Thioredoxin Suppresses Parkin-associated Endothelin Receptor-like Receptor-induced Neurotoxicity and Extends Longevity in *Drosophila*<sup>[S]</sup>

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Parkin-associated endothelin receptor-like receptor (Pael-R) is a substrate of the E3 ubiquitin ligase Parkin, which has been implicated in the pathogenesis of Parkinson disease. Misexpression of human Pael-R in *Drosophila* has been shown to induce selective loss of dopaminergic neurons, a symptom of Parkinson disease. Using this model, we investigated whether thioredoxin (TRX), an evolutionarily conserved antioxidant and molecular chaperone, could suppress the neurotoxicity induced by Pael-R. The *Drosophila* genome contains three TRX-encoding genes, namely TrxT, Trx-2, and dld. When each of the TRX genes was overexpressed together with Pael-R in all neurons, the number of dopaminergic neurons and level of locomotor activity were significantly increased compared with control flies. To assess the role of the antioxidant activity of TRX in this context, we generated redox-defective mutants, TrxT(C35A) and TrxT(D26A/K57I), and coexpressed each of them with Pael-R. The mutants suppressed the Pael-R neurotoxicity similarly to wild-type TrxT, although the extent of the rescue was slightly reduced for the locomotor activity. We confirmed that both mutants remained active as chaperones, suggesting that this activity may be the major cause of the suppression. In the absence of Pael-R, overexpression of TRX in all neurons increased the level of locomotor activity in aged flies and extended the mean longevity by 15%. Furthermore, overexpression of TRX suppressed neurotoxicity in a *Drosophila* model of Machado-Joseph disease expressing polyglutamine. These results establish that *Drosophila* TRX can function as an anti-aging agent and as a suppressor of Pael-R- and polyglutamine-induced neurotoxicity.

Parkinson disease (PD)<sup>[3]</sup> is a neurodegenerative disorder that is characterized by a progressive loss of dopaminergic (DA) neurons in the substantia nigra, dopamine depletion in the striatum, and impaired motor functions<sup>[1]</sup>. A number of point mutations and deletions in parkin have been shown to be associated with autosomal recessive juvenile PD<sup>[2]</sup>. Parkin encodes an E3 ubiquitin ligase, a component of the ubiquitin-proteasome pathway that degrades damaged or misfolded proteins<sup>[3]</sup>. Several proteins have been identified as substrates for Parkin, including α-synuclein<sup>[4]</sup>, Parkin-associated endothelin receptor-like receptor (Pael-R)<sup>[5]</sup>, CDCrel-1<sup>[3]</sup>, the synaptic vesicle protein synphilin-1<sup>[6]</sup>, and DJ-1<sup>[7,8]</sup>. Among these, α-synuclein<sup>[9]</sup>, Pael-R<sup>[5]</sup>, and DJ-1<sup>[10]</sup> have also been implicated in familial forms of PD. Parkin is also associated with Hsp70 and carboxyl terminus of Hsc70-interacting protein (CHIP; a chaperone-dependent E3 ligase), which together control the ubiquitination and degradation of an insoluble form of Pael-R<sup>[11]</sup>. Pael-R has been shown to exhibit neurotoxicity in *Drosophila*, because misexpression of human Pael-R caused selective loss of DA neurons with aging and this phenotype was suppressed by overexpression of human Parkin (hPk)<sup>[12]</sup>. Recently, CHIP was found to be a component of Lewy bodies in the human brain, where it colocalizes with α-synuclein and Hsp70<sup>[13]</sup>. In a cell culture model, overexpression of CHIP inhibited the formation of α-synuclein inclusion bodies and reduced the α-synuclein protein level. These observations support the hypothesis that the Parkin-linked ubiquitin-proteasome pathway plays a central role in the pathogenesis of PD, perhaps also including late-onset cases.

Thioredoxin (TRX), a dithiol-reducing enzyme, is induced by a variety of oxidative stimuli, including UV irradiation, inflammatory cytokines, and chemical carcinogens and plays crucial roles in the regulation of cellular responses such as gene expression, cell proliferation, and apoptosis<sup>[14]</sup>. The sequence containing the redox active site, Cys-Gly-Pro-Cys-Lys, is conserved among all TRX family proteins<sup>[15]</sup>. A wide variety of cellular functions of TRX have been reported in various cells and organisms, including a cytoprotective effect against oxidant damage<sup>[16]</sup>.
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Plasmid Construction and Molecular Biology—Poly(A) RNA was isolated from third instar larvae using a Fast RNA kit (Bio101, Vista, CA) and a Quick Prep Micro mRNA purification kit (Amersham Biosciences) and then reverse-transcribed using a Ready-To-Go T-Primed First-Strand kit (Amersham Biosciences). TrxT (24), dhd (22), and thioredoxin T (TrxT) (24), all of which contain the conserved redox active site. We found that overexpression of each of the Drosophila TRXs could suppress the Pael-R-induced phenotypes, such as the selective loss of DA neurons and reduced locomotor activity, as efficiently as human Parkin. Because the redox-defective TRX mutants were also able to suppress the Pael-R-induced phenotypes, the antioxidant activity is unlikely to be responsible for the suppression. The neuroprotective function of TrxT was also observed in a Drosophila model of Machado-Joseph disease (MJD) expressing polyglutamine (25). Furthermore, we demonstrated that overexpression of TrxT in the absence of Pael-R improved locomotor activity in aged animals and extended adult longevity.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Molecular Biology—Poly(A) RNA was isolated from third instar larvae using a Fast RNA kit (Bio101, Vista, CA) and a Quick Prep Micro mRNA purification kit (Amersham Biosciences) and then reverse-transcribed using a Ready-To-Go T-Primed First-Strand kit (Amersham Biosciences). TrxT (24), dhd (22), and Trx-2 (23) cDNAs were amplified by PCR using a KOD-PLUS DNA polymerase kit (Toyobo), and the resulting cDNA fragments were subcloned into the EcoRI/XhoI sites of pBluescript SK (+) (Stratagene, La Jolla, CA) and sequenced with a BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, CA). A redox-defective mutant, TrxT(C35A), was generated by PCR mutagenesis using the TrxT cDNA as a template and primers M1 (5'-TGCCTGACATGCCAAGAAAGGC-3') and R1 (5'-TGCTTGGAGCAATGCCTTGCAAGGC-3'); dhd, F2 (5'-CTAGAATTCATGCACTCCGAGAGGAG-3') and R2 (5'-TACCATGATCCCACCTCCACACCGTG-3'); Trx-2, F3 (5'-ACTGAATCAGCTGCGCATGCGAAGAA-3') and R3 (5'-TGCTGCAGTAGAATACTTTCCGCA-3'). The obtained PCR products were ligated with EcoRI and Xhol (Toyobo), and the resulting cDNA fragments were subcloned into the EcoRI/Xhol sites of pBluescript SK+ (Stratagene, La Jolla, CA) and sequenced with a BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, CA). A redox-defective mutant, TrxT(C35A), was generated by PCR mutagenesis using the TrxT cDNA as a template and primers M1 (5'-TGCCTGACATGCCAAGAAAGGC-3') and M2 (5'-CCAGTCTGCATAAAATCAATTAACGCTTGCAAGGA-3'). Another redox-defective mutant, TrxT(D26A/K57I), was generated by PCR mutagenesis using the TrxT cDNA as a template with primers M3 (5'-AAGTGGGTGGTTAACGTTTCTTATGCA-3') and M4 (5'-GACCTCGGGCAGTTAAGGCTTGTCG-3') for the D26A mutation and M5 (5'-GTACCCAGTACGATACGACCGCATGCGAAGAA-3') and M6 (5'-AGACTAAACACCTACTCGTTATATGCA-3') for the K57I mutation. The wild-type and mutant TrxT cDNAs were subcloned into pGEX-4T-1 to create GST fusion proteins and pUAST (26) to produce transgenic flies expressing TrxT under GAL4 control. The constructs were introduced into the genome by P-element-mediated transformation (27).

Fly Stocks—Flies were reared on glucose cornmeal agar medium at 25 °C. Strain y w^67c23 Df(1)w^67c23 was used as a recipient for transgenic flies. P[UAS-GFP.S65T]T2 was obtained from the Kyoto Stock Center (Kyoto, Japan). P[UAS-Pael-R] and P[UAS-human parkin] were gifts from B. Lu (Rockefeller University, New York, NY) (12). P[UAS-MID2-tr-Q78] was from N. Bonini (University of Pennsylvania, Philadelphia, PA) (25). P[GALA4-elav.L]2 and P[GALA4-elav.L]3 were obtained from the Bloomington Stock Center (Bloomington, IN). P[GALA4-elav.L]2 was combined with P[UAS-Pael-R] to make elav > Pael-R stocks, which facilitate further genetic crosses with various upstream activating sequence (UAS) transgenes. P[GALA4-elav.L]3 was used for other crosses as elav-GAL4 driver. dhd^28 and dld^28 were gifts from H. Salz (Case Western Reserve University, Cleveland, OH) (22). Both mutants were backcrossed six times into y w^67c23 Df(1)w^67c23.

Insulin Disulfide Reduction Assay—TRX activity was determined using an insulin precipitation assay as previously described (28). GST-TrxT fusion proteins were expressed in Escherichia coli BL21 (DE3) and purified using glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer’s protocol. The protein concentrations were determined using a Bio-Rad protein assay system with bovine serum albumin as the standard. After preincubation of the recombinant proteins in 100 μl of assay mixture containing 0.1 μg/ml bovine insulin (Sigma), 2 mM EDTA, and 0.1 μM potassiu m phosphate buffer, pH 7.0, for 15 min at room temperature, the reaction was initiated by adding 0.4 μl of 10 mM dithiothreitol. Increases in the absorbance at 595 nm were monitored at 25 °C using a Model U-1100 spectrophotometer (Hitachi, Tokyo, Japan). Assays were performed with concentrations of 50 and 75 μg/ml for TrxT and 50 μg/ml for TrxT(C35A) and TrxT(D26A/K57I).

Chaperone Activity—The chaperone activity of TrxT was determined by monitoring the refolding of citrate synthase (Sigma) as described previously (21, 29). Citrate synthase at a concentration of 1 μM was denatured in 8 μl urea and 13.3 mM dithiothreitol for 1 h at 25 °C. Renaturation was initiated by 24-fold dilution in 149 μM Tris- HCl, pH 8.0, and 0.74 mM EDTA for 90 min at 25 °C in the presence or absence of GST fusion proteins. The citrate synthase activity was determined by adding a 40-μl aliquot of each citrate synthase mixture to 800 μl of assay mixture containing 158 mM Tris- HCl, pH 8.0, 0.023 mM acetyl CoA, 0.5 mM oxaloacetic acid, and 0.12 mM 5,5′-dithio- bis-2-nitrobenzoic acid and measuring the absorbance at 412 nm using a Model UV-160A spectrophotometer (Shimadzu, Kyoto, Japan) for 30 s.

Climbing Activity—Climbing activity assays were performed as described previously (30). Briefly, a total of 10–20 flies were bumped down to the bottom of a graduated cylinder (diameter 2 cm; length 20 cm), and pictures were taken at 18 s after the bumping to observe the distances that individual flies had climbed up the wall of the cylinder. In total, 10 trials were carried out for each time point and genotype, and the mean
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(± S.E.) distance was calculated. Experiments involving TRX transgenes were carried out using at least two independent transgenic lines for each construct.

**Immunohistochemistry**—Adult brains were dissected from flies in phosphate-buffered saline (PBS) and immediately fixed in 4% formaldehyde/PBS for 1 h. Next, the tissues were washed in PBS containing 0.03% Triton X-100 (PBST) for 10 min, incubated in methanol for 20 min, washed four times in PBST for 10 min each, blocked in 10% goat serum/PBST for 2 h at room temperature, and incubated with an anti-tyrosine hydroxylase antibody (31) at a dilution of 1:1000 in 10% goat serum/PBST at 4°C overnight. After three washes in PBST for 10 min each, the tissues were incubated with an Alexa 568-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR) at a dilution of 1:2000 in PBST for 2 h at 25°C. The samples were then washed with PBS and mounted with GEL/MOUNT (Cosmo Bio, Tokyo, Japan). Between 5 and 10 fly heads were examined for each genotype per time point.

**Hydrogen Peroxide Resistance**—S2 cells were transfected with pUAS-GFP, pUAS-TrxT, or TrxT(D26A/K57I) using Effectene (Qiagen, Hilden, Germany) together with pUAS-lacZ and pWAGAL4 (a gift from Dr. Y. Hiromi, National Institute of Genetics, Mishima, Japan). At 48 h after transfection, the cells were exposed to 20 mM H2O2 for 16 h and then lysed with 250 μl of lysis buffer (100 mM potassium phosphate buffer, pH 7.8, 0.2% Triton X-100, and 1 mM dithiothreitol). Debris was removed by centrifugation, and the β-galactosidase activity in the supernatant was determined. Briefly, 30 μl of each cell extract was added to 270 μl of reaction solution (100 mM sodium phosphate buffer, pH 7.5, 1 mM MgCl2, and 350 μM 2-nitrophenyl-β-D-galactopyranoside) and incubated at 37°C for 30 min. The reaction was stopped by adding 500 μl of 1 M Na2CO3, and the absorbance at 420 nm was measured. Viability was determined as the percentage of β-galactosidase activity in H2O2-treated cells relative to that in untreated cells.

**Longevity**—Newly eclosed males of appropriate genotypes were collected within 24 h and transferred to new vials containing food every 2–3 days until all the flies had died. A total of 90 flies/genotype (30 flies/vial) was used for longevity assays, and the number of dead flies was counted at the time of each transfer. Experiments were performed at 25°C.

**RESULTS**

**Drosophila TRX Genes**—The Drosophila genome contains three genes that encode TRX-like proteins, namely Dhd (22), Trx-2 (23), and TrxT (24). These three TRX proteins have a conserved redox active site consisting of Cys-Gly-Pro-Cys-Lys (supplemental Fig. S1). TrxT consists of 157 amino acid residues with a carboxyl-terminal extension of ~50 amino acids compared with the other two proteins and is expressed at a high level in the testes (24). However, its biochemical properties are poorly characterized. To examine whether TrxT has insulin-reducing activity similar to other TRX proteins, we cloned a cDNA encoding TrxT and expressed it as a GST fusion protein in E. coli. The purified fusion protein was subjected to insulin disulfide reduction assays as described under “Experimental Procedures” (Fig. 1a). To assess the role of the redox active site, we generated redox-defective mutants, TrxT(C35A) and TrxT(D26A/K57I), in which cysteine, glutamate, and lysine residues were replaced with alanine or isoleucine at the indicated positions (supplemental Fig. S1). The wild-type TrxT fusion protein showed insulin disulfide-reducing activity, whereas the redox-defective mutants did not. These results demonstrate that Drosophila TrxT is an active redox protein and further confirm that the conserved active site residues, including Cys-35, Asp-26, and Lys-57, are essential for its redox function.

Another biochemical property of TRX is a chaperone activity. TRX from E. coli has also been shown to function as a chaperone (21). Therefore, we examined whether Drosophila TRX has a chaperone activity using citrate synthase-refolding assays as described under “Experimental Procedures.” As shown in Fig. 1b, chaperone activity of TrxT was detected in a dose-dependent manner, whereas no activity was detected with GST alone or ovalbumin as controls. Furthermore, the redox-defective mutants TrxT(C35A) and TrxT(D26A/K57I) showed similar high chaperone activities to wild-type TrxT. These results are consistent with a previous study using a redox-defective
TRX from E. coli (21). Therefore, these mutants are useful for assessing the contribution of the antioxidant activity of TRX without affecting its chaperone activity.

Panneural Expression of TrxT Inhibits Pael-R Neurotoxicity in Drosophila—Because oxidative stress and dysfunction of the ubiquitin-proteasomal system have been considered to promote PD symptoms, we examined whether TRX could inhibit the neurodegenerative process in a Drosophila model. To achieve this, we used a transgenic Drosophila model that misexpresses human Pael-R, a substrate of the E3 ubiquitin ligase Parkin, which has been associated with autosomal recessive juvenile PD (5, 12). These flies show age-dependent selective degeneration of DA neurons. Although the behavioral phenotype of the model has not yet been characterized, it is highly likely that motor function declines in an age-dependent manner, as observed in another PD Drosophila model that misexpresses α-synuclein (32). We used climbing assays to assess the locomotor function of the Pael-R-misexpressing flies. On day 5, the average climbing activity of control flies coexpressing GFP was 18.3 ± 0.2 cm, and this declined to 4.7 ± 0.3 cm on day 50 (Fig. 2a). In co-overexpression experiments, hPK was included as a positive control (12). On day 5, no dramatic change was observed in the climbing activity of flies co-overexpressing TrxT, Trx-2, dhd, or hPK, although the activities of the latter three genotypes were slightly higher than that of control flies (p < 0.05). However, coexpression of each of these proteins dramatically improved the climbing activity in 50-day-old flies, producing average activities of 10.3 ± 0.4, 9.9 ± 0.7, 10.6 ± 0.4, and 10.1 ± 0.8 cm for TrxT, Trx-2, dhd, and hPK, respectively. These results indicate that overexpression of any of the Drosophila TRX genes can suppress the Pael-R-induced defects in climbing activity in aged animals.

Next, we examined the number of DA neurons, because these are selectively lost in Pael-R-misexpressing flies. Adult brains of the various genotypes were dissected and immunohistochemically stained using an anti-tyrosine hydroxylase antibody to visualize DA neurons. We counted the number of DA neurons in the dorsomedial clusters (Fig. 2, b–e). The average number of DA neurons in young (5-day-old) control flies coexpressing GFP was ~19, of which 80% on average had been lost in 50-day-old flies. The phenotype was clearly rescued by coexpression of the TRX transgenes, producing mean numbers of DA neurons of 12.5 ± 0.8, 10.3 ± 0.6, and 10.9 ± 1.3 for TrxT, Trx-2, and dhd, respectively. The level of rescue was again equivalent to that obtained in positive control flies coexpressing hPK (10.3 ± 0.8). These results establish that overexpression of any of the Drosophila TRX genes can suppress the Pael-R-induced neurotoxicity in Drosophila.

Redox-defective TrxT Mutants Suppress Pael-R Toxicity—TRX functions, at least, as an antioxidant and a molecular chaperone. To assess the contribution of its antioxidant activity to the suppression of Pael-R-induced neurotoxicity, each of the redox-defective TrxT constructs was introduced into the Drosophila genome and coexpressed with Pael-R. As shown in Fig. 3a, overexpression of either mutant form of TrxT improved the climbing activity similar to wild-type TrxT at 50 days of age. Specifically, the average climbing activities were 10.3 ± 0.4, 8.7 ± 0.1, and 8.9 ± 0.3 cm for flies coexpressing TrxT, TrxT(C35A), and TrxT(D26A/K57I), respectively. The slightly lower levels of climbing activity observed for the mutants may suggest that there is some contribution of the antioxidant activity. With respect to the numbers of DA neurons, similar levels of rescue were observed for the wild-type TrxT and redox-defective TrxT mutants. Specifically, the average numbers of DA neurons were 12.5 ± 0.9, 11.8 ± 0.9, and 11.7 ± 0.8 for TrxT, TrxT(C35A), and TrxT(D26A/K57I), respectively (Fig. 3b). These results suggest that the antioxidant activity of TRX is unlikely to be the major cause of the suppression of Pael-R neurotoxicity in Drosophila.

TRX Promotes the Viability of Cells Exposed to Hydrogen Peroxide—We next investigated the extent to which TRX can protect cells from the oxidative stress induced by hydrogen per-
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oxide, using cultured S2 cells. Cell viability was monitored by the activity of β-galactosidase, which was cotransfected with wild-type TrxT or redox-defective TrxT mutants (Fig. 4). Wild-type TrxT increased the cell viability compared with control expressing GFP (29.7% versus 41.6%). The TrxT mutants lacking redox activity were also capable of increasing the cell viability (39.5 and 33.6% for TrxT(C35A) and TrxT(D26A/K57I), respectively). However, there were significant differences in the viabilities between the wild-type TrxT and TrxT mutants, suggesting that the antioxidant activity of TRX also contributes to cell viability.

Mutations in Trx Family Genes Alter Resistance to Pael-R—To address the functional redundancy of TRX family genes, we examined whether loss-of-function mutations in TRX genes could influence the Pael-R-induced phenotype. When elav > GAL4 was combined with dhd<sup>l8</sup>, null allele of dhd, the average climbing ability (2.4 ± 0.2 cm) was significantly reduced compared with those with wild-type (7.4 ± 0.3 cm) (Fig. 5). When dhd<sup>l8</sup> was combined, the climbing activity was further reduced (0.8 ± 0.1 cm) from those with dhd<sup>d</sup> (t test, p < 0.01). Because
Effects of overexpression of MJD model. Drosophila with overexpressed TRX transgenes in a rotoxicity in other models of neurodegenerative disease, we examined whether TRX, an antioxidant molecule, could inhibit the age-dependent loss of DA neurons and higher levels of locomotor activity compared with those bearing the GAL4 driver only (t test, *p < 0.05).

Overexpression of Trx Suppresses MJD-derived Polyglutamine Toxicity—To examine whether TRX can suppress neurotoxicity in other models of neurodegenerative disease, we overexpressed TRX transgenes in a Drosophila MJD model expressing a carboxyl-terminal fragment of ataxin-3 that contains the glutamine tract (25). As shown in Fig. 6, the climbing ability was significantly improved in flies coexpressing either of the TRX family genes, including those mutated with redox active sites. Therefore, TRX can also suppress polyglutamine-mediated neurotoxicity.

TrxT Promotes the Longevity of Flies—Oxidative stress is thought to be the major cause of aging and to reduce longevity. We examined whether overexpression of TRX could promote longevity in the absence of Pael-R. Fig. 7a shows the survival curves of flies expressing wild-type TrxT in all neurons and control flies carrying the GAL4 transgene only. The mean longevity of flies overexpressing TrxT was 39.1 ± 0.6 days, representing a 15.3% extension compared with that of control flies (33.9 ± 1.1 days). These results demonstrate that panneural expression of TRX can promote the longevity of flies in normal aging.

We also determined the climbing activity of flies expressing the UAS-TrxT transgene (Fig. 7b). The activity was slightly higher than that of control flies at 30 days of age (6.03 ± 1.2 and 3.98 ± 0.5 cm for TrxT and control flies, respectively). These results demonstrate that TrxT can improve the age-related deterioration of motor function in normal aging.

**DISCUSSION**

*Drosophila* represents a powerful *in vivo* model for human neurodegenerative diseases, which can be mimicked by either disrupting the fly homologs of human disease genes or introducing the dominant alleles of human disease genes (33). To date, three types of *Drosophila* PD models have been constructed involving misexpression of human α-synuclein (32, 34), misexpression of human Pael-R (12), and disruption of *Drosophila* parkin (35–38), respectively. These models serve as excellent systems for identifying genetic and environmental factors that modify the disease progression, thereby facilitating the development of new therapeutic approaches. Indeed, Hsp70 and Parkin have been shown to suppress the phenotype of the α-synuclein model (12, 34, 39), whereas the Pael-R model was used to demonstrate the function of Parkin in promoting protein degradation through the ubiquitin-proteasome pathway (12).

In the present study, we investigated whether TRX, an antioxidant molecule, could inhibit the age-dependent loss of DA neurons and locomotor dysfunction in the Pael-R model. Overexpression of TrxT, Trx-2, or dhd was found to suppress the Pael-R-induced neurotoxicity, resulting in flies with increased numbers of DA neurons and higher levels of locomotor activity compared with age-matched control flies that only misexpressed Pael-R.
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Experiments involving loss-of-function mutations in Trx genes suggested that they function additively. We also demonstrated that TRX could also suppress the neurotoxicity mediated by polyglutamine in a Drosophila model of MJD. Thus, our present results establish that TRX can function as a suppressor of neurotoxicity induced by Pael-R or polyglutamine.

TRX is known to be an antioxidant molecule that is capable of removing hydrogen peroxide (40), particularly in the presence of methionine sulfoxide reductase (41) or the TRX peroxidase system (42). TRX in the submicromolar range has been shown to protect human neuroblastoma cells (SH-SY5Y) from the oxidative stress-induced apoptosis caused by serum deprivation or 1-methyl-4-phenylpyridinium (17). In addition, TRX can induce Mn-SOD and inhibit hydroxyl radical production, lipid peroxidation, and apoptosis. Importantly, only reduced TRX appeared to be capable of protecting SH-SY5Y cells from serum deprivation-induced apoptosis, because inhibition of Trx reductase was found to abolish the protective effects of Trx-S2 (17).

Panneural expression of Pael-R causes age-dependent selective degeneration of DA neurons (12). The selective neurotoxicity in PD has been explained by the vulnerability of DA neurons to oxidative stress (43). There are several components that increase the level of oxidative stress in DA neurons. Increased dopamine turnover by the enzyme monoamine oxidase in the early stage of PD may generate excess hydrogen peroxide and subsequent formation of hydroxyl radicals, leading to death of DA neurons (44). Dopamine may be oxidized to quinones, either enzymatically or via the reduction of ferric iron or other metals, and these metabolites may mediate the synthesis of hydroxyl radicals and oxysterol radicals (45). Therefore, it is possible that TRX inhibits Pael-R-induced toxicity through inhibiting the accumulation of reactive oxygen species via its redox activity.

To assess the role of the redox activity of TRX in suppressing Pael-R-induced neurotoxicity in flies, we generated redox-inactive TrxT mutants and coexpressed each of them with Pael-R. There were no significant differences in the inhibitory effects between wild-type TrxT and the TrxT mutants for either climbing ability or number of DA neurons. These findings indicate that the redox activity of TRX is not responsible for the suppression of Pael-R-induced neurotoxicity, at least under the present experimental conditions. We also examined the effects of TRX on the viability of Drosophila cultured cells after treatment with hydrogen peroxide. Again, the redox-inactive TrxT mutants increased viability similarly to wild-type TrxT. However, climbing activity and cell viability were slightly lower for redox-defective TrxT than for wild-type TrxT. These findings may suggest that TRX protects cells from death through its antioxidant activity to some extent. It is possible that misexpression of Pael-R or hydrogen peroxide treatment was too drastic, such that the antioxidant activity was not sufficient to protect the cells.

It is noteworthy that the redox-defective mutants still functioned as molecular chaperones. The chaperone activity of TRX is critical for supporting cell survival against the cellular damage caused by various stresses, including oxidative stress. Chaperones promote the refolding of misfolded proteins to prevent the aggregation of cellular proteins. Therefore, neurotoxicity suppression may be mediated by the chaperone activity of TRX. Similar functional aspects of TRX have been demonstrated in the regulation of apoptosis signal-regulating kinase 1 protein stability. Although the redox activity of TRX was originally thought to be essential for inhibiting the activity of apoptosis signal-regulating kinase 1 ubiquitination and degradation independently of its redox activity (20).

The role of molecular chaperones in cell survival is obvious, because Hsp70 is a potent suppressor of both polyglutamine disease and PD in Drosophila (34, 47) and also suppresses 1-methyl-4-phenylpyridinium toxicity (48). From a therapeutic point of view, activation of molecular chaperones by external stimuli would be helpful. Geldanamycin has been shown to suppress α-synuclein neurotoxicity in Drosophila through the activation of stress response mechanisms (49). Moreover, it is important to understand the mechanisms by which protein ubiquitination and degradation are regulated in neurons in response to external stimuli.

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