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Fatty acid methyl esters as a potential therapy against cerebral ischemia

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Abstract – Saturated fatty acids have been traditionally thought to be detrimental in circulation. However, we describe the beneficial effects of palmitic and stearic acid methyl esters as it relates to neuroprotection and cerebral vasodilatory properties in global and focal cerebral ischemia. The methyl esterification of fatty acids is not prominent in nature. However, it seems to have biological activity related to neuroprotection/vasodilation. We will discuss the etiology of cerebral ischemia such as stroke and cardiac arrest in terms of current and future treatment modalitiesas it relates to fatty acid-based therapies. This review is based on the meeting presentation at the "The Journées Chevreul, Lipids & Brain III 2015" in Paris on 16–18 March, 2015.

Keywords: Cerebral ischemia / cerebral blood flow / neuroprotection / palmitic acid methyl ester / protein arginine methyltransferase

Résumé – Les esters méthyliques d'acides gras comme possible thérapie contre l'ischémie cérébrale. Les acides gras saturés ont été de longue date considérés comme délétères pour la circulation sanguine. Cependant, nous décrivons dans cet article les effets bénéfiques d'esters méthyliques d'acides palmitique et stéarique en ce qui concerne la neuroprotection, ainsi que les propriétés de vasodilatation au niveau cérébral face à l'ischémie cérébrale globale et focale. L'estérification méthylique d'acides gras n'est pas très répandue dans la nature. Cependant, elle semble avoir une activité biologique neuroprotectrice/vasodilatatrice. Nous discutons, dans cet article, de l'étiologie de l'ischémie cérébrale de type AVC et arrêt cardiaque, et ce en termes de modalités de traitement, actuels et futurs, en ce qui concerne les thérapies à base d'acide gras. Cette revue est basée sur la présentation faite à l'occasion de la conférence « Les Journées Chevreul, Lipids & Brain III 2015 » qui s'est tenue à Paris les 16–18 Mars 2015.

Mots clés : Ischémie cérébrale / circulation sanguine cérébrale / neuroprotection / ester méthylique d'acide palmitique / arginine méthyltransférase

1 Introduction

Cerebrovascular disease (*i.e.* cerebral ischemia) is one of the major causes of death and disability in the U.S. affecting up to 691 000 individuals each year (Go *et al.*, 2014; Guzauskas *et al.*, 2012). Interruption of blood supply to the brain causes neuronal cell death in the brain (*i.e.* cortex, hippocampus, and striatum) resulting in inadequate delivery of oxygen, glucose, and metabolites to the brain. There are nearly 2 million patients who have survived from cerebral ischemia (*i.e.* stroke)

in the US, but continuously suffer from prolonged neurocognitive deficits. The estimated cumulative cost of care after stroke is $36.5 billion dollars annually (Go *et al.*, 2014). However, most therapies except for thrombolytic agents and hypothermia have failed to reduce neurological deficits and mortality rate after ischemic stroke due to the fact that mechanisms underlying stroke-induced neuronal cell death are multi-faceted and remains unclear. Therefore, discovering novel therapies against ischemia is greatly needed.

One of the hallmarks of cerebral ischemia is cerebral blood flow (CBF) derangements following ischemia (Manole *et al.*, 2009; Miller *et al.*, 1980; Sabri *et al.*, 2013). CBF

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derangements play a crucial role in the pathological progression of neuronal cell death and neurocognitive deficits caused by cerebral ischemia. Therefore, developing novel therapies that can effectively alleviate post-ischemic CBF derangements is the major challenge in the treatment of cerebral ischemia. We describe a new vasodilator (palmitic acid methyl ester, PAME) and neuroprotective agent (stearic acid methyl ester, SAME) originating from the sympathetic nervous system.

2 Cerebral ischemia

Cerebral ischemia results in insufficient CBF to meet metabolic demand. This leads to poor oxygen supply (hypoxia) resulting in neuronal cell death (infarction). There are two types (focal and global) of cerebral ischemia. Focal cerebral ischemia is confined to a specific region of the brain; it can be caused by lipohyalinosis, thrombosis, or embolism while global cerebral ischemia occurs in wide areas of brain with an overall reduction in CBF. The most common cause of global ischemia is cardiac arrest; other causes are hypovolemic shock, chronic carbon monoxide intoxication, and repeated episodes of hypoglycemia (Raichle, 1983).

2.1 Focal ischemia

Ischemic (focal) stroke represent 70–80% of all strokes. These are caused by sudden blockage of CBF resulting in a corresponding loss of neurological function (Tegos *et al.*, 2000). Reduction of blood supply and oxygen to the brain causes hypoxia and ischemia which may lead to infarction (Jamieson, 2009; Tegos *et al.*, 2000). Clinical manifestations of stroke are variably dependent on the type of vessel obstruction and location. The two major types of ischemic stroke are thrombotic and embolic (Tegos *et al.*, 2000). A platelet thrombi that develops over a disrupted atherosclerotic plaque causes thrombotic (atherosclerotic) stroke (Jamieson, 2009; Tegos *et al.*, 2000). The most common locations are the middle cerebral artery, internal carotid artery near the bifurcation, and the basilar artery. The main risk factors associated with thrombotic stroke are atherosclerosis, uncontrolled hypertension, diabetes mellitus, and transient ischemic attack (Jamieson, 2009; Tegos *et al.*, 2000). The gross and histological findings of atherosclerotic stroke are extension to the peripheral cerebral cortex, loss of demarcation between white and grey matter, and gliosis. Reperfusion does not usually occur; therefore the majority of thrombotic strokes are pale infarctions (Jamieson, 2009; Tegos *et al.*, 2000). There is also a development of a cystic area 10 days to 3 weeks after the initial event due to liquefactive necrosis. Embolic stroke is due to emboli from a blood clot, fat, amniotic fluid, or air, which travels through the bloodstream and lodges in the cerebral blood vessel (most commonly in the middle cerebral artery). After lysis of embolic material, blood reperfusion produces hemorrhagic infarction, and may extend to the periphery of the cerebral cortex (Jamieson, 2009; Tegos *et al.*, 2000).

2.2 Global ischemia

Cardiac arrest (a form of global cerebral ischemia) occurs when CBF to the brain and other organs is stopped or drastically reduced (Geocadin *et al.*, 2008; Huang *et al.*, 2014; Stub *et al.*, 2011). If circulation is restored in a short period of time neurological symptoms may be transient. However, at times, brain damage may be permanent and irreversible if a significant amount of time passes (more than 5 min) before restoration of CBF. The most common causes of cardiac arrest in 60–70% of cases are ventricular fibrillation or ventricular tachycardia due to ischemic heart disease (Geocadin *et al.*, 2008; Huang *et al.*, 2014; Stub *et al.*, 2011). The most critical factors determining overall patient survival during cardiac arrest are effective CPR, prompt cardiac rhythm analysis, and early defibrillation in cases of ventricular fibrillation.

There is widespread and diffuse injury to the brain post cardiac arrest, resulting in watershed infarcts at the junction of arterial territories. The most vulnerable areas to hypoxic injury are the CA1 region of the hippocampus; layers 3, 5 and 6 of the cerebral cortex, and cerebellar Purkinje cells (Geocadin *et al*., 2008). Global cerebral ischemia results in widespread neuronal damage via loss of adenosine triphosphate (ATP) production leading to dysfunction of Na^+/K^+ ATPase pumps. Subsequent excitotoxicity occurs when activation of N-methyl-D-aspartate (NMDA) receptors by glutamate increases intracellular calcium. Elevated intracellular calcium is detrimental for cellular survival increasing oxygen-free radicals by interfering with the mitochondrial respiratory chain. Reperfusion provides oxygen as a substrate for several enzymatic oxidation reactions that produce free radicals, increasing excitotoxicity. These reactive oxygen species are known to cause damage through lipid peroxidation, protein oxidation, and DNA fragmentation, all contributing to neuronal apoptosis (Geocadin *et al*., 2008; Stub *et al*., 2011).

2.3 Cellular metabolism

Post-ischemic CBF is defined by an initial hyperemia (vasodilation leading to an increase in CBF) followed by prolonged hypoperfusion (vasoconstriction leading to a decrease in CBF). Although hyperemia causes excessive release of activated catabolic enzymes and free radicals (Colbourne *et al.*, 1999; Wang *et al.*, 2011; Werner and Engelhard, 2007) resulting in neuronal cell injury shortly after ischemia, hypoperfusion induced mitochondrial depletion and excessive release of excitatory neurotransmitter, reactive oxygen species, and peroxynitrite (Brown and Borutaite, 2006; Murphy and Gibson, 2007; Rodrigo *et al.*, 2005; Takizawa *et al.*, 2002) all can play a crucial role in the pathogenesis of neuronal cell death following ischemia (Sarti *et al.*, 2002). Neurons in the CA1 region of the hippocampus can play a critical role in the spatial learning/memory formation, which is most vulnerable to ischemia (Abe *et al.*, 1995). Therefore, patients surviving from ischemia can suffer from mild to severe neurocognitive deficits.

Oxygen/glucose deprivation caused by hypoperfusion has been shown to induce cellular death. ATP derived from the mitochondria play a crucial role in cell fates. Mitochondrial dysfunction during/after cerebral ischemia can lead to the

pathogenesis of necrotic or apoptotic cell death (Huang *et al.*, 2014; Kochanek *et al.*, 2015) in the brain. In particular, depletion of ATP during ischemia inhibits the Na^+/K^+ pump (Na^+/K^+ ATPase) leading to an imbalance of ion (K^+, Na^+, Ca^{2+}) gradients (Kochanek *et al.*, 2015). The ionic imbalance caused by cerebral ischemia further induces plasma membrane depolarization to trigger massive release of excitatory neurotransmitter release such as glutamate from presynaptic nerve terminals (Sun *et al.*, 2008). Excessive activation of post-synaptic NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors via glutamate allows massive influx of Ca^{2+} into postsynaptic neurons. The abnormal accumulation of cytoplasmic Ca^{2+} following an ischemic event ultimately reaches toxic levels to induce irreversible neuronal cell death via caspase-9-dependent apoptosis and necrosis (Kochanek *et al.*, 2015; Saatman *et al.*, 1996).

2.4 Current therapies against ischemia

Besides hypothermia for the treatment against global ischemia (*i.e.* injecting cold fluids and the use of ice pack surrounding the patient to lower body temperature to 32–34 °C (Geocadin *et al.*, 2008)), the use of intravenous thrombolytic agents, such as recombinant tissue plasminogen activator, is standard therapy for ischemic stroke within 3 h after the onset of symptoms (The National Institute of Neurological Disorders, 1995; Wardlaw *et al.*, 2014). Thrombolytics dissolve the arterial brain clot reestablishing CBF, protecting neurons from damage while improving neurological function (1995; Wardlaw *et al.*, 2014). In spite of their advantageous effect in reestablishing CBF, thrombolytics are associated with increased incidence of symptomatic intracerebral bleeding, premature death and death, 3–6 months after stroke (1995; Wardlaw *et al.*, 2014). In addition to these complications, thrombolytics have a narrow therapeutic time-window, possess no vasodilatory properties; neither do they prevent post-ischemic hypoperfusion.

3 Fatty acids

Saturated fatty acids such as palmitic acid (PA) have been accepted as fats that are not beneficial to cardiovascular health. This 16 carbon fatty acid is one of the most common in nature derived from coconut/palm oils and can also be found in meats and dairy. Stearic acid, (SA, C-18) is also a common saturated fatty acid ubiquitous in animals and vegetables (Abbadi *et al.*, 2004). The synthesis of PA and SA is well understood through fatty acid-CoA synthase. These fatty acids are derived from acetyl-CoA with subsequent additions of carbon chains through fatty acid-CoA synthase (Abbadi *et al.*, 2004). Lin *et al.*, 2008, 2014 reported that PAME but not SAME is a vasoactive substance causing vasodilation while SAME is not a vasoactive substance but can provide neuroprotection against ischemia along with PAME in both animal models of global and focal ischemia. It is interesting to note that the synthesis of PA/SA is well-known but the methyl esterification of PA/SA to form PAME/SAME is not prevalent in nature and therefore, not well-understood (Lin and Perez-Pinzon, 2013).

Only recently has PAME and SAME been presented as a novel component in neuroprotection during cerebral ischemia (Lin and Perez-Pinzon, 2013; Lin *et al.*, 2014). PAME and SAME are simultaneously released from the sympathetic nervous system (Lin *et al.*, 2008). More specifically, PAME has been shown to induce aortic vasodilation and neuroprotection, while SAME causes neuroprotection in cardiac arrest and middle cerebral artery occlusion (MCAO) models of ischemia. More specifically, PAME/SAME was first discovered to be released from the superior cervical ganglion (SCG) upon electrical/chemical depolarization. Endogenous application of PAME induced aortic vasodilation and was found to be 3000 times more potent than other known nitric oxide (NO) donors (Lin *et al.*, 2008). With proper timing and dosage, administration of PAME and SAME can be a successful therapy against cerebral ischemia.

3.1 Vascular reactivity

Lin *et al.* (2008) first reported that PAME released from SCG (the largest cervical ganglion in the sympathetic tract (Lee *et al.*, 2011a, 2011b; Lee *et al.*, 2012; Wu *et al.*, 2014)) is a novel vasodilator (Lin *et al.*, 2008). Exogenous application of PAME directly onto the rabbit or rat thoracic aorta induced vasodilation in a concentration-dependent manner (Lin *et al.*, 2008; Lee *et al.*, 2010; Lee *et al.*, 2011c). The EC_{50} value (the concentration that induces 50% of the maximum relaxation) for PAME-elicited aortic vasodilation is 0.19 nM which is at least 178 times (*vs.* 34.8 nM) lower than that of sodium nitroprusside (NO donor)-induced vasodilation (Brizzolara-Gourdie and Webb, 1997; Lin *et al.*, 2008) indicating that PAME is a potent vasodilator.

Due to the novelty of PAME, the physiological significance of PAME on the regulation of systemic or CBF is relatively unknown. However, it has also been shown that PAME spontaneously released from the retina is described as the retina-derived relaxing factor (Lee *et al.*, 2010) which induces potent vasodilation in the pre-contracted rat aorta suggesting an important role in retinal circulation (Lee *et al.*, 2010). In addition, PAME is also released from perivascular adipose tissue (PVAT), which has been reported to be PVAT–derived relaxing factor (Lee *et al.*, 2011c) regulating systemic blood pressure. It is interesting to note that the reduction of PAME released from PVAT can be observed in genetic hypertensive rats (Lee *et al.*, 2011c). Furthermore, exogenous application of PA failed to induce aortic vasodilation (Blondeau *et al.*, 2007; Lin *et al.*, 2008) indicating that the methylation of PA is essential for vasodilation and PAME's bioactivity.

The release of PAME from the SCG, PVAT, and retina, however, dramatically decreased in calcium-free Krebs' solution (Lee *et al.*, 2010, 2011c; Lin *et al.*, 2008) indicating that calcium may be important for PAME release. Furthermore, the release of PAME from the SCG was reduced in the present of myosin light chain inhibition (Lin *et al.*, 2008), which prevents synaptic vesicle pool mobilization (Ryan, 1999) suggesting that the release of PAME is possibly linked to synaptic vesicle exocytosis. The exact mechanisms of PAME-induced vasodilation are not well-known. However,

PAME-induced aortic vasodilation is inhibited by voltage-gated potassium (Kv) channel blockers such as tetraethylammonium and 4-aminopyridine in a concentration-dependent manner (Lee *et al.*, 2010, 2011c) indicating that PAME may induce aortic vasodilation via opening Kv channels on vascular smooth muscle. The vasodilatory properties of PAME offer a potential therapeutic opportunity in the treatment against cerebral ischemia-induced hypoperfusion consequently promoting brain injury. Pre-treatment with PAME reduced brain infarct volume and neuronal cell death caused by MCAO in the rat (Lin *et al.*, 2014). In addition, pre-treatment with PAME enhanced CBF and brain perfusion 24 h after asphyxial cardiac arrest-induced global ischemia to further attenuate neuronal cell death (Lin *et al.*, 2014) indicating that PAME provides neuroprotection against cerebral ischemia *via* alleviating hypoperfusion following ischemia.

SAME, a C18:0 saturated fatty acid, was co-released with PAME from SCG and the retina (Lee *et al.*, 2010; Lin *et al.*, 2008). Unlike PAME, exogenous administration of SAME was unable to induce aortic vasodilation (Lin *et al.*, 2008). Therefore, the exact physiological significance of SAME still needs further investigation. Although SAME does not possess any vasodilatory properties, SAME can provide neuroprotection against brain injury caused by MCAO or cardiac arrest without affecting post-ischemic CBF. PAME and SAME diminished cardiac arrest and MCAO-induced brain injury. Mechanisms underlying SAME-induced neuroprotection still need to be defined. However, SAME reduces brain injury following cerebral ischemia possibly through stabilization of cellular membranes suggesting neuroprotection against traumatic brain injury *via* stabilizing damaged cellular membranes by decreasing degenerating axons (Koob *et al.*, 2005, 2008; Liu-Snyder *et al.*, 2007).

3.2 Regulatory factor(s) of PAME and SAME

PAME and SAME were released from the SCG in the presence of arginine analogs such as N^ω-nitro-L-arginine (nitric oxide synthase (NOS) inhibitor) (Lin *et al.*, 2008). In fact, the release of PAME and SAME were also promoted in the presence of L-arginine, D-arginine, and nitro-D-arginine. These results suggest that there is an interaction in the release of PAME/SAME and arginine analogs. These findings suggest that endogenous methylation of PA by a yet-to-be-determined arginine reaction may be crucial for the vasodilatory effects derived from arginine analogs (Lin *et al.*, 2008; Lin and Perez-Pinzon, 2013).

With this in mind, we are beginning to screen compounds or enzymes that have the ability to methylate other compounds propagated by arginine. We discovered that protein arginine methyl transferases (PRMT) meet the criteria. Posttranslational modification of proteins or other organic compounds via phosphorylation are not the only major controlling element in terms of function. Methyl groups also operate as a major controlling element. Protein methylation can occur on amino acids such as lysine, arginine, histidine, proline, and carboxy groups (Bedford and Richard, 2005; Wolf, 2009). This section will focus on the methylation of arginine via PRMTs.

The modification of the arginine containing guanidino group is quantitatively one of the most extensive protein methylation reactions in eukaryotic cells (Bedford and Clarke, 2009). Arginine is a positively charged amino acid known to mediate hydrogen bonding and amino-aromatic interactions (Bedford and Richard, 2005). The amide of arginine within polypeptides can be posttranslationally modified *via* methylation (*i.e.* arginine methylation) resulting in regulation of physiological functions of nuclear (histone methylation (Bedford and Richard, 2005)) and cytoplasmic (Wolf, 2009) proteins.

The methylation of arginine residues are catalyzed by PRMTs, which are ubiquitously expressed with various isoforms generated by alternative splicing (Bedford, 2007; Bedford and Richard, 2005; Bedford and Clarke, 2009; Wei *et al.*, 2014; Wolf, 2009). Typical PRMTs methylate glycine and arginine rich patches (GAR motifs) within their substrates. PRMTs remove one methyl group, from S-adenosyl methionine to generate S-adenosyl homocysteine. Methylation of omega (ω) guanidine group in eukaryotes occurs in three different ways: monomethylarginine, symmetric dimethylarginine, and asymmetric dimethylarginine (ADMA) (Bedford, 2007; Bedford and Richard, 2005; Bedford and Clarke, 2009; Wei *et al.*, 2014; Wolf, 2009). The accumulations of these end products play a role in certain vascular disorders, cancers, and cerebral ischemia in subarachnoid hemorrhage (SAH).

Thus far, 10 PRMT isoenzymes have been identified in humans classified as PRMT1-10. PRMT 1-10 differ in sequence and length of their N-terminal region, but all possess a conserved core region that includes a methyltransferase domain, which is a 7 β-barrel and dimerization arm (Wei *et al.*, 2014). It is important to note that the difference in N-terminal regions is not fully understood, but it is hypothesized that it may play a role in the enzymatic activity of PRMTs as well as impairment in stability (Wolf, 2009). Imbalances in the regulation of arginine methylation have clinical implications for many vascular disorders, such as hypercholesterolemia, myocardial infarction, and cerebral vasospasm in subarachnoid hemorrhage (Bedford and Richard, 2005; Bedford and Clarke, 2009; Leiper *et al.*, 2007; Wei *et al.*, 2014). PRMT type I metabolism forms ADMA, which can inhibit L-arginine derived NOS preventing L-arginine to L-citrulline conversion forming NO (Bode-Boger *et al.*, 2007) having consequences in the vascular endothelium, immune system, and neurotransmission in the central/peripheral nervous system (Bedford and Richard, 2005; Bedford and Clarke, 2009; Leiper *et al.*, 2007; Wei *et al.*, 2014). To prevent accumulation of ADMA, dimethylarginine dimethylaminohydrolase (DDAH) enzymes work to eliminate free methylarginines. An imbalance in this pool caused by DDAH dysfunction or by dysfunction in PRMT type I can produce ADMA accumulation and decrease NOS activity (Bedford and Richard, 2005; Bedford and Clarke, 2009; Leiper *et al.*, 2007; Wei *et al.*, 2014). This creates the potential risk for endothelial dysfunction, systemic vascular resistance, and elevated systemic and pulmonary blood pressure (Bedford and Richard, 2005; Bedford and Clarke, 2009; Leiper *et al.*, 2007; Wei *et al.*, 2014). Current dogma suggest that elevated ADMA concentration in blood plasma constitutes a possible risk factor associated with premature cardiovascular disease and death (Leiper *et al.*, 2007). In addition, the accumulation

of ADMA has been shown to have a strong correlation with other disease states such as multiple sclerosis, spinal muscular atrophy, atherosclerosis, hypertension, myocardial infarction, renal failure, and stroke (Bedford and Richard, 2005; Bedford and Clarke, 2009; Leiper *et al.*, 2007; Wei *et al.*, 2014).

PRMT metabolism via ADMA has consequences in cerebral ischemia. For example, delayed cerebral vasospasm and ischemia in SAH has three pathophysiological phases (Jung *et al.*, 2004; Pluta and Oldfield, 2007). First, release of oxyhemoglobin from the subarachnoid clot destroys neuronal NOS (nNOS) containing neurons at the perivascular space due to its neurotoxic effects. Second, there is temporary endothelial NOS (eNOS) inhibition due to accumulation of ADMA. ADMA inhibits l-arginine-induced NO production. The increase in ADMA levels are due to further metabolism of hemoglobin to bilirubin-oxidized fragments (BOXes). These BOXes upregulate PRMT activity increasing ADMA production and inhibit DDAH decreasing hydrolysis of ADMA. The enhanced concentration of ADMA and consequent decreased levels of L-citrulline (product of both ADMA hydrolysis and NO production) in cerebral spinal fluid are correlated with the degree and time course of delayed cerebral vasospasm (Jung *et al.*, 2004). Thus, in the last phase there is resolution of the vasospasm due to elimination of BOXes that result in reduction of ADMA levels and increase in NO production by eNOS. This results in the recovery of endothelial-dependent vasodilatory activity despite the persistent absence of nNOS (Jung *et al.*, 2004; Pluta and Oldfield, 2007).

Similar to α-linolenic acid and docosahexaenoic acid, the saturated fatty acids PAME and SAME are released and enhanced in the presence of arginine analogs from the SCG neurons possessing neuroprotective properties in experimental models of focal and global cerebral ischemia (Lin and Perez-Pinzon, 2013). Due to the dependence of arginine analogs in the release of PAME and SAME, we suggest the regulation of PAME/SAME may be involved directly or indirectly *via* PRMTs. Our studies signify the importance of PRMTs in cerebral pathological circumstances. However, more studies regarding PAME and SAME as it relates to PRMTs are needed to delineate the possible regulatory factors involved in the ischemic brain.

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