Peroxiredoxin 6 Overexpression Induces Anxiolytic and Depression-Like Behaviors by Regulating the Serotonergic Pathway in Mice

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Abstract
Peroxiredoxin 6 (PRDX6) is a bifunctional protein with both glutathione peroxidase and calcium-independent phospholipase activity. Recently, we reported that PRDX6 plays an important role in dopaminergic neurodegeneration in Parkinson’s disease. However, the relationship between PRDX6 function and emotional behavior remains elusive. In the present study, we examined depression- and anxiety-like behaviors in PRDX6-overexpressing transgenic (PRDX6-Tg) mice using the forced swim test, tail suspension test, open field paradigm, and elevated plus-maze. PRDX6-Tg mice exhibited depression-like behaviors and low anxiety. In particular, female PRDX6-Tg mice exhibited anxiolytic behavior in the open field test. Furthermore, the serotonin content in the cortex and 5-hydroxytryptophan-induced head twitch response were both reduced in PRDX6-Tg mice. Interestingly, levels of dopa decarboxylase expression in the cortex were decreased in male PRDX6-Tg mice but not in female mice. Our findings provide novel insights into the role of PRDX6 in 5-HT synthesis and suggest that PRDX6 overexpression can induce depression-like behaviors via downregulation of the serotonergic neuronal system.

Key Words: Peroxiredoxin 6, Depression, Anxiety, L-amino acid decarboxylase, Serotonin

INTRODUCTION

Peroxiredoxin 6 (PRDX6), the only 1-Cys member of the peroxiredoxin (PRDX) family (Chae et al., 1994), is a bifunctional enzyme with glutathione peroxidase (GPx) and phospholipase A2 (PLA2) activities, called aiPLA2 (Fisher et al., 1999; Manevich et al., 2004). Recently, we reported that PRDX6 plays an important role in dopaminergic neurodegeneration in a mouse model of Parkinson’s disease (PD) (Yun et al., 2015). Moreover, an imbalance in the redox system can be associated with psychiatric symptoms (Vaccarino et al., 2008). However, the potential role of PRDX6 in psychological behavior remains poorly clarified. Insufficiency of the serotonergic central nervous system (CNS) may play a key role in the pathophysiology of depression (Stockmeier, 2003). Patients with depression exhibit reduced cerebrospinal fluid levels of 5-hydroxyindoleacetic acid (5-HIAA), as well as tryptophan depletion-induced transient relapse during successful treatment with selective serotonin (5-hydroxytryptamine, 5-HT) reuptake inhibitors (SSRIs) (Owens and Nemeroff, 1994). The pharmacological management of depression currently involves drugs that frequently target monoamine transporters, including SSRIs, noradrenaline (NA) inhibitors (NRIs), or a combination of both (SNRIs) (Hillhouse and Porter, 2015).

The association between 5-HT receptors, depression, and anxiety has been well-established. Abnormalities in the synthesis, degradation, and transport of neurotransmitters can lead to diverse neurological manifestations, including developmental delay, motor disorders, epilepsy, autonomic dysfunction, and neuropsychiatric features, which might be associated with neurotransmitter synthetic enzymes or transporters such as tyrosine hydroxylase, aromatic L-amino acid decarboxylase

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(AADC), and dopamine transporter (DAT) (Ng et al., 2014; Siu, 2015). AADC is a homodimeric pyridoxal phosphate-dependent enzyme responsible for synthesizing dopamine and 5-HT. AADC deficiency is reportedly associated with PD (Zou et al., 2016). In the present study, we examined depression- and anxiety-like behaviors in PRDX6-overexpressing transgenic (PRDX6-Tg) mice. Furthermore, we investigated the association between AADC and neurotransmitter imbalances in PRDX6-Tg mice.

MATERIALS AND METHODS

Animals
PRDX6-Tg (C57BL/6J) mice were purchased from Jackson Laboratory (ME, USA) and C57BL/6 (non-Tg) mice were purchased from DBL (Eumseong, Korea). Animals were maintained in conventional housing at 23 ± 2°C under a controlled 12 h light/dark cycle, with drinking water and rodent chow provided throughout the experiment. Behavioral analyses were conducted on mice aged 2-5-months. Animals were used for each behavioral test only once in the following order: one group of mice was subjected to the open field test, elevated plus-maze test, tail suspension test, grip strength test, and rotarod test; the second group of mice was subjected to the open field test, elevated plus-maze, inclined screen test, forced swim test, and the floor was approximately 10 cm. Mice were suspended by their tails using a string attached to the tails with adhesive tape (~1 cm from the tip of the tail), and the string was looped around a hook. The distance between the tip of the nose of each mouse and the floor was approximately 10 cm. Mice were suspended for 6 min. Video files were transmitted directly from the camera to a connected computer to analyze the swimming duration. We used on-screen stopwatch software (Xnote Stopwatch, dnSoft Research Group, Cheboksary, Russia) for the time measurements. Immobility time was calculated as follows:

\[
\text{Immobility time (s) = Total time} - \text{swimming time}
\]

Tail suspension test
The tail suspension test was performed according to a previously described method, with minor modifications (Steru et al., 1985; Cryan et al., 2005; Tomida et al., 2009). Each mouse was placed in a transparent rectangle (20 (width)×12 (length)×50 (deep) cm). Mice were suspended by their tails using a string attached to the tails with adhesive tape (~1 cm from the tip of the tail), and the string was looped around a hook. The distance between the tip of the nose of each mouse and the floor was approximately 10 cm. Mice were suspended for 6 min. Video files were transmitted directly from the camera to a connected computer to analyze the mobility duration. We used on-screen stopwatch software (Xnote Stopwatch, dnSoft Research Group) for time measurements. Immobility time was calculated as follows:

\[
\text{Immobility time (s) = Total time} - \text{mobility time}
\]

5-HT and dopamine in the cortex
The 5-HT metabolism was measured as previously described (Miyamoto et al., 2002; Kasahara et al., 2006). The mice were sacrificed after behavioral analysis, and levels of 5-HT and dopamine were determined using high-pressure liquid chromatography (HPLC). Each frozen brain sample was weighed and homogenized with an ultrasonic processor in 0.2 M perchloric acid containing isoproterenol as an internal standard. The homogenates were placed on ice and centrifuged at 20,000 g for 15 min. The supernatants were mixed with 1 M sodium acetate to adjust the pH to 3.0 and injected into an HPLC system (Shiseido, Tokyo, Japan), equipped with a reversed-phase ODS column (SC-5ODS, 150×2.1 mm, EICOM, Kyoto, Japan) and an electrochemical detector (EICOM). 

5-HTP-induced head twitch response
The head twitch response was measured according to a previously described method with minor modifications (Nabeshima et al., 1992). Mice were treated with 5-HTP (150 mg/kg, i.p.) or vehicle (1% dimethyl sulfoxide). Head twitch responses were recorded for 2 min at 10, 20, and 30 min after injection.

Quantitative real-time PCR (qPCR)
For mRNA quantification, total RNA was extracted using an easy-Spin™ total RNA extraction kit (iNtRON Biotech, Daejeon, Korea). Complementary DNA was synthesized from the total isolated RNA using a SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA).
qPCR was performed using the SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen) specific for glyceraldehyde 3-phosphate dehydrogenase (Gapdh, 5'-TGCTCAAGCT-CATTTCCGTG-3' and 5'-CTTACTCTCGAGGACCATG-3'), tryptophan hydroxylase 2 (Tph2, 5'-ATGCCTAATCCACCTTTCTC-3' and 5'-TTCTCTTGCTGGTGT-3'), dopa decarboxylase (Ddc, 5'-CTGGTCACTGGGCTGygCC-3' and 5'-ACTGGGACTTTCCCTGATA-3'), 5-hydroxytryptamine receptor 1A (Htr1a, 5'-TGAGACAGGGTGAGGACGAC-3' and 5'-GATTCGTCATGCAGTTCACCA-3'), 5-hydroxytryptamine receptor 2A (Htr2a, 5'-CCGCTTCAACTCCAGAACCAC-3' and 5'-AAGTTGTCATCGGCGAGCAG-3') and 5-hydroxytryptamine receptor 2C (Htr2c, 5'-TGCCATGTTGGGCTACAATCA-3' and 5'-CGAAGGACCCGATGAAGC-3'). All reverse transcription reactions were run in an iCycler IQ5 (Bio-Rad, Hercules, CA, USA) using universal cycling parameters (10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C). cDNA was included in a 25 µL volume PCR reaction with the following components: 0.125 µL each of forward and reverse primer, 12.5 µL SYBR green, and 0.5 µL cDNA with sterilized water. The results were normalized to GAPDH and quantified relative to expression in control samples. For relative quantification calculation, the 2^(-ΔΔCt) formula was used.

ΔΔCt=(Ct_target–Ct_GAPDH) experimental sample–(Ct_target–Ct_GAPDH) control sample

Statistics

Data represent mean ± standard error (SE). Data were analyzed using Student’s t-test or two-way repeated-measures ANOVA followed by Bonferroni post-hoc t-test using Sigma-Plot 14.5 software (Systat Software, San Jose, CA, USA).

RESULTS

Anxiety-like behaviors were decreased in PRDX6-Tg mice

In the open field test, the time spent in the center of the open field, an indicator of decreased anxiety-like behavior (Crawley, 1999), was increased in PRDX6-Tg mice when compared with the non-Tg mice (Fig. 1A). To further investigate anxiety-like behavior in PRDX6-Tg mice, the mice were subjected to the elevated plus-maze test, which also measures anxiety-like behavior based on the natural aversion of rodents to open and elevated areas. PRDX6-Tg mice spent more time in the open arm than non-Tg mice (Fig. 1B), whereas the time spent in closed arms did not differ between non-Tg and PRDX6-Tg mice (Fig. 1C). These results suggested that PRDX6-Tg mice show decreased anxiety-like behaviors in these tests.

Depression-like behaviors were increased in PRDX6-Tg mice

Oxidative stress is also associated with depression-like be-
behavior; however, the behavioral results from open field tests in animal models of depression remain controversial (McHedlidze et al., 2011). Therefore, to examine the depression-like responses of non-Tg and PRDX6-Tg mice, we performed forced swim and tail suspension tests. PRDX6-Tg mice showed an elevated duration of immobility when compared with non-Tg mice in the forced swim and tail suspension tests (Fig. 2A, 2B). These results suggested that PRDX6-Tg mice show increased depression-like behavior in these tests.

**5-HT content was reduced in the cortex of PRDX6-Tg mice**

We speculated that the increased depression-like behavior of PRDX6-Tg mice might be related to alterations in the 5-HT content. We assessed 5-HT levels in the cortex, as depression-like behavior might be associated with dysfunctions in 5-HT neurotransmission (Mouri et al., 2012). The 5-HT content in the cortex of PRDX6-Tg mice was significantly lower than that in the cortex of non-Tg mice (Fig. 3A). In addition, the dopamine content was found to be slightly lower in the cortex of PRDX6-Tg mice than that in non-Tg mice, but the difference was not significant (Fig. 3B).

**5-HTP-induced head twitch response was inhibited in PRDX6-Tg mice**

Serotonergic dysfunction is well-known to be associated with depression (Mouri et al., 2012). The head twitch response in rodents induced by 5-HTP, a precursor of 5-HT, is considered a specific behavioral model for the activation of serotonergic neurons (Come et al., 1963; Schreiber et al., 1995). Therefore, we measured the 5-HTP-induced head twitch response in non-Tg and PRDX6-Tg mice. PRDX6 overexpression did not alter the total head twitch response (Fig. 4A); however, PRDX6-Tg mice showed a lower head twitch response than non-Tg mice 30 min after 5-HTP injection (Fig. 4B; Gene condition: F(1,24)=2.57, p=0.135; time condition: F(2,24)=12.832, p<0.001; interaction: F(2,24)=1.37, p=0.337).

**AADC expression level was decreased in PRDX6-Tg mice**

Serotonergic neuronal function is associated with several factors, including tryptophan hydroxylase (TPH), serotonin transporter, and serotonin receptors. 5-HT is synthesized from 5-HTP via the AADC. Therefore, we compared mRNA levels of 5-HT in the cortex of non-Tg and PRDX6-Tg mice; however, we detected no significant changes in total mRNA levels in female and male mice (Fig. 5A). When separated by sex, mRNA levels of Htr2c and Ddc were significantly regulated by PRDX6 overexpression (Fig. 5B, 5C). In particular, PRDX6-Tg mice exhibited reduced Ddc mRNA expression. Compared with male non-Tg mice, male PRDX6-Tg mice, but not female mice, displayed significantly reduced Ddc mRNA levels in the cortex.

**DISCUSSION**

Redox balance is closely associated with several psychological disorders. Among antioxidant enzymes, PRDX6 exhibits its unique bifunctional activity. We have previously reported that PRDX6 plays a role in dopaminergic neurodegeneration by modulating 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in a PD model (Yun et al., 2015). However, we demonstrated that PRDX6-Tg mice exhibit emotional behavioral phenotypes. In the elevated plus-maze test,
the time spent in the open arm was higher in PRDX6-Tg mice than in non-Tg mice. Interestingly, female mice exhibited more anxiolytic behavior than male mice. To elucidate the mechanism underlying this behavior, we measured expression levels of serotonin receptors in the cortex. Htr2c mRNA levels were increased in PRDX6-Tg female mice but not in male mice. According to a previous study, Htr2c knockout mice display anxiety-like behaviors (Rosenzweig-Lipson, 2011). In addition, 5-HT2C receptor agonists induce anxiolytic-like activity in the elevated plus-maze test (Nic Dhonnchadh et al., 2003). Therefore, we postulated that the anxiolytic behaviors of PRDX6-Tg mice could be associated with upregulated Htr2c levels. In particular, the profound increase in Htr2c in female PRDX6-Tg mice might explain their marked anti-anxiety behaviors when compared with those of male PRDX6-Tg mice.

We also observed that PRDX6-Tg mice exhibited increased depression-like behavior in the forced swim and tail suspension tests. Furthermore, 5-HT levels were reduced in the cortex of PRDX6-Tg mice. Deficits in 5-HT neuronal transmission are well-known to be associated with depression (Jacobson et al., 2012). In the cortex, the 5-HT content was decreased in PRDX6-Tg mice, while dopamine levels were unaltered. Furthermore, the head twitch response, a typical behavior induced by 5-HTergic neuronal activation, was reduced in PRDX6-Tg mice (Canal et al., 2010; Canal and Morgan, 2012). These results suggest that depression-like behaviors in PRDX6-Tg mice are related to downregulated 5-HT neurotransmission.

We further measured AADC expression levels in PRDX6-Tg mice, as the head twitch response is evoked by 5-HT metabolized from 5-HTP (a precursor) via Ddc in our model (Colpaert and Janssen, 1983; Darmani, 1996). Ddc mRNA levels were lower in male PRDX6-Tg mice than in non-Tg mice but not in female mice. Low Ddc expression in male PRDX6-Tg mice may be associated with greater depression-like behavior observed in the tail suspension test. The precise molecular mechanism remains unknown; however, PRDX6 transcription of Ddc may be regulated by the POU protein, which is associated with PRDXs (Millevoi et al., 2001; Oliviero et al., 2015). In the present study, the levels of PRDX6 expression in the cortex of male and female mice did not differ significantly. Furthermore, the natural expression pattern of PRDX6 has been reported in both males and females (Birzniece et al., 2002; Balasiner et al., 2010; Buonora et al., 2015). One human study has reported that males exhibit approximately 20% higher plasma levels of Prdx6 than females (Buonora et al., 2015). The physiological and pathophysiological roles of the sex-specific expression patterns of PRDX6 need to be elucidated in future studies. Collectively, our results suggest that PRDX6 regulates the expression of anxiolytic and depression-like behaviors by modulating 5-HTergic neurotransmission in the cortex.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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