Estrogen, Insulin, and Dietary Signals Cooperatively Regulate Longevity Signals to Enhance Resistance to Oxidative Stress in Mice*

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To investigate the biological significance of a longevity mutation found in daf-2 of Caenorhabditis elegans, we generated a homologous murine model by replacing Pro-1195 of insulin receptors with Leu using a targeted knock-in strategy. Homozygous mice died in the neonatal stage from diabetic ketoacidosis, whereas heterozygous mice showed the suppressed kinase activity of the insulin receptor but grew normally without spontaneously developing diabetes during adulthood. We examined heterozygous insulin receptor mutant mice for longevity phenotypes. Under 80% oxygen, mutant female mice survived 33.3% longer than wild-type female mice, whereas mutant male mice survived 18.2% longer than wild-type male mice. These results suggested that mutant mice acquired more resistance to oxidative stress, but the benefit of the longevity mutation was more pronounced in females than males. Manganese superoxide dismutase activity in mutant mice was significantly up-regulated, suggesting that the suppressed insulin signaling leads to an enhanced antioxidant defense. To analyze the molecular basis of the gender difference, we administered estradiol to mutant mice. It was found that the survival of mice under 80% oxygen was extended when they were administered estradiol. In contrast, mutant and wild-type female mice showed shortened survivals when their ovaries were removed. The influence of estrogen is remarkable in mutant mice compared with wild-type mice, suggesting that estrogen modulates insulin signaling in mutant mice. Furthermore, we showed additional extension of survival under oxidative conditions when their diet was restricted. Collectively, we show that three distinct signals; insulin, estrogen, and dietary signals work in independent and cooperative ways to enhance the resistance to oxidative stress in mice.

Recent experimental evidence suggests that oxidative stress is a principle cause of aging (1). Biochemical data further support the hypothesis that the oxidative damage accumulated in macromolecules such as DNAs, lipids, and proteins leads to the physiological decline in aging tissues. In a genetic analysis of invertebrate models, longevity mutants have been identified that show extension of lifespan associated with an enhanced resistance to oxidative stress (2). In Caenorhabditis elegans, a mutation of the daf-2 gene that encodes an insulin/insulin-like growth factor type 1 (IGF-1)* receptor significantly enhanced resistance to oxidative stress and extended lifespan (3). These phenotypes are regulated by the insulin/IGF-1 signaling pathway from DAF-2 to DAF-16, a homologue of the hepatocyte nuclear factor-3/forkhead transcription factor (4, 5). Furthermore, expression of sod3 encoding manganese superoxide dismutase (MnSOD) is up-regulated in daf-2 mutants (5). In insulin-like signaling mutants of flies, it is suggested that the increased expression of SOD genes confers resistance to oxidative stress and the extension of lifespan (6). Thus, the regulation of SOD levels is critical for the determination of lifespan, which is modified by the insulin/IGF-1 signaling pathway. This signaling pathway has evidently been conserved from C. elegans to Drosophila (5, 6).

Holzenberger et al. (7) reported that a heterozygous deficiency for the IGF-1 receptor in mice enhances resistance to oxidative stress and extends lifespan. Because in IGF-1 receptor deficient mice heterozygous females exhibited a greater increase in lifespan than males, the beneficial effect of reduced IGF-1 signaling is also modulated by gender difference. However, it is unclear whether insulin signaling controls tolerance to oxidative stress and the extension of lifespan in mammals or is modulated by gender difference. In humans, the life expectancy of women is generally longer than that of men. For example, in Japan, with the highest life expectancy in the world, life expectancy was 78.36 years for men and 85.33 years for women in 2003. However, in laboratory animal models, the relationship of gender to longevity is variable, with lifespan being dependent on other factors like breeding and diet (8). Borras et al. (9) paid attention to the gender difference in rats, reporting that the expression and enzyme activity levels of MnSOD as well as glutathione peroxidase are higher in females than males. Ho et al. (10) reported that human MnSOD transgenic mice showed a significant increase in survival when exposed to 90% oxygen. Furthermore, in pulmonary epithelial cells of mice the overexpression of human MnSOD confers protection

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The abbreviations used are: IGF-1, insulin-like growth factor type 1; AL, ad libitum; Cu/ZnSOD, copper/zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; DR, dietary restriction; E2, 17β-estradiol; IR, insulin receptor; OVX, ovariectomy; ROS, reactive oxygen species; wt, wild type; kb, kilobase; RT, reverse transcription.
against exposure to hyperoxygen (11). The regulation of anti-
oxidant enzymes including MnSOD plays an important role in the determination of lifespan, suggesting that the gender difference in antioxidant enzymes explains the difference in life expectancy between females and males.

In the present study we generated a homologous murine model similar to the daf-2 mutant (I(p1195L/wt)) mouse in order to investigate the biological significance of longevity mutations found in the daf-2 gene of C. elegans. We demonstrated that the mutant mice acquired an enhanced resistance to oxidative stress. Furthermore, we also revealed that the influence of gender difference and dietary restriction (DR) on the acquired resistance involved defective insulin signaling.

**EXPERIMENTAL PROCEDURES**

**Generation of Insulin Receptor (IR) Mutant Mice**—The 129 mouse genomic library in AFIXII (Stratagene, La Jolla, CA) was screened with rat IR cDNA containing exon 19–21 as a probe. Three overlapping clones covered exon 15–22 of the mouse IR gene. The 1.1-kb fragment containing exon 20 of the gene was PCR-amplified with two Spel-anchored primers (5′-GGA CTA GTA GCA TGG AGA ACT GGA-3′ and 5′-AGT GAG TGG TAC TGT GAC CAG C-3′) and an Smal-anchored primer (5′-CGG CCG GAC ATA ACT TCG TAT AAT G-3′) and was mutagenized with a 19-bp mutagenic oligonucleotide (5′-GGA TGT CAC TCG AGG CTC T-3′) using the pALTER system (Promega, Madison, WI). The introduced mutation, P1195L, was confirmed present by sequencing. The 2.6-kb short 3′ homologous fragment was inserted into a XhoI/ApaI-restricted pBluescript II SK (pBSII SK, Stratagene). Germline transmission was confirmed by PCR amplification with the chimeric mice by the aggregation method as described (13). The clone with the expected homologous recombination was used for generating the knock-in allele.

**Southern Blotting and PCR Analysis**—Genomic DNAs used for the electroporation of embryonic stem cells. Genomic DNAs from day1 neonates were solubilized in 1% Triton X-100, 50 mM HEPES (pH 7.6), 150 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The insoluble material was separated by ultracentrifugation at 100,000 × g for 1 h at 4 °C. The supernatant was applied to a wheat germ lectin-agarose column pre-equilibrated with buffer A containing 0.1% Triton X-100, 50 mM HEPES (pH 7.6), 150 mM NaCl, and protease inhibitors. The column was extensively washed using buffer A, and the bound glycoproteins, such as IR, were eluted in buffer A containing 0.3 M N-acetylglucosamine. Aliquots of eluate were incubated in the absence or presence of 0.1 mM insulin (Eli Lilly) for 1 h at room temperature. Thereafter, phosphorylation was initiated by the addition of 0.1 mM ATP (0.1 mM ATP in buffer containing 0.1 M NaCl and 1 mM EDTA). After 15 min of room temperature, the reaction was stopped by the addition of stopping solution containing 0.1 mM NaF, 4 mM NaVO₃, 1 mM EDTA, and 1 mM sodium pyrophosphate. Samples were immunoprecipitated with anti-IR antibody (kindly provided by Dr. Ebina, Tokushima University) (15) and protein A-Sepharose CL-4B (Amersham Biosciences). Labeled phosphoproteins were separated on sodium dodecyl sulfate, 10% polyacrylamide gels and detected by gel imaging or by autoradiography.

**Measurement of Protein Kinase B Phosphorylation**—Mice were injected intravenously with 0.75 units kg⁻¹ insulin or equal volume of vehicle. All tissues were collected in liquid nitrogen 5 min after the injection. Frozen tissues were homogenized with 6 (for muscle) or 10 (for liver and kidney) times the volume of the tissue in homogenization buffer (1% Triton X, 50 mM HEPES, pH 7.4, 100 mM sodium pyrophosphate, 1 mM EDTA, and 10 mM sodium phenylmethylsulfonyl fluoride). Livers from day1 neonates were solubilized in 1% Triton X, 50 mM HEPES, pH 7.4, 100 mM sodium pyrophosphate, 1 mM EDTA, and 10 mM sodium phenylmethylsulfonyl fluoride. Phosphorylation at Ser-473 of protein kinase B in tissue extracts was analyzed with anti-phosphoprotein kinase B Ser-473 and anti-protein kinase B antibodies (Cell Signaling) as described (16).

**Oxidative Stress**—Twenty (4-month-old) mice were continuously exposed to 80% oxygen for 14 days in a chamber (50 × 30 × 50 cm). The concentration of oxygen was monitored with a oxygen analyzer (G-101-Y, Iijima Products, Gamagori, Japan) and maintained with a constant flow of 80% oxygen gas (3 liters/min). Exposure was continuous for the time indicated except for a few minutes when the chamber was opened for housekeeping purposes weekly. The mice were fed food and water ad libitum and kept on a 12-h light/dark cycle at 25 °C. Wistar rats were obtained from SLC. As a model of chemical oxidative stress, the mice (4-month-old) were intraperitoneally injected with paraquat (Sigma) prepared in phosphate-buffered saline at a dose of 70 mg/kg of body weight. We checked the survival of mice every 2 h until 120 h after the injection.

**Analytical Procedures**—Blood glucose levels were determined in fed mice (3, 6, and 12 months old) using an automatic monitor, Gluco-card (Hoechst Marion Rousell). Serum insulin levels were measured in 16-week-old mice. Serum obtained from fed mice was analyzed for insulin using a mouse insulin enzyme-linked immunosorbent assay kit (Shibayagi, Japan). In glucose tolerance tests fasting (4-month-old) mice were injected intraperitoneally with 0.75 units kg⁻¹ insulin or equal volume of vehicle. Thereafter, phosphorylation was initiated by the addition of 0.1 mM ATP (0.1 mM ATP in buffer containing 0.1 M NaCl and 1 mM EDTA). After 15 min of room temperature, the reaction was stopped by the addition of stopping solution containing 0.1 mM NaF, 4 mM NaVO₃, 1 mM EDTA, and 1 mM sodium pyrophosphate. Samples were immunoprecipitated with anti-IR antibody (kindly provided by Dr. Ebina, Tokushima University) (15) and protein A-Sepharose CL-4B (Amersham Biosciences). Labeled phosphoproteins were separated on sodium dodecyl sulfate, 10% polyacrylamide gels and detected by gel imaging or by autoradiography.

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**Reverse Transcription (RT)-PCR**—Tissues were homogenized in Trizol reagent (Invitrogen). Total RNA was extracted according to the manufacturer's instructions. cDNA was synthesized by reverse transcriptase from 1 μg of total RNA using a RNA PCR kit (Takara Bio Inc., Japan). Mouse MnSOD, Cu/ZnSOD, and glyceraldehyde-3-phosphate dehydrogenase cDNAs were amplified using specific oligonucleotide primers (MnSOD, 5'-GAC CTG CCT TAC GAC TAT GG-3' and 5'-GAC CCT CCT CCT TAT GAG-3'; Cu/ZnSOD, 5'-ATG AAA GCG GTG GTG GTG ATG-3' and 5'-AAT CAC TCC AGA GCC CAA GCC CAA GCC CAA-3'; glyceraldehyde-3-phosphate dehydrogenase, 5'-TCG GTC TGA ACG GAT TGG GC-3' and 5'-ATT TCT CCT CTT GCT CTT CTA CAC CC-3'). PCR products were electrophoresed and visualized using ethidium bromide.

**Ovariectomy (OVX) and Estradiol (E2) Administration**—At 12 weeks of age, female mice were ovariectomized or sham-operated via a midline incision under anesthesia with pentobarbital (40 mg/kg, intraperitoneal injection). After 4 weeks, treated mice were exposed to 80% oxygen or 20% oxygen and feeding was adjusted based on the oxygen level. Insulin and estradiol treatment groups were established, with half of the mice receiving insulin (10 units/kg) and the other half receiving estradiol (20 mg/kg per week, Sigma) in corn oil (Ajinomoto, Japan). After 4 weeks of treatment, the mice were exposed to 80% oxygen or 20% oxygen. For the insulin and estradiol treatment groups, the mice were exposed to 80% oxygen and the other half received estradiol (20 mg/kg per week, Sigma) in corn oil (Ajinomoto, Japan). After 4 weeks of treatment, the mice were exposed to 80% oxygen or 20% oxygen.

**RESULTS**

**The Generation of Modified IR Mutant Mice**—To elucidate the biological significance of the longevity mutation found in the daf-2 mutant of *C. elegans*, we generated a homologous murine model by replacing Pro-1195 of IR with Leu by targeted knock-in of the genomic IR gene in mice. As shown in Fig. 1A, we first isolated an ES cell carrying a mutation homologous to daf-2 (e1391) (3), i.e. Leu-1195 in exon 20 of the IR genomic gene by homologous recombination with the target vector. Germline transmission was confirmed in the F1 offspring of the chimeric and C57BLACK6 mice by Southern blotting. As shown in Fig. 1B, the blot showed that the 3' probe detected a 6.2-kb polymorphic EcoRV allele in wild-type (*IrP1195L/wt*) as well as heterozygous (*IrP1195L/wt*) mice, whereas the probe detected a 7.5-kb mutant EcoRV allele in heterozygous and homozygous (*IrP1195L/wt*) mice. To delete the neomycin resistance gene from the germline, we cross-bred *IrP1195L/wt* mice with CAG-Cre mice (14). We successfully deleted the neomycin resistance gene from the germline by cross-breeding CAG-Cre mice as schematized in the bottom of Fig. 1A. To detect the wild-type and mutant alleles, we amplified the genomic fragment harboring exon 20 and 21 by PCR with a sense primer (P1) and an antisense primer (P2). The PCR products were then differentiated based on the EcoRI site that is specifically introduced with

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**FIG. 1. Generation of IR mutant mice.** A, strategy used to knock-in the IR mutation (P1195L) in the murine IR gene locus. Diagrams of the murine IR genome locus, targeting vector, knock-in allele, and mutated allele are presented. The open boxes indicate the neomycin resistance cassette (neo) and DT-A gene. Exons are indicated as solid boxes. Open triangles represent loop sequences. After the cross-breeding with Cre-expressing transgenic mice, the neo cassette was popped out from the genome (mutated allele). The probe for Southern blotting is shown as a black box. The arrows indicate primers P1 and P2 for PCR. RI, EcoRI; RV, EcoRV; B, Southern blot analysis of EcoRV-digested liver DNAs in embryos. The knock-in allele yielded a 7.5-kbp EcoRV fragment, whereas the wild-type allele yielded a 6.2-kbp EcoRV fragment. C, PCR-based genotyping of IR mutant mice. The neo cassette was popped out from the genome (mutated allele). The black box indicates the neomycin resistance cassette (neo) and DT-A gene. Exons are indicated as solid boxes. Open triangles represent loop sequences. After the cross-breeding with Cre-expressing transgenic mice, the neo cassette was popped out from the genome (mutated allele). The probe for Southern blotting is shown as a black box. The arrows indicate primers P1 and P2 for PCR. RI, EcoRI; RV, EcoRV; B, Southern blot analysis of EcoRV-digested liver DNAs in embryos. The knock-in allele yielded a 7.5-kbp EcoRV fragment, whereas the wild-type allele yielded a 6.2-kbp EcoRV fragment. D, inulin-induced IR phosphorylation in liver, kidney, and muscle of IR mutant mice. 1, insulin-induced protein kinase B (PKB) phosphorylation in liver, kidney, and muscle of IR mutant mice. 2, insulin-induced IR phosphorylation in liver, kidney, and muscle of IR mutant mice.
the knock-in allele (Fig. 1C). A genotypic analysis of the off-

spring showed that the frequency of wild-type, heterozygous and homozygous mice was 31:61:0 at the 4-week-old stage. Further observation, however, showed that some mice were identified as homozygous at 1 day old, which apparently showed that the growth retardation was complicated by the diabetic ketoacidosis. The result suggested that the homozygous mice failed to thrive due to diabetic ketoacidosis.2 The result indicated that the resistance to oxidative stress, we measured SOD activities in the liver, kidney, and heart of both female and male mutant mice (Fig. 1D). As with IR autophosphorylation in IR mutant mice, MnSOD activity was up-regulated by 22.9% in IR mutant female mice compared with that of wild-type female mice (Fig. 3C). In contrast, we did not detect any pronounced difference in MnSOD activity between IR mutant and wild-type mice. The beneficial effects in male mutant mice were less pronounced in comparison to those in female mutant mice (Fig. 2D). These results suggested that IR mutant mice of both genders survived longer than wild-type mice in oxidative conditions. It is also of note that there is a gender difference in the resistance to oxidative stress in IR mutant mice. This data suggested that the insulin signaling may be modulated by sex hormones (i.e., either by the beneficial effects of estrogen or by the deleterious effects of testosterone).

MnSOD Plays a Role in the Acquisition of Resistance to Oxidative Stress—Because MnSOD and Cu/ZnSOD play an important role in the intracellular defense against reactive oxygen species (ROS), we measured SOD activities in the liver of IR mutant mice. As shown in Fig. 3A, the MnSOD activity of IR mutant female mice was significantly up-regulated by 39.9% compared with that of wild-type female mice (484.9 ± 96.1 units/mg of protein for IR mutant mice versus 346.5 ± 51.7 units/mg of protein for wild-type mice, p < 0.05). As for male mutant mice, MnSOD activity was up-regulated by 22.9% in comparison with that of wild-type male mice, but the up-regulation was less significant than that found in wild-type female mice (Fig. 3B). In contrast, we did not detect any pronounced difference in Cu/ZnSOD activity between IR mutant and wild-type mice of either sex (Fig. 3, C and D). To analyze the molecular basis for the up-regulation of MnSOD, mRNAs for MnSOD and Cu/ZnSOD were analyzed by RT-PCR (Fig. 3E). We found that the transcription of MnSOD was up-regulated in liver, brain, kidney, and heart of both female and male mutant mice, whereas that of the Cu/ZnSOD and glyceraldehyde-3-phosphate dehydrogenase genes was not up-regulated in mutant or wild-type mice (Fig. 3E). Furthermore, to investigate the contribution of other antioxidant enzymes to resistance to oxidative stress, we measured activities of glutathione peroxi-

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2 T. Baba, T. Shimizu, Y. Suzuki, H. Koseki, and T. Shirasawa, manuscript in preparation.
Cu/ZnSOD activity was not up-regulated in mutant mice. and heart of from at least five mice per genotype. *, GAPDH (GAPDH) gene as an internal control is shown. Each bar represents the mean ± S.D. in each group from at least five mice per genotype. *p < 0.05; IrP1195L/wt mice versus wt/wt mice.

**FIG. 3.** The expression and activities of antioxidant enzymes. A, MnSOD activity was significantly stronger in IrP1195L/wt female mice than in wt/wt female mice. B, IrP1195L/wt male mice showed higher levels of MnSOD activity than wt/wt male mice but not significantly so. C and D, Cu/ZnSOD activity was not up-regulated in mutant mice. E, RT-PCR analysis. Expression of the MnSOD gene was stronger in liver, brain, kidney, and heart of IrP1195L/wt male and female mice than wt/wt mice. Expression of the Cu/ZnSOD gene was not up-regulated in mutant mice. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control is shown. Each bar represents the mean ± S.D. in each group from at least five mice per genotype. *p < 0.05; IrP1195L/wt mice versus wt/wt mice.

First, we investigated the oxidative tolerance under hyperoxic conditions and the activity of MnSOD in the liver of O VX mice. As shown in Fig. 4, A and B, we compared the survival rate of O VX mice with that of non-O VX mice in both the IR mutant mice and wild-type strains. O VX mutant mice died significantly earlier than non-O VX mutant mice, i.e., O VX mutant mice had a 50% survival rate at 6.5 days compared with 12 days for non-O VX mutant mice (Fig. 4A). O VX wild-type mice died earlier than non-O VX wild-type mice, i.e., O VX wild-type mice had a 50% survival rate at 5.5 days compared with 7 days for non-O VX wild-type mice (Fig. 4B). The shortening of the survival rate was more pronounced in IR mutant mice than the wild-type mice, when both were ovariectomized. To clarify the molecular mechanisms of shortened survival in O VX mice, we investigated the gene expression and enzyme activity of MnSOD in the liver of mutant mice. As shown in Fig. 5A, the MnSOD activity of IR mutant mice was significantly down-regulated upon O VX (484.9 ± 96.1 units/mg of protein for mutant mice versus 387.3 ± 88.9 units/mg of protein for O VX mutant mice, p < 0.05). The down-regulation of MnSOD activity was less pronounced in wild-type O VX mice. In contrast, we did not detect any difference in Cu/ZnSOD activity upon O VX (Fig. 5C). The transcription of MnSOD was significantly down-regulated in O VX mice (Fig. 5F).

Second, to investigate the influence of exogenous estrogen on male mice, we administered E2. After exposure to 80% oxygen, we compared the survival of males treated with E2 to that of untreated male of both IR mutant mice and wild-type mice. Interestingly, mutant males administered E2 survived significantly longer than untreated mutant mice (Fig. 4E). The extension of survival was more pronounced in IR mutant mice than wild-type mice, when both were administered estrogen (Fig. 4, E and F). As shown in Fig. 5B, MnSOD activity was up-regulated by 20.6% in IR mutant mice treated with E2. The MnSOD activity of wild-type male mice was also up-regulated by 15.3% upon administration of E2. In contrast, we found no up-regulation of Cu/ZnSOD activity with E2 administration. (Fig. 5D). The transcription of MnSOD was significantly up-regulated in male mice administered E2 (Fig. 5F).
Finally, to exclude the possibility that the deficiency of testosterone caused by the feedback regulation of E2 administration is attributable to the phenotype of E2-treated males, we investigated the influence of exogenous estrogen on OVX mice. After 4 weeks of E2 administration, OVX mice were exposed to 80% oxygen. OVX mutant mice treated with E2 survived significantly longer than untreated mutant mice; the E2-treated group had 50% survival at 10.5 days compared with 6.5 days for the untreated group (Fig. 4C). OVX wild-type mice treated with E2 also survived longer than untreated wild-type male mice but not significantly so (Fig. 4D). We demonstrated here that mutant mice administered E2 survived longer with hyperoxygen as well as showed an up-regulation of MnSOD expression. Interestingly, the influence of estrogen was remarkable on IR mutant mice compared with wild-type mice.

The Gender Difference of Glucose Metabolism in IR Mutant Mice—To determine whether the suppression of IR signaling induces an impairment of glucose metabolism, levels of glucose (Fig. 6A) and insulin (Fig. 6B) were analyzed in blood samples. In a fed state, IR mutant mice had almost the same blood glucose concentrations as wild-type mice. However, about 6% of mutant male mice developed hyperglycemia (more than 200 mg/dl) (Fig. 6A). This rate was the same as in mice heterozygous for insulin receptor knock-out (25). In contrast, we did not detect any differences in urinary glucose between mutant and wild-type mice (data not shown). Serum insulin concentrations in the fed state were significantly increased in both female and male mutant mice compared with wild-type mice (Fig. 6B).
Although we failed to detect any gender difference in insulin concentrations of wild-type mice, insulin concentrations were significantly increased in IR mutant males (6.40 ± 1.49 ng/ml) compared with IR mutant females (2.96 ± 1.63 ng/ml) (Fig. 6B).

To determine the physiological consequence of mutated IR, glucose and insulin tolerance tests were performed on 4-month-old mice. Both female and male IR mutant mice showed normal fasting blood glucose concentrations as compared with control mice (Fig. 6, C and D). However, IR mutant male mice showed a moderately impaired glucose tolerance as compared with control male mice (Fig. 6D), whereas IR mutant female mice showed normal glucose tolerance (Fig. 6C). In addition, IR mutant females were more sensitive to blood glucose-lowering effects than wild-type mice when insulin was administered (Fig. 6E). In contrast, IR mutant male mice showed resistance to blood glucose-lowering effects in the insulin tolerance test (Fig. 6F). Although IR signaling was suppressed in IR mutant mice, mutant females sustained insulin sensitivity with hyper-insulinemia. This gender difference suggested that sex hormones may modulate the insulin signaling in IR mutant mice.

Then, to investigate whether sex hormones influence glucose metabolism, insulin and insulin tolerance tests were performed on female OVX mice and male mice treated with E2. Upon OVX, the glucose tolerance of IR mutant mice showed no significant changes as compared with that of wild-type mice (Fig. 6G). However, OVX mutant mice showed impaired insulin sensitivity compared with non-OVX mutant mice (Fig. 6, E and I). Furthermore, E2 administration improved glucose intolerance and insulin resistance in IR mutant male mice (Fig. 6, H and J compared with D and F). In contrast, wild-type mice showed no significant changes upon the administration of E2.

As for the mechanism of action of estrogen, Pedersen et al. (26) reported that IR binding was increased in adipocytes from rodents treated with estrogen. In that case, IR binding seemed to be due to an increased number of IRs (26). Yu et al. (27) reported that an IR signaling pathway is involved in estrogen-mediated retinal neuroprotection. The influence of estrogen is remarkable on IR mutant mice when compared with wild-type mice, suggesting that estrogen partially enhances its effect through insulin signaling.

**DR Additively Enhances the Resistance against Oxidative Stress in IR Mutant Mice—**Dietary restrictions that facilitate a reduction in body weight, mainly fat mass, are known to prolong lifespan by enhancing resistance against oxidative stress in mammals (28–30). Bluher et al. reported that fat-specific insulin receptor knock-out mice showed reduced adiposity, suggesting that the reduction of fat mass may be beneficial for the extension of lifespan (31, 32). To clarify whether there is a reduction of adipose tissue induced by mutation in the IR gene, we observed whole body and organ weights of IR mutant mice. Both male and female IR mutant mice sustained a significant 10–15% reduction in body weight compared with wild-type mice (Fig. 6, A and B). The fat mass of IR female mutant mice decreased significantly by 41.3% when compared with wild-type mice (446.6 ± 36.1 versus 761.4 ± 183.0 mg, p < 0.05), whereas that of IR male mutant mice dropped significantly by 57.5% compared with wild-type mice (522.9 ± 54.7 versus 1230.0 ± 196.0 mg, p < 0.01). We found no difference in the weights of other organs between IR mutant and wild-type mice (Fig. 6, A and B). To exclude the possibility that IR mutant mice show reduced food intake, we measured the food intake of mice at age of 16 weeks. We did not detect any significance difference in food intake between wild-type and mutant mice (female; 3.37 ± 0.1 versus 3.45 ± 0.18 g/day, male; 3.54 ± 0.11 versus 3.57 ± 0.15 g/day). We demonstrated that IR mutant mice specifically exhibited a reduction in fat mass, suggesting that the phenotype of IR mutant mice is very similar to that of fat-specific insulin receptor knock-out mice (31, 32).

To elucidate the influence of DR in IR mutant mice, the diet was restricted to 65% that consumed by the mice fed *ad libi-*
Insulin Signaling Regulates Stress Resistance in Mice

**DISCUSSION**

*MnSOD Is Up-regulated in Vivo in IR Mutant Mice—*Dafl2 is one of the longevity mutants found in *C. elegans* (3). The causative mutation was found in the gene encoding the insulin-like receptor, suggesting that the *daf-2* mutant has a defective insulin-like signaling, which eventually triggers the dauer formation as well as the extension of lifespan in *C. elegans* (3, 4). The mutation of *daf-16*, a major suppressor mutant for *daf-2*, was found in the gene encoding the forkhead transcription factor (34, 35). Biochemical analyses revealed that DAF-16, or dFOXO of the fly ortholog, played a pivotal role as a transcription factor by regulating the longevity genes downstream of the insulin signaling pathway (34–36). The signaling pathway triggered by insulin or insulin-like ligands is well conserved among various animal species, including mammals and invertebrates such as *D. melanogaster*, in which mutants for the insulin receptor, *IR*, or IRS-1, chico, also exhibit the longevity phenotype (6, 20). One of the candidate longevity genes in the downstream of *daf-16* is the *MnSOD* gene (37), whose expression is specifically up-regulated in *daf-2* mutants (5). Because biochemical studies show that MnSOD catalyzes the superoxide endogenously generated in the mitochondrial matrix, the balance between the generation of ROS and its degradation capacity in mitochondria may be critical for the determination of lifespan (38), which favors the free radical theory of aging (39, 40). Kops *et al.* (21) recently demonstrated in * vitro* that FOXO3a, a homologue of DAF-16, actually induces MnSOD expression, which enhances the antioxidative defense system in cultured mammalian cells challenged with oxidative stress. In the present study we demonstrated for the first time that MnSOD expression is up-regulated in vivo by defective insulin signaling in IR mutant mice. Because IR mutant mice carry a mutation homologous with *daf-2* with respect to the severely suppressed signaling of insulin, the up-regulation of MnSOD suggests that one of the longevity signals is sent to the anti-oxidative defense system, which is well conserved between invertebrates and mammals. Although further analyses are needed for elucidation of the molecular basis for the regulation of MnSOD, we found the transcriptional up-regulation of the MnSOD gene in IR mutant mice, suggesting the direct involvement of IR mutant mice in the regulation of MnSOD gene.

**Estrogen Confers Resistance to Oxidative Stress by Up-regulating the MnSOD Gene**—As shown in Fig. 4, the acquired
In the present study we demonstrated that in IR mutant mice resistance to oxidative stress was further enhanced by DR or E2. We are still finishing an analysis of the lifespan of IR mutant mice under normoxic conditions. We also expect a gender difference in the lifespan of IR mutant mice based on the analysis in oxygen chambers. Female mutant mice may have an enhanced defense system due to the estrogen secreted by the ovaries during the reproductive period. We demonstrated here that three distinct signals, insulin, estrogen, and dietary signals, work in different and independent ways and together increase resistance to oxidative stress and MnSOD levels in mice.

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Fig. 7. The influence of DR in IR mutant mice. A and B, comparison of mean body weights (BW) and organ weights between $P_1^{+/1195L/wt}$ and $I^+/+I$ mice. $P_1^{+/1195L/wt}$ mice had significantly lower body weights than $I^+/+I$ mice. The reduction in fat mainly causes the reduction in body weight. C and D, R additively enhances the resistance against hyperoxygen in $I^+/+I$ mice. Each bar represents the mean ± S.D. in each group from at least five mice per genotype. * $p < 0.05$; ** $p < 0.01$; $I^+/+I$ mice versus $I^+/+I$ mice.
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