Effects of Sphingosine 1-Phosphate, a Naturally Occurring Biologically Active Lysophospholipid, on the Rat Cardiovascular System

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ABSTRACT—Recent studies have indicated that sphingosine 1-phosphate (Sph-1-P) is released into the blood flow from activated platelets upon stimulation to exhibit a wide spectrum of biological functions. The purpose of the present study was to assess the acute cardiovascular effects of circulating Sph-1-P in the *in vivo* rat model. Intravenous administration of Sph-1-P decreased the heart rate, ventricular contraction and blood pressure, while it hardly affected the atrioventricular and intraventricular conduction. Sph-1-P did not affect the adenylate cyclase activities of the membrane preparations made from the right atrium and left ventricle. These results suggest that functional receptors like lysophospholipid receptor Edg-1, which can inhibit adenylate cyclase via G_i protein, are lacking in the rat heart. Moreover, these observations will provide a clue to better understand the various types of Sph-1-P-related pathophysiological processes.

Keywords: Sphingosine 1-phosphate, Heart rate, Blood pressure, Contraction, Adenylate cyclase

Sphingosine 1-phosphate (Sph-1-P) is formed intracellularly by phosphorylation of sphingosine by sphingosine kinase, and it is degraded by Sph-1-P lyase in most living cells (1). While Sph-1-P has been implicated as an intermediary metabolite in sphingolipid metabolism cascade in the cells, recent studies have shown that Sph-1-P exhibits a wide spectrum of biological functions besides its role as an intermediary lipid metabolite (1–4).

Platelets lack Sph-1-P lyase activity, but possess strong sphingosine kinase activity, resulting in the accumulation of Sph-1-P (2, 3). It has been postulated that Sph-1-P released from activated platelets by an agonist like thrombin is involved in a variety of physiological and pathophysiological processes including hemostasis, thrombosis and atherosclerosis (1–5). However, precise analysis of the acute cardiovascular effects of Sph-1-P is still lacking, although such knowledge is obviously of major clinical importance.

The purpose of the present study was to assess the effects of Sph-1-P on the heart rate and systemic and left ventricular blood pressure in addition to the electrocardiogram (ECG) using the *in vivo* rat model (6). Since Sph-1-P has been reported to inhibit cyclic AMP production via G_i -coupled receptors in HEK cells (7) and Swiss 3T3 fibroblasts (8) and known to activate atrial muscarinic potassium current $I_{K(ACh)}$ of guinea pig (9), the effects of Sph-1-P on

the adenylate cyclase activity were examined in the cardiac tissue of rats to analyze the signal transduction systems of Sph-1-P.

MATERIALS AND METHODS

Animals were obtained through the Animal Laboratory for Research of Yamanashi Medical University. All experiments were performed in accordance with Guidelines for Animal Experiments of Yamanashi Medical University.

Assessment of the cardiovascular effects of Sph-1-P

Male Sprague-Dawley rats weighing 200–300 g were anesthetized by intraperitoneal injection of 60 mg/kg of pentobarbital sodium (6). The trachea was cannulated for artificial respiration with room air. Heparinized catheters were placed in the aorta and left ventricle via the right and left carotid arteries for continuous monitoring of the systemic and left ventricular pressure, respectively. The maximum upstroke velocity of the left ventricular pressure (LVdP/dt) and left ventricular end-diastolic pressure (LVEDP) were measured to estimate the contractility and preload of the left ventricle, respectively. The surface lead II ECG was obtained from the limb electrodes. Another heparinized catheter was inserted to the right femoral vein for the Sph-1-P or vehicle solution administration. The

ECG together with the systemic and left ventricular pressure was continuously monitored with a polygraph system (RM-6200; Nihon-Kohden, Tokyo) and recorded on a thermal recorder (WS-682G, Nihon-Kohden) at a paper speed of 200 mm/s.

The experiment was performed in the following order: The low dose of 1 $\mu\text{g}/\text{kg}$ ($=2.6 \text{ nmol}/\text{kg}$) of Sph-1-P solution was injected and the cardiovascular effects were observed for 3 min. Fifteen minutes after the initial injection, the vehicle solution of 1 $\mu\text{g}/\text{kg}$ of Sph-1-P solution was injected and the cardiovascular effects were observed for 3 min. After another 15 min, the same protocol was applied to assess the effects of the middle dose of 10 $\mu\text{g}/\text{kg}$ (26.3 nmol/kg) of Sph-1-P and its vehicle. Finally, 30 min after the assessment of the middle dose of Sph-1-P, the same protocol was used to examine the high dose of 100 $\mu\text{g}/\text{kg}$ (263.5 nmol/kg) of Sph-1-P and its vehicle. Because the effects of Sph-1-P were relatively short-lasting, which usually disappeared within 5 min except for the high dose, multiple doses of the substance were studied in the same animal.

Measurement of adenylate cyclase activity

Male Sprague-Dawley rats weighing 200–300 g were anesthetized by intraperitoneal injection of 60 mg/kg of pentobarbital sodium, and then their hearts were excised. The right atrium and left ventricle were trimmed, placed in the microcentrifugation tube and homogenized with 1 ml of ice cold SET buffer (0.25 M sucrose, 0.1 mM EDTA, 5.0 mM Tris-HCl) using a pellet mixer. The homogenate was filtered through a Nitex filter (Tetko, Los Angeles, CA, USA) and centrifuged at $10,000 \times g$ for 5 min at 4°C. The pellet was resuspended in 1 ml of SET buffer and the mixture was centrifuged three more times. The final pellet was resuspended in 500 μl of SET buffer. Protein analysis was performed using a commercially available protein assay reagent (Pierce, Rockford, IL, USA). The membrane suspension was diluted with SET buffer to a concentration of 3–5 mg protein/ml.

The adenylate cyclase activity of the membrane preparation was measured with an enzymatic fluorometric assay technique, as those previously described (10–12). A 10- μl aliquot of adenylate cyclase mix (100 mM Tris-acetate, pH 7.4; 20 mM KCl; 10 mM MgCl_2 ; 20 mM phosphoenolpyruvate; 2 mM ATP; 20 μM GTP; 2 mM dithiothreitol; 0.4 mg/l bovine serum albumin; 0.1 mM 3-isobutyl-1-methylxanthine (IBMX); 100 $\mu\text{g}/\text{ml}$ pyruvate kinase) was added to each microcentrifugation tube in duplicate with or without either of Sph-1-P (2×10^{-8} to 2×10^{-5} M), forskolin (2×10^{-4} M) or forskolin (2×10^{-4} M) plus Sph-1-P (2×10^{-8} to 2×10^{-4} M). Next, 10 μl membrane suspension was added to each tube. The reaction mixture and membrane suspension, both before and after being mixed, were

maintained at 4°C to ensure the same starting time for all assay tubes. The reaction was initiated by placing the tubes in a water bath maintained at 37°C. After 30 min, the reaction was terminated by heating at 90°C for 3 min. The mixture was vortexed 3 times and centrifuged at $10,000 \times g$ for 5 min. A 5- μl aliquot of the supernatant was transferred to a 10 $\times$ 75 mm disposable assay tube (Iwaki Lab Ware, Tokyo) in triplicate. For the cyclic AMP standard, 5 μl of a known amount of cyclic AMP was added to the tubes in triplicate. The cyclic AMP concentration was assayed using the enzymatic fluorometric method as previously described (10–12).

Drugs and biochemicals

The following drugs were purchased: pentobarbital sodium (Tokyo-Kasei, Tokyo), heparin calcium (Mitsui, Tokyo) and Sph-1-P (Biomol, Plymouth Meeting, PA, USA). Apyrase, phosphoenolpyruvate, phosphodiesterase, IBMX, sucrose, fructose, dithiothreitol and forskolin were purchased from Sigma Chemical Company (St. Louis, MO, USA). Sph-1-P (MW: 379.5) was dissolved in phosphate-buffered saline with 4 mg/ml of bovine serum albumin (Sigma) at a concentration of 100 $\mu\text{g}/\text{ml}$ and then diluted with the saline to prepare solutions of 10 $\mu\text{g}/\text{ml}$ and 1 $\mu\text{g}/\text{ml}$. The injected volume of the Sph-1-P solution was 0.2–0.3 ml. All other enzymes and substrates were obtained from Boehringer-Mannheim Company (Indianapolis, IN, USA).

Data analysis and statistics

The data are presented as the mean $\pm$ S.E.M. The statistical comparisons of mean values within a group were carried out using one-way repeated-measures analysis of variance (ANOVA) followed by Contrast for statistical analysis between basal values and others, whereas those between the groups were evaluated by the unpaired *t*-test as described before (13). A *P*-value less than 0.05 was considered significant.

RESULTS

Each control value was not significantly different between Sph-1-P ($n=9$) and vehicle administered groups ($n=9$). After the administration of each vehicle solution, no significant change was detected in each parameter during the observation period, as shown in Figs. 1–3.

Effects of Sph-1-P on blood pressure and heart rate

The time courses of the effects of Sph-1-P on the heart rate and the mean blood pressure are summarized in Fig. 1 ($n=9$). The basal heart rate (beats/min) and mean blood pressure (mmHg) for the low dose of 1 $\mu\text{g}/\text{kg}$ of Sph-1-P were 392 ± 8 and 122 ± 6 , respectively. After the adminis-

Fig. 1. The time course of effects of Sph-1-P on the heart rate (HR) and the mean blood pressure (mBP) (circles, $n=9$). The results of each vehicle are depicted by a shadowed line (squares, $n=9$). The data are presented as the mean $\pm$ S.E.M. Closed symbols represent significant differences ($P<0.05$) from the pre-drug control value (C).

Fig. 2. The time course of effects of Sph-1-P on the maximum upstroke velocity of the left ventricular pressure (LVdP/dt) and left ventricular end-diastolic pressure (LVEDP) (circles, $n=9$). The results of each vehicle are depicted by a shadowed line (squares, $n=9$). The data are presented as the mean $\pm$ S.E.M. Closed symbols represent significant differences ($P<0.05$) from the pre-drug control value (C).

tration of 1 µg/kg of Sph-1-P, no significant change was detected in these parameters. The basal heart rate (beats/min) and mean blood pressure (mmHg) for the middle dose of 10 µg/kg of Sph-1-P were 392 ± 9 and 121 ± 6 , respectively. After the administration of 10 µg/kg of Sph-1-P, the mean blood pressure decreased at 30 s, while no significant change was detected in the heart rate. The basal heart rate (beats/min) and mean blood pressure (mmHg) for the high dose of 100 µg/kg of Sph-1-P were 385 ± 10 and 116 ± 7 , respectively. After the administration of 100 µg/kg of Sph-1-P, the mean blood pressure decreased for

30 s to 1 min, and the heart rate decreased for 30 s to 2 min.

Effects of Sph-1-P on LVdP/dt and LVEDP

The time courses of the effects of Sph-1-P on the LVdP/dt and LVEDP are summarized in Fig. 2 ($n=9$). The basal LVdP/dt (mmHg/s) and LVEDP (mmHg) for the low dose of Sph-1-P were 6378 ± 288 and 1.4 ± 0.6 , respectively. After the administration of 1 µg/kg of Sph-1-P, no significant change was detected in these parameters during the observation period. The basal LVdP/dt

Fig. 3. The time course of effects of Sph-1-P on the PR interval and QRS width of the surface lead II electrocardiogram (circles, $n=9$). The results of each vehicle are depicted by a shadowed line (squares, $n=9$). The data are presented as the mean $\pm$ S.E.M.

(mmHg/s) and LVEDP (mmHg) for the middle dose of Sph-1-P were 6156 ± 316 and 1.6 ± 1.1 , respectively. After the administration of $10 \mu\text{g/kg}$ of Sph-1-P, no significant change was detected in these parameters during the observation period. The basal LVdP/dt (mmHg/s) and LVEDP (mmHg) for the high dose of Sph-1-P were 5956 ± 454 and 0.7 ± 0.8 , respectively. After the administration of $100 \mu\text{g/kg}$ of Sph-1-P, the LVdP/dt decreased for 30 s to 3 min, while no significant change was detected in the LVEDP.

Effects of Sph-1-P on ECG

The time courses of the effects of Sph-1-P on the PR interval and QRS width are summarized in Fig. 3 ($n=9$). The basal PR interval (ms) and QRS width (ms) for the low dose of Sph-1-P were 49 ± 2 and 28 ± 1 , respectively. No significant changes were observed in the PR interval and QRS width at any dose of Sph-1-P.

Effects of Sph-1-P on the adenylate cyclase activity

The effects of Sph-1-P, forskolin and forskolin plus Sph-1-P on the adenylate cyclase activity in membrane preparations obtained from the right atrium and left ventricle are summarized in Table 1 ($n=5$). Forskolin (10^{-4} M) significantly increased the adenylate cyclase activities of the membrane preparations of the right atrium and left ventricle, while Sph-1-P in concentrations of 10^{-8} to 10^{-5} M did not affect the adenylate cyclase activity of these membrane preparations. More importantly, Sph-1-P at concentrations of 10^{-8} to 10^{-5} M did not suppress the forskolin (10^{-4} M)-stimulated adenylate cyclase activities.

Table 1. Effects of Sph-1-P on the adenylate cyclase activity (pmol/mg per minute)

Forskolin (M)	Sph-1-P (M)	RA	LV
(-)	(-)	39.2 ± 3.8	44.9 ± 5.1
(-)	10^{-8}	38.0 ± 3.7	40.4 ± 4.4
(-)	10^{-7}	39.3 ± 4.1	44.8 ± 5.6
(-)	10^{-6}	38.3 ± 4.3	42.1 ± 5.5
(-)	10^{-5}	38.8 ± 4.2	45.0 ± 5.3
10^{-4}	(-)	$80.0 \pm 10.0^*$	$103.7 \pm 13.9^*$
10^{-4}	10^{-8}	$77.9 \pm 10.9^*$	$102.9 \pm 14.0^*$
10^{-4}	10^{-7}	$77.6 \pm 8.8^*$	$101.5 \pm 12.3^*$
10^{-4}	10^{-6}	$79.2 \pm 9.5^*$	$102.0 \pm 13.9^*$
10^{-4}	10^{-5}	$78.5 \pm 8.3^*$	$102.7 \pm 14.8^*$

The data are presented as the mean $\pm$ S.E.M. ($n=5$). * $P<0.01$, vs. vehicle solution (forskolin (-), Sph-1-P (-)). RA: right atrium, LV: left ventricle, Sph-1-P: sphingosine 1-phosphate, and (-): not added.

DISCUSSION

The present study was designed to assess the cardiovascular effects of Sph-1-P in the in vivo rat model. As clearly demonstrated in this study, Sph-1-P exerted negative chronotropic and inotropic effects and hypotensive action, while it hardly affected the atrioventricular and intraventricular conduction. We also assessed the effects of Sph-1-P on the adenylate cyclase activity for the first time in the membrane preparations made from the adult rat heart and found that Sph-1-P in a broad range of concentrations did not affect the adenylate cyclase activities of the right atrium or left ventricle.

It has been reported that Sph-1-P can activate the atrial muscarinic potassium current $I_{K(ACh)}$ of the guinea pig with an EC_{50} of 1.2 nM (9) and has been speculated that Sph-1-P can regulate the heart rate in vivo (1, 7, 14). In accord-

ance with these previous studies, Sph-1-P exerted the negative chronotropic effects in this study, but it hardly inhibited the adenylate cyclase activity of the right atrium. On the other hand, the negative inotropic action of Sph-1-P was observed, which has not been reported elsewhere, but Sph-1-P did not affect the adenylate cyclase activity of the left ventricular tissue, either. The lack of the effect of Sph-1-P on the adenylate cyclase activity as demonstrated in this study is in sharp contrast with the previous reports describing that Sph-1-P inhibits cyclic AMP production via G_i -coupled receptors in HEK cells with an EC_{50} of 2 nM (7) and Swiss 3T3 fibroblasts at 5 μ M (8). These contradictory observations may be due to the species difference in the tissue distribution of the Sph-1-P receptor-coupled signal transduction system, and they may indicate that functional receptors like lysophospholipid receptor Edg-1, which can inhibit the adenylate cyclase via G_i protein, are lacking in the rat heart (14, 15).

On the other hand, Sph-1-P has been shown to potently increase calcium concentration in human arterial smooth muscle cells (4). Contrary to what we expected from this previous knowledge, Sph-1-P decreased the mean blood pressure in a dose-related manner in vivo, which has not been reported elsewhere. Thus, the present results at least indicate that Sph-1-P may play an important role in the regulation of the vascular tone and blood pressure in vivo. However, more precise molecular mechanisms for this vascular effect must be determined.

In summary, Sph-1-P exerted negative chronotropic and inotropic effects in addition to hypotensive action without affecting the cardiac conduction in the in vivo rat, but Sph-1-P hardly affected the basal and forskolin-stimulated adenylate cyclase activity of the heart. These results suggest that functional receptors like Edg-1 are lacking in rat heart. Moreover, these observations will provide a clue to better understand the role of Sph-1-P in the various types of pathological conditions, such as the thrombotic disorders and blood transfusion toxicity (16), and may help to develop new diagnostic and therapeutic agents for these diseases.

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