Methylated Arginine Analogues: Their Potential Role in Atherosclerosis and Cognition Using the Poloxamer 407-induced Mouse Model of Dyslipidemia

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**Methylated Arginine Analogues: Their Potential Role in Atherosclerosis
and Cognition Using the Poloxamer 407-induced
Mouse Model of Dyslipidemia**

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ABSTRACT

An experimental mouse model of dyslipidemia and atherosclerosis was utilized to study the generation of methylarginines in vivo, as well as any potential behavioral changes in mice associated with the production of excess methylarginines. Following 14 weeks of poloxamer 407 treatment, mice developed atherosclerosis and the plasma concentrations of monomethylarginine and asymmetric dimethylarginine were found to be significantly greater than corresponding concentrations in control mice. This finding may have contributed to the development of aortic atherosclerotic lesions in poloxamer-treated mice by interfering with nitric oxide availability and hence, normal function of vascular endothelium. Poloxamer 407-treated mice also showed a significant decrease in locomotor and exploratory activity, together with signs of emotional stress and anxiety relative to controls. Passive/avoidance testing to assess learning and memory provided suggestive evidence that poloxamer-treated mice could potentially be characterized as having undergone a disruption in the process of forgetting about an aversive event; specifically, a foot shock, when compared to control mice. Thus, it is also suggested that the increase in both plasma monomethylarginine and asymmetric dimethylarginine in poloxamer-407-treated mice may somehow influence learning and memory, since endothelial dysfunction caused by reduced nitric oxide availability has been hypothesized to negatively influence cognitive function.

KEYWORDS

Atherosclerosis
Dyslipidemia
L-arginine
Methylarginines
Nitric oxide

INTRODUCTION

At the end of the last century, it had been determined that disturbances in nitric oxide (NO) metabolism triggered the following sequence of events; decreased NO availability → endothelial dysfunction → atherosclerosis (**Boger et al. 1996; Cooke 1996**). According to this hypothesis, an important role was identified for methylated forms of L-arginine (Arg), which is a substrate of NO synthase. Recent studies have confirmed the critical role of NO and asymmetric dimethylarginine (ADMA) in endothelial dysfunction and the development of atherosclerosis (**Landim et al. 2009; Napoli et al. 2006**). These previous studies were conducted, in part, to clarify the mechanism by which methylarginines (MA) participate in the development of atherosclerosis (**Gilinsky et al. 2015; Jacobi et al. 2010; Loland et al. 2013; Naruse et al. 1994**).

In contrast to the numerous studies that have been conducted to investigate the relationship between ADMA and atherosclerosis, only a few publications have been devoted to evaluating the effect(s) that ADMA has on the brain vascular system and, in turn, on behavior and memory. Presently, there is intense interest in cognitive impairment originating from disturbances to the vasculature. Until recently, the study of cognitive impairment as a manifestation of cerebrovascular disease has been hampered by the lack of common standards for assessment. The term “vascular cognitive impairment” encompasses all levels of cognitive decline associated with cardiovascular diseases from mild deficits in one or more cognitive domains to crude dementia syndrome (**Di Legge and Hachinski 2010**). Therefore, the objectives of the present study were to 1) measure the plasma concentration of arginine, as well as its methylated analogues, using a mouse model of experimentally-induced dyslipidemia and atherosclerosis, and 2) demonstrate any potential changes in animal behavior, including learning and memory, that may associated with the elevated plasma MA levels.

In order to evaluate the effects of ADMA and other MA, which are themselves considered possible mediators to changes in vascular function seen with atherosclerosis, we used a well-characterized and well-documented mouse model of dyslipidemia and atherogenesis (**Johnston**

2004; Johnston et al. 2000, 2002; Korolenko et al. 2012; Palmer et al. 1998). This animal model involves prolonged treatment (approx. 12-16 weeks) of mice with a nonionic surfactant known as poloxamer 407 (P-407). The elevation of plasma cholesterol and triglycerides (TG) is dose-dependent (Johnston et al. 1999; Johnston and Palmer 1993; Palmer et al. 1998; Wout et al. 1992). Fatty streaks are microscopically visible by 4 weeks, although the formation of fully-developed aortic atherosclerotic lesions reaches a maximum in both number and density after 12-16 weeks of P-407 treatment (Johnston et al. 2000, 2002; Korolenko et al. 2012; Palmer et al. 1998). The present model was selected to investigate MA and ADMA production and their possible effect(s) on atherosclerosis, because 1) the poloxamer 407-induced mouse model of dyslipidemia and atherosclerosis recapitulates many of the physiological and biochemical processes observed in humans with atherosclerosis (Johnston 2004; Palmer et al. 1998), and 2) NO production by macrophages *in vitro* is unaffected by P-407 (Johnston et al. 2003). Based on the finding that P-407 does not modulate NO production by macrophages *in vitro*, this animal model of dyslipidemia and atherosclerosis appeared to be an appropriate animal model with which to test our hypothesis concerning the production of arginine, and its methylated analogues, and their possible association with animal behavior by interfering with biochemical reactions involving NO.

METHODS

Animals.

Six groups of male CBA mice (body mass of 25–30 g) from the Animal Care Unit of the Scientific Research Institute of Physiology and Basic Medicine (SRIPBM) were used for the biochemical and behavioral experiments. Mice had free access to water and laboratory food. The experimental protocols were approved by the SRIPBM ethical committee and conducted in accordance with the European Community Council Directive 86/609/EEC.

Mice used for the biochemical experiments consisted of the following groups. Group 1 mice ($n=14$) were administered 0.5 mL of poloxamer 407 (0.5 g/kg) by intraperitoneal (i.p.) injection every third day for 14 weeks according to Johnston et al. (**Johnston 2004**). Group 2 mice ($n=10$) received saline (0.5 mL) by i.p. injection every third day for 14 weeks and were used as controls. Group 3 mice ($n=9$) were administered 0.5 mL of poloxamer 407 (0.5 g/kg) by i.p. injection every third day for 2 weeks. Lastly, Group 4 mice ($n=6$) were administered saline (0.5 mL) by i.p. injection every third day for 2 weeks.

Male CBA mice were also used in behavioral tests and consisted of two groups. Group 5 ($n=10$) and Group 6 ($n=14$) mice were administered either saline (controls) or poloxamer 407, respectively, as described above for mice contained in Groups 1 and 2. Two series of experiments were performed on the same mice between 6 and 14 weeks of saline or poloxamer 407 administration.

Measurement of serum lipids

Serum was obtained after centrifugation of blood samples at 3000 x g for 20 min at 4 °C (Eppendorf Centrifuge 5415R; Eppendorf, Hamburg, Germany) and stored at -70 °C until analysis of total cholesterol and triglycerides. Total cholesterol and triglyceride concentrations were determined from serum, using *de novo* triglyceride and *de novo* cholesterol synthesis kits (Vector-Best, Novosibirsk Region, Russia). Photometry of the samples was performed on a

5010 semiautomatic photometer (Robert Riele, Germany) with a temperature-controlled, flow-through cuvette.

Measurement of L-arginine and methylarginines.

All procedures were performed between 0930 and 1500. Between the behavioral tests, there was one day of rest. This was enough time for the mice to rest (**Lad et al. 2010**), except for the passive avoidance test, in which case, the mice received 2 days of rest.

Animals were euthanized 24 hours after the last dose of poloxamer 407. Blood was collected in EDTA vacutainers, and plasma was separated by centrifugation. Concentrations of Arg and its methylated analogs in plasma were measured by high-performance liquid chromatography after solid-phase extraction (SPE) as described by Teerlink (**2005**). Briefly, plasma was stirred and centrifuged at 9100 x g for 10 min. The sample (200 μ L) was then mixed with 100 μ L of a 40 μ M solution of the internal standard (homoarginine) and 700 μ L of pH 7.0 phosphate-buffered saline and subsequently applied to the SPE columns (Oasis MCX 1cc, 30 mg; Waters, MA), which had previously been conditioned with 1 mL of methanol and 1 mL of water. Columns were consecutively washed with 1 mL of 100 mM HCl and 1 mL of methanol. Analytes were eluted with 1 mL of concentrated ammonia/water/methanol/1 M NaOH (10/40/50/0.5). After evaporation of the eluent under nitrogen, the amino acids were redissolved in 200 μ L of water. Then 50 μ L of the solution was derivatized with the same volume of orthophthaldialdehyde reagent containing 3-mercaptopropionic acid.

The derivatives were separated using an isocratic reversed phase chromatography on a Luna C18(2) column (3- μ m particle size, 100*2 mm; Phenomenex, Torrance, CA) with an injection volume of 20 μ L (auto-sampler SIL-10AD, Shimadzu, Kyoto, Japan). Fifty mM KH₂PO₄ buffer (pH 6.4) containing 8.7% acetonitrile was used as the mobile phase at a flow rate of 0.2 mL/min (LC-10ADvp pump, Shimadzu, Kyoto, Japan) with a column temperature of 45°C. Fluorescence detection was used at excitation and emission wavelengths of 340 and 455 nm, respectively (RF-

10A detector, Shimadzu, Kyoto, Japan). Our chromatograms provide full resolution of the amino acids (see Figure 1). Homoarginine was used as an internal standard (IS) at a concentration of 20-40 $\mu\text{mol/l}$ during sample processing and 20-40 $\mu\text{mol/l}$ in the set of standards (depending on the quality of the column). Such a high concentration of the IS was used to distinguish its presence from the normal plasma concentration of endogenous homoarginine (0.3 – 0.7 $\mu\text{mol/l}$) found in wild-type mice as measured by LC/MS/MS (Cordts et al. 2015). It is strongly suggested that homoarginine be used as the internal standard when both methylarginine inhibitors of NO synthase (*i.e.*, MMA and ADMA) are to be measured (Teerlink, 2005). As shown previously, using MMA as the internal standard results in the percent of amino acids lost during the solid-phase extraction step to decrease from about 15% to approximately 1% (Teerlink et al. 2002). We used the same procedure.

Concentrations of the analytes were calculated using the peak area of the standards (100 μM of Arg, 10 μM each of MMA, ADMA, and SDMA) as described by Sukhovshin and Gilinsky (Sukhovshin and Gilinsky 2013).

Histology studies

For morphological study of heart tissue, samples were fixed with 10% neutral buffered formalin. Specimens were embedded in paraffin and 5 μm cross-sections of tissues were stained with hematoxylin and eosin according to standard methods. The slices of heart tissue were evaluated using a STAR Carl Zeiss light microscope (Germany).

Behavioral tests.

Open field:

The “open field” test for the evaluation of locomotor activity, exploratory, and anxious behavior was carried out in a chamber (Photobeam activity system, San Diego Instruments, CA, USA) having the dimensions of 40 x 40 x 37.5 cm with photobeams (16 x 16). Mice were

placed in the center of the open field and moved freely throughout the arena. Their activity (number of beam breaks) in the center and peripheral areas, as well as the number of rearing and defecations were recorded for 5 min.

Plus-maze:

The elevated plus-maze test is probably the most popular of all currently available animal tests with which to assess anxiety and is based on unconditioned or spontaneous behavior (Rotgers and Dalvi 1997). The maze consists of two closed and two open arms (plus-maze, San Diego Instruments). A mouse was placed in the center of the maze with its nose to the closed arm. The following parameters were analyzed: the time spent on the open arms and in the center, the total number of open arm entries, the number of times that a mouse was peeking out of the closed arms, and the number of transitions from one closed arm to another closed arm. Additionally, the levels of defecation are recorded over a 5-minute test period.

Light/dark test:

The light/dark test was used to assess the behavioral response to a novel situation, including locomotor activity, exploratory, and anxious behavior. The test was carried out using a dark/light camera. Mice were initially placed in the light compartment. The latency time for the first passage from the light compartment to the dark compartment, the number of transitions between the two compartments, the time spent in the light compartment, the number of times that a mouse was peeking out, and the number of times a mouse was peeking into the dark compartment were recorded during a 3-minute test period. Lastly, rearing and defecation were measured.

Acoustic startle response:

Acoustic startle response (ASR) and prepulse inhibition (PPI) tests were used to evaluate unconditioned fear and sensorimotor gating using an SR-LAB ABS system (San Diego

Instruments). Mice were placed in the cylinder, which was mounted on a platform equipped with an ultrasensitive piezoelectric motion sensor. After a 3-min adaptation period, a series of two blocks of acoustic stimuli were presented. The first block consisted of the main startle acoustic stimuli (115 dB, 60 msec duration, 10 stimuli). When the PPI test was performed, a weak acoustic stimulus (70 to 85 dB, 20 msec) preceded the main one in the second block. The time interval between the prestimulus and main stimulus was 100 msec. The interval between stimuli was programmed in random mode from 5 to 25 sec, with a mean value of 15 sec. All stimuli were presented as white noise pulses on a background of white noise (65 dB). The amplitude of the startle reaction was determined as the peak voltage, which was proportional to the rate of displacement of the platform piezoelectric accelerometer within 100 msec after stimulus onset. Data were expressed in relative units. The mean value of the prestimulus inhibition was calculated using the same type of prestimulus intensity and, subsequently, it was calculated for each group of mice.

Passive avoidance:

The one-trial passive avoidance test was used to evaluate memory trace formation following an aversive stimulus. The step-through inhibitory avoidance apparatus was an automated camera with dark and light compartments (Gemini avoidance system, San Diego Instruments). This task requires the transition of the mouse from one compartment to another compartment. On the day of training, the mouse was placed in the light section of the apparatus, and after entry into the dark compartment with all four feet, the door was automatically closed and a foot shock was delivered through the stainless steel floor grid (0.5 mA, 2 s). After the foot shock, the mouse was then immediately returned to the home cage. Passive avoidance retention was tested 24 hours after training by placing the mouse in the light compartment and noting the latency of entry into the dark compartment (step-through latency). The maximum duration for the experiment was 180 seconds.

Statistical analysis.

Statistical analysis was performed using one- or two-way analysis of variance (ANOVA) for repeated measures (the 1st factor for passive avoidance test – group; the 2nd factor- testing time) followed by a *post hoc* analysis using the Newman-Keuls test. When only two mean values were compared for statistical significance, we utilized the Student's *t*-test and considered a *p* value less than 5% ($p < 0.05$) to indicate a statistical difference between the two mean values.

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RESULTS

Blood Lipids Following Poloxamer 407 Treatment

After 14 weeks of poloxamer-407 or saline administration, the serum concentration of total cholesterol in P-407-treated mice was found to be significantly ($p < 0.001$) greater than corresponding mean values for control mice (380 ± 68.1 mg/dL vs. 125 ± 8.1 mg/dL). The same result was obtained concerning the serum triglycerides ($2,440 \pm 813$ mg/dL vs. 144 ± 23.9 mg/dL). A statistical analysis was performed to determine if there was a positive correlation between the plasma concentration of monomethylarginine (MMA) and total cholesterol, as well as with plasma MMA and triglycerides. There was a highly-significant correlation between plasma MMA and total cholesterol concentrations ($r = 0.63$; $p < 0.002$), but the correlation between plasma MMA and triglycerides, while still significant, was not as strong ($r = 0.46$; $p < 0.03$). However, interestingly, as it pertains to ADMA, a significant positive correlation was found only for the plasma concentration of ADMA and triglycerides ($r = 0.50$; $p < 0.02$), but not total cholesterol ($r = 0.48$; $p < 0.25$).

Plasma Concentration of Methylarginines During and After Poloxamer 407 Treatment

As can be noted in Figure 2, the time over which both saline and poloxamer 407 were administered to mice did not appear to influence the plasma concentration of either L-Arg, SDMA, or ADMA. The one exception was the plasma concentrations of MMA, because there was a significant ($p < 0.05$) decrease in the plasma concentrations of MMA for both control (0.28 ± 0.06 $\mu\text{mol/L}$ (2 wk) vs. 0.059 ± 0.02 $\mu\text{mol/L}$ (14 wk)) and poloxamer 407-treated (0.27 ± 0.06 $\mu\text{mol/L}$ (2 wk) vs. 0.10 ± 0.02 $\mu\text{mol/L}$ (14 wk)) mice from 2 weeks to 14 weeks (Fig. 2B). Additionally, at 14 weeks, the plasma concentrations of both MMA and ADMA in P-407-treated mice remained significantly ($p < 0.05$) greater than the corresponding plasma concentrations for their respective controls (Fig. 2B and 2D). Interestingly, there was a significant ($p < 0.05$) increase in the plasma concentration of SDMA for poloxamer 407-treated mice at 2 weeks (0.37

$\pm 0.04 \mu\text{mol/L}$) when compared to controls ($0.24 \pm 0.04 \mu\text{mol/L}$), however, this difference in plasma concentrations of SDMA between poloxamer 407-treated and control mice disappeared by 14 weeks. Figure 2C illustrates the unchanged symmetric dimethylarginine. Stability of the SDMA provided us assurance that the recorded or observed changes in MA were not due to a change in the sensitivity of the chromatographic assay.

Histological Analysis

Heart tissue of P-407-treated mice exhibited numerous changes in the blood vessels typical of early atherosclerosis development (Fig. 3). Specifically, we observed an infiltration of xanthomatous cells of the muscle layer of arteries, swelling, and the destruction of intima and elastic fibrils (Fig. 3.2). Inside of the left ventricle space, there existed numerous thrombi localized along the blood vessels. Atherocalcinosis of some vessels, with calcification localized between elastic fibers, was noted (Fig. 3.2 and 3.3). In most cardiomyocytes, changes consisted primarily of damage to the contractile components

Animal Behavior

After 6 and 14 weeks of poloxamer 407 administration, the poloxamer-treated mice exhibited a significant ($p < 0.05$) decrease in locomotor and exploratory activity, as reflected by a decrease in the mean values for 'central arena' and 'rear' in the "open-field" test, when compared to the corresponding mean values for control mice (Table 1). The significant ($p = 0.0001$) increase in defecation observed with P-407-treated mice relative to controls suggests emotional stress and anxiety. In the "plus-maze" test, the P-407-treated mice demonstrated a highly significant reduction in the number of open arm entries and open arm duration. The transitions from one closed arm to another closed arm were reduced as well compared to controls, although it did not reach statistical significance. Nevertheless, the decrease in the number of crossings between closed arms in the "plus-maze" test would, at a minimum, suggest that P-407-treated mice appeared to exhibit a decrease in exploratory and motor activity when

compared to controls. Relative to control mice, P-407-treated mice spent significantly ($p < 0.05$) less time in the light compartment (*i.e.*, light time) in the “light/dark” test. Table 1 demonstrates a significant ($p < 0.05$) reduction in the number of crossings (transitions) between compartments in the “light/dark” test, which could be explained by a decrease of exploratory activity. All of the above mentioned behaviors indicate an increased level of emotional stress and anxiety for P-407-treated mice, as well as a decrease in exploratory activity, when compared to controls.

Passive avoidance; specifically, one-way passive avoidance demonstrated no difference between P-407-treated and control mice during the training period as assessed by the ‘step-through latency’ time (*i.e.*, the time required to enter the “dark” compartment of the 2-compartment apparatus) (Fig. 4A). On day 0 of training, the time necessary to step through and into the dark compartment (where the foot shock was administered) was approximately 37 ± 2 sec for both P-407-treated and control mice, whereas the time required to enter the dark compartment (‘step-through latency’) increased to approximately 178 ± 3 sec and 150 ± 9 sec for P-407 and control mice, respectively, on day 1 of training, which indicates that the mice had learned to passively avoid the aversive stimulus (Fig. 4A). To determine whether the mice would demonstrate recollection (memory) of the shock, the mice were tested 45 days later. As can be noted in Fig. 4B, when mice were retested for step-through latency, control mice demonstrated that they had partially forgotten about the aversive shock, since they had a mean step-through latency time of only 72 ± 21 sec, whereas the P-407-treated mice had a significantly ($p < 0.01$) longer mean step-through latency time of 138 ± 12 sec, which would suggest interference with the process of forgetting about an aversive event or stimulus (Fig. 4B).

DISCUSSION

Similar to previous findings by Johnston et al. (Johnston 2004; Johnston et al. 2000, 2002; Korolenko et al. 2012; Palmer et al. 1998), we also have successfully shown that the chronic (~14 wk) administration of P-407 to mice not only results in sustained hyperlipidemia,

but also the formation of aortic atherosclerotic lesions. Because Johnston et al. previously reported that the production of NO by macrophages *in vitro* was not affected by P-407 (Johnston et al. 2003), this appeared to be a suitable animal model of hyperlipidemia and atherosclerosis with which to test our hypothesis concerning the production of arginine, and its methylated analogues, and their possible influence on animal behavior by interfering with biochemical reactions involving NO.

The precise mechanisms by which aortic atherosclerotic lesions are formed in mice chronically treated with P-407 are still being elucidated twenty years after the introduction of this experimental animal model of atherogenesis. The findings of the present study might suggest an additional mechanism that contributes to the formation of atherosclerotic lesions in P-407-treated mice. We have previously shown that the elevation of plasma triglycerides is more sensitive than the rise in plasma total cholesterol following P-407 administration to mice (Palmer et al. 1998; Wout et al. 1992). However, as previously reported, the primary lipoprotein cholesterol fraction elevated with P-407 treatment is low-density-lipoprotein (LDL) cholesterol (Johnston et al. 1999). It is well-established that this cholesterol lipoprotein fraction represents so-called “bad cholesterol” and is the target for the class of drugs known as the “statins” (Murphy et al. 2008). In the P-407 mouse model, the increase in the LDL cholesterol fraction is made even more injurious to vascular endothelium, because the LDL cholesterol undergoes subsequent oxidation to form oxidized LDL (ox-LDL) (Johnston et al. 2003; Johnston and Zhou 2007). This is of particular interest to this study, because 1) ox-LDL cholesterol has been shown to interfere with the biological activity of NO *in vitro* (Jacobs et al. 1990; Liao et al. 1995), and 2) an increase in MMA and ADMA also causes an increase in the oxidation of LDL cholesterol (Asif et al. 2013). When taken together with the elevation of plasma MMA and ADMA observed in the present study, the bioavailability of NO could be reduced, since it has long been established that MMA and ADMA are endogenous competitive inhibitors of NO synthase (Vallance et al. 1992a,b). Additionally, it is well-documented that an

increase in plasma ADMA is associated with endothelial vasodilator dysfunction and probably subsequent cardiovascular disease; specifically, atherosclerosis (**Boger 2003; Sydow et al. 2005**). In fact, endothelial dysfunction has been observed in morphologically intact vessels even before the onset of clinically manifest vascular disease (**Harrison 1993; Sydow et al. 2005**).

Of relevance to the present work, it has been shown that chronic elevation of ADMA causes atherosclerotic lesions in mice (**Suda et al. 2004**) and that increased plasma levels of ADMA, together with a concomitant reduction in the L-arginine/ADMA ratio, has been observed in patients with hypercholesterolemia (**Eid et al. 2003**). Using a hereditary postprandial hypertriglyceridemic rabbit model (PHT rabbit) in which triglycerides are elevated to a much greater extent than cholesterol, which is similar to the P-407 mouse model of dyslipidemia used in this study, Matsumoto et al. recently demonstrated that a marked elevation of postprandial plasma triglycerides caused visceral fat accumulation, fatty degeneration of the liver, early atherosclerotic intimal thickening, and rapid onset of vascular dysfunction in endothelial cells (**Matsumoto et al. 2014**). Thus, it is now widely accepted that ADMA functions as an endogenous regulator of NO synthesis and that it becomes dysregulated in various disease states, which, in turn, causes endothelial dysfunction (**Stuhlinger et al. 2001, 2002, 2003**).

One intriguing aspect of endothelium-derived NO is that it exerts a tonic vasodilator tone, prevents cellular adhesion to the vessel wall, inhibits platelet activation, and retards the development of atherosclerosis in experimental animals (**Vallance 2001**). If NO is truly involved with preventing or inhibiting cells from adhering to vessel walls, then it could be postulated that NO function has been disrupted or modified in the P-407-induced mouse model of atherosclerosis, because we have previously shown that the shed, soluble forms of three cellular adhesion molecules; namely, ICAM-1, VCAM-1, and E-selectin were significantly increased after a single injection of P-407 to mice relative to controls (**Johnston 2009**). Thus, it would appear that NO was unable to prevent the three soluble cell adhesion molecules

mentioned above from adhering to vessel walls, although it must be emphasized that Johnston measured the soluble, shed forms of these three adhesion molecules, which are typically thought to be a surrogate marker for the cellular-bound fraction (**Johnston 2009**). Nevertheless, the fact that these cell adhesion molecules were upregulated in the P-407 mouse model of atherosclerosis would suggest that they could subsequently facilitate the adhesion of cells and platelets to the endothelial cells that line the luminal side of the vessel. Importantly, and as mentioned above, while NO retards the development of atherosclerosis in experimental animal models, it would seem to suggest that NO's function is compromised in the P-407 mouse model of atherosclerosis, because mice do, in fact, develop aortic atherosclerotic lesions after 14 weeks of P-407 treatment as shown in the present study and as reported previously (**Johnston et al. 2000, 2002; Korolenko et al. 2012; Palmer et al. 1998**).

One important question that arises from the present findings is why the plasma levels of ADMA remain significantly greater than corresponding plasma ADMA levels for controls after 14 weeks of P-407 administration. There exist two main pathways for ADMA clearance. The first is by renal excretion and the second is through metabolism by dimethylarginine dimethylaminohydrolase (DDAH) (**Ogawa et al. 1987**). The reduced clearance of ADMA in renal failure is associated with severe endothelial vasodilator dysfunction, which can be reversed by intravenous administration of the NO precursor L-arginine (**Vallance et al. 1992b**) or by dialysis which removes plasma ADMA (**Gilinsky et al. 2012; Kielstein et al. 1999**). However, in hypercholesterolemia, ADMA accumulation seems to be due to an impairment of DDAH activity (**Ito et al. 1999**). Therefore, in the present investigation, perhaps the elevated level of plasma ADMA at 14 weeks in P-407-treated mice relative to controls is a result of partial DDAH inactivation by, as yet, an undetermined mechanism, although this hypothesis will serve to guide future experiments to ascertain the exact mechanism. This proposed mechanism, while speculative at this point in time, would result in the accumulation of plasma ADMA in P-407-treated mice relative to plasma ADMA levels we determined in control mice.

ADMA and MMA accumulation causes reduced NO synthesis and promotes atherosclerosis (**Asif et al. 2013**). Interestingly, we have previously shown that administration of a statin drug (atorvastatin) was able to halt the formation of aortic atherosclerotic lesions in P-407-treated mice (**Johnston et al. 2000**). This is extremely significant for two reasons. First, the reduction in the plasma concentration of LDL-cholesterol achieved with administration of atorvastatin to P-407-treated mice presumably also caused a simultaneous decrease in ox-LDL, which, in turn, eliminated, or at least minimized, the injurious inflammation to vessel walls; specifically, vascular endothelial cells. Secondly, statin drugs, including atorvastatin, have been shown to enhance the activity of DDAH and thereby reduce ADMA concentrations, such that ADMA (and maybe MMA) cannot function as a NOS inhibitors and limit the availability of NO (**Maas 2005; Wadham and Mangoni 2009**). Therefore, the reduction in both LDL-cholesterol and ox-LDL, together with an atorvastatin-mediated enhancement in DDAH activity, potentially contributed to the absence of aortic atherosclerotic lesions previously observed in P-407-induced hyperlipidemic mice simultaneously receiving atorvastatin (**Johnston et al. 2000**).

The second major finding in the present study was that animal behavior, as well as learning and memory (as assessed using a passive avoidance test), was altered. It may be that the common link between the cardiovascular consequences of endothelial dysfunction (*i.e.*, atherosclerosis) and impaired learning and memory is the excessive accumulation of ADMA. It is well-recognized that virtually all known vascular risk factors are associated with increased plasma concentrations of ADMA (**Cooke 2005; Vallance and Leiper 2004**). Thus, it is entirely possible that ADMA and MMA are directly involved in the pathogenesis of cognitive impairment and dementia, in addition to its established role in adverse cardiovascular and cerebrovascular conditions (**Sibal et al. 2010**). Furthermore, one possible mechanism that could be advanced for the changes in behavior and 'learning and memory' that were observed with P-407-treated mice in the present study might include the sustained elevation of plasma ADMA

and MMA relative to controls, although further experimentation in the very-specialized field of “animal cognitive deficit assessment” is required to unequivocally prove this hypothesis.

It should be emphasized that we did not perform any formal univariate or multivariate correlation analyses between the elevations in serum MA (MMA and ADMA) and either cognitive impairment, or changes in animal behavior, due to the limited volume of plasma obtainable from the mouse model we employed. Hence, like in the most of similar studies only associations, but no statements implying causality, have been advanced in the present investigation with regard to elevated serum MA levels and either cognitive impairment, or changes in animal behavior.

It would seem worthwhile to discuss the putative mechanism(s) that might produce a cognitive deficit in mice with regard to learning and memory as it relates to ADMA accumulation and NO availability, although, to date, our laboratory only has preliminary evidence (*i.e.*, only passive avoidance experiments) to suggest that mice chronically administered P-407 have actually manifested a decline in cognitive function. Impaired NO synthesis and/or availability results in endothelial dysfunction, vasoconstriction, and remodeling, thus favoring atherosclerosis and thrombosis. The result of these phenomena is an impaired blood flow regulation and supply to peripheral organs and tissues (**Bian and Murad 2003**). There is strong evidence that both impaired NO synthesis and availability exert detrimental effects to the cerebral circulation (**Asif et al. 2013**). This can manifest either as acute ischemic events (stroke) and/or sub-acute or chronic hypoperfusion states (*e.g.*, white matter lesions). Both of these abnormalities reduce cognitive function as it pertains to learning and memory and increase the risk of dementia (**Debette and Markus 2010; Gottesman and Hillis 2010**). In fact, Arlt *et al.* reported that increased circulatory ADMA was associated with impaired cognition in patients with Alzheimer’s disease (**Arlt et al. 2008**). Additionally, Huang et al. recently reported that the accumulation of ADMA in the plasma of rats that had undergone a bile duct ligation demonstrated significant impairment of motor coordination and cognition (spatial memory)

performance when compared to controls (**Huang et al. 2010**). Thus, as stated above, one of our future research goals will be to determine whether the sustained elevation of plasma MMA and ADMA we observed in mice treated with P-407 for 14 weeks, relative to controls, contributes wholly, or in part, to deficits in locomotor and exploratory activity, spatial learning, and specific aspects of memory including acquisition, consolidation, retention, and recall.

CONCLUSION

Dyslipidemia and atherosclerosis were demonstrated following 14 weeks of P-407 administration to mice. In addition, we determined that 14 weeks of P-407 administration to mice resulted in significantly greater plasma concentrations of MMA and ADMA relative to controls; both compounds, of which, are known endogenous inhibitors of NO synthase and are thought to contribute to deficits in cognitive processes. The inhibition of NO synthase by MA in P-407-treated mice would be expected to limit the availability of NO and perturb numerous aspects of normal endothelial function. Therefore, it is suggested that elevated plasma MA and, specifically, ADMA and MMA, following 14 weeks of P-407 treatment in mice potentially limited the availability of NO *in vivo*, which, in turn, may have partially contributed to endothelial dysfunction and the subsequent atherosclerosis we observed in the P-407-treated mice, although further experimentation aimed at assessing NOS activity and brain histology (cerebrovascular endothelium) is required to establish causality. Additionally, several behavioral tests were performed in our mice to determine whether elevated levels of plasma MMA and ADMA might potentially influence locomotor and exploratory activity, as well as learning and memory using a passive avoidance test. Poloxamer 407-treated mice showed a significant decrease in locomotor and exploratory activity, together with signs of emotional stress and anxiety relative to control mice. The passive avoidance test provided suggestive evidence that

P-407-treated mice could potentially be characterized as having undergone a disruption in the process of forgetting about an aversive event; specifically, a foot shock, when compared to control mice. In other words, the memory trace of the aversive or fearful event (foot shock) is 'well-stored' in the P-407-treated mice 45 days later, which is indicative of a disturbance or disruption in memory, because it prevents the formation of a new memory trace that might involve or suggest; "*no danger/aversive-stimulus exists now*". While speculative at this time due to our limited data, it is hypothesized that the elevated plasma ADMA and MMA observed in P-407-treated mice, relative to control mice, following 14 weeks of P-407 treatment may somehow influence learning and memory.

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Table 1: Behavior of Control and Poloxamer 407- treated Mice Following Six and Fourteen Weeks of Poloxamer 407 Administration.

<i>Parameters</i>	6 weeks			14 weeks		
	Control	Poloxamer	<i>p</i>	Control	Poloxamer	<i>p</i>
	Open-field (evaluates locomotor and exploratory activity and anxious behavior)					
Central arena, (<i>n</i>)	94±19	50±6	0.02	110±20	49±5	0.001
Periphery, (<i>n</i>)	288±25	221±21	0.06	255±24	230±23	0.07
Rear, (<i>n</i>)	5±1	1±0.5	0.002	6±1.5	1±0.3	0.001
Defecation, (<i>n</i>)	1±0.3	5±0.4	0.0001	1±0.2	6±0.5	.0001
	Plus-maze (evaluates animal anxiety)					
Open arm time, (sec)	120±30	32±13	0.01	97±12	39±8	0.001
Open entries, (<i>n</i>)	2±0.3	0.7±0.2	0.001	3±0.2	1±0.3	0.0003
Crossing, (<i>n</i>)	2±0.5	0.8±0.3	0.05	2±0.7	1±0.4	0.06
Peeking out, (<i>n</i>)	8±2	4±0.8	0.05	8±0.9	2±0.4	0.0001
Defecation, (<i>n</i>)	4±0.6	5±0.6	0.3	4±0.6	4±0.9	0.9
	Light/dark (evaluates locomotor and exploratory activity and anxious behavior associated with a novel situation)					
Step-through latency, (sec)	41±5	64±10	0.08	52±7	71±8	0.07
Light time, (sec)	68±6	41±5	0.006	81±9	44±5	0.0008
Crossing, (<i>n</i>)	9±0.7	5±0.6	0.0008	11±0.7	6±0.5	0.0001
Rear, (<i>n</i>)	11±1	6±0.7	0.001	9±0.6	5±0.5	0.0001
Peeking in, (<i>n</i>)	2±0.4	1.5±0.3	0.09	2±0.3	2±0.4	0.1
Peeking out, (<i>n</i>)	4±0.5	2±0.4	0.05	5±0.6	2±0.3	0.006
Defecation, (<i>n</i>)	0.5±0.3	0.8±0.4	0.6	2±0.4	3±0.6	0.5
	Prepulse inhibition of startle response (evaluates unconditional fear and sensorimotor gating)					
PPI 78 dB	28±4	36±5	>0.05	32±4	36±2	>0.05
PPI 86 dB	39±3	44±3	>0.05	46±4	48±2	>0.05
Startle amplitude	1948±192	2068±127	>0.05	1601±94	1966±57	>0.05

Indications: Open arm time is the time spent in open arms. Open arm entries are the number of entries into open arms. Crossing is the number of transitions from one closed arm to the

other closed arm (plus maze), the number of transitions from the light compartment to dark one (light/dark). Peeking out is the number of peeking out the closed arms (plus maze) and the dark compartment (light/dark). Step-through latency is the time for the first entry to the dark compartment. Light time is the time spent in the light compartment. Peeking in is the number of peeking in the dark compartment.

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LEGENDS for Figures

Fig.1

Chromatograms of standards and plasma of the control and Poloxamer treated mice.

Line A-A' indicates the increase of sensitivity 8 times as compared to first 10 min

Arg – L-Arginine; **MMA** – Monomethylarginine; **IS** – Internal Standard (Homoarginine); **ADMA** – Asymmetric dimethylarginine; **SDMA** – Symmetric dimethylarginine

Fig.2.

Concentrations of L-arginine and methylarginines after different periods of Poloxamer 407 administration.

NOTE. 14 weeks administration of Poloxamer 407 results in the significant increase of monomethylarginine and asymmetric dimethylarginine (B, D), but not of L-arginine and symmetric dimethylarginine (A, C).

* - data of Poloxamer and control groups differ significantly ($p < 0.05$);

- data received at 6 and 14 weeks differ significantly .

Figure modified from Gilinsky, M.A., Sukhovshin, R.A., and Cherkanova, M.S. 2015.

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Fig.3. Mouse Heart after Poloxamer 407 treatment.

Fig.3.1. Heart of mouse after 14 weeks of Poloxamer 407 treatment. Calcium deposits in the vessel walls and lumen in the form of a dark blue substance. H & E stain. Magn. 400 .

Fig.3_2,3. Heart of mouse after poloxamer treatment (fragments of Fig.1). Calcium deposits in the vessel walls and lumen in the form of a dark blue substance. H & E stain. Magn. 1000 with immersion.

H – hematoxylin, E – eosine.

Fig.4.

Passive avoidance task.

Training took place at the days 0 and 1. After 45 days animals were tested on fear trace retention.

The same chromatograms but magnification is 4 times higher.

Figure 1.

Figure 2.

Fig.3.1

Fig.3.2

Fig.3.3

Figure 4.