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Elevated prostacyclin biosynthesis in mice impacts memory and anxiety-like behavior

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Abstract

Prostacyclin is an endogenous lipid metabolite with properties of vasodilation and anti-platelet aggregation. While the effects of prostacyclin on the vascular protection have been well-documented, the role of this eicosanoid in the central nervous system has not been extensively studied. Recently, a transgenic mouse containing a hybrid enzyme, of cyclooxygenase-1 linked to prostacyclin synthase, was developed that produces elevated levels of prostacyclin in vivo. The goal of this study was to investigate whether increased prostacyclin biosynthesis could affect behavioral phenotypes in mice. Our results uncovered that elevated levels of prostacyclin broadly affect both cognitive and non-cognitive behaviors, including decreased anxiety-like behavior and improved learning in the fear-conditioning memory test. This study demonstrates that prostacyclin plays an important, but previously unrecognized, role in central nervous system function and behavior.

Keywords

Prostacyclin; eicosanoid; behavior; memory; anxiety

1. Introduction

Prostacyclin (PGI₂) is synthesized in vascular endothelial cells when arachidonic acid (AA) is converted to PGI₂ through a three-step catalytic reaction by two enzymes, cyclooxygenases-1 or 2 (COX-1, COX-2) and PGI₂ synthase (PGIS). Prostacyclin mediates biological actions through its G-protein-coupled receptor (IP receptor), where it is known to cause vasodilation and anti-platelet aggregation as one of the major vasoprotectors [1, 2] and the endocrine systems [3, 4], and is used as a vascular protective agent and treatment option in pulmonary hypertension [5, 6]. Although PGI₂ is known to be generated in cerebral arteries [7, 8], the consequences of increased PGI₂ secretion within central nervous system (CNS) have not been extensively characterized.

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Early studies on PGI₂ indicated a reduction in cerebral blood flow after PGI₂ intravenous infusion in normotensive humans and rats [9–12], implicating a possible role in the maintenance of cerebrovascular tone. This is probably of importance since in cases of brain injury, PGI₂ may potentially be therapeutic. For example, 48 hours following fluid percussion brain injury in rats, PGI₂ treatment reversed blood flow reductions in the injured cortex of rats [13], indicating an improvement in cortical perfusion. Additionally, administration of PGI₂ agonists TTC-909 [14] and ONO-1301 [15] both reduce infarct volume after middle cerebral artery occlusion (MCAO) in rats, with ONO-1301 reducing neurological deficits. TTC-909 was also shown to reduce memory impairments in rats with cerebral embolism [16], supporting the notion that PGI₂ may play a role in cognitive functions.

Interestingly, there is evidence for a possible prostacyclin receptor isoform within the CNS, IP₂, which is pharmacologically distinguished from the peripheral receptor, IP₁ [17, 18]. IP₂ receptor expression has been identified in the cerebral cortex, striatum, thalamus, and hippocampus of the rat brain [18]. Additionally, activation of the IP₂ receptor by the novel ligand, 15R-TIC, was found to be neuroprotective after MCAO in monkeys. 15R-TIC significantly reduced infarct volume and increased the cerebral metabolic rate of oxygen, which was shown to not be as a result of increased cerebral blood flow to the damaged regions [19].

The discovery of IP₂ receptor expression in the brain supports the possibility that PGI₂ directly acts in the CNS and could potentially contribute to cognitive functioning. CP-Tg mice overexpress the TriCat enzyme [20, 21], a hybridized enzyme linking COX-1 and PGIS via a transmembrane domain. Overexpression of this hybrid results in a three-fold increase in anabolism of PGI₂ in cell lines and shows an increased ability to efficiently convert AA into PGI₂ [22]. CP-TG mice expressing this enzyme express show resistance to induced thrombotic stroke and cardiac arrest [21]. Our aim in this study was to investigate whether PGI₂ could beneficially affect cognitive-like behaviors in PGIS-overexpressing mice.

2. Materials and methods

2.1. Animals

Heterozygous CP-Tg, containing a hybrid enzyme of COX-1 linked to PGIS (COX-1-10aa-PGIS; N = 20), and non-transgenic (NTg; N = 9), mice used in experiments were maintained in the Animal Care Facility at the University of Houston. They were kept in groups of one to five mice per cage in a room with a 12 hour light cycle (lights on at 6:00AM and off at 6:00PM) at 23C with food and water available ad libitum. Male mice and female mice were randomized across groups. 3–6 months were used for all behavioral assays. All experiments were conducted in accordance with the University of Houston IACUC guidelines using approved protocols. CP-Tg mice were genotyped using PGIS-specific primers (BD2442-1: GGATTCCAAGTCTCCACCC; BD2442-2: GCAGTCACACTGGTAGCGG) [21].

2.2. PGI₂ Activity Assay

PGI₂ activity in CNS tissue was quantified as previously reported [23]. To determine the activities of the enzyme that converted arachidonic acid (AAs) were homogenized with cold PBS (50mg / 100 µl). 0.1 µl of [¹⁴C] AA (1 µM) was added to the homogenized samples in a total reaction volume of 0.1 mL. After a 5 minute incubation, the reaction was terminated by adding 200 µl of solvent containing 0.1% acetic acid in 35% acetonitrile and 65% deionized H₂O (solvent A). After centrifugation at 13,000 rpm for 10 minutes, the

supernatant was injected into a reverse phase C18 column (Varian Microsorb-MV 100-5, 4.5×250 mm) using the solvent A with a gradient from 35% to 100% of acetonitrile for 40 min at a flow-rate of 1.0 mL/min. The [¹⁴C]-labeled AA metabolites, including [¹⁴C]-6-keto-PGF1a (degraded PGI₂) were monitored directly by a flow scintillation analyzer (Packard 150TR). A positive control for PGI₂ production was performed using cell lysates containing 100,000 HEK293 cells transiently transfected with COX-1-10aa-PGIS cDNA; production of PGI₂ and metabolites were analyzed by HPLC, as previously reported [22].

2.3. Open Field test

Anxiety-like and non-cognitive behavior was assessed by the open field test at three and six month periods for all mice. Each mouse was allowed to explore a novel environment in a clear chamber for 30 min while being monitored (OptoMax, Columbus Instruments) with standard room lighting conditions [24].

2.4. Rotarod test

Motor learning and coordination were evaluated by an accelerating cylindrical drum Rotamex Rotarod machine (Columbus Instruments; Columbus, OH) at three and six months of age. Each mouse was placed on a horizontal accelerating rod (4–40 rpm) and subjected to 4 trials a day for two days with 15 min. intervals between each trial. A trial ended when the mouse fell off the rod, time elapsed 200 seconds, or became inverted twice in the same trial without falling.

2.5. Light dark (LD) exploration

Mice were subjected to light-dark exploration test to evaluate anxiety-like behavior at three and six months of age. The light-dark box consisted of a light compartment (27 cm × 27 cm × 27 cm) and a dark compartment (27 cm × 18 cm × 27 cm) separated by a partition with a single opening (7 cm × 7 cm) to allow passage between compartments as previously described [25, 26]. Each trial lasted 5 min and time was measured manually as previously described [24].

2.6. Elevated-plus maze

Anxiety-like behavior was measured using the Elevated plus maze at three and six months of age. The test consists of an X-shaped apparatus with two arms closed off from observation on the sides and end by large blinders, and two arms open to light with a central area between the arms not enclosed. Standard lighting was used during testing. The mice were placed in the center area facing an open arm and were recorded by a video camera for 5 minutes. Time and number of transitions were documented manually.

2.7. Fear conditioning

Contextualized and Cued fear conditioning to test for short and long-term memory and learning was conducted at six months as previously described [27, 28].

2.8. Statistical analyses

Quantitative results were expressed as mean ± standard error of mean (SEM). For contextualized and cued fear conditioning trials was analyzed using repeated measures ANOVA (p 0.05 was considered statistically significant). All other data was analyzed using one-way ANOVA and Fisher LSD post hoc test to determine significant differences between groups (p 0.05 was considered statistically significant).

Results

3.1. Analysis of PGI₂ Production in Brain

A functional activity assay was used to demonstrate enhanced PGI₂ production in the CP-Tg mouse line. As shown in Figure 1A, incubation of arachidonic acid resulted in a peak of 6-Keto-PGF1 α at ~6 minutes in HPLC in CP-Tg mice, and a notable enhancement in 6-Keto-PGF1 α production compared to the NTg control. Brain tissue homogenates from Ntg and CP-Tg mouse cortex showed a mean 60.4% increase in PGI₂ production in the CP-Tg line, compared to control (n=3, $p < 0.05$).

3.2. Effect of PGIS overexpression on non-cognitive behavior

Motor functions were assessed by the open-field activity and a motorized rotarod. PGIS overexpression had no effect on spontaneous activity at 3 months (Fig. 2A), but at 6 months displayed hypo-activity (Fig. 2B, $p < 0.05$). At 3 months of age PGIS mice had an increased latency to fall in trials 1, 2, and 3 indicating increased coordination and balance (Fig. 2C, $p < 0.05$). At six months of age PGIS mice had normal motor coordination, balance and learning (Fig. 2D).

3.3. Analysis of anxiety-like behavior in open field assay

To assess anxiety-like behavior in mice time spent in the center and the perimeter was measured. At 3 months of age, CP-Tg mice showed a significant increase in time spent in the perimeter with a concomitant decrease in time spent in the center), an indication of anxiety-like behavior in rodents (Fig. 3A, $p < 0.05$). There were no differences observed at 6 months (Fig. 3B).

3.4. Light Dark test

The light dark (LD) test was used to further assess anxiety-like behavior by observing the naturalistic conflict between the tendency to explore a novel environment rather than the aversive brightly lit open-field. At 3 months, there were no significant differences in either the time spent in the lit area or number of transitions (Fig. 4A and 4B). At six months of age PGIS mice significantly spent more time in the lit are and made fewer transitions (Fig. 4C and 4D, $p < 0.05$).

3.5. Elevated Plus Maze

To further assess anxiety-like behavior in the CP-Tg mice the elevated plus maze (EPM) was used in addition to the LD test. This test introduces height and openness in the arms which are not present in the LD. There were no significant differences in time spent in the open arms, but a significant increase in number of transitions at 3 months (Fig. 5A and 5B, $p < 0.05$). There were no significant differences in time spent in the open arms or number transitions at 6 months (Fig. 5C and 5D).

3.6. PGIS and Fear Conditioning

All mice responded to the electric shocks during the training phase. Compared to the NTg group, CP-Tg mice showed a higher percentage freezing during the third shock compared to NTg mice during the training trial (Fig. 6A, $p < 0.05$). There were no differences in total percent freezing time during the contextual fear conditioning trial (Fig. 6B). CP-Tg mice exhibited an abnormal freezing pattern, as indicated by within subject significance (Fig. 6B, $p < 0.004$), with elevated percentage freezing from 3–6 minutes, in contrast to NTg mice that showed a relatively sustained freezing pattern during the duration of the whole trial.

4. Discussion

This report is the first characterization of the effect of PGIS overexpression on cognitive and non-cognitive behaviors in mice. Prostacyclin synthase is abundantly expressed peripherally and throughout the CNS, within the cerebrovasculature, in neurons and in glia cells [29]. PGI₂ has long been recognized as a vasoprotective agent and it has been well-established that PGI₂ plays an important role in inflammation [1, 2], but the question of whether PGI₂ might exert a significant influence on CNS function has not been addressed. Previous work has shown that loss of the peripheral IP1 prostacyclin receptor appears to reduce both inflammatory and pain responses [1, 30].

We found that the increased levels of PGI₂ in CP-Tg mice did not result in any significant differences in body weight or alterations in general development. Additionally, increased PGI₂ did not appear to be lethal, as all animals survived the duration of the study. In CP-Tg mice transgenic expression of the hybrid enzyme, COX-1-10aa-PGIS, occurs in all major organ systems, including the CNS [21]. Consequently, it is possible that the changes in cognitive and non-cognitive behaviors we observed in CP-Tg mice could result from increased PGI₂ production from both within the CNS and peripherally.

Assessments of motor coordination indicated that 3 month PGIS mice were more coordinated than wild-type mice, while at 6 months there was no difference, an indication that PGIS overexpression did not negatively impact motor coordination. In terms of general exploratory activity, open field testing revealed that PGIS overexpression resulted in significantly decreased exploratory behavior at six months. No differences were observed at 3 months. While a reduction in these behaviors could be interpreted as impaired locomotor activity, our results from rotarod testing indicated otherwise. Additionally, increased amount of time spent at the perimeter versus the center of the open field at 3 months was observed, indicative of anxiety-like behavior [31]. However, at 6 months no effect on anxiety-like behavior in the open field was observed.

Since one behavioral assay may not completely capture anxiety-like behavior in rodents [32], we tested the effect of the PGIS genotype on two other widely-used anxiety assays: elevated-plus maze and the light/dark box. Interestingly, in the elevated-plus maze there were no significant differences between CP-Tg mice (3- and 6-month old) and the controls in time spent in the open arm, an indication that the PGI₂ had no effects on anxiety-like behavior in this assay. However, in the light/dark box assay, PGIS expression resulted in a decrease in anxiety-associated behavior at 6 months, with more time spent in the light, but there was no significant effect at 3 months, raising the possibility that PGI₂ could act to mediate anxiety. Collectively, the tests on anxiety show that the effects of elevated PGI₂ are task-dependent, and that elevated endogenous PGI₂ levels may be anxiogenic in young mice, while being anxiolytic in older mice.

Several areas of the cortex have been shown to be involved in anxiety including prelimbic and infralimbic cortices [33, 34], orbitofrontal cortex [35] and prefrontal cortex [36–38]. Additionally, the hippocampus [36, 39–41] and striatum [42–44] are thought to also regulate anxious behaviors in rodents. Since the PGI₂ receptor, IP₂, is expressed in the cerebral cortex, hippocampus, and striatum [18], this certainly raises the possibility that increased activation or desensitization of the IP₂ receptor (due to elevated PGI₂ levels) in the cortex, hippocampus and striatum could mediate anxious-like behavior in mice.

Our work also shows that elevated PGI₂ levels impact associative learning. We introduced CP-Tg mice to cued and contextual fear conditioning to assess memory of a conditioned stimulus (cue) and spatial memory of the environment (context). Our results indicated that testing of contextual-associated memory after one hour of training was significantly

increased in 6-month-old CP-Tg mice, while at 24 hours post-training the performance of CP-Tg mice was identical to their wild-type counterparts. Short-term memory was improved, however, long-term memory remained unchanged, PGI₂ is likely to play a role in short-term memory. Since IP₂ receptor expression has been found in the hippocampus [18], and contextual fear conditioning is hippocampal-dependent [45–48], it is conceivable that PGI₂ could be involved in hippocampal-dependent memory acquisition and/or consolidation. These observations complement the recent findings by Muramatsu et al [49] who reported that prostacyclin elevates cAMP levels through activation of the IP receptor on cultured neurons *in vitro*, promoting axonal remodeling.

To our knowledge, this is the first evidence of the involvement of PGI₂ in anxiety and contextual fear conditioning. Considering the limited degree of understanding of the role of PGI₂ and its associated receptor action within the CNS, the findings in this study warrant further attention. Future work will corroborate the behavioral findings presented here with histological and molecular confirmation of CNS IP₂ receptor expression and PGI₂ levels in PGIS mice, as well as understanding some of the potential underlying mechanisms of PGI₂-induced anxiolytic behavior and facilitation of short-term memory. Elucidating the role of PGI₂ in cognitive functioning will broaden our understating of its role in the CNS.

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Highlights

- Prostacyclin-overexpressing mice display enhanced motor coordination at 3 months of age.
- Prostacyclin-overexpressing mice exhibit alterations in anxiety-associated behavior
- Six-month old transgenic-mice displayed improved short-term memory in the contextual fear-conditioning test.
- Our observations suggest that prostacyclin expression can modulate anxiety and memory

A **B** **Figure 1. Measurement of PGI $_2$ production in brain**

(A) Examples showing 6-Keto-PGF 1α detected using HPLC, using a positive control (COX-1-10aa-PGIS positive cell lysate), non-transgenic mouse, and TG-Cp mouse brain tissue. (B) This figure demonstrates that CP-Tg mouse tissue exhibits enhanced conversion of arachidonic acid to PGI $_2$ ($n=3$, for each group, $p<0.05$), compared with control.

Figure 2. PGIS overexpression had significant effect on non-cognitive behavior

(A) Exploratory behavior measured by the open field activity in 3 month old mice shows no significant differences. (B) At 6 months of age, exploratory behavior measured in CP-Tg mice is significantly decreased compared to NTg mice. (MOVE) = Total time moving, (REST) = time at rest, (STEREO) = time moving in stereotypic fashion. (C). In 3 month old mice, CP-Tg showed improved coordination on the rotarod, compared with NTg mice on trials 1, 2, and 4. (D) At 6 months of age, there was no significant difference between groups. * $p < 0.05$.

A **B**

Figure 3. Effect of PGIS on anxiety-like behavior measured by the open field activity

(A) Three month old CP-Tg mice exhibit greater time spent on the periphery of the open field, compared with NTg. (B) At 6 months of age, there is no significant difference between NTg and CP-Tg mice between the perimeter and the center of the open field). * $p < 0.05$.

A **B**

Figure 4. PGIS overexpression decreased anxiety-like behavior at six months in light dark exploration

(A) At three months of age, there was no significant difference in time or number of transitions between the two groups. (B) At six months of age, CP-Tg mice spent a significantly greater time in light, and had a reduced number of transitions, compared to NTg mice. * $p < 0.05$.

A **B**

Figure 5. Anxiety-like behavior measured by elevated plus maze

(A) Time spent in open arms is similar between CP-Tg and NTg lines in three month old mice, but CP-Tg mice show significantly more transitions than NTg mice. (B) In six month old mice, the time spent in open arms and the number of transitions is similar between the two groups. * $p < 0.05$.

Figure 6. PGIS overexpression had a significant effect on associative learning

(A) CP-Tg has a significant increase in percentage freezing time during the third shock of the training trial. (B) CP-Tg mice exhibited an increased average motion index compared with NTg mice. Mice were tested in both cue and context trials. (C) CP-Tg exhibited an enhanced response in the short-term memory component in the contextual fear conditioning ($p < 0.05$). (D) No significant differences were seen in long term memory contextual fear conditioning. (E) No significant differences were seen in short term cue fear conditioning. (F) No significant differences were observed in long term cue fear conditioning.