JNK Signaling Confers Tolerance to Oxidative Stress and Extends Lifespan in *Drosophila*

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Summary

Changes in the genetic makeup of an organism can extend lifespan significantly if they promote tolerance to environmental insults and thus prevent the general deterioration of cellular function that is associated with aging. Here, we introduce the Jun N-terminal kinase (JNK) signaling pathway as a genetic determinant of aging in *Drosophila melanogaster*. Based on expression profiling experiments, we demonstrate that JNK functions at the center of a signal transduction network that coordinates the induction of protective genes in response to oxidative challenge. JNK signaling activity thus alleviates the toxic effects of reactive oxygen species (ROS). In addition, we show that flies with mutations that augment JNK signaling accumulate less oxidative damage and live dramatically longer than wild-type flies. Our work thus identifies the evolutionarily conserved JNK signaling pathway as a major genetic factor in the control of longevity.

Introduction

Reactive oxygen species (ROS) damage biological macromolecules (lipids, nucleic acids, and proteins) by oxidizing them and altering their structure and function. Oxidative damage to DNA and proteins has been implicated in a variety of degenerative diseases (among them Alzheimer’s disease, amyotrophic lateral sclerosis, Parkinson’s disease, and rheumatoid arthritis; Stadman, 2001). In addition, accumulation of genomic defects caused by ROS throughout the lifetime of an organism may result in increased cancer risk (Beckman and Ames, 1998; Feig et al., 1994; Grollman and Moriya, 1993). According to the “free radical theory of aging” (Beckman and Ames, 1998; Harman, 1956), the collective damage wrought by ROS is also believed to be a major cause for the general deterioration of cell and tissue function with progressing age (Tissenbaum and Guarente, 2002). To counteract these injuries, aerobic organisms employ a variety of molecules and mechanisms that prevent or relieve damage inflicted by ROS, some of which have been shown to extend lifespan (Beckman and Ames, 1998; Finkel and Holbrook, 2000). It is as yet not fully elucidated how the activity of this protective system is regulated following oxidative insults. Stress-sensing signaling pathways include several MAP kinase cascades involving the “stress-activated protein kinases” (SAPKs) p38 and JNK (Davis, 2000; Paul et al., 1997; Stronach and Perrimon, 1999). The JNK signaling cascade is triggered by a variety of insults, including UV radiation and oxidative stress. The consequences of this activation in vivo are not well understood. At the cellular level, it may serve a protective function (Minamino et al., 1999) but may also promote apoptosis (Tourier et al., 2000). Here, we analyze the role of JNK, and its downstream genetic program, in oxidative stress tolerance and aging of the organism.

Results and Discussion

JNK Induces a Gene-Expression Program of Oxidative Stress Response in the Organism

JNK phosphorylates a variety of transcription factors and enhances their transcriptional activation potential. Thus, insight into the biological consequences of stress-activated JNK signaling might be gained by analyzing the relevant downstream genetic programs. We chose *Drosophila* as a model organism for such studies, as its JNK pathway is genetically very tractable. The multiplicity of homologous and functionally at least partially redundant kinases (three mammalian JNK genes produce at least ten protein isoforms; Davis, 2000) has impeded similar analyses in mammals. We recently mapped the genomic region to JNK signaling in the *Drosophila* embryo using serial analysis of gene expression (SAGE; Jasper et al., 2001). Among the genes induced in embryos with increased JNK signaling, we identified a group with tentative functions in cellular stress responses as well as several genes that were known to be activated in response to oxidative damage (Figure 1, and Supplemental Table S1 available online at http://www.developmentalcell.com/cgi/content/full/5/5/811/DC1; Jasper et al., 2001). In an independent experiment, we found similar genes upregulated in response to JNK signaling in differentiating photoreceptors (H.J., unpublished data and Supplemental Table S2). These findings suggested that JNK signaling activates a gene expression program that confers tolerance to oxidative stress in a variety of cell types. To test this hypothesis, we monitored the expression of four representative genes (*hsp68, gstD1, fer1HCH, and mtmA*), which were identified as JNK dependent in our SAGE experiments, in the adult fly using quantitative real-time RT-PCR. The induction of the respective mRNAs in response to oxidative stress, artificially brought on by treatment with the drug paraquat, was measured in wild-type flies and in hemizygotes for *hep*, a hypomorphic allele of the *Drosophila* JNKK gene, *hemipterous* (*hep*; Glise et al., 1995). Paraquat, a compound widely used to apply oxidative stress to cells and organisms, leads to continuous intracellular generation of O₂⁻ radicals (Arking et al., 1991). It efficiently activates JNK in the fly, as indicated by the transcriptional activation of a *puckered* (*puc*), one of the prototypical target genes of JNK signaling in *Drosophila* (Figure 1). *puc* encodes a JNK-specific phosphatase that downregulates the pathway, thus establishing a negative feedback loop (Martin-Blanco et al., 1998). RT-PCR data show that JNK signal-
Oxidative Stress Tolerance Induced by JNK Signaling in Drosophila

To examine the relevance of JNK signaling for the sensitivity of the organism to oxidative stress, we exposed adult flies to paraquat for a prolonged period of time and monitored their survival (Figure 2). Compared to wild-type animals, flies with decreased JNK signaling potential (hemizygotes for hep, or heterozygotes for a hypomorphic allele of the Drosophila JNK gene basket, bsk) were more sensitive to moderate doses of paraquat. Conversely, flies gained resistance to paraquat when signal flow through the kinase cascade was promoted by overexpression of Bsk or Hep. Similarly, boosting JNK signal transduction by reducing the gene dose of puc, conferred strong paraquat resistance in a hep- and bsk-dependent fashion (Figure 2). Flies heterozygous for puc exhibit elevated levels of JNK activity, as inferred by the dosage sensitivity of JNK-mediated apoptotic phenotypes in the developing wing (Adachi-Yamada et al., 1999), as well as by rescue of developmental defects normally observed in flies carrying hep and kay mutations (Zeitlinger and Bohmann, 1999). Constitutive overexpression of one of the identified JNK-inducible stress response genes, Hsp68, also protects flies against oxidative stress, suggesting that JNK’s downstream genetic program mediates the observed protection (Figure 2). The observed differences in sensitivity to paraquat were not due to feeding abnormalities or a general tolerance to toxic compounds of the tested genotypes, as they are similarly sensitive to G418 toxicity (Figure 2B).

Tissue-specific overexpression of superoxide dismutase (SOD) in motoneurons increases the resistance to oxidative stress and extends the lifespan of Drosophila (Parkes et al., 1998). This result suggests neurons as the “weakest link” in the organism’s tolerance to oxidative insults and as a cell type in which protective mechanisms would be most critical. To investigate whether JNK signaling in neurons could play a role in such mechanisms, we examined fly strains in which Hep overexpression was directed either to the nervous system or to muscle tissue in an RU486-inducible manner (using the “gene-switch Gal4” driver; Osterwalder et al., 2001). The toxicity of paraquat was reduced significantly when Hep was expressed in the nervous system (ELAV Gal4) and as a cell type in which protective mechanisms are shown. Activation of puc is indicative of JNK signaling activity. Metallothionein A (MtnA) is induced by a variety of environmental stresses and is believed to play an important antioxidant role (Nath et al., 2000). Heat shock protein 68 (Hsp68) is closely related to Hsp70. Its induction after oxidative stress (Gosslau et al., 2001) may relieve protein damage by ROS. Glutathione S Transferase D1 (GSTD1) is induced in aging and paraquat-exposed flies (Zou et al., 2000). Ferritin 1 heavy chain (Fer1HCH) scavenges free iron, a major generator of ROS through the Fenton reaction. Induction of ferritin after oxidative stress as well as its regulation by AP-1 has been described in the mouse (Tsujii et al., 1998). The lack of induction of these genes in hep hemizygotes indicates a requirement for JNK signaling in the oxidative-stress response in Drosophila.

Importantly, the inducibility of the JNK effect by RU486 in this system rules out variations in the genetic back-
Levels of JNK Signaling

According to the free radical theory of aging (Harman, 1956), one genetic determinant for the lifespan of an organism is its sensitivity to oxidative stress. We asked whether the protection against oxidative damage that is brought about by an increase in JNK signaling potential might be sufficient to extend Drosophila’s life expectancy. We examined flies heterozygous for puc to test this hypothesis, since our experiments demonstrated that the tolerance of flies to oxidative stress increases with decreasing gene dose of puc. Flies heterozygous for either one of two different loss-of-function alleles of puc \((puc^{c244.1}\) or \(puc^{c123}\); Martin-Blanco et al., 1998) showed dramatic extensions of median and maximum life expectancy compared to wild-type flies and to flies of an isogenic control strain (Figures 3A, 3C, and 3D). The difference in the degree of lifespan extension by the two alleles correlates well with their described allelic strength (McEwen et al., 2000). Our results thus suggest a direct relationship between the decrease of Puc activity in the mutants and the resulting lifespan extension. Since biochemical and genetic data indicate that the activity of Puc is limited to the JNK signaling pathway (as opposed to other MAPK pathways; Martin-Blanco et al., 1998), the lifespan extension in puc mutants is likely to be caused by higher levels of JNK signaling. We tested the requirement for a functional JNK pathway in the longevity of puc mutants directly by comparing the lifespan of puc\(^{c244}\) heterozygous males in a wild-type background to puc\(^{c123}\) heterozygotes in a hep\(^{1}\) hemizygous background (Figure 3B). Heterozygosity for puc leads to an only modest increase in mean and maximum lifespan of hep\(^{1}\) hemizygous flies, indicating that a functional JNK cascade is required for efficient lifespan extension in puc mutants. These results strongly support the notion that the longevity phenotype observed in puc mutants is due to an increase in JNK signaling activity.

Our genomic experiments suggested that elevated JNK signaling activity causes higher basal levels of protective genes. We tested whether constitutive overexpression of one of the identified JNK target genes, hsp68, would be sufficient to extend lifespan of Drosophila (Figure 3E). In agreement with our hypothesis, we observed small but significant increases in mean and maximum lifespan in flies that overexpress hsp68 compared to isogenic wild-type controls. This experiment is consistent with earlier observations in which increased expression of chaperones extended the lifespan of Drosophila (Tatar et al., 1997).
Figure 3. Increased JNK Signaling Potential Extends Lifespan in Drosophila

(A) Heterozygotes for pucE69 (green diamonds) have a longer life span than flies that are otherwise isogenic (10× backcrossed, black squares). Note that lifespan in these experiments was generally shorter than in the following ones, due to cultivation at 29°C.

(B) The extension of lifespan by puc heterozygosity requires functional Hep. hep1 hemizygous males (black squares) exhibit lifespan comparable to wild-type flies. Reduction of the puc gene-dose only marginally extends lifespan in these flies (orange circles). Compared to puc mutants in a wild-type background, hep1 reduces lifespan.

(C and D) Males heterozygous for two different puc alleles (progeny from crosses between puc/TM3 and OreR) show significant extension of mean and maximal lifespan when compared to wild-type flies. Females show qualitatively similar lifespan data, even though the extension of lifespan in pucE69 females is less pronounced than in males (data not shown).

(E) Constitutive overexpression of the JNK target gene hsp68 extends lifespan. w1118;P{w11001mCarmGal4} flies were crossed to w1118 or w1118;P{w11001mCUASHsp68}, and survival of the male progeny was monitored at 25°C. In all cases, survival in three or more independent cohorts of about 100 flies each was monitored over time. Here, these data are combined into one graph for clarity. Statistical analysis of the combined survival data was performed using the log rank test. Cohort sizes and p values are as follows. (A) puc, n = 232; ry, n = 192; p < 0.001. (B) hep, n = 258; hep,puc, n = 267; puc, n = 338; p < 0.001. (C) puc, n = 333; OreR, n = 252; p < 0.001. (D) puc, n = 268; OreR, n = 325; p < 0.001. (E) armGal4, n = 679; armGal4 > Hsp68, n = 663; p < 0.001. (F) elavGal4, n = 360; elav > Hep, n = 320; p < 0.001.

Unless stated otherwise, flies were reared at 25°C.

Providing higher JNK signaling levels in neuronal tissue is sufficient to increase oxidative stress tolerance (Figure 2C). To test whether neuronal-specific protection would also be sufficient to extend lifespan of the organism, we monitored survival in flies that overexpress Hep constitutively in neuronal tissue under the control of ELAV Gal4. Neuronal overexpression of Hep extended lifespan significantly, indicating that the level of JNK activity in neuronal tissue determines not only the fly’s oxidative stress tolerance, but also its lifespan. Importantly, these results confirm, independently of puc mutations, that JNK signaling promotes longevity.

Several genetically determined changes in physiology have been associated with extended lifespan in Drosophila. Such changes include reduced reproductive activity, dwarfism, delays in development, as well as stress...
tolerance (Clancy et al., 2001; Tu et al., 2002). We examined whether the JNK pathway might affect parameters indicative of such physiological changes (Figure 4), puc heterozygotes and wild-type controls exhibit roughly equivalent sizes (as determined by body weight, Figure 4E), reproductive activities (fecundity, Figure 4A), as well as developmental timing (Figure 4B). In contrast, oxidative stress tolerance and tolerance to starvation differ markedly between wild-type and puc heterozygous flies (Figures 2 and 4C). Importantly, 10-day-old puc heterozygotes contain significantly decreased levels of oxidized proteins (Figure 4D). The quantity of protein oxidation products, such as polypeptides carrying carbonyl groups, is a measure for the accumulated oxidative damage suffered by an organism (Stadtman, 2001). Taken together, our results suggest that increased JNK signaling is sufficient to reduce oxidative damage throughout the lifetime of a fly and that this beneficial effect may be the cause of the longevity phenotype of gain-of-function mutants for this signaling pathway.

This work identifies the JNK signaling pathway as a significant genetic determinant of longevity in Drosophila. Activation of JNK in response to oxidative challenge and to other environmental insults has been well described in a number of model systems and was proposed to trigger the expression of genes that could mediate protective functions on the organism at least in certain cell types. Against this backdrop, resulting in the prediction that JNK signaling would protect the organism from oxidative challenge (which we show here to be correct), it may seem surprising that, until now, no evidence has been produced that links JNK signaling to an extended lifespan. Evidently, experimental limitations of mammalian systems, including increased functional complexity and genetic redundancy, have precluded clear-cut experiments to address this question.

While we cannot exclude unidentified functions of JNK signaling that might be relevant to the aging process, it seems plausible (and the free radical theory of aging would predict) that the observed protection against oxidative insults decisively delays aging and thus causes the longevity phenotype of puc heterozygotes. Earlier observations, as well as our experiments, support this notion: Hsp70, and its JNK-inducible relative Hsp68, extend lifespan when overexpressed in Drosophila (Figure 3G and Tatar et al., 1997). These chaperones have been implicated in oxidative stress resistance (Gossiau et al., 2001) and may have repair functions downstream of JNK signaling. The reduced level of oxidative damage in aging puc heterozygotes (Figure 4E) further supports this view. JNK signaling thus emerges as an evolutionarily conserved gene-regulatory network that limits oxidative damage in the organism and its impact on aging.

Experimental Procedures

Genetics and Fly Handling
Fly strains used were OreR, from Bloomington stock center; heps1/FM6, gift from S. Noselli; pucG司法TM3, and pucG司法TM2, gifts from E. Martin-Bianco; UASHep and UASBsk, gifts from M. Miotzik; elav-Gal4, elav-GSGal4, and MHC-GSGal4, gifts from M. Rama-swami. Fly lines carrying UASHsp68 transgenes were generated by P element-mediated transformation of w1118 flies. The Hsp68 cDNA was cloned using PCR to amplify the full-length cDNA sequence (as annotated in Flybase) and ligating it into the NotI and BglII sites of pUAST. Unless stated otherwise, flies were reared at 25°C and 65% humidity on cornmeal- and molasses-based food. All experiments were conducted with flies that developed at equal larval densities.

For paraquat treatments, flies were starved for 6 hr and then transferred to vials containing filter paper soaked in 5% sucrose solution with or without 15 mM paraquat (Methyl Viologen, Sigma). Survival was assessed after 16 hr. Flies were kept in the dark at all times.

RU486-induction was performed by feeding flies with 400 μg/ml RU486 in 5% sucrose on filter paper for 1.5 hr. Filter paper was then exchanged for filter paper soaked with 400 μg/ml RU486 and 20 mM paraquat in 5% sucrose. Survival was assessed after 20 hr.

Compared genotypes were always treated in parallel.

To monitor lifespan, cohorts of about 100 males or females were separated after mating for 2 days after hatching and transferred into fresh vials at defined densities (100 flies per 50 ml food). Flies were transferred into fresh vials every 4 days. To generate an isogenic control line for puc mutants, pucG司法TM3 flies were backcrossed 10 times into the ryP background using the ryP P element inserted in the puc locus as a marker. In additional experiments (Figure 3C and 3D), the lifespan of puc+/ ♂ male progeny from crosses of puc/TM3 mutants with OreR wild-type controls was monitored. The reference background for transgenic lines (elavGal4, armGal4, UASHepwt, and UASHSP68) was w1118. Driver lines were crossed...
with either w1118 or with the corresponding UAS lines and the lifespan
of male progeny was assessed.

Fecundity was measured by counting the number of eggs laid
each day for the first ten days after mating. Genotypes were com-
pared by counting eggs laid by puc,ry/ry females that were mated
with puc,ry/ry males; or by ry/ry females mated with ry/ry males.
Fifty males and one hundred females were allowed to mate for 3
days, then groups of 15 females were separated into individual vials,
and the number of eggs laid was determined every 24 hr.

Developmental time was assessed by recording the cumulative
number of adults emerged as a function of time. Thirty-five puc102,ry/ry
females were crossed with 20 ry/ty males for 5 days. Starting at
10 days after mating and for an additional 15 days, the number of
adults from each vial was counted daily.

Protein Carbonyl Quantification

Protein carbonyl content was measured as previously described
(Levine et al., 1999), using 30 10-day-old male flies per genotype
homogenized in 500 μl 5 mM phosphate buffer (pH 7.5).

Real-Time RT-PCR

Total RNA was isolated from ten paraquat-treated male flies using
Trizol (GibcoBRL). First-strand cDNA was prepared from 5
μl total RNA using Superscript II Reverse Transcriptase (Invitrogen) and
oligo (dT)12–18 primer in 50 μl reaction volume. Real-time quantitative
PCR was performed on a 7500 Thermal Cycler (Applied Biosystems)
following the manufacturer’s suggestions using SYBR Green-based detection
of PCR products. Melting curves were examined after amplification
as to transcript levels in mock-treated flies.

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