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Sphingolipid Therapy in Myocardial Ischemia-Reperfusion Injury

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Abstract

Sphingolipids are known to play a significant physiological role in cell growth, cell differentiation, and critical signal transduction pathways. Recent studies have demonstrated a significant role of sphingolipids and their metabolites in the pathogenesis of myocardial ischemia-reperfusion injury. Our laboratory has investigated the cytoprotective effects of *N*,*N*,*N*-Trimethylsphingosine chloride (TMS), a stable *N*-methylated synthetic sphingolipid analogue on myocardial and hepatic ischemia reperfusion injury in clinically relevant *in vivo* murine models of ischemia-reperfusion injury. TMS administered intravenously at the onset of ischemia reduced myocardial infarct size in the wild-type and obese (ob/ob) mice. Following myocardial I/R, there was an improvement in cardiac function in the wild-type mice. Additionally, TMS also decreased serum liver enzymes following hepatic I/R in wild-type mice. The cytoprotective effects did not extend to the ob/ob mice following hepatic I/R or to the db/db mice following both myocardial and hepatic I/R. Our data suggests that although TMS is cytoprotective following I/R in normal animals, the cytoprotective actions of TMS are largely attenuated in obese and diabetic animals which may be due to altered signaling mechanisms in these animal models. Here we review the therapeutic role of TMS and other sphingolipids in the pathogenesis of myocardial ischemia reperfusion injury and their possible mechanisms of cardioprotection.

Keywords

myocardial infarction; *N*,*N*,*N*-Trimethylsphingosine; Diabetes; Obesity; db/db; ob/ob

Acute Myocardial Infarction

Cardiovascular disease is expected to be the major cause of death globally due to its increasing prevalence in developing countries and the rising incidence of obesity and diabetes in the Western world. Cardiovascular disease, including coronary artery disease is responsible for 38 percent of all deaths in North America and is the most common cause of death in European men under 65 years of age and the second most common cause in women [1]. Approximately 1.5 million individuals in the United States suffer from myocardial infarction annually and approximately one third of them die [2]. The estimated direct and indirect costs of coronary artery disease in the United States amount to $ 142.5 billion [2]. Given the enormous public health problem that it represents, basic science and clinical research studies have focused

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extensively on understanding the pathophysiology of myocardial injury in coronary artery disease and myocardial infarction.

Myocardial infarction is characterized by irreversible cell injury caused due cessation of coronary blood flow. Advances in modern medicine have now made it possible to reperfuse the ischemic myocardium early through pharmacological and surgical strategies. However, although reperfusion of ischemic myocardium is important in prevention of necrosis and minimizing tissue injury, evidence from animal studies suggests that reperfusion of ischemic areas, in particular the readmission of oxygen contributes to further tissue damage [3,4]. Ischemia-Reperfusion (I/R) injury is characterized by the formation of oxygen radicals upon re-introduction of molecular oxygen to ischemic tissue, resulting in widespread lipid and protein oxidative modifications, mitochondrial injury, and cell death [4–6]. It is also characterized by an inappropriate inflammatory response in the microcirculation, resulting in an increase in leukocyte-endothelial cell interactions mediated by the up-regulation of both leukocyte and endothelial cell adhesion molecules [7,8]. The resulting increase in leukocyte accumulation and activation leads to massive release of tissue proteases and NADPH mediated free radical production further exacerbating endothelial cell, smooth muscle and microvascular injury [9]. The myocardial injury is further compounded by the “no reflow” phenomenon caused by endothelial cell swelling, impaired nitric oxide release and capillary occlusion with platelets and neutrophils [10]. All of these mechanisms lead to myocardial necrosis following I/R. It has been suggested that cell death during myocardial ischemia occurs by two different mechanisms, necrosis and apoptosis [11]. Many cells that are alive by the end of ischemia eventually die by apoptosis during reperfusion [12,13]. Experimental studies suggest that prolonged ischemia may lead to intracellular changes that initiate apoptosis and normalization of cellular metabolism through the restoration of blood flow may cause the progression of the process that exacerbates tissue injury. Intensive research efforts have focused on the amelioration of various pathophysiological components of I/R injury to limit the extent of myocardial injury from necrosis and apoptosis.

The present review article will summarize the current knowledge regarding the efficacy of sphingolipid therapy in experimental models of myocardial ischemia-reperfusion injury.

Sphingolipid Classification

Sphingolipids are ubiquitous components of animal cell membranes and are also associated with intracellular organelles [14]. They have been found to play an important role in both intra-cellular and extra-cellular signaling pathways and are a key component of membrane rafts or microdomains [15]. Structurally, sphingolipids are composed of a long chain sphingoid base, an amide linked fatty acid, and a polar head at the 1-position [16]. There are three main types of sphingolipids: ceramides, sphingomyelins, and glycosphingolipids. Except for ceramide, which has a hydroxyl at the 1-position and for sphingomyelin, which has a phosphorylcholine head group, all other sphingolipids contain carbohydrate head groups and are hence designated glycosphingolipids [16]. Glycosphingolipids can be further subdivided in neutral (cerebrosides) or acidic fractions (gangliosides and sulfatides). It is become evident that the biological activity of sphingolipids resides not only in their complex structures (e.g., gangliosides and sulfatides) but also in their derivatives and metabolites such as ceramide, sphingosine and *N*-methylated products (i.e, *N*,*N*-dimethylsphingosine (DMS) [17]. Sphingolipid and its metabolites have been found to play an important role in a number of biological and pathological processes including myocardial ischemia reperfusion injury [18].

Sphingolipids and Myocardial I/R Injury

Numerous glycolipids and sphingolipids have been investigated in the setting of of myocardial I/R injury. Gangliosides are a class of sialic acid containing glycosphingolipids that are

particularly abundant in the central nervous system [19]. They have been implicated in several biological processes including cell-cell recognition, regulation of cell growth and differentiation and signal transduction [20]. The ganglioside GM-1 has previously been shown to act as a survival factor in both neuronal cells and cardiac fibroblasts [21,22]. Maulik *et al*. [23] initially showed that GM-1 can reduce I/R injury in an isolated rat heart by Langendorff technique and had suggested that the likely mechanisms included inhibition of calcium overloading and scavenging of free radicals generated during the reperfusion of myocardium. Recent studies by Jin *et al.* [24] have confirmed that GM-1, which enhances endogenous sphingosine-1-phosphate production, reduces cardiac injury through PKCε-dependent intracellular pathways in an isolated mouse heart Langendorff model of ischemia-reperfusion injury.

Sulfatides are sulfated galactosylceramides found in various mammalian tissues, which show structural and physiologic similarities to gangliosides [25]. Sulfatides have been found to have anti-inflammatory properties and have been found to inhibit platelet adhesion and aggregation through P-selectin interactions [26]. It has been previously shown that P-selectin expression plays an important role in the exacerbation of myocardial-ischemia reperfusion injury [27]. Yamada et al. [28] have shown that administration of sulfatide prior to ischemia reduces the extent of myocardial infarction in an *in vivo* rat model of ischemia reperfusion injury through a blockade of leukocyte adhesion to the endothelium via P- and L-selectins. These studies suggest that the complex sphingolipids that were once believed to function only as structural components to cell membranes may play an important role in signal transduction. Through the organization of signaling molecules in microdomains or as reservoirs of lipid-derived secondary messengers these complex sphingolipids modulate several biological and pathological processes involved in I/R injury.

Sphingomyelin is predominantly found in the outer leaflet of the plasma membrane and is broken down into ceramide and sphingosine under conditions of stress [29]. The sphingolipid metabolites ceramide, sphingosine and sphingosine 1-phosphate (S1P) play an important role in cell proliferation, survival and cell death [30]. Ceramide is the main second messenger, and is produced by sphingomyelinase induced hydrolysis of sphingomyelin and also by *de novo* synthesis [31,32]. Sphingosine is formed by deacylation of ceramide. The deacylation is catalyzed by ceramidases, which are important regulators of ceramide and sphingosine levels during agonist stimulation. Sphingosine in turn can be rapidly phosphorylated to S1P by sphingosine kinase (SK). Ceramides and sphingosine usually inhibit proliferation and promote apoptosis, while the further metabolite S1P stimulates growth and suppresses apoptosis [33, 34]. Because these metabolites are interconvertible, it has been proposed that it is not the absolute amounts of these metabolites but rather their relative levels that determines cell fate.

Ceramide has been shown to mediate apoptosis via activation of CD95 and tumor necrosis factor receptors [35,36]. Accumulation of ceramide and its cell permeable analogs occurs in various cell types during apoptosis. Bielawska *et al*. [37] have also shown that ceramide signaling pathway may be involved in ischemia-reperfusion induced death of cardiomyocytes using both *in vitro* and *in vivo* rat models. Therefore mechanisms that increase the production of ceramide may lead to increase in apoptotic cell death following myocardial I/R. Similarly studies have shown that sphingosine, an inhibitor of protein kinase C [38], which is a major product of ceramide catabolism also inhibits cell growth and induces apoptosis through caspase-3 like proteases in hepatoma cells [39]. SK generates S1P by phosphorylation of sphingosine and is the rate-limiting step in the cellular synthesis of S1P. Unlike ceramide and sphingosine, S1P promotes cell growth, viability, mitosis and angiogenesis either through S1P receptors or intracellularly as a secondary messenger [34]. Jin *et al*. [24] have shown that the cardioprotection conferred by the monoganglioside GM-1, which stimulates SK, is cardioprotective in part through increased generation of S1P. They have also shown that the

cardioprotection conferred by myocardial ischemic preconditioning may also be mediated through PKCε mediated increase in SK activity [40]. Furthermore, Theilmier *et al*. [41] have also shown that intravenous administration of S1P 30 min prior to ischemia reduces myocardial injury in an *in vivo* model of myocardial I/R injury. These studies suggest that the activation of SK facilitates the conversion of ceramide and sphingosine to S1P thereby removing pro-apoptotic signals and creating a survival signal in the cell. The regulation of this "sphingolipid rheostat" as described by Cuvillier *et al*. [42] may therefore be a critical factor in determining cell fate during myocardial I//R injury.

Sphingosine and its *N*-Methylated derivatives, have been known to be negative modulators of transmembrane signaling through the inhibition of protein kinase C (PKC) [43]. *N*,*N*-dimethylsphingosine (DMS) is formed by *N*-methylation of sphingosine endogenously by N-methyltransferase [44]. It is a competitive inhibitor of SK and is widely used in experimental studies [45]. Previously Jin *et al*. [40] have shown that high dose DMS inhibits the cardioprotection observed in ischemic preconditioning through inhibition of SK via inhibition of PKCε activity in isolated murine hearts. However, subsequent experiments demonstrated that DMS has a biphasic effect on cardioprotection and while a high dose of DMS is inhibitory, low dose DMS protects murine hearts against myocardial I/R injury via activation of PKCε-SK-S1P-Akt pathway [46]. In addition to modulating PKC activity, DMS also activates tyrosine kinase [47], particularly the EGF receptor kinase and since EGF receptors are known to be involved in pharmacological preconditioning [48] and protection against myocardial I/R injury, activation of EGF receptor kinase may be another probable mechanism of DMS mediated cardioprotection.

N,N,N-trimethylsphingosine and Myocardial I/R Injury

N,*N*,*N*-trimethylsphingosine is a stable synthetic analogue of sphingosine which has been shown to modulate protein kinase C (PKC) activity and exert a number of important biological effects including: inhibition of tumor cell growth and metastasis [43], inhibition of neutrophil respiratory burst [49], inhibition of leukocyte migration [49], and inhibition of platelet aggregation [50], suggesting potent anti-inflammatory properties. Murohara *et al*. [51] have previously demonstrated that TMS therapy reduces myocardial infarct size in an *in vivo* feline model of myocardial I/R injury. Campbell *et al*. [52] also demonstrated in a Langendorff perfused heart model of myocardial I/R that administration of TMS significantly maintained post-reperfusion coronary flow and left ventricular developed pressure.

Recently, our laboratory has studied the effects of TMS during myocardial I/R extensively in clinically relevant *in vivo* models of ischemia-reperfusion injury in the heart and the liver. In these studies we have investigated the effects of TMS therapy on myocardial infarct size and cardiac function following longer periods of myocardial reperfusion. We also investigated its therapeutic effect across varying dosages (0.001mg/kg to 1mg/kg) demonstrating a complete dose response relationship. These studies demonstrated a significant cardioprotective effect of TMS following myocardial I/R in healthy mice as evidenced by a profound reduction in myocardial infarct size. In addition, we also measured the effect of TMS on cardiac function. High-resolution two-dimensional echocardiography was used to assess cardiac function by measuring left ventricular ejection fraction (difference between the end-diastolic volume and end-systolic volume divided by the end-diastolic volume) and fractional shortening (difference between the end-diastolic dimension and end-systolic dimension divided by the end-diastolic dimension). TMS therapy during MI/R led to a preservation of left ventricular ejection fraction and fractional shortening resulting in significant attenuation of cardiac dysfunction. Furthermore, we investigated the effects of TMS therapy in obese (ob/ob) and diabetic (db/db) mice subjected to myocardial ischemia-reperfusion injury. In these animals, we failed to observe the same degree of cardioprotection as compared to the wild-type animals. We further

tested the effect of TMS therapy in the liver following hepatic I/R. TMS reduced the serum liver enzymes levels in the wild-type mice following hepatic I/R at a dose of 0.025 mg/kg. These cytoprotective effects again did not extend to the ob/ob and db/db mice. Our data suggests that TMS-activated cytoprotective signaling pathways have attenuated or altered function in obese and diabetic animals.

Studies have previously shown that TMS attenuates the surface expression of P-selectin on coronary endothelial cells during reperfusion of the ischemic heart [53] and attenuates leukocyte-endothelium interactions [49] leukocyte migration and a subsequent inflammatory response [54], inhibiting the generation of oxygen derived free radicals. Previous studies have also shown that PKC–mediated signal transduction pathways are involved in P-selectin upregulation on platelet and endothelial cell surfaces [55] and inhibitors of PKC activation cause attenuation of leukocyte migration, platelet aggregation [56–60] and oxygen derived free radicals production [61]. Since TMS is a potent inhibitor of PKC activity [47], this may be an important underlying signaling mechanism in the protection against acute inflammatory injury and may be responsible for the beneficial effects of TMS therapy following I/R.

Diabetes and obesity are metabolic disease states with aberrant regulation of cellular signaling pathways [62]. Hyperglycemia during diabetes mellitus increases non-enzymatic glycation, characterized by the binding of glucose or its by-products to amino groups of proteins. This reaction leads to the formation of complex compounds, advanced glycation end products (AGEs), which alter structure and functions of proteins [63]. Glycation and oxidative stress are closely linked, and both phenomena are referred to as “glycoxidation” [64]. All steps of glycoxidation result in the generation of oxygen free radical, some of them being common with lipidic peroxidation pathways. In addition, glycated proteins activate membrane receptors such as RAGE through AGEs, and induce an intracellular oxidative stress and a pro-inflammatory status [65]. Glycated proteins, therefore, may modulate functions of cells involved in oxidative metabolism and induce inappropriate signaling responses [66]. Hyperglcemia also activates the glycolytic pathway and increases the production of diacylglcerols (DAG) [67]. Increases in intracellular DAG levels activate protein kinase C (PKC) [67]. Activation of the DAG-PKC pathway may therefore lead to up-regulation of endothelial cell adhesion molecules, accumulation of leukocytes and platelets, increased production of ROS, increased endothelial permeability and endothelial dysfunction, all of which may contribute towards an increase cell death following I/R injury. Although it would seem plausible that the inhibition of PKC by TMS would negate these effects and promote cell survival, the apparent lack of benefit in hyperglycemic mice suggests that acute administration of TMS fails to inhibit the chronic activation of DAG/PKC transduction pathway in hyperglycemic states.

In the ob/ob obese mouse we observed a reduction in myocardial infarct size following I/R, however this reduction was not as significant as was observed in the wild-type mice. Although both diabetes and obese models are characterized by hyperphagia, obesity, hyperglycemia and hyperinsulemia associated with characteristic pancreatic lesions, diabetes causes the more severe condition. The significantly lower fasting blood glucose in the ob/ob compared to the db/db [68] may explain the sustained benefit of TMS following myocardial I/R in the ob/ob model. The liver of these ob/ob mice however is characterized by higher levels of triglycerides and an increased severity of steatosis [69]. Hyperlipidemia can lead to an increase in the intracellular accumulation of DAG and subsequent activation of the DAG/PKC pathway [70]. Therefore, activation of the DAG-PKC pathway in the liver in the ob/ob mice may have accounted for the lack of any benefit with TMS therapy following hepatic I/R in these mice.

The signaling mechanisms involved in TMS action in the heart and the liver are complex and appear to be dependent on the intra- and extra-cellular milieu of the animal. Therefore, further studies are needed to completely elucidate the signaling mechanisms in these clinically relevant

animal models before the translation of pre-clinical results with TMS as a therapeutic agent to patient populations.

Summary and Conclusion

The evolutionary conservation of sphingolipids, their metabolizing enzymes and their receptors suggest that the sphingolipid pathways play a crucial role in modulating cell function and its response to injury. Although emerging evidence has shown that sphingolipid mediated signal transduction pathways play a significant role in cardiovascular pathophysiology, further investigations are necessary to clarify the complex mechanisms involving this function. Further studies are required to address the complete characterization of the enzymes involved in sphingolipid metabolism, the structural and functional relationships of sphingolipid receptors and the molecular targets of sphingolipids and their mediators. This may be accomplished with the use of enzyme and receptor inhibitors or through the use of animal models with modifications of genes involved in sphingolipid metabolism and their molecular targets. A better understanding of these mechanisms would allow for the development of strategies aimed at modifying the signal transduction pathways towards achieving preservation of cellular function following a noxious injury as sustained during ischemia and reperfusion of the myocardium.

Currently pharmacological inhibition can be achieved through various mycotoxins that inhibit sphingomyelinase and other enzymes of ceramide synthesis and through synthetic drugs such as PDMP and *N* -butyl-deoxynojirimycin, which block ceramide glucosylation, however the safety and efficacy of these inhibitors in humans has not been tested. Further enzymatic studies may lead to the development of other activators of enzymes such as SK, which have been shown to be cardioprotective. An additional therapeutic strategy would be the use of specific agonists of S1P receptors. Mandala *et al*. [71] have recently demonstrated that activation of S1P receptors by an immunosuppressive agent FTY720 inhibits leukocyte recirculation leading to immunosuppression. FTY720 has now passed through pre-clinical testing into clinical trials and early data suggests that it may be an important treatment option for relapsing multiple sclerosis.[72] The successful translation of sphingolipid-based therapies in immunological research therefore leads us to believe that decoding the signaling pathways mediated by sphingolipids and its mediators in the myocardial, endothelial and humoral cells will allow for the identification of novel targets for therapeutic intervention in the heart.

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