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A polysaccharides MDG-1 augments survival in the ischemic heart by inducing S1P release and $S1P_1$ expression

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ABSTRACT

Ophiopogon japonicus is a traditional Chinese medicine used to treat cardiovascular disease. Recent studies have confirmed the anti-ischemic properties of a water-soluble β-D-fructan (MDG-1) from *O. japonicus*. The sphingosine 1-phosphate (S1P) signaling pathway is involved in its cytoprotective effects. Herein, we explore the role of the S1P signaling pathway in the anti-ischemic effect of MDG-1 and assess one possible mechanism by which it induces S1P release and sphingosine 1-phosphate receptor 1 ($S1P_1$) expression in human microvascular endothelial cells (HMEC-1) and cardiomyocytes. Our evidence demonstrates that MDG-1 promotes sphingosine kinase (SPHK) activity in HMEC-1 cells. An analytical method for measuring the mass of S1P using ESI/MS/MS was developed and we found that MDG-1 increases intracellular S1P levels. Meanwhile, MDG-1 is protective during hypoxia and ischemia through mechanisms that require $S1P_1$ receptor activation, which was confirmed both in oxygen glucose deprivation (OGD) and coronary artery ligation models by using transfection of cloned human $S1P_1$ receptor and RNA interference. These data indicate that the increase of intracellular S1P generation, particularly by activation of the SPHK enzyme, coupled with the autocrine and paracrine stimulation of cell surface S1P receptors, is a potential mechanism in the anti-ischemic and cell protective effect of MDG-1.

1. Introduction

Ophiopogon japonicus is widely distributed in Southeast Asia and is a traditional Chinese medicine which has been used to treat cardiovascular disease and diabetes for thousands of years. Although clinical evidence of its efficacy is lacking, this medicine has been confirmed in various experiments to have anti-ischemic and anti-arrhythmic properties. It inhibits platelet aggregation, protects the endothelium from apoptosis, and improves microcirculation [1,2]. Previous work suggested that a water-soluble polysaccharide, MDG-1, extracted from *O. japonicus* might have an important role in preventing myocardial ischemia and hypoglycemic effects [3,4]. MDG-1, a β-D-fructan with an average molecular weight of 3400 Da, constitutes approximately 4% of the total plant. MDG-1 contains a backbone composed of Fruf (2→1), and a side chain of Fruf (2→6) Fruf (2→) per average 2.8 of main chain residues and contains trace of α-D-Glc, which may connect to its reducing terminal (Fig. 1) [5]. MDG-1 protects myocardial cells from ischemia-induced death that occurs after coronary artery ligation in rats, protects cardiomyocyte from the damage induced by hypoxia/reoxygenation and restores cardiac contraction and coronary bloodflow. It also controls the increased heart rate caused by ischemia-reperfusion of isolated rat myocardium [3,6].

Sphingosine 1-phosphate (S1P), part of an intracellular signaling pathway involved in cell survival and angiogenesis, is critical to the development and therapy of ischemic disease [7]. Sphingosine kinase (SPHK) promotes the phosphorylation of sphingosine to form S1P. Two isoforms of SPHK (SPHK1 and SPHK2) have been characterized. Many agonists activate and induce translocation of SPHK1 to the plasma membrane, causing a rapid increase in S1P production. S1P interacts with five specific S1P receptors belonging to the G protein coupled receptors (GPCR) family [8,9]. Recent studies demonstrated that S1P protects the heart against ischemia-reperfusion injury when administered prior to the index ischemia (preconditioning) and mediates myocardial ischemic preconditioning in isolated mouse hearts [10–12]. This protection requires SPHK1, which is the key enzyme that catalyzes the formation of S1P, and is likely to depend on exported S1P to initiate a cell survival signaling cascade [13]. Activation of SPHK1 and S1P receptor

Abbreviations: HMEC-1, human microvascular endothelial cells-1; OGD, oxygen glucose deprivation; S1P, sphingosine 1-phosphate; SPHK, sphingosine kinase; $S1P_1$, sphingosine 1-phosphate receptor 1; ERK, extracellular signal regulated kinase; SH, sham operation; AMI, acute myocardial ischemia.

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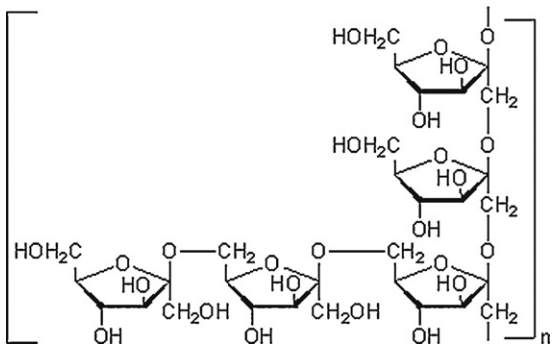

Fig. 1. The repeating unit structure of the MDG-1 from *Ophiopogon japonicus*, and MDG-1 is β-D-fructosan with an average molecular weight of 3400 Da, containing a backbone composed of Fruf (2→1), and a branch of Fruf (2→6) Fruf (2→) per average 2.8 of main chain residues and trace of α-D-Glc, which maybe connect to its reducing terminal.

1 ($S1P_1$) is important to prevent apoptosis and promote angiogenesis [14–16]. We recently reported that MDG-1 upregulates SPHK1 and $S1P_1$ expression. MDG-1 shows anti-ischemic activity and protects cardiomyocytes and HMEC-1 cells from ischemia-induced cell damage *via* the S1P/bFGF/Akt/ERK/eNOS signaling pathway [6].

To explore the role by which the S1P signaling pathway plays an anti-ischemic effect of MDG-1, the present study focused on one possible mechanism by which MDG-1 may induce S1P release and $S1P_1$ expression in human microvascular endothelial cells (HMEC-1). Microvascular endothelium is associated directly in angiogenesis and is distributed widely throughout cardiac tissue and on cardiomyocytes. Our evidence demonstrates that MDG-1 induces SPHK activation and endogenous S1P production, and the $S1P_1$ plays an important role in the cytoprotective effect of MDG-1.

2. Materials and methods

2.1. Preparation of MDG-1

MDG-1 was prepared by extraction from *O. japonicus* (Cixi, Zhejiang Province, China) and purified as previously described [5]. Briefly, soluble components of the tuberous roots of *O. japonicus* were extracted with ten volumes of water at 95–100 °C followed by dialysis. The concentrated and dialyzed extract was precipitated by the addition of four volumes of 95% ethanol. The resulting precipitate was redissolved and centrifuged. Crude polysaccharide was lyophilized from the supernatant and further purified by ultrafiltration (membrane MW cut-off: 10,000) under pressure (0.3 MPa). Products with molecular weights estimated to be less than 10,000 were fractionated on DEAE Sepharose Fast Flow and eluted with distilled water. The major fraction was pooled, dialyzed, concentrated, lyophilized and MGD-1 was purified using a Sephadex G-25 column eluted with H_2O.

2.2. Cell culture

Human microvascular endothelial cells were maintained in MCDB131 medium containing 10% FBS (v/v), 2 mM L-glutamine, 5 ng/ml epidermal growth factor (EGF) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) at 37 °C in a 5% CO_2 incubator.

2.3. Cell treatment, membrane preparation, and measurement of SPHK activity

Cells were uniformly seeded (5×10^5 cells/well) in 6-well plates. After MDG-1 stimulation with the indicated concentration for 24 h, cells were washed twice with ice-cold PBS and then scraped into 100 μl lysis buffer [20 mm Tris (pH 7.5), 10 nm EDTA, 2 mm EGTA, 250 mm sucrose, 1 mm PMSF, 10 nm okadaic acid, 1 mm dithiothreitol, 5 mm NaF, 1 mm Na_3VO_4, 0.5 mm 4-deoxypyridoxine, and 10 μg/ml leupeptin and aprotinin]. Cell suspensions were freeze-thawed several times, and cell homogenates were clarified by centrifugation at $700 \times g$ for 10 min. Homogenates were separated into membrane and cytosolic fractions by centrifugation at $100{,}000 \times g$ for 60 min at 4 °C. The membrane fractions were resuspended in SPHK buffer. Sphingosine kinase activity in cell membrane lysates (5 μg protein) was measured using a SPHK biochemical fluorescence based assay kit (Gyrasol Technologies, Santa Fe, NM, USA) according to the manufacturer's instructions. The SPHK activity was determined by comparing fluorescence using 540 nm excitation and 580 nm emission.

2.4. Mass measurements of S1P

Human microvascular endothelial cells were seeded (5×10^5 cells/well) in 6-well plates. After MDG-1 stimulation with the indicated concentration for 24 h, cells were lysed with an equal volume of RIPA buffer. Briefly, 90 μl of lysate added with 5 μl ammonia water was transferred to a clean 96-well plate, 2-fold volume of buspirone solution (acetonitrile containing 0.14 N HCl). The mixture was centrifuged at 3500 rpm for 5 min. The supernatant was mixed with equal volume of water and 10 μl of the final mixture was injected for LC–MS/MS analysis. A Shimadzu LC-20A liquid chromatographic system equipped with a DGL-20A vacuum degasser, a dual pump, and a SIL-20A autosampler (Shimadzu) was used. Detection was performed on an ABI 4000 mass spectrometer equipped with TurboIonSpray (ESI) Interface (Applied Biosystems, Concord, Ontario, Canada). Analyst 1.5 software packages (Applied Biosystems) were used to control the LC–MS/MS system, as well as for data acquisition and processing. Chromatographic separation was achieved on the Thermo Hypersil Gold C18 (50 mm × 2.1 mm, 5 μm) column. The mobile phase consisted of eluent A (water/formic acid [100:0.1, v/v]) and eluent B (acetonitrile/formic acid [100:0.1, v/v]). The gradient was as follows: From $t = 0$ to 1.0 min A/B 90:10, followed from $t = 1.0$ to 1.3 min by a linear gradient from 90:10 to 10:90, then from $t = 1.3$ to 2.2 min 10:90, from $t = 2.2$ to 2.5 min a linear gradient from 10:90 to 90:10 and finally from $t = 2.5$ to 3 min A/B 90:10. The flow rate was set at 1.0 ml/min. S1P and the internal standard buspirone eluted at 1.9 and 1.77 min, respectively. The mass spectrometer was operated in the positive ion mode with an electrospray voltage of 4000 V at 500 °C. Precursor-to-product ion transitions of m/z 380.4→264.4 for S1P (collision energy 23 eV), m/z 386.2→122.4 for buspirone (45 V).

2.5. Cloning of human $S1P_1$ receptor and cell transfection

$S1P_1$ was amplified by PCR from human full length cDNA clone (imaGene, Berlin, Germany) using Ex Taq polymerase (Takara Biomedicals, Otsu, Japan) using primers 5′-CATTGAAGCTTCCACCATGGGGCCCACCAGCTG and 5′-CATTGTC TAGACTGGAAGAAGAGTTGA, which include terminal Xba and *Hind*III restriction sites, respectively. Using these sites the amplified fragment was cloned into the expression vector pcDNA3.1b (Clontech) in-frame; the amino acid sequence of the insert was identical to the $S1P_1$ protein sequence in SwissProt (P21453). HMEC-1 cells were grown to ~70% confluency on 35-mm plates. Transfection was performed with FuGENE HD transfection reagent (Roche Applied Science) according to the manufacturer's instructions. Human microvascular endothelial cells were transfected with the empty vector pcDNA3.1b or the expression construct, S1P1-pcDNA3.1b, for 48 h.