

Review article

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CARDIOPROTECTIVE ROLE OF SPHINGOSINE-1-PHOSPHATE

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Sphingosine-1-phosphate (S1P) has been shown to exert potent cardioprotective action. It binds to the plasma membrane receptors. Three S1P receptors are present in the heart: 1, 2 and 3. Exogenous S1P increases viability of cardiomyocytes incubated under hypoxic conditions. It also reduces the infarct size in isolated, perfused rat heart after ischemia/reperfusion. Formation of S1P in the heart is catalyzed by the enzyme sphingosine kinase 1 and its catabolism by the enzyme sphingosine 1-phosphate lyase. Reduction in the activity of sphingosine kinase 1 or knocking out its gene eliminates cardioprotective effect of ischemic preconditioning in mice. Knocking out the sphingosine-1-phosphate lyase gene exerts potent cardioprotective effect against ischemia/reperfusion in the mouse heart. The following mechanism of cardioprotection by S1P is suggested: S1P binds to its membrane receptors and the complex activates protein G. Activated protein G activates PI3K which in turn activates pro-survival Akt kinase. S1P activates also Stat3, a powerful cardioprotectant. It should be added that ischemia/reperfusion increases markedly the myocardial content of two other sphingolipids, namely sphingosine and ceramide. Sphingosine, a direct precursor of S1P, in a high dose is cardiotoxic. Ceramide (the key sphingolipid, a precursor of sphingosine) is claimed to be responsible for activation of apoptosis during ischemia/reperfusion. Therefore, the two latter sphingolipids may counteract, to a certain degree, the cardioprotective action of S1P. It is suggested, that development of specific S1P agonists and compounds modifying activity of the two enzymes could provide important therapeutic tools in the treatment of the heart infarct.

Key words: *ceramide, heart infarct, ischemia/reperfusion, ischemic preconditioning, post-ischemic preconditioning, sphingolipids, sphingosine-1-phosphate, sphingosine-1-phosphate lyase, sphingosine kinase*

Abbreviations used: S1P – sphingosine-1-phosphate; SPH – sphingosine; S1P1-5 – S1P1-5 receptors; I/R – period of ischemia followed by reperfusion; IPC – ischemic preconditioning (it combines several short cycles: ischemia-reperfusion before ischemia); IPOST – ischemic postconditioning (it combines several very short cycles: ischemia-reperfusion between ischemia and reperfusion); LVDP – left ventricle developed pressure; LVEDP – left ventricle end diastolic pressure; GM-1 – ganglioside activating sphingosine kinase; SK – sphingosine kinase; S1PL – sphingosine-1-phosphate lyase; PI3K – phosphatidylinositol 3-kinase; PKC – protein kinase C; VPC – VPC23019 (it is a compound blocking cardiac S1P receptors 1, 2 and 3); FTY – FTY720 (it is a nonspecific agonist of S1P1 and 3 cardiac receptors); SEW – SEW2871 (it is a specific agonist of cardiac S1P1 receptor)

INTRODUCTION

Sphingolipids are present in all eukaryotic cells. The backbone of all sphingolipids is ceramide. It is composed of a long chain sphingoid base and a fatty acid residue. Complex sphingolipids contain also other residues. Ceramide is located on the crossroads of sphingolipid metabolism. It is formed either from sphingomyelin or on *de novo* synthesis pathway. Ceramide may be

converted either back to sphingomyelin, or to ceramide-1-phosphate, glucosylceramide and galactosylceramide. The first step of ceramide catabolism is its deacylation and release of free sphingosine. Sphingosine can be phosphorylated to form sphingosine-1-phosphate (1, 2). A scheme of sphingolipid metabolism including names of the enzymes is presented in *Fig. 1*.

Certain sphingolipids, mainly ceramide, sphingosine-1-phosphate, sphingosine, sphinganine and ceramide-1-phosphate exert broad biological effects (3-5). In recent years, many data have been collected indicating the role of some bioactive sphingolipids, namely sphingosine-1-phosphate, sphingosine and ceramide in cardioprotection. Sphingosine-1-phosphate (S1P) exerts very strong cardioprotective effect.

SOURCES OF SPHINGOSINE-1-PHOSPHATE IN PLASMA

Erythrocytes are the main source of S1P in the plasma. Other sources of S1P in the plasma are platelets and endothelial cells (6, 7). The concentration of S1P in human plasma ranges from 200 to 1000 nM (8-11). 50–70% of total S1P in the plasma is transported by high density lipoproteins (HDL), about 30% by albumin and <10% by low density lipoproteins (LDL) and very low density lipoproteins (VLDL) (9-11).

of S1P (20). It was shown that infusion of TNF- α , ceramide and S1P reduces the infarct size after ligation of the coronary artery to similar degree as IPC. The presence of N-oleoylethanolamine, an inhibitor of ceramidase (*Fig. 1*) in the perfusion medium considerably reduces the protective role of IPC, TNF- α and ceramide on the infarct size and left ventricular developed pressure (LVDP) after I/R. Inhibition of ceramide catabolism results, most likely, in elevation of its content in the heart. Contrary to ceramide itself, the protective effect ceramide + the inhibitor was very weak. It strongly indicates that the cardioprotective effect of TNF- α was finally mediated by S1P, the product of ceramide catabolism and not by ceramide itself. Also, adding of S1P to the perfusion medium during reperfusion after ischemia increases the recovery of LVDP to over 80% of the control value and reduces the infarct size of the left ventricle to 8%. In comparison, the respective values with S1P-free perfusion medium are ~8 and ~45% (21). Lower dose of the compound was less effective (22). The results obtained on isolated, perfused mouse heart (23) were also similar to the results obtained in isolated cardiomyocytes (18). The hearts were perfused before I/R either with S1P or GM-1 (it is a ganglioside activating sphingosine kinase and increasing the content of S1P). Each compound reduced the infarct size by about 50%, accelerated the recovery of LVDP and reduced left ventricular end diastolic pressure (LVEDP). Additionally, they reduced the liberation of creatine kinase from the heart after I/R. More data on an involvement of S1P in the cardioprotection of isolated, perfused heart are presented below in the section describing a role of particular enzymes and receptors in the cardioprotection induced by S1P.

INVOLVEMENT OF PARTICULAR SPHINGOSINE-1-PHOSPHATE RECEPTORS IN CARDIOPROTECTION

As it has already been mentioned (6, 13), there exist three S1P receptors in the heart, namely S1P1, S1P2 and S1P3. It raises a question on the role of the particular receptors in the process of cardioprotection. However, the present data regarding this question are rather incomplete. In mouse, either S1P2 or S1P3 or both receptors were knocked out and the animals were subjected to I/R procedure *in vivo*. Knocking out only one receptor (either S1P2 or S1P3) did not affect the infarct size after I/R. However knocking out the two receptors increased the infarct size by over 50%. Knocking out S1P1 receptor is lethal at the embryonic stage of life so that S1P1 knockout mice were unavailable for the experiment (24). Theilmeier *et al.* (25) showed in the mice that intravenous infusion of S1P before I/R reduced the infarct size by 32-40%, depending on a dose of the compound. Knocking out of S1P3 receptor blocks this protective action of intravenous S1P on the infarct size. Another approach to study the role of S1P receptors was to use their agonists and blockers. It was shown that blockade of S1P1 and 3 receptors by means of a compound VPC23019 (VPC) markedly reduced the protective effect of both IPC and IPOST (ischemic postconditioning) on the infarct size as well on LVDP after I/R in isolated, perfused rat heart. The compound also reduced the protective effect of IPC on viability of isolated rat cardiomyocytes after *in vitro* I/R (19). Both unspecific S1P receptor agonist (FTY720, FTY; in the heart it binds to S1P1 and 3 receptors) and specific agonist of the S1P1 (SEW2871, SEW) exerted a protective action similar to S1P in isolated cardiomyocytes subjected to the I/R procedure. These effects of the agonists were blocked by VPC (18, 26). The results obtained with the use of agonists in isolated, perfused rat heart are somewhat puzzling. FTY added at the beginning of reperfusion, after a period of ischemia, did not influence the infarct size but

accelerated recovery of LVDP, reduced the level of LVEDP and the number of apoptotic cardiomyocytes. SEW added under the same conditions did not affect either the infarct size, the hemodynamic parameters or the level of apoptosis (26). FTY introduced during reperfusion additionally accelerated the recovery of contractility of isolated human atria stripes (SEW was not studied in this case) (26). *In vivo* study confirmed the lack of influence of FTY on the infarct size in rat heart after I/R. It was further shown that the compound increased the mortality of the animals as a consequence of fatal arrhythmias (27). The above data indicate that the outcome of stimulation of particular S1P receptors during I/R may depend on the type of receptor. The particular receptors bind to different G proteins and it may be a reason for the differences between the effect of particular agonists (28). Certainly, much more data are needed to get broader knowledge on this topic. Nevertheless, it is clear that specific functions played by particular heart S1P receptors should be taken into account in the prospective studies.

ROLE OF ENZYMES OF SPHINGOSINE-1-PHOSPHATE METABOLISM IN CARDIOPROTECTION

The level of S1P in most cells is regulated by the activity of two enzymes: sphingosine kinase (SK) and sphingosine-1-phosphate lyase (S1PL) (*Fig. 1*).

Sphingosine kinase

Sphingosine kinase (SK) catalyzes the conversion of sphingosine to S1P. There are two isoforms of SK: 1 and 2. Isoform 1 prevails in the heart muscle (30). Preincubation of isolated neonatal rat cardiomyocytes with N,N-dimethylsphingosine (DMS, an inhibitor of SK) markedly increases the number of dead cardiomyocytes cultured under normoxic conditions. This action of DMS is counteracted by S1P and ganglioside GM-1, an activator of SK (17). IPC activates SK1, increases the level of S1P and reduces the infarct size after I/R in isolated, perfused mouse heart. Inhibition of SK by DMS and in consequence the inhibition of elevation in the level of S1P by IPC eliminates the beneficial effect of IPC on the infarct size as well as LVDP and LVEDP after I/R (31). In further studies, DMS used in a much lower dose showed a cardioprotective effect in isolated perfused mouse heart. This effect was absent in PKC ϵ knockout mice. It was speculated that DMS, at low dose, activates PKC ϵ which in turn activates cytosolic SK1 and increases intracellular content of S1P (32). In the isolated, perfused heart of the rat ischemia was shown to inhibit SK1 activity by 61% and it remained on this level after subsequent reperfusion. IPC reduced this drop in the enzyme activity by half and partially prevented the reduction in the S1P level in the heart after I/R. It also reduced the infarct size and accelerated the recovery of LVDP. Also, adding S1P to the perfusion medium during reperfusion improved the recovery of LVDP (22). Knocking out SK1 gene in mice reduces the viability of isolated cardiomyocytes incubated under hypoxic conditions. Also, it increases the amount of cytochrome C released from mitochondria. Adding S1P to the incubation medium under the same conditions increased the viability of cardiomyocytes obtained both from wild and SK1 knockout mice. However, the effective dose of the compound in case of cardiomyocytes obtained from the wild animals was lower than in case of cardiomyocytes obtained from SK1 knockout mice. GM-1 also increases the survival of cardiomyocytes obtained from the wild mice but it does not have an effect in cardiomyocytes obtained from the SK1 knockout mice. Both VPC and inactivation of G $_i$ protein (by means of the pertussis toxin) eliminates the influence

of GM-1. These data indicate that S1P generated by GM-1 leaves the cell and acts either in autocrine or paracrine way (33). The results obtained in isolated, perfused heart of the mouse confirmed these observations (34). Also, SK1 knockout mice did not respond to the cardioprotective impact of either IPC or IPOST during I/R studied in isolated, perfused heart (35). On the other hand, in rats, overexpression of SK1 gene attenuated the reduction in LVDP and elevation in LVEDP after ligation of the left coronary artery (36). Taken together, the data on a role of SK1 in cardioprotection clearly show that formation of S1P from sphingosine by this enzyme plays a crucial role in the process.

Sphingosine-1-phosphate lyase

Sphingosine-1-phosphate lyase (S1PL) catalyzes the irreversible breakdown of S1P (*Fig. 1*). Recently, the data were presented showing that S1PL is involved in cardioprotection. The experiments were carried out on isolated, perfused hearts of wild and S1PL knockout mice. Ischemia increased the activity of the enzyme in the heart of wild mice. This elevation was prevented by IPC. In the S1PL gene knockout mice, the heart S1P content was elevated compared to the controls. Knocking out the gene reduced the infarct size and accelerated the LVDP recovery after I/R. Inhibition of S1PL by means of THI (tetrahydroxybutylimidazole) additionally increased the level of S1P. It also reduced the infarct size and accelerated the recovery of LVDP after I/R. These data indicate that the reduced activity of S1PL, same as the increased activity of SK1, elevates S1P content and exerts cardioprotective action against I/R (37). It is suggested that manipulation in the activity of the two enzymes may to be a promising therapeutic target (38).

INTRACELLULAR PATHWAYS OF SPHINGOSINE-1-PHOSPHATE ACTION IN CARDIOPROTECTION

The intracellular pathways of the cardioprotective action of S1P are only poorly investigated. Available data indicates that Akt kinase plays a key role in mediating the cardioprotective action of S1P. It was shown that hypoxia itself as well as I/R activates very strongly Akt both in cultured cardiomyocytes and in isolated, perfused hearts of mice. This activation of Akt is accompanied by reduction in the number of apoptotic cardiomyocytes, reduction of infarct size, acceleration of recovery in LVDP and reduction in LVEDP in the mouse heart after I/R (18, 25, 39, 40). S1P and an antibody named 4B5.2, a selective activator of S1P1 receptor, markedly increase the activity of the enzyme in isolated mice cardiomyocytes (18, 25). Knocking out either S1P2 or S1P3 in the mice is accompanied only by a minor reduction in the activation of Akt by S1P. However, knockout of both receptors attenuates the process (25). Knocking out both receptors also blocks activation of Akt by I/R (25). Also, blockade of S1P1 receptors (by VPC at concentration of 100 nmol/l at which it does not interfere with S1P3 receptors) blocks activation of Akt by S1P in isolated mice cardiomyocytes (18). Inactivation of protein G₁ (by pertussis toxin) or inhibition of PI3K (by wortmannin) blocks activation of Akt both by S1P and hypoxia (18). These data confirm the key role of S1P in activation of Akt in the process of cardioprotection. They also indicate that the ways of activation of Akt through S1P receptors are different. It is certainly due to the activation of different G proteins by particular S1P receptors (41). Hypoxia, S1P and 4B5.2 also block the elevation in the activity of glycogen synthase kinase-3 β (they cause its phosphorylation) which has protective effect on mitochondria. These data suggest the following sequence of events: activation of S1P receptors activates protein G₁ which in

turn activates PI3K. Activated PI3K activates Akt and inactivates glycogen synthase kinase-3 β (18). Inhibition of protein kinase C (by chelethryne) blocks the protective action of S1P in cultured neonatal rat cardiomyocytes against hypoxia (17). Other studies showed that it is the PKC _{ϵ} isoform of protein kinase C which is responsible for cardioprotection (23). However, knocking out PKC _{ϵ} gene did not affect the cardioprotective impact of S1P. It indicates that S1P exerts its action independently of PKC _{ϵ} (23). It was suggested, that the previously (17) observed effect of protein kinase C blockade by chelethryne was a result of an unspecific action of this compound. I/R also activates ERK, JNK and p38 MAP kinases. However, removal of both S1P2 and S1P3 receptors did not affect the heart response to I/R. It indicates that the S1P receptors are not involved in the activation of the kinases by I/R (25). The data collected by Boengler *et al.* (42) clearly show that activation (phosphorylation) of the signal transducer and activator of transcription 3 (Stat3) exerts strong cardioprotective action against I/R injury. The mechanism of Stat3 activation during I/R was elucidated by Frias *et al.* (43). They showed in isolated neonatal rat cardiomyocytes that S1P as well as reconstituted HDL (rHDL) enriched in S1P activates Stat3. They further showed using agonists and antagonists of different S1P receptor types that S1P activates Stat3 mainly through the S1P2 receptor. The latter data further support the role of S1P in cardioprotection and show that the compound exerts its effect not only by activation of Akt but also Stat3.

A ROLE OF OTHER BIOACTIVE SPHINGOLIPIDS IN ISCHEMIA/REPERFUSION INJURY

So far, two other sphingolipids, namely sphingosine and ceramide, were shown to be involved in I/R injury.

Sphingosine

Sphingosine (SPH) is a product of hydrolysis of ceramide and a precursor of S1P (*Fig. 1*). Ischemia of isolated, perfused rabbit heart as well as hypoxia of isolated cardiomyocytes obtained from an adult rat results in a several-fold elevation in the content of SPH (44). Vessey *et al.* (45) showed, that SPH in a high dose (5 μ M) in a perfusion medium increases the infarct size after I/R in isolated, perfused rat heart. It indicates that elevation in the content of sphingosine during I/R may be cardiotoxic. However, it should be added that physiological concentration (0.4 μ M) of SPH added either before ischemia or during reperfusion reduced the infarct size from over 45% to only 6% of the left ventricle. It also markedly accelerated the recovery of LVDP. However, blockade of S1P1 and 3 receptors with the VPC does not prevent the cardioprotective action of this compound (contrary to S1P) in the perfused rat heart. Also, inhibition of the PKC does not block the cardioprotective action of SPH. However, this effect is blocked by inhibition of protein kinase A or G. It clearly indicates that SPH is acting on a different pathway than S1P, namely on the pathway involving cyclic nucleotides and not the S1P receptors (45).

Ceramide

Ceramide plays very important role in the heart pathology. Accumulation of ceramide was claimed to be responsible for lipoapoptosis, cardiomyopathy and loss of myocardial function in obese rats (46). In LpL^{OP1} mice (mice with overexpression of a glycosylphosphatidylinositol membrane- anchored form of lipoprotein lipase in cardiomyocytes) the heart ceramide content is elevated and dilated cardiomyopathy develop.

Inhibition of *de novo* ceramide synthesis is accompanied with improved cardiac function, reduction in utilization of fatty acids, increased oxidation of glucose as well with reduced mortality (47). Myocardial ceramide metabolism is much regulated by PPAR α . Cardiac-specific overexpression of this receptor in high-fat fed mice results in accumulation of ceramide in the myocardium (48). Also, in high-fat fed rats, pharmacological stimulation of PPAR α (with WY-14643, a selective PPAR α agonist) resulted in elevation in the content of the heart of ceramide (49). It should also be added, that the content of ceramide in myocardium of obese and diabetic human subjects did not differ from that in the lean ones. (50). It was repeatedly shown that I/R increased the concentration of ceramide *in vivo* in the hearts of rats and rabbits (51, 52), in the perfused rat heart (53, 54) and in isolated cardiomyocytes (55). It was also shown in isolated rat heart that I/R did not elevate each of the 14 identified ceramides but only 7 of them. The ceramides differ in the fatty acid residues (53). Biological importance of this fact has not been explained so far. Inhibition of sphingomyelinase, the enzyme which hydrolyzes sphingomyelin to ceramide (Fig. 1), reduces the increase in the content of ceramide after I/R (51, 54). Ceramide is a potent activator of apoptosis of different cell types including cardiomyocytes (3, 4, 5, 55). The elevation in the content of ceramide is accompanied by augmentation of apoptosis in the ischemic area of the isolated heart (51, 54) as well as in the hypoxic isolated cardiomyocytes (55). It suggests that elevation in the content of ceramide could contribute to the augmentation of apoptosis after I/R. IPC reduces the content of ceramide during I/R and it may add to the mechanisms of cardioprotection by IPC (54). Dear *et al.* (56) put forward a possibility of another mechanism of the action of ceramide. They examined the effect of IR and IPC/IR on the level of ceramide, S1P and the expression of endothelial isoform of nitric oxide synthase (eNOS) in calveolae isolated from *ex vivo* perfused rat heart. I/R increased several fold the content of ceramide and the expression of eNOS bound to protein named caveolin-1 and did not increase the content of S1P in the calveolae. IPC reduced the elevation in the content of ceramide and the expression of eNOS bound to caveolin-1 after I/R but elevated manifold the content of S1P in the calveolae. It would suggest that I/R inactivates the eNOS in calveolae by increasing the binding of the enzyme with caveolin 1. This binding is augmented by ceramide. Subsequently, the availability of active eNOS and as result production of nitric oxide are diminished. Reduction in the content of ceramide and elevation in the content of S1P by IPC prevents the binding of eNOS to caveolin 1 and in consequence increases the production of cardioprotective nitric oxide.

BIOACTIVE SPHINGOLIPIDS IN THE HEART INFARCT IN HUMAN BEINGS

In patients, early after the myocardial infarction the concentration of S1P in the plasma decreases by about 50% and of sphinganine-1-phosphate by about 40% whereas the concentration of ceramide, sphinganine and sphingosine remains stable. In most patients, further reduction in the concentration of S1P and sphinganine-1-phosphate was observed on the fifth day after the infarction. As already mentioned, platelets are a source of S1P. Treatment of the patients with aspirin, which has antiplatelet effect, could be at least one reason responsible for the reduction in the concentration of S1P. The reduction in the concentration of S1P in the plasma after the infarction reduces, undoubtedly, its cardioprotective action (8). To date, there are no data on a role of sphinganine-1-phosphate present in the plasma in cardioprotection.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Sphingosine-1-phosphate (S1P) has emerged as a very powerful cardioprotective factor. Exogenous S1P increases viability of cardiomyocytes incubated under hypoxic conditions and reduces the infarct size in isolated, perfused rat heart after I/R. It also mediates the beneficial effect of pre- and post-conditioning in the heart subjected to I/R. Formation of S1P in the heart is catalyzed by the enzyme sphingosine kinase 1 and its catabolism by the enzyme sphingosine-1-phosphate lyase. Reduction in the activity of sphingosine kinase 1 or knocking out its gene eliminates cardioprotective effect of ischemic preconditioning in mice. Knocking out the sphingosine-1-phosphate lyase gene has a very potent cardioprotective effect against I/R injury in the mouse heart. It is postulated that S1P exerts its cardioprotective action by activation of Akt kinase and Stat3.

The great variety of biological effects of S1P tempted to develop specific compounds acting on the activity of SK1 or SPL or binding to particular S1P receptors with an idea to introduce them to therapy (57). So far, a compound called Fingolimod (FTY720) was shown to have strong immunomodulating properties and has been approved by US Food and Drug Administration as a drug for treatment of multiple sclerosis (58, 59). It is to believe that development new drugs mimicking the cardioprotective properties of S1P is under the way.

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