JNK/FOXO-mediated Neuronal Expression of Fly Homologue of Peroxiredoxin II Reduces Oxidative Stress and Extends Life Span*§

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Activation of c-Jun N-terminal kinase (JNK) signaling in neurons increases stress resistance and extends life span, in part through FOXO-mediated transcription in Drosophila. However, the JNK/FOXO target genes are unknown. Here, we identified Jafrac1, a Drosophila homolog of human Peroxiredoxin II (hPrxII), as a downstream effector of JNK/FOXO signaling in neurons that enhances stress resistance and extends life span. We found that Jafrac1 was expressed in the adult brain and induced by paraquat, a reactive oxygen species-generating chemical. RNA interference-mediated neuronal knockdown of Jafrac1 enhanced, while neuronal overexpression of Jafrac1 and hPrxII suppressed, paraquat-induced lethality in flies. Neuronal expression of Jafrac1 also significantly reduced ROS levels, restored mitochondrial function, and attenuated JNK activation caused by paraquat. Activation of JNK/FOXO signaling in neurons increased the Jafrac1 expression level under both normal and oxidative stressed conditions. Moreover, neuronal knockdown of Jafrac1 shortended, while overexpression of Jafrac1 and hPrxII extended, the life span in flies. These results support the hypothesis that JNK/FOXO signaling extends life span via amelioration of oxidative damage and mitochondrial dysfunction in neurons.

FOXO transcription factors are key regulators of growth, metabolism, life span, and stress resistance in various organisms, including Drosophila (1, 2). FOXO is regulated by the insulin signaling pathway and the stress-induced JNK signaling pathway (3, 4). Oxidative stress activates the stress-responsive JNK, which promotes FOXO nuclear localization and up-regulates expression of antioxidant proteins (5, 6).

In Drosophila, neuronal activation of JNK/FOXO signaling confers resistance to oxidative stress and extends life span (4, 7). Neurons are particularly susceptible to oxidative damage because of their high levels of ROS production and relatively low levels of antioxidant enzymes (8). Thus, activation of the JNK/FOXO pathway in neurons may extend life span through up-regulation of anti-oxidative stress genes. However, little is known regarding the JNK/FOXO target genes in neurons.

Thiol-reducing systems are important reducers of many oxidative stressors, such as peroxide (9). Peroxiredoxin (Prx), also called thioredoxin peroxidase, eliminates hydroperoxide with thioredoxin as an immediate hydrogen donor and reduces ROS levels (10). Among six distinct mammalian Prxs (I–VI), Prx II is exclusively expressed in the brain (11), suggesting that Prx II may play an important role in response to oxidative stress in neurons. However, the regulation of PrxII expression in neurons has not been elucidated. In this study, we demonstrated that neuronal expression of Jafrac1, a Drosophila homologue of human Prx II (hPrxII), was regulated by JNK/FOXO signaling, promoted resistance to oxidative stress, and extended the life span of the flies.

EXPERIMENTAL PROCEDURES

Drosophila Culture and Mutants—Drosophila melanogaster were kept at 25 °C and cultured using standard methods. Wild-type Oregon-R, w–, elav-Gal4 (pan-neuron driver), Actin5C-Gal4 (ubiquitous driver), Cha-Gal4 (cholinergic neuron driver), repo-Gal4 (glial cell driver), elavGS-Gal4 (pan-neuro-

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‡‡‡ The abbreviations used are: JNK, c-Jun NH2-terminal kinase; JNKK, JNK kinase; Prx, peroxiredoxin; ROS, reactive oxygen species; PBS, phosphate-buffered saline; mtDNA, mitochondrial DNA; RNAi, RNA interference; pJNK, phospho-JNK.
nal GeneSwitch driver), and UAS-dFOXO.WT flies were obtained from the Bloomington Stock Center. Jafrac1^{G1104} mutants, which carries an EP element (12) inserted at 5’-untranslated region of the jafrac1, were purchased from GenExel, Inc. (Daejeon, Korea). The JNK signaling related genes were examined using the Student’s t test (Microsoft Excel) and p < 0.05 was accepted as statistically significant.

**Measuring ROS Levels in Drosophila**—To measure the intercellular ROS level in Drosophila, we used the method described by Strayer et al. (15). Non-fluorescent 2,7-dichlorofluorescein di-acetate (Molecular Probes) is a cell permeable dye and can be converted into 2,7-dichlorofluorescein by interacting with hydrogen peroxide (16). Flies (3 days old) were treated with 20 mM paraquat for 24 h and then fixed in 4% paraformaldehyde in PBS for 1 h at room temperature and incubated with the pJNK antibody (1:200) or FOXO antibody (1:500) and, subsequently, with Alexa Fluor 594-conjugated anti-rabbit IgG (1:200, Molecular Probes). Fluorescence images were acquired using an Axiovert 200M microscope (Carl Zeiss, Germany).

**Life Span Assay**—For longevity experiments, 1- and 2-day-old adult male or female flies were collected (10 per vial), transferred to fresh medium every 2 days, and scored for survivors. The starting population for each genotype was 100 flies. Three replicates were tested for each genotype. Survival, $I_x$, was estimated as $N_x/N_0$, where $N_x$ is the number of flies alive at the beginning of each census interval, and $N_0$ is the initial cohort size. Significant differences in survival between pairs of cohorts were tested using the log-rank test.

**RESULTS**

*Drosophila Jafrac1 Is an Ortholog of Human PrxII*—Based on amino acid sequence similarity, we identified a Drosophila peroxiredoxin II gene (Jafrac1, CG1633, Dpx-4783) that belongs to the 2-Cys peroxiredoxin subfamily. The amino acid sequence of the Jafrac1 protein shows significant homology to hPrxII, including conserved cysteine motifs (Fig. 1A), and the overall amino acid similarity of 83% between Jafrac1 and hPrxII (157/188). There are six *Drosophila* homologs of human peroxiredoxins that share at least 60% amino acid identity. Among these, the molecular characters of Jafrac1 are most similar with hPrxII, including protein size, the number of conserved cysteine residues, and subcellular localization (supplemental Table 2).

Jafrac1 mRNA is expressed throughout development, and in the third instar larvae, Jafrac1 mRNA is abundant in brain, imaginal discs, and Malpighian tubules (supplemental Fig. S1).

**Neuronal Expression of Jafrac1 or hPrxII ROS-induced Lethality**—The expression of Jafrac1 was induced in wild-type flies treated with 20 mM paraquat for 24 h (Fig. 1B). To test the effect of Jafrac1 overexpression on oxidative stress,
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we generated transgenic fly lines carrying the Jafrac1 or hPrxII genes under the control of the UAS promoter. Reverse transcription-PCR (Jafrac1) and Western blot analysis (hPrxII) confirmed that both lines exhibited Gal4-dependent up-regulation of Jafrac1 transcripts and hPrxII proteins, respectively (supplemental Fig. S2).

Ubiquitous expression of Jafrac1 using Actin5C-Gal4 demonstrated that Jafrac1 expression reduced oxidative stress-induced lethality (Fig. 1C). We next tested whether neuronal overexpression of Jafrac1 is sufficient to confer resistance to paraquat treatment. Using elav-Gal4, which drives expression in all postmitotic neurons (19), and Cha-Gal4, which drives expression primarily in cholinergic neurons (20), we found that elav>Jafrac1 and Cha>Jafrac1 adult flies exhibited significantly reduced paraquat-induced lethality, comparable to Actin5C>Jafrac1 flies (Fig. 1C). Interestingly, Jafrac1 overexpression in glial cells using repo-Gal4 was not protective. In contrast, neuronal knockdown of Jafrac1, but not knockdown of Jafrac1 in glial cells, sensitized flies to paraquat-induced lethality (data not shown). These results suggest that Jafrac1 plays a protective role against oxidative stress in neurons.

Importantly, ROS hypersensitivity of RNAi-mediated knockdown of Jafrac1 in flies was rescued by overexpression of human PrxII (elav>Jafrac1-RshPrxII) (Fig. 1C), suggesting that human PrxII is a functional homolog of Drosophila Jafrac1.

To test the role of Jafrac1 in ROS metabolism, we measured ROS levels using a 2,7-dichlorofluorescein-DA dependent fluorescence

FIGURE 1. Neuronal expression of Jafrac1, a Drosophila ortholog of hPrxII, enhances resistance to oxidative stress. A, amino acid sequence alignments of PrxII and Jafrac1 show amino acid homologies (dark backgrounds) and conserved cysteine residues (boxes). B, expression of Jafrac1 in flies treated with 20 mm paraquat treatment for 24 h. rp49 mRNA was used as a control. C, paraquat-induced lethality of flies overexpressing Jafrac1 or with Jafrac1 knockdown driven by tissue-specific enhancers. Neuronal overexpression of hPrxII rescued the increased lethality by elav>Jafrac1-RNAI (elav>Jafrac1-RNAI; hPrxII). Each bar represents the mean ± S.E. from four independent experiments (n = 200 per experiment; *, p < 0.05; Student’s t test). D, ROS levels after paraquat treatment. Neuronal overexpression of Jafrac1 (elav>Jafrac1) or hPrxII (elav>hPrxII) reduced the ROS level, whereas inhibition of Jafrac1 expression in neurons (elav>Jafrac1-RNAI) increased the ROS level compared with the wild-type control. Mean ± S.E. from three independent experiments (n = 50 per each experiment; *, p < 0.05; **, p < 0.001; Student’s t test).

FIGURE 2. Neuronal expression of Jafrac1 or hPrxII restores oxidative stress-induced mitochondrial dysfunction. ATP levels in flies exposed to 20 mm paraquat. Flies with neuronal overexpression of Jafrac1 (elav>Jafrac1) or hPrxII (elav>hPrxII) exhibit higher ATP levels compared with wild-type controls (WT), whereas ATP levels were reduced in the Jafrac1−/− mutant and neuronal Jafrac1 inhibition (elav>Jafrac1-RNAI) flies. Data are expressed as mean ± S.E. from four independent experiments (n = 10 per each experiment; *, p < 0.05; **, p < 0.001; Student’s t test). Copy numbers of mitochondrial marker genes (Co I: cytochrome c oxidase subunit I; Co III: cytochrome c oxidase subunit III; and Cyt C: cytochrome c) in the flies after paraquat treatment. The copy number reduction was significantly aggravated in the Jafrac1−/− mutant and neuronal Jafrac1 inhibition (elav>Jafrac1-RNAI) flies and restored by neuronal overexpression of Jafrac1 or hPrxII (elav>Jafrac1 or elav>hPrxII). Mean ± S.E. from three independent experiments (n = 5 per each experiment; *, p < 0.05; **, p < 0.001; Student’s t test).
When wild-type flies were exposed to 20 mM paraquat, intracellular ROS levels were significantly increased in a time-dependent manner: 3-fold and 3.5-fold after 6- and 12-h exposures, respectively. The intracellular ROS levels were dramatically reduced by neuronal overexpression of Jafrac1 or hPrxII (elav>Jafrac1 or elav>hPrxII) (Fig. 1D). Conversely, flies with neuronal knockdown of Jafrac1 (elav>Jafrac1-Ri) showed increased ROS levels compared with wild-type control flies (Fig. 1D). These results indicate that both Jafrac1 and hPrxII can modulate intracellular ROS levels in Drosophila.

Neuronal Expression of Jafrac1 or hPrxII ROS-induced Mitochondrial Dysfunction—To test whether Jafrac1 has a protective effect on mitochondrial function under oxidative stress, we measured ATP levels in flies with neuronal overexpression of Jafrac1 or hPrxII after paraquat treatment. In the control flies, 20 mM paraquat treatment resulted in a 50% reduction in the ATP level. Neuronal overexpression of Jafrac1 or hPrxII (elav>Jafrac1, elav>hPrxII) markedly restored ATP production, whereas the reduction of ATP levels after paraquat treatment was enhanced in loss-of-function Jafrac1 mutants (Jafrac1G1104) and flies with neuronal knockdown of Jafrac1 (elav>Jafrac1-Ri) (Fig. 2A).

We next quantified mitochondrial abundance by measuring the mitochondrial DNA (mtDNA) copy number. Treatment with 20 mM paraquat caused a marked reduction in the levels of mtDNA, and this reduction in mtDNA levels induced by paraquat treatment was restored by Jafrac1 or hPrxII overexpression in neurons (Fig. 2B). The reduction in mtDNA levels after paraquat treatment was enhanced in loss-of-function Jafrac1 mutants (Jafrac1G1104) and flies with neuronal knockdown of Jafrac1 (elav>Jafrac1-Ri). These data indicate that neuronal expression of Jafrac1 or hPrxII restores oxidative stress-induced mitochondrial function.
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Neuronal Expression of Jafrac1 Inhibits Oxidative Stress-induced JNK Activation—Oxidative stress can activate the stress-responsive JNK/FOXO signaling pathway (3, 4, 6, 7). To examine JNK and FOXO activation by oxidative stress in cholinergic neurons, we marked cholinergic neurons with green fluorescent protein (Cha>GFP) and immunostained with the phospho-JNK (pJNK) and FOXO antibody to detect the active form of JNK and FOXO, respectively, in the neuromuscular junctions of third instar larvae. Activated JNK was observed in the cholinergic neurons treated with 20 mM paraquat (Fig. 3B, yellow), but not in the controls (Fig. 3A). Similarly, FOXO protein was localized in the nuclei of cholinergic neurons treated with 20 mM paraquat (Fig. 3D, arrow), but not in the controls (Fig. 3C).

Jafrac1 expression (Cha>Jafrac1) reduced the number of pJNK-positive neurons (Fig. 3E). Western blot analysis following treatment with 20 mM paraquat revealed that the treatment induced activation of JNK, but overexpression of Jafrac1 in neurons (elav>Jafrac1 and Cha>Jafrac1) suppressed JNK activation (Fig. 3F). These results suggest that oxidative stress-induced JNK activation can be suppressed by Jafrac1 in neurons.

Peroxiredoxin (Prx) enzymes modulate oxidative stress via its evolutionary conserved cysteine (Cys) residues (21). Under oxidative stress condition, cysteine sulfenic acid of Prx is oxidized. To test whether a Drosophila Prx homolog, Jafrac1, is also oxidized under oxidative stress condition, we performed Western blot analysis with oxidized Prx specific antibody, anti-Prx-SO3. In wild-type flies, the level of oxidized Prx homolog was increased after 20 mM paraquat for 24 h, indicating that the oxidation of Cys residue is conserved in Jafrac1. Increase of oxidation of Jafrac1 was enhanced in a hemizygous mutation of JNKK

FIGURE 3. Neuronal expression of Jafrac1 inhibits oxidative stress-induced JNK activation. Cha>GFP marked cholinergic neurons in Drosophila third instar larve (green) and 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclear DNA in muscle (blue). A and B, activated JNK (p-JNK) in cholinergic neurons after paraquat treatment (yellow, white arrows). C and D, nuclear translocation of FOXO transcription factors after paraquat treatment in cholinergic neurons (arrows). E, number of p-JNK-positive neurons after paraquat treatment. 60% of cholinergic neurons were p-JNK-positive in the wild type compared with 25% in flies overexpressing Jafrac1 (Cha>Jafrac1). Mean ± S.E. from five independent samples (*, p < 0.05; **, p < 0.001; Student’s t test). F, Western blotting with anti-p-JNK (top) and anti-JNK (middle). A representative blot and the quantification of signals as the ratio of p-JNK and JNK (mean ± S.E., n = 3) are shown (bottom). Neuronal overexpression of Jafrac1 (elav>Jafrac1 and Cha>Jafrac1) suppressed the oxidative stress-induced phosphorylation of JNK. G and H, oxidation of peroxiredoxin in wild-type or JNK/FOXO mutants under oxidative stress condition. G, Western blot analysis with oxidized peroxiredoxin-specific antibody, anti-Prx-SO3. H, Coomasie Blue staining image of transferred gel used in Western blot analysis confirms equal protein loading.
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![Graph showing survival rate over days for different genotypes: WT, elav-gal4, elav-Jafac1, elav-Jafac1-Ri, elav-Jafac1-1, and Jafac1-G1104.](image)

FIGURE 5. Neuronal expression of Jafac1- or hPrxII-extended life span. A, a neuronal overexpression of Jafac1 or hPrxII (elav>Jafrac1 or elav>hPrxII) extended the life span of the flies, whereas the Jafac1mutant and neuronal knockdown of Jafrac1 (elav>Jafrac1-RNAi) reduced the life span compared with the wild-type and elav-Gal4 controls (n = 200 per each genotype). B and C, extended life span in flies with neuronal overexpression of Jafrac1 induced in the adult stage using the GeneSwitch driver (elav-Gal4). Male and female flies with Jafrac1 expression in adult neurons induced by RU486 show increased average, median, and maximum life spans compared with untreated control flies (log rank test, male, \( \chi^2 = 1.50, p < 0.0001, n = 189; \) female, \( \chi^2 = 0.91, p < 0.0001, n = 197 \)). D, a model of the JNK/FOXO pathway. Oxidative stress by the paraquat treatment induces mitochondrial damage and activates the JNK/FOXO pathway. This stress induces neuronal expression of Jafrac1, which in turn suppresses oxidative stress-induced lethality and extends life span.

Next, we tested the effect of genetic manipulation of FOXO on Jafrac1 gene expression. To induce FOXO expression in neurons in the adult stage, we used the RU486-inducible elav-Gal4 driver (elavGS-Gal4). Neuronal overexpression of wild-type FOXO (elavGS>dFOXO + RU) or the insulin-insensitive nuclear form of FOXO (elavGS>dFOXO.TM + RU) increased the expression level of jafrac1 by >2-fold compared with the controls, whereas the expression of Jafrac1 was reduced in a FOXO mutant (Foxo21/25) (Fig. 4B). In addition, the expression of Jafrac1 was over 3-fold increased in wild-type control flies treated with 20 mM paraquat for 24 h, but this increase was significantly reduced in a hemizygous mutation of JNKK (Hep1/Y) or transallelic mutation of dFOXO21/25 (Fig. 4C). These results indicate that JNK/FOXO signaling regulates Jafrac1 expression in neurons.

Neuronal Expression of Jafrac1 or hPrxII Extends Life Span in Flies—Because it is well established that activation of JNK/FOXO signaling increases life span (2, 4, 7), we examined the role of neuronal Jafrac1, a target gene of JNK/FOXO signaling, in the control of the fly life span. Neuronal overexpression of Jafrac1 or hPrxII in neurons (elav>Jafrac1 or elav>hPrxII) significantly increased life span, while neuronal knockdown of Jafrac1 (elav>Jafrac1-Ri), as well as the loss-of-function mutation (Jafrac1mutant), caused a reduction in life span (Fig. 5A). To test whether neuronal expression of Jafrac1 in the adult stage is sufficient to extend life span, we used the RU486-inducible elav-Gal4 driver (elavGS-Gal4) to express Jafrac1 in adult neurons (22). It has been reported that RU486 feeding does not affect life span of flies (2). Expression of Jafrac1 in adult neurons extended life span by 26% in females (Fig. 5B) and 29% in males (Fig. 5C), compared with the control flies.

DISCUSSION

Multiple lines of evidence point to the activation of the JNK/FOXO pathway as a common cellular response to oxidative damage across animal phyla (4, 6, 7, 13, 23). In Drosophila, JNK confers tolerance to oxidative stress and extends life span by inducing a protective gene expression program. Increased JNK

JNK/FOXO Signaling Regulates Jafrac1 Expression under Both Normal and Oxidative Stressed Conditions—JNK/FOXO signaling activates expression of several genes involved in cellular stress responses (4). To test whether JNK/FOXO signaling regulates Jafrac1 expression, we first examined the effect of genetic manipulation of JNK activity in neurons on Jafrac1 gene expression. Neuronal overexpression of the constitutively active form of hemipterus (hep), a Drosophila JNKK gene (elav>hepCA and cha>hepCA), markedly increased the expression level of Jafrac1. In contrast, Jafrac1 mRNA levels are reduced in flies carrying one copy of a loss of function mutation of the Drosophila homolog of JNK, Basket (Bsk'), in the hemizygous hep' mutant (hep'/Y) background (Fig. 4A).
activity in neurons is sufficient to promote stress tolerance and extend life span in flies (4, 7). However, whether this effect is due to the specific protection of neurons against oxidative damage, or whether JNK activation in neurons may induce a humoral response that regulates longevity systemically, is unclear (1, 5).

In this study, we demonstrated that JNK/FOXO signaling is required for the expression of Jafrac1 in brains under both normal and oxidative stressed conditions (Fig. 4). There are two putative FOXO consensus binding sites (RWWAACA) in the promoter region of Jafrac1 (data not shown), suggesting that the transcription factor FOXO may bind to the Jafrac1 promoter and directly activate Jafrac1 transcription. We also demonstrated that neuronal knockdown of Jafrac1 enhances, and neuronal overexpression of Jafrac1 reduces, ROS-induced lethality. Furthermore, the neuronal knockdown of Jafrac1 shortened, while overexpression of Jafrac1 extended, the life span of the flies. These results support the hypothesis that, in Drosophila, the JNK/FOXO pathway protects neurons from oxidative stress and extends life span by induction of antioxidan genes, including Jafrac1 in neurons (Fig. 5D).

Peroxiredoxins (Prxs) are identified by their ability to neutralize cellular hydroperoxides in mammals (24). A family of five Prx genes has been identified and characterized in D. melanogaster (25). All Drosophila Prxs have peroxidase activities, and their expressions are induced by oxidative stress. Prx overexpression enhances resistance to oxidative stress by hydrogen peroxide and paraquat in cultured Drosophila cells (25, 26). In Drosophila, overexpression of Jafrac1 has been shown to counteract the enhanced susceptibility of immune-regulated catalase knockout flies to natural infections (14). Moreover, mitochondrial peroxiredoxin (Dpx-5037, mTPx) has been reported to restore wild-type life span in a Drosophila model for Friedreich’s ataxia (27).

We have demonstrated that neuronal expression of Jafrac1 and hPrxII significantly reduces the ROS level and restores mitochondrial function in paraquat-treated flies (Figs. 1D and 2). Several studies in Drosophila show that expression of specific mitochondrial proteins can increase resistance to oxidative stress as well as extend life span (28–30), suggesting that mitochondrial function plays an important role in determining life span. Collectively, Jafrac1 or hPrxII may extend life span by acting as a guardian for neuronal mitochondria under age-associated oxidative stress conditions. Furthermore, because mitochondrial dysfunction is associated with many neurodegenerative diseases (8), induction of Jafrac1/PrxII in neurons may also be protective against age-associated neurodegenerative diseases.

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