes observed in our study has recently been observed in the in vivo regenerating liver by Ikeda et al. (26), confirming our assumption that the enhancement of the S1P activation of phospholipase C in the regenerating liver may also be due to increased expression of S1P₂ in vivo. Similar changes of S1P₂ expression were observed in other cell types: S1P₂ expression was down-regulated in F9 embryonic carcinoma cells when the cells were differentiated by the treatment of retinoic acid (27), and myogenic differentiation in C2C12 myoblasts, was accompanied with down-regulation of S1P₂ expression (28). Thus, S1P₂ expression seems to be up-regulated in proliferating status and down-regulated in differentiated status. On the other hand, decrease of S1P₁ expression in proliferating hepatocytes observed in our study is compatible with up-regulation of S1P₁ expression during differentiation of endothelial cells (29). Thus, S1P₁ expression seems to be up-regulated in differentiated status and down-regulated in proliferating status.

A loose cell-to-cell interaction has been suggested to favor cell proliferation and disappearance of liver-specific characteristics in the primary culture system (23–25, 30). In our study, the enhancement of S1P-induced phospholipase C activation was cell-density-dependent; there was a marked enhancement at low cell density but only a slight increase at high cell density. Thus, the loss of cell-to-cell contact through plasma membranes at low cell-density appears to be a trigger for the enhancement of S1P-induced actions during primary culture. However, we do not know at present which component in the plasma membrane induces cell cycle transition (G0-G1). Since responses to other Ca²⁺-mobilizing agents were shown to be diminished in primary-cultured hepatocytes at low cell density (30) and other signaling molecules, such as G proteins and phospholipase C, were not functionally changed or diminished during the primary culture or by the hepatectomy, the enhancement phenomenon could be explained by the increased expression of S1P₂ in cultured hepatocytes. However, two things remain, curiously; increased expression of S1P₂ receptor in 3-h-cultured hepatocytes is not observable as enhanced S1P-induced PLC activation at that time point and decreased expression of S1P₁ receptor in 24-h-cultured hepatocytes was not shown as decreased S1P response on adenylyl cyclase (17). Such discrepancies might be explained by differential couplings of S1P receptors and G proteins in further studies. Previously, Kajiyama and Ui reported differential coupling between β-adrenergic receptors and Gs proteins in primary cultured hepatocytes (30).

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