

Review

The Acid Sphingomyelinase/ Ceramide System as Target for Ischemic Stroke Therapies

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Key Words

Stroke • Acid sphingomyelinase • Ceramide

Abstract

In this review, we summarize implications of the acid sphingomyelinase/ ceramide system in ischemic stroke. Acid sphingomyelinase catalyzes the formation of the bioactive sphingolipid ceramide which coalesces into membrane platforms and has a pivotal role in inflammation, cell signaling and death. Cerebral ischemia increases acid sphingomyelinase activity and elevates brain ceramide levels, which has been associated with the exacerbation of ischemic injury and deterioration of stroke outcome. In view of the fact that lowering acid sphingomyelinase activity and ceramide level was shown to protect against ischemic injury and ameliorate neurological deficits, the acid sphingomyelinase/ ceramide system might represent a promising target for stroke therapies.

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Stroke*Background*

Ischemic stroke, defined as an acute neurological deficit caused by the thromboembolic occlusion of a brain-supplying artery, is a leading cause of death and disability among adults worldwide [1]. The available treatment strategies for acute ischemic stroke (AIS) aim for recanalization of the occluded vessel by means of systemic thrombolysis and/ or mechanical thrombectomy. Recombinant tissue-plasminogen activator (rtPA) is a thrombolytic which is delivered intravenously (i.v) and the only pharmacological intervention approved by the US Food and Drug Administration (FDA) in 1996 after rtPA treatment was shown to reduce disability and mortality when delivered within up to 4.5 hours following stroke [2, 3]. Because of this short time window, only a small percentage of stroke patients (up to 30 percent, depending on health care system) benefits from intravenous thrombolysis. Recanalization can also be achieved by endovascular mechanical thrombectomy with a

stent retriever which is indicated in case of proximal large vessel occlusions [4]. The first device used for this procedure received FDA approval in 2004 [5]. Thrombectomy is mostly performed in addition to thrombolysis [6]. According to American Heart Association/ American Stroke Association guidelines from 2018 this procedure is recommended within 6 hours after onset of stroke symptoms [7]. Both intravenous thrombolysis and mechanical thrombectomy may induce side effects, such as brain hemorrhages [8, 9]. There is a clear need for treatments that allow promoting stroke recovery when the time window for acute treatments has exceeded.

Pathophysiology of ischemic stroke

The occlusion of a brain-supplying artery results in focal cerebral ischemia. Focal cerebral ischemia is characterized by a central core region with strongly compromised cerebral blood flow (CBF) that is surrounded by the so called penumbra, an area that is still viable and has a CBF that is below functional thresholds [10].

Major pathological events that occur during cerebral ischemia are massive excitatory neurotransmitter release (specifically of glutamate) associated with peri-infarct depolarizations occurring within minutes, inflammatory responses peaking after 24 hours to a few days and delayed neuronal injury that is most prominent in the first days but progresses over weeks resulting in secondary neurodegeneration and brain atrophy [11].

These pathophysiological events are directly initiated by the cerebral hypoperfusion. The impaired delivery of glucose and oxygen to the brain leads to mitochondrial dysfunction and a reduced synthesis of adenosine triphosphate (ATP) [12]. The depletion of ATP causes an impaired function of Na⁺/K⁺-ATPases. Consequently, neurons and glia cells depolarize and excitatory amino acids such as glutamate are released which accumulate in the synaptic space since uptake is also compromised, leading to an overactivation of N-methyl-D-aspartate (NMDA) receptors and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Further, as Na⁺ and Cl⁻ enter the cell, water follows passively which leads to cell swelling. The overactivation of glutamate receptors induces a cellular calcium influx that triggers the activation of calcium-dependent proteases, endonucleases and lipases [13]. The calcium-mediated activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase catalyzes superoxide production which mediates cell death [14]. The release of damage-associated molecular pattern (DAMP) molecules from dying neurons activate resident microglia that produce inflammatory cytokines such as the tumor necrosis factor α (TNF- α), interleukin 1 (IL-1) and interleukin 6 (IL-6) [15-20]. The expression of adhesion molecules such as the intercellular adhesion molecule 1 (ICAM-1) on cerebral endothelial cells is increased allowing the infiltration of inflammatory cells such as neutrophils that further exacerbate brain damage [21, 22]. Further, the matrix metalloproteinase 9 (MMP9) disrupts the integrity of the blood-brain-barrier (BBB) by degradation of the basal lamina and tight junctions, consequently leading to edema formation and possibly hemorrhagic transformation [23-27].

Failed strategies for ischemic stroke treatment

Despite extensive research and promising data from pre-clinical stroke model, a plethora of supposedly neuroprotective compounds targeting specific elements of the ischemic cascade have failed in clinical trials. For reasons of space, we only touch a few examples here. Several trials assessed the potential benefit of calcium antagonists after preclinical data indicated that blocking of calcium channels reduces infarct size and brain edema [28, 29]. The Very Early Nimodipine Use in Stroke (VENUS) trial has approached this with nimodipine, a calcium channel blocker that is used to prevent the occurrence of subarachnoid hemorrhage (SAH) related vasospasms. The VENUS trial enrolled 454 patients, which were treated within 6 hours after stroke onset. No differences were observed between the treated group and the placebo group at trial termination [30]. Likewise, the Flunarizine in Stroke Treatment (FIST) trial analyzed the effect of the calcium channel blocker flunarizine, which is clinically used for treating migraine. 331 patients were enrolled and treatment started within 24 hours

after stroke onset. Flunarizine was not superior to placebo in this study [31]. Other studies similarly evaluated nimodipine or flunarizine, all with lack of clinical benefits [32].

NMDA receptors antagonists have been designed because of the high glutamate concentrations released in ischemic tissue that exacerbate brain injury [33-35]. The efficacy of the NMDA antagonist aptiganel hydrochloride was investigated in a trial with 628 patients who were treated within 6 hours after stroke. The treatment was not efficacious and due to cognitive side effects raised serious safety concerns [36]. The Glycine Antagonist in Neuroprotection (GAIN) trial examined the efficacy of another NMDA antagonist called gavestinel and enrolled 1367 patients that were treated within 6 hours after symptom onset. Also, gavestinel failed to alleviate functional outcome [37], as did studies with other NMDA receptor antagonists [32].

Since inflammation is a crucial element in stroke pathology, efforts have been made to attenuate the inflammatory response after stroke. Based on observations that mice deficient for ICAM-1 were protected from ischemia-reperfusion injury [38], a monoclonal murine ICAM-1 antibody called enlimomab was tested in 625 patients who received the antibody or placebo within 6 hours after stroke onset. Enlimomab was not effective, but was associated with an elevated mortality rate [39]. Another attempt to dampen the inflammatory response after stroke was the delivery of a humanized antibody called rovelizumab which targets the $\beta 2$ -subunit of the lymphocyte function-associated antigen-1 (LFA-1) and macrophage-1 antigen (Mac-1) which bind to ICAM-1. The antibody was well tolerated but the trial was halted at an interim analysis because a beneficial effect could not be detected [40]. In the meantime, concerns have been raised about the mouse mutants used for examining effects of ICAM-1 knockout. While membrane bound ICAM-1 is not detectable, soluble forms of ICAM-1 are still present in the serum of these mice [41]. Based on these insights, ICAM-1 null mice with a deletion of the entire coding region of ICAM-1 have been developed, which did not reveal any beneficial effects of ICAM-1 deficiency in ischemic stroke models. Specifically, there was no difference in infarct size compared to wildtype mice and the disruption of the blood-brain-barrier was even more pronounced in ICAM-1 null than wildtype mice [42].

Additionally, a large variety of neuroprotective agents acting including free radical scavengers, nitric oxide inhibitors, AMPA antagonists, GABA agonists, sodium channel blockers or gangliosides have been studied in clinical stroke trials, all without benefits [43].

Acid sphingomyelinase/ ceramide system

Synthesis and structure of the acid sphingomyelinase

Acid sphingomyelinase (ASM) is a lysosomal phosphodiesterase which is encoded by the sphingomyelin phosphodiesterase gene (*SMPD1*) that is expressed on chromosome 11p15.1-p15.4 [44].

ASM is synthesized as an N-glycosylated 75 kDa pre-pro-enzyme, which is targeted to the endoplasmic reticulum (ER) where the N-terminal signal peptide is cleaved which leads to the formation of the 72 kDa pro-enzyme [45, 46]. This precursor is post-translationally subjected to glycosylation inside the Golgi complex [47]. Lysosomal ASM (L-ASM) acquires high mannose oligosaccharides, whereas secretory ASM (S-ASM) has a more complex glycosylation composition [45, 48]. L-ASM is targeted to the lysosome via mannose 6-phosphate receptor or sortilin-mediated pathways and binds Zn^{2+} ions during this trafficking process [45, 48, 49]. It is further proteolytically processed at the C-terminus which is necessary for the enzyme to obtain its catalytic activity [50]. S-ASM is released from the cell via the Golgi secretory pathway and additionally requires extracellular Zn^{2+} for its activation [48]. The crystal structure of mature ASM revealed that the enzyme is composed of an N-terminal saposin domain, a C-terminal metallophosphoesterase catalytic domain with two Zn^{2+} ions and a prolin-rich connector domain [51, 52]. Sphingomyelin binds to ASM

by positioning its ceramide-phosphate group at the Zn^{2+} center [51]. The catalytic activity of ASM is dependent on an acidic pH and its attachment to membrane surfaces that is mediated by electrostatic forces [51].

Functional inhibitors of ASM (FIASMs)

Several antidepressants such as amitriptyline, fluoxetine and nortriptyline are functional inhibitors of ASM (FIASMs) [53]. Antidepressants were long believed to act via inhibition of monoamine reuptake. While fluoxetine is a selective serotonin reuptake inhibitor, amitriptyline preferentially inhibits serotonin and noradrenalin reuptake and nortriptyline preferentially inhibits noradrenalin reuptake [54]. However, tianeptine is a serotonin reuptake enhancer [55]. The monoamine mechanism for the induction of antidepressant effects was challenged by observations that amitriptyline and fluoxetine reduced ASM activity and ceramide concentration, inducing neurogenesis and behavioral recovery in a mouse model of depression in wildtype mice but not ASM deficient mice [56]. These experiments clearly identified a critical role of the ASM/ ceramide system as target for antidepressants.

Despite their structural heterogeneity, FIASMs have lipophilic and weakly basic properties in common [53]. As lysosomotropic compounds, FIASMs passively enter the lysosome through the lysosomal membrane, become protonated in this acidic compartment, accumulate and interfere with the attachment of ASM to the lysosomal membrane, consequently leading to a proteolytic degradation of ASM by lysosomal proteases [53, 57-59]. Treatment with FIASMs does not induce complete ASM degradation [56, 60]. It is not clear yet if the remaining ASM activity originates from lysosomes but a basal ASM activity might be necessary to prevent pathologies resembling Niemann-Pick disease that is caused by genetic ASM deficiency [53, 61]. The simultaneous treatment with multiple FIASMs results in an amplified inhibition of ASM [62].

FIASMs do not generally abrogate the activity of all lysosomal hydrolases. Yet, desipramine, chloroquine and chlorpromazine were also shown to inhibit the lysosomal enzymes acid ceramidase (AC), acid lipase and phospholipases A and C [53]. Since FIASMs are capable of passively diffusing through the blood-brain barrier (BBB) [62], they are attractive tools not only in the treatment of depression, but potentially also of ischemic and degenerative brain disease.

ASM and ceramide signaling in experimental ischemic stroke

The sphingomyelinase pathway is activated by stress stimuli for instance in response to irradiation, acute systemic inflammation induced by lipopolysaccharide (LPS) or upon release of reactive oxygen species (ROS) or pro-inflammatory cytokines such as the tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) [63-67]. Upon activation, ASM is translocated from the lysosomes to the extracellular leaflet of the cell membrane, bringing it in close proximity to its substrate sphingomyelin [68, 69]. ASM then hydrolyzes sphingomyelin producing ceramide and phosphorylcholine. Due to its biophysical properties ceramide coalesces into microdomains that fuse and form large ceramide-enriched membrane platforms with a diameter of 200 nanometer up to several micrometer [70]. Ceramide-enriched membrane platforms induce the spatial reorganisation and clustering of membrane proteins and receptors, leading to the amplification of their elicited cell signals [71]. Ceramide-rich membrane platforms are critically involved in induction of apoptosis and growth inhibition, besides others [70].

Experimental studies provided evidence for the significance of the ASM/ ceramide system in ischemic stroke (Table 1). In mice subjected to 60 minutes transient proximal middle cerebral artery occlusion (MCAO), ASM activity and ceramide level were significantly increased in the ischemic cerebral cortex after 6 hours reperfusion [72]. Ceramide was not increased upon MCAO in ASM knockout mice [72]. ASM deficiency reduced infarct size,

improved neurological deficits and inhibited the production of inflammatory cytokines [72]. Primary cortical neuronal cultures from ASM knockout mice exhibited less vulnerability to glutamate-induced toxicity [72]. Intracerebroventricular administration of xanthogenate tricyclodecan-9-yl (D609), an inhibitor of the phosphatidylcholine (PC)-specific phospholipase C that catalyzes the formation of ASM-activating diacylglycerol (DAG) [73, 74], 30 minutes before MCAO and 2 hours after reperfusion prevented the increase of ASM activity and ceramide level, dampened pro-inflammatory cytokine production, attenuated neuronal damage and improved post-stroke behavior [72]. Interestingly, the ceramide increase in ischemic stroke is strictly linked to reperfusion. Thus, ischemic mice revealed this increased ceramide level in ischemic tissue only when transient (30 minutes) proximal but not permanent proximal MCAO was induced [75]. Yu et al. did not observe any alterations in sphingomyelin content, thus excluding sphingomyelin hydrolysis as the ceramide source in this case of mild focal cerebral ischemia and postulating that ceramide was rather generated by *de novo* biosynthesis because intermediate products of this alternative pathway for ceramide generation such as dihydrosphingosine and dihydroceramide were increased [75]. In rats exposed to 90 minutes transient proximal MCAO, ceramide levels increased in the thalamus by 190% and in the entorhinal cortex by 175% after 6 hours reperfusion, which was prevented when FK506 was administered 5 minutes after MCAO induction [76]. Complementary findings were made in an *in vitro* model of ischemia. Human neuroblastoma cells exposed to low oxygen concentrations and serum starvation revealed increased ASM activity and ceramide level after 30 minutes of reoxygenation under conditions resulting in apoptotic cell injury [76]. Preincubation with FK506 counteracted the ceramide increase and apoptosis induction [76]. FK506 is neuroprotective in several experimental stroke models [77-79]. FK506 has prominent immunomodulatory effects [80]. In the ischemic brain, FK506 was shown to downregulate pro-inflammatory cytokines and dampen the activation of astrocytes [81, 82]. In retinal ischemia, FK506 reduced leukocyte accumulation [83]. In a model of chronic bilateral cerebral ischemia induced by bilateral carotid artery occlusion (BCAO) in rats, ASM activity and ceramide level were unchanged at day 1 after BCAO and significantly increased at day 3, 7 and 14 in the frontal cortex, corpus callosum, internal capsule and globus pallidus [84]. Ceramide was found in glial fibrillary acidic protein (GFAP)-positive astroglia [84]. An increase of ceramide and a decrease of sphingomyelin was noted in the hippocampus 30 minutes and 24 hours after 5 minutes of BCAO in gerbils [85], indicating that ceramide was produced via the sphingomyelinase pathway. Ceramide

Table 1. ASM and ceramide signaling in experimental ischemic stroke

Reference	Species	Stroke model	Main observations
[72]	Mice	60 minutes transient proximal MCAO	Ceramide and ASM activity increased in the cerebral cortex of wildtype mice after 6 hours reperfusion. ASM deficiency or ASM inhibition by D609 protected from ischemia/ reperfusion (I/R) injury.
[75]	Mice	30 minutes transient proximal MCAO or permanent proximal MCAO	Ceramide increased in the ischemic tissue after 24 hours reperfusion due to <i>de novo</i> synthesis but not after permanent MCAO.
[76]	Rats	90 minutes transient proximal MCAO	Ceramide increased in the thalamus and entorhinal cortex after 6 hours reperfusion. FK506 prevented ceramide production and apoptosis.
[84]	Rats	Chronic BCAO	Ceramide and ASM activity increased in the frontal cortex, corpus callosum, internal capsule and globus pallidus at day 3, 7 and 14.
[85]	Gerbils	2 minutes or 5 minutes transient BCAO	Ceramide increased and sphingomyelin decreased in the hippocampus 30 minutes and 24 hours after 5 minutes BCAO but not after 2 min BCAO.

accumulation depends on the severity of the ischemic episode. No ceramide changes were observed after 2 minutes of BCAA [85].

Effects of ASM inhibitors in experimental ischemic stroke

Experimental studies have also investigated the utility of FIASMAS in stroke treatment (Table 2). In rats exposed to 60 minutes transient proximal MCAO, fluoxetine reduced infarct volume and ameliorated motor deficits [86]. A low dose of fluoxetine (1 mg/kg) was neuroprotective when administered (i.v.) at 30 minutes post-stroke, but at 9 hours post-stroke a high dose (10 mg/kg) was necessary for the induction of tissue survival and motor recovery [86]. Even more delayed treatment after 12 did not confer neuroprotection or enhance motor deficits [86]. In rats treated with fluoxetine at 5 mg/kg or 10 mg/kg (i.v.) 6 hours post-stroke, inflammatory responses were reduced, demonstrated by a decrease in the activation of the nuclear factor kappa light-chain enhancer of activated B-cells (NF-κB), a reduction in microglia activation and a reduction in brain neutrophil infiltration [86]. The authors confirmed the anti-inflammatory effects of fluoxetine in primary microglial cells or primary neutrophils treated with lipopolysaccharide (LPS) and reported a fluoxetine-mediated reduction in NF-κB activation and pro-inflammatory cytokine gene expression levels [86]. Post-ischemic hyperactivity in gerbils was reduced by pretreatment with fluoxetine intraperitoneally (i.p.) administered at doses of 10 mg/kg, 20 mg/kg or 40 mg/kg 30 minutes before 5 minutes of BCAA [87]. High dose fluoxetine pretreatment at 40 mg/kg (i.p.) for 3 consecutive days before BCAA also increased neuronal survival in the CA1 hippocampal area [87]. Mice receiving fluoxetine in the drinking water (120 mg/l) from day 3 to day 28 after photothrombotic stroke revealed a reduction in final stroke size [88]. In the peri-infarct cortex, the ceramide level was increased at day 3 and 7 post-stroke due to *de novo* synthesis and not ASM activation [88]. In this study, fluoxetine did not improve neurological recovery [88]. After focal cerebral ischemia in rats induced by endothelin-1 injection fluoxetine delivery via miniosmotic pumps (10 mg/kg) for 4 weeks starting on day 7 did not enhance motor function [89]. Neurological outcome and infarct volume were

Table 2. Effects of ASM inhibitors in experimental ischemic stroke

Reference	Species	Stroke model	Main observations
[86]	Rats	60 min transient proximal MCAO	Fluoxetine administration 9 hours post stroke at 10 mg/kg (i.v.) protected from I/R injury and improved behavioral outcome at day 2.
[87]	Gerbils	5 minutes transient BCAA	Pretreatment with fluoxetine at 40 mg/kg (i.p.) for 3 days before stroke promoted neuronal survival in the hippocampus and pretreatment with fluoxetine 30 minutes before stroke at 10 mg/kg, 20 mg/kg or 40 mg/kg (i.p.) reduced post-ischemic hyperactivity at day 4.
[88]	Mice	Photothrombotic stroke	Ceramide increased at day 3 and 7 in the peri-infarct cortex due to <i>de novo</i> synthesis. Fluoxetine delivered via drinking water (120 mg/l) from day 3 to 28 reduced the infarct size but did not improve functional deficits at day 7 and 28.
[89]	Rats	Intracortical and striatal injections of endothelin-1	Fluoxetine administration from day 7 at 10 mg/kg (via miniosmotic pumps) did not improve functional deficits after 2 and 4 weeks of treatment.
[90]	Rats	120 minutes transient proximal MCAO	Fluoxetine administration from day 2 at 5 mg/kg did not affect the infarct size and behavioral outcome after 10 days of treatment.

not altered in rats exposed to 120 minutes transient proximal MCAO that were treated with fluoxetine at 5 mg/kg (i.p.) for 10 days from day 2 [90].

Effects of ASM inhibitors in clinical stroke trials

Due to the lack of safety concerns, ASM inhibitors have been studied in clinical trials. In a placebo-controlled study in ischemic stroke patients exhibiting hemiparesis or hemiplegia, fluoxetine (20 mg/day) initiated between day 5 and day 10 post-stroke and continued for 90 days in addition to physiotherapy enhanced motor recovery [91], suggesting that fluoxetine (20 mg/day) modulates brain plasticity. Indeed, another study in patients with hemiparesis showed that even a single dose of fluoxetine (20 mg) 14 days after stroke modulated cerebral motor activation and enhanced hand motor function [92]. The effect on motor function after a single bolus injection excludes that motor effects are attributed to the enhancement of the patients' mood that normally requires a longer treatment time [91]. Further, treatment with fluoxetine (10 mg/day gradually increased to 40 mg/day) or nortriptyline (25 mg/day gradually increased to 100 mg/day) for 12 weeks within the first 6 months after stroke increased the probability of survival irrespective of whether the patient suffered from depression at enrollment or not [93]. In patients with stroke-associated neuropathic pain without depressive symptoms, 4 weeks of amitriptyline treatment (25 mg/day gradually increased to 75 mg/day) induced pain relief [94].

There have also been studies lacking recovery promoting effects. In stroke patients with persistent neurological deficits treated with placebo or fluoxetine (20 mg/day) starting 2 days to 15 days post-stroke for 6 months, fluoxetine reduced the incidence of depression but increased the incidence of bone fractures [95]. Importantly, fluoxetine did not influence neurological outcome or stroke survival [95]. A study comparing the efficacy of fluoxetine (10 mg/day gradually increased to 40 mg/day) or nortriptyline (25 mg/day gradually increased to 100 mg/day) for 12 weeks in depressed and non-depressed patients that had a stroke within 6 months before, found that nortriptyline ameliorated post-stroke depression, whereas both antidepressants did not enhance stroke-related physical and cognitive impairments [96].

Conclusion

There is compelling evidence that the ASM/ ceramide system is critically involved in ischemic stroke pathogenesis. A variety of experimental studies demonstrated beneficial effects of ASM inhibition and ceramide lowering for ischemic brain injury. Solid evidence was obtained that FIASMs reduce ischemic injury in the acute stroke phase and in addition experimental studies suggested that ASM inhibition may also induce neurological recovery in the post-acute stroke phase. Unfortunately, these studies so far did not elucidate whether these restorative actions were specifically attributed to the modulation of the ASM/ ceramide system. Further studies are needed to address this question.

FIASMs have been used in clinics for decades which provides the advantage that possible side effects, toxicity and contraindications are well known. Due to their broad mechanisms of action, it at this stage remains speculative to assign recovery promoting actions in stroke patients to their inhibitory effect on ASM. Specific ASM inhibitors with negligible off-target effects are not available for use in humans so there is currently no alternative to achieve ASM inhibition in patients other than by FIASMs [53]. Since clinical trials using FIASMs were inconsistent, a generalized statement on the efficacy of FIASMs in stroke patients cannot be made.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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