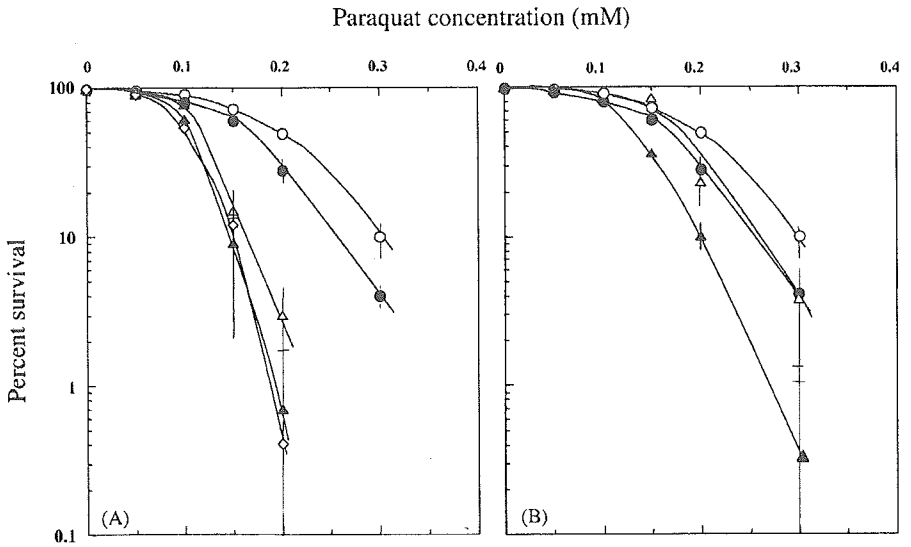

Fig. 4. Paraquat sensitivities of wild type, *daf-16*, p38-related mutants (*nsy-1* and *sek-1*) and JNK-related mutants (*jnk-1* and *mek-1*). L1 larvae were cultured on seeded nematode growth (NGM) agar plates containing various concentration of paraquat. Four days later, survival was determined by counting the number of L4 larvae and adults. (A) Wild-type animals: (○); *daf-16*: (●); *nsy-1*: (△); *sek-1*: (▲); *sek-1* and *daf-16*: (◇). (B)Wild-type animals: (○); *daf-16*: (●); *jnk-1*: (△) and *mek-1*: (▲). Each value is the mean ± S.D. of three different experiments.

addition, the JNK-related *jnk-1* and *mek-1* mutants were slightly hypersensitive to paraquat (Fig. 4B).

4. Discussion

We have explored the possibility that pathways in addition to the *daf-2*-akt/sgk pathway mediate the response of DAF-16 to oxidative stress. The DAF-16 is translocated to the nucleus when animals are subjected to oxidative stress in the form of paraquat (Fig. 3). We questioned whether ROS directly activate the DAF-2 signal pathway. Therefore, we examined signaling pathways that are known to respond to ROS in other organisms to see if they play a role in activation of DAF-16 in *C. elegans*. Under low levels of oxidative stress, largely cytoplasmic localization of DAF-16 was seen in wild type and in three mutants [*jnk-1* (JNK-related MAPK), *mek-1* (JNK-related MAPKK) and *sek-1* (p38-related MAPKK)], each of which is defective in one predicted signaling pathway (Fig. 3). Conversely, DAF-16 was also found in the nuclei of wild-type animals as well as *jnk-1* and *mek-1* mutants when they were subjected to oxidative stress in the form of paraquat (Fig. 3). Thus, oxidative stress has no effect on the localization of DAF-16 via the JNK-related MAPK and MAPKK. In the *sek-1* mutant, however, DAF-16 remained cytoplasmic even in the face of oxidative stress (Fig. 3). The fact that DAF-16 translocation is blocked by *sek-1* suggests that the p38 signal transduction pathway acts upstream to activate DAF-16 in response to ROS. Alternatively, *sek-1* could, like *akt-1* and *sgk-1*, phosphoinactivate DAF-16, and ROS could prevent this. The fact that mutation of *sek-1* alone does not cause nuclear localization of DAF-16 could reflect the action of other, additional effectors of ROS. Heat or starvation did not prevent nuclear localization in *sek-1*, indicating that the upstream pathway for oxidative stress may be different than the pathway for other stresses such as heat or starvation (data not shown).

Both the p38-related *nsy-1* (MAPKKK) and *sek-1* (MAPKK) mutants are hypersensitive to oxidative stress as presented by paraquat (Fig. 4A). Conversely, *daf-16* was only slightly hypersensitive, suggesting that the p38 pathway regulates resistance to ROS by downstream elements in addition to *daf-16*. This is consistent with fact that the *daf-16*; *sek-1* showed the same degree of hypersensitivity as the *sek-1* single mutant. It also indicates that, at least in terms of regulating resistance to oxidative stress, the p38 pathway plays a major and perhaps exclusive role in regulating *daf-16* activation. In addition, the JNK-related *jnk-1* and *mek-1* mutants were slightly hypersensitive to paraquat (Fig. 4B). This suggests that the JNK signal transduction pathway may play a role, albeit minor, in regulating resistance to oxidative stress. It is known that an apoptosis signal-regulating kinase (ASK)-1 acts upstream of JNK and p38 MAPK in mammals and corresponds to NSY-1 (MAPKKK) in *C. elegans*. NSY-1 is regulated by the redox response of thioredoxin binding with ASK-1 (Saitoh et al., 1998; Tobiume et al., 2001). As the *nsy-1* mutant is hypersensitive to paraquat (Fig. 4A), thioredoxin may also regulate the p38-signaling pathway in *C. elegans*. Kim et al. (2002) showed that signaling of p38 MAP kinase pathway including *nsy-1* and *sek-1* is require for pathogen resistance or cellular immune response to bacteria. It suggests that signals by oxidative stress may trigger immune responses through this pathway. Alternatively, this pathway may be required for defense to oxidative stress caused during the process of the immune response.

Much attention has focused on the insulin-like signaling pathway in *C. elegans* because of its pivotal role in life-span determination and oxidative stress resistance. While the genetics of the pathway itself have been well elucidated, comparatively little is known about additional upstream inputs into the pathway, or the possibility of additional DAF-16 regulated output. The experiments reported in this letter implicate p38 action involves DAF-2 or that it acts independently of this pathway. This observation should better enable future research to determine the exact mechanisms by which ROS activate signal transduction pathways to effect transcriptional regulation. It has been reported in mammals that insulin mRNA levels were reduced by oxidative stress, suggesting that insulin signaling pathway regulates oxidative stress (Kaneto et al., 2002). In *C. elegans*, insulin levels under oxidative stress may be regulated by p38 signaling pathway via SEK-1 (Tanaka-Hino et al., 2002). This occurs primarily in chemosensory neurons. Food might provide the trigger for this secretion (Pierce et al., 2001). In the case of *C. elegans*, the study may be difficult to reproduce because there are at least 38 candidate genes (*ins-1–ins-38*) that encode insulin-like peptides in terms of its predicted structure. INS-1 most closely resembles human insulin in terms of its predicted interaction with DAF-2 insulin-like receptor (Kenyon, 1996, worm base: http://www.wormbase.org/), its physiological function is still unclear. In addition, a complete understanding of these signaling pathways requires consideration of not only reductions in the insulin-like peptides by oxidative stress but also dephosphorylation of PI3 kinase or AKT and inhibition of 14-3-3-dependent or -independent pathways (Lin et al., 1997; Murphy et al., 2003).

The current state of our understanding is summarized in Fig. 5.

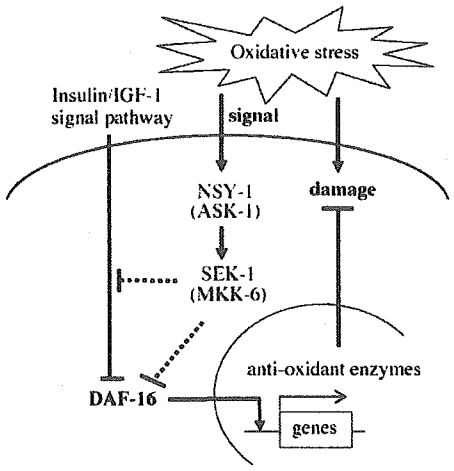

Fig. 5. Schematic diagram of cellular responses to ROS. Key components of the insulin-like signaling pathway are shown, as elucidated by others. In addition, a likely location for the oxidative-stress mediated input of p38 is also indicated.

Acknowledgement

This work is founded in part by grants of the Virtual Research Institute of Aging of Nippon Boehringer Ingelheim to N. Ishii.

References

Brenner, S., 1974. The genetics of *Caenorhabditis elegans*. Genetics 77, 71–94.

Byrd, D.T., Kawasaki, M., Walcoff, M., Hisamoto, N., Matsumoto, K., Jin, Y., 2001. UNC-16, a JNK-signaling scaffold protein, regulates vesicle transport in *C. elegans*. Neuron 32, 787–800.

Cahill, C.M., Tzivion, G., Nasrin, N., Ogg, S., Dore, J., Ruvkun, G., Alexander-Bridges, M., 2001. Phosphatidylinositol 3-kinase signaling inhibits DAF-16 DNA binding and function via 14-3-3-dependent and 14-3-3-independent pathways. J. Biol. Chem. 276, 13402–13410.

Emmons, S.W., Klass, M.R., Hirsh, D., 1979. Analysis of the constancy of DNA sequences during development and evolution of the nematode *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U.S.A. 76, 1333–1337.

Henderson, S.T., Johnson, T.E., 2001. *daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*. Curr. Biol. 11, 1975–1980.

Hertweck, M., Gobel, C., Baumeister, R., 2004. *C. elegans* SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span. Dev. Cell. 6, 577–588.

Honda, Y., Honda, S., 1999. The *daf-2* gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*. FASEB J. 13, 1385–1393.

Ip, Y.T., Davis, R.J., 1998. Signal transduction by the c-Jun N-terminal kinase (JNK)—from inflammation to development. Curr. Opin. Cell Biol. 10, 205–219.

Kaneto, H., Sharma, A., Suzuma, K., Laybutt, D.R., Xu, G., Bonner-Weir, S., Weir, G.C., 2002. Induction of c-Myc expression suppresses insulin gene transcription by inhibiting neuroD/BETA2-mediated transcriptional activation. J. Biol. Chem. 277, 12998–13006.

Kenyon, C., 1996. Environmental factors and gene activities that influence life span. In: Riddle, D.L., Blumenthal, T., Meyer, B.J., Priess, J.R. (Eds.), *C. elegans* II. Cold Spring Harbor Press, New York, pp. 791–813.

Kim, D.H., Feinbaum, R., Alloing, G., Emerson, F.E., Garsin, D.A., Inoue, H., Tanaka-Hino, M., Hisamoto, N., Hatsumoto, K., Tan, M.-W., Ausubel, F.H., 2002. A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity. Science 297, 623–626.

Koga, M., Zwaal, R., Guan, K.-L., Avery, L., Ohshima, Y., 2000. A *Caenorhabditis elegans* MAP kinase kinase, MEK-1, is involved in stress responses. EMBO J. 19, 5148–5156.

Kyriakis, J.M., Avruch, J., 1996. Protein kinase cascades activated by stress and inflammatory cytokines. Bioassays 18, 567–577.

Lin, K., Dorman, J.B., Rodan, A., Kenyon, C., 1997. *daf-16*: an HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. Science 278, 1319–1322.

Lin, K., Hsin, H., Libina, N., Kenyon, C., 2001. Regulation of the *Caenorhabditis elegans* longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat. Genet. 28, 139–145.

Murphy, C.T., McCarroll, S.A., Bargmann, C.I., Fraser, A., Kamath, R.S., Ahringer, J., Li, H., Kenyon, C., 2003. Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. Nature 424, 277–284.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G.I., Lee, L., Tissenbaum, H.A., Ruvkun, G., 1997. The fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. Nature 389, 994–999.

Paradis, S., Ailion, M., Toker, A., Thomas, J.H., Ruvkun, G., 1999. A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase

signals that regulate diapause in *Caenorhabditis elegans*. Genes Dev. 13, 1438–1452.

Pierce, S.B., Costa, M., Wisotzkey, R., Devadhar, S., Homburger, S.A., Buchman, A.R., Ferguson, K.C., Heller, J., Platt, D.M., Pasquinelli, A.A., Liu, L.X., Doberstein, S.K., Ruvkun, G., 2001. Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse *C. elegans* insulin gene family. Genes Dev. 15, 672–686.

Robinson, M.J., Cobb, M.H., 1997. Mitogen-activated protein kinase pathways. Curr. Opin. Cell Biol. 9, 180–186.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, N., Miyazono, F., Ichijo, H., 1998. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J. 17, 2596–2606.

Sulston, J.E., Brenner, S., 1974. The DNA of *Caenorhabditis elegans*. Genetics 77, 95–104.

Tanaka-Hino, M., Sagasti, A., Hisamoto, N., Kawasaki, M., Nakano, S., Ninomiya-Tsuji, J., Bargmann, C.I., Matsumoto, K., 2002. SEK-1 MAPKK mediates Ca^{2+} signaling to determine neuronal asymmetric development in *Caenorhabditis elegans*. EMBO Rep. 3, 56–62.

Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K.-I., Takeda, K., Minowa, O., Miyazono, K., Noda, T., Ichijo, H., 2001. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO Rep. 2, 222–228.

Villanueva, A., Lozano, J., Morales, A., Lin, X., Deng, X., Hengartner, M.O., Kolesnick, R.N., 2001. *jkk-1* and *mek-1* regulate body movement coordination and response to heavy metals through *jnk-1* in *Caenorhabditis elegans*. EMBO J. 20, 5114–5128.