Short Communication

Study on Action Mode of Sphingosine 1-Phosphate in Rat Hepatocytes

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Abstract. Sphingosine-1-phosphate (S1P) acts on a set of G protein-coupled receptors in the plasma membrane and also as a second messenger in certain cell types. There are two possible pathways to mobilize intracellular Ca^{2+} concentration by S1P. One is through phospholipase C, and the other is through intracellular Ca^{2+} channels operated by S1P. The Mn^{2+} quenching method was applied to elucidate the action mode of S1P-induced Ca^{2+} mobilization in rat hepatocytes. In permeabilized hepatocytes, inositol trisphosphate induced Mn^{2+} quenching, and it was blocked by heparin. However, S1P did not induce Mn^{2+} quenching. Results suggest that S1P did not mobilize Ca^{2+} through intracellular Ca^{2+} channels.

Keywords: hepatocyte, sphingosine 1-phosphate, second messenger

Sphingosine 1-phosphate (S1P), a bio-active sphingo-lipid metabolite, is highly abundant in platelets and is released from activated platelets, and it has been shown to be involved in many cellular functions, including cell growth, differentiation, and programmed cell death (1). 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) (1, 2) and also as a second messenger in certain cell types (3). G-protein-couplings and intracellular signaling of each plasma membrane receptors have been investigated by using recombinant DNAs (1, 2). Stimulations of S1P_2 and S1P_3 result in an elevation of intracellular Ca^{2+} concentration via the Gq-phospholipase C (PLC) pathway (4, 5).

On the other hand, the molecular target(s) or action mechanism of S1P as a second messenger has not yet been discovered. Transient activation of sphingosine kinase by several stimulants (such as PDGF, $\text{Fc}\epsilon\text{RI}$ antigen, and $\text{TNF-}\alpha$) and subsequent production of S1P have been observed in several cell types (6–10).

Because S1P acts on its receptors in the plasma membrane as a first messenger, it is not easy to distinguish the two roles. S1P, produced in the cytosol, could act on the receptors in the membrane after its release. In permeabilized cells of the DDT₁MF-2 smooth muscle cell line, however, S1P induced a large release of Ca^{2+} directly from Ins-P_3 -sensitive and insensitive Ca^{2+} stores (6); in Swiss 3T3 fibroblasts, S1P mobilized Ca^{2+} from internal stores primarily through a mechanism independent of inositol lipid hydrolysis (8); and in RBL-2H3 rat mast cell line, Ca^{2+} was mobilized through S1P production via sphingosine kinase when the cells were stimulated by $\text{Fc}\epsilon\text{RI}$ antigen (9). Thus, S1P has two mechanisms to increase $[\text{Ca}^{2+}]_i$, i.e., directly through the interaction of the lipid with Ca^{2+} channels on intracellular Ca^{2+} pool and indirectly through receptor activation and subsequent production of Ins-P_3 which interacts with the pool and releases Ca^{2+} .

Action and signaling of S1P in rat hepatocytes have been previously investigated (11). Although a robust increase of $[\text{Ca}^{2+}]_i$ by S1P was observed in rat hepatocytes primarily-cultured for 24 h, the precise action mode of S1P in Ca^{2+} increase has not been elucidated. In order to clarify the action site of S1P to mobilize Ca^{2+} in

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hepatocytes, the effect of S1P on Ca^{2+} stores in permeabilized hepatocytes was examined by measurement of Mn^{2+} quenching.

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 (11). Freshly prepared hepatocytes were suspended at a density of 1.92×10^5 cells/ml in the William's E medium (pH 7.4) containing 5% fetal calf serum, 1 nM insulin, 1 nM dexamethasone, 100 units/ml penicillin G, and 100 μg /ml streptomycin sulfate and maintained at a density of 4×10^4 cells/cm² on plastic Petri dishes, each containing a collagen-coated cover glass, at 37°C under 5% CO_2 and 95% air for 1.5 h or 24 h.

For measurement of Mn^{2+} quenching of compartmentalized fura-2, hepatocytes cultured for the indicated hours were loaded with fura-2 by incubation with 5 μM fura-2/AM for 60 min at 37°C. Extracellular dye was washed out twice with permeabilization medium. The fura-2-loaded cells were permeabilized in permeabilization medium containing 10 μg /ml saponin, 5 μg /ml oligomycin, 1 μg /ml rotenone, 2 μM thapsigargin, and 1 μg /ml each of the protease inhibitors, pepstatin and leupeptin, for 10 min at 37°C. The permeabilization medium consisted of 120 mM KCl, 20 mM Hepes, 10 mM NaCl, 1 mM KH_2PO_4 and 0.2 mM MgCl_2 , pH 7.2. Cell permeabilization was assessed with trypan blue and was always greater than 99%. The cover glass was removed and fixed on a sample chamber (SC-20 model; Nihonbunko, Tokyo) for perfusion method measurement. Addition of Mn^{2+} or Ins-P_3 was achieved by changing the flow medium to an agent-containing one. The time course of quenching of compartmentalized fura-2 fluorescence by Mn^{2+} was monitored, using the Ca^{2+} -insensitive excitation wave length of 360 nm with emission at 510 nm (CAM-230 system, Nihonbunko). The Mn^{2+} concentration was 4 μM .

In hepatocytes, the Mn^{2+} quenching method was previously used to examine the kinetics of Ca^{2+} release from Ins-P_3 -sensitive intracellular Ca^{2+} stores (12). As shown in Fig. 1, in saponin-permeabilized hepatocytes, Ins-P_3 induced Mn^{2+} quenching in a dose-dependent manner. Heparin, which is a competitive inhibitor of Ins-P_3 binding to its receptor, inhibited the Mn^{2+} quenching induced by Ins-P_3 (Fig. 2). Adrenergic responses in hepatocytes switch from α -receptor-dominant to β -receptor-dominant during liver regeneration in vivo and primary culture in vitro (13), and the decrease of α -adrenoceptor-mediated response during primary culture occurs concurrently with total decrease of Gq protein-mediated responses, that is, vasopressin-mediated and angiotensin II-mediated (13). In the present study, Ins-P_3 -induced Mn^{2+} quenching was weakened during

the primary culture (Figs. 1 and 2): The dose of heparin to completely inhibit Ins-P_3 (2 μM)-induced quenching was 200 μg /ml in fresh cells and was 50 μg /ml in 24-h cultured cells (Fig. 2). The decrease of Mn^{2+} quenching response to Ins-P_3 in 24-h cultured cells might explain the decrease of Gq-coupled receptor-mediated responses during the primary culture. Next, the effect of S1P on Mn^{2+} quenching in fresh and 24-h cultured hepatocytes was measured. However, no significant effect of S1P on the Mn^{2+} quenching was observed (Fig. 3).

In our previous studies, we showed that enhancement of S1P-induced PLC activation during hepatocytes culture was accompanied by increases of S1P-induced Ca^{2+} response and phosphorylase activation (11) and also possible involvements of G-proteins and S1P_2 receptor in the S1P signaling pathway (11, 14). However, S1P action on intracellular Ca^{2+} stores was not directly measured. In the present study, by using the Mn^{2+} quenching method, we directly investigated whether the action site of S1P was present in the plasma membrane or on intracellular stores and found that S1P did not evoke Ca^{2+} release from Ca^{2+} pools in permeabilized hepatocytes, excluding a possibility that S1P acts as a second messenger to mobilize Ca^{2+} through intracellular Ca^{2+} channels.

Saponin-permeabilized cells of DDT₁MF-2 smooth muscle cell line and Swiss 3T3 fibroblast cell line were used for analysis of Ca^{2+} release by sphingosine derivatives, and S1P was proposed to mobilize Ca^{2+} from Ins-P_3 -sensitive Ca^{2+} pools. Heparin blocked Ins-P_3 -induced Ca^{2+} release but not S1P-induced Ca^{2+} release in Swiss 3T3 fibroblasts (6, 8). In the previous studies, Ca^{2+} release from intracellular Ca^{2+} stores was measured by monitoring $^{45}\text{Ca}^{2+}$ or fluorescence of fura-2 in the cytosolic side (6, 8). In our study, quenching of fura-2 inside the intracellular Ca^{2+} stores was monitored, and Ins-P_3 -induced Ca^{2+} release and blockage by heparin were reproduced in hepatocytes. However, S1P-induced Ca^{2+} release was not observed. The discrepancy between our data and the data of the previous reports might result from the difference of studied cells and/or difference in monitoring methods. Furthermore, S1P degradation in the assay medium could be a possibility for the failure of S1P action. However, the permeabilized cells are washed with the cytosol-like permeabilization buffer twice. Therefore, the cytosolic side is full of the buffer and no cytosolic constituents would be remaining in the intact cells. Still, there is a possibility that S1P-degrading enzymes are attached on the intracellular organelles. However, S1P is delivered in the flowing cytosol-like medium. When S1P is degraded in the assay medium, S1P is also contacting the putative Ca^{2+} channels simultaneously. Thus, such a possibility is

Fig. 1. Dose-response curves of Ins-P_3 -induced Mn^{2+} quenching in fresh and 24-h cultured permeabilized hepatocytes. Hepatocytes cultured for 1.5 h (fresh) or 24 h were loaded with fura-2/AM ($5 \mu\text{M}$) for 60 min. The fura-2-loaded cells were permeabilized and incubated with Mn^{2+} and Ins-P_3 . The fluorescence of compartmentalized fura-2 in intracellular organelles was monitored by 360-nm excitation and 510-nm emission.

Fig. 2. Inhibition of Ins-P_3 -induced Mn^{2+} quenching by heparin in fresh and 24-h cultured permeabilized hepatocytes. The fura-2-loaded cells were permeabilized and incubated with Mn^{2+} and Ins-P_3 ($2 \mu\text{M}$) in the presence or absent of the indicated doses of heparin. The fluorescence of compartmentalized fura-2 in intracellular organelles was monitored by 360-nm excitation and 510-nm emission.

Fig. 3. Effect of S1P on Ins-P_3 -sensitive Ca^{2+} stores in fresh and 24-h cultured permeabilized hepatocytes. The fura-2-loaded cells were permeabilized and incubated with Mn^{2+} , S1P, and Ins-P_3 . The fluorescence of compartmentalized fura-2 in intracellular organelles was monitored by 360-nm excitation and 510-nm emission.

quite low.

It is well known that the liver is regenerated after 70% partial hepatectomy. The primary culture of hepatocytes has been used as an *in vitro* model of liver regeneration, because characteristic changes during liver regeneration have been reproduced during the primary culture of hepatocytes such as a transition from differentiated state to proliferating state. One of the phenotypes is to switch adrenergic responses from α -receptor to β -receptor (13). We demonstrated that not only α -adrenoceptor-

mediated signaling but also other Gq protein-coupled receptor-mediated signalings were weakened during the primary culture (13). Furthermore, we observed the decrease of Ins-P_3 -binding sites on intracellular stores during the primary culture (15). In the present study, we demonstrated that Ins-P_3 -induced Mn^{2+} quenching was weakened during the primary culture, confirming the previous observation that Ins-P_3 -binding sites were decreased during the primary culture (15). In the previous paper, we observed a discrepancy between lyso-

phosphatidic acid (LPA)-induced inositol phosphate production and Ca^{2+} mobilization in cultured hepatocytes (11): During the primary culture, LPA-induced inositol phosphate production was not decreased, whereas LPA-induced Ca^{2+} mobilization was decreased by about 50% compared with that in fresh hepatocytes. This discrepancy, however, could now be explained by the decrease of Ins-P_3 -binding sites on intracellular stores during the primary culture (15), evidenced by the decrease of Ins-P_3 -induced Mn^{2+} quenching in 24-h cultured hepatocytes. S1P-induced Ca^{2+} mobilization was increased in 24-h cultured hepatocytes, whereas Ins-P_3 -binding sites were decreased in the cells (11, 15). Increased expression of S1P₂ receptor and increased production of Ins-P_3 could explain the increased Ca^{2+} mobilization by S1P in 24-h cultured hepatocytes, although future studies need to elucidate why such an inefficient signaling pathway was used by S1P in 24-h cultured hepatocytes (11, 14).

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