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Original Article

Peroxiredoxin2 Deficiency Aggravates Aging-Induced Insulin Resistance and Declines Muscle Strength

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Abstract

This study examined the role of peroxiredoxin2 (Prx2) in aging-induced insulin resistance and reduction in skeletal muscle function in young (2-month-old) and old (24-month-old) Prx2 knockout (KO) and wild-type mice. Plasma insulin levels increased with aging in Prx2 KO mice but not in wild-type mice. Insulin sensitivity in the whole-body and skeletal muscle as assessed with the hyperinsulinemic-euglycemic clamp was lower in Prx2 KO mice than in wild-type mice in the old group but was not significantly different between the two genotypes in the young group. Insulin-induced activation of intracellular signaling molecules was also suppressed in old Prx2 KO mice compared to their wild-type littermates. Oxidative stress, inflammation, and p53 expression levels in skeletal muscle were higher in Prx2 KO mice than in wild-type mice in the old group but were not different between the two genotypes in the young group. p53 expression was negatively correlated with skeletal muscle insulin sensitivity in old mice. Skeletal muscle mass was similar between the two genotypes but grip strength was reduced in old Prx2 KO mice compared to old wild-type mice. These results suggest that Prx2 plays a protective role in aging-induced insulin resistance and declines in muscle strength by suppressing oxidative stress.

Keywords: Oxidative stress, Skeletal muscle, Hyperinsulinemic-euglycemic clamp, p53

A rapidly growing aging population has led to an increased prevalence of chronic diseases such as type 2 diabetes, cardiovascular disease, and Alzheimer's disease. Insulin resistance is common feature of these diseases, as it is a primary pathophysiological feature of type 2 diabetes mellitus and a major risk factor for cardiovascular diseases and Alzheimer's disease (1–3). The incidence of insulin resistance increases with age and was recently independently associated with leukocyte telomere length (4), suggesting that insulin resistance is closely linked to aging.

Oxidative stress is one of the important mechanisms underlying insulin resistance. The levels of oxidative stress markers are higher in the plasma and peripheral tissues of insulin-resistant humans (5,6). Increased expression of superoxide dismutase 2 (SOD2) in skeletal muscles attenuates high-fat diet-induced insulin resistance in mice (7), whereas genetic ablation of methionine sulfoxide reductase A aggravates diet-induced insulin resistance (8). Increased oxidative stress is not only an important cause of aging but also a significant factor in aging-associated insulin resistance (9). Oxidative stress activates c-Jun N-terminal kinases (JNKs), leading to the suppression of the insulin-stimulated activation of insulin receptor substrate 1 and its downstream signaling pathway; however, the suppression of JNKs abates insulin resistance in mice (10). Reduced skeletal muscle strength and mass are also closely associated with oxidative stress and may contribute to the development of insulin resistance with age because skeletal muscle is responsible for 70%–80% of insulin-stimulated glucose uptake (11,12)

The peroxiredoxins (Prxs) are a family of small thiol-dependent peroxidases and consist of six isoforms (13). Prxs catalyze reduction of the H_2O_2 and peroxynitrite to water and nitrite, respectively (13). Accumulating evidence suggests that Prxs are critical antioxidants

in cell defense against oxidative stress, as they are ubiquitously and highly expressed and have scavenging activity comparable to that of catalase and glutathione peroxidase (13). Prxs affect physiological functions by regulating reactive oxygen species levels and are closely associated with a variety of pathologies, including aging (14,15).

Prx2 is mainly present in cytosol (13), and its protective role in aging has also been well described previously. The expression levels of Prx2 are reduced in the plasma of aged mice (16). Embryonic fibroblasts from Prx2-deficient mice display accelerated cellular senescence by activating the extracellular signal-regulated kinase (ERK) pathway, which is correlated with organismal aging of mouse skin (14,17). Additionally, Prx2 is a critical antioxidant for the maintenance of the normal life span of *Caenorhabditis elegans* (15). Genetic modulation of Prx3, 4, and 6 has been shown to be closely related to insulin resistance (18–21), but the role of Prx2 in insulin resistance remains unknown.

Based on the fact that Prx2 protects against oxidative stress and aging, we hypothesized that Prx2 may play a protective role in aging-induced insulin resistance and declines in muscle mass and strength. To address this hypothesis, we examined whether Prx2 deficiency induced insulin resistance and affected skeletal muscle mass and strength in young and old Prx2 knockout (KO) and wild-type mouse littermates.

Methods

Animals

Prx2 KO mice on the C57BL/6 background were a kind gift from Dr. Dae Yeul Yu (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea) and Goo Taeg Oh (Ewha Womans University, Seoul, Korea). Prx2 KO and wild-type littermates aged 2 (young) and 24 months (old) were housed in a room under a 12:12-hour light/dark cycle. Only male mice were used to eliminate the effects of estrogen and estrous cycle on insulin sensitivity. The mice were fed a chow diet and given ad libitum access to water. They were anesthetized via an intraperitoneal injection of 250 mg/kg tribromoethanol (avertin). The study was conducted in strict accordance with the guidelines and protocols approved by the Institutional Animal Care and Use Committee of the Yeungnam University College of Medicine (YUMC-AEC2015-029).

Hyperinsulinemic-euglycemic Clamp

Insulin sensitivity was measured in conscious mice using the hyperinsulinemic-euglycemic clamp technique, as described previously (22). Briefly, hyperinsulinemic-euglycemic clamp was performed for 2 h with a continuous infusion (15 pmol•kg^{-1}•min^{-1}) of regular human insulin (Lilly, USA). A constant glucose level of approximately 6 mM was maintained by the infusion of 20% glucose. [3-^{3}H] glucose (PerkinElmer Life and Analytical Sciences, USA) was infused continuously (0.1 μCi/min) to measure whole-body glucose turnover, and 2-deoxy-D-[1-14C] glucose (PerkinElmer Life and Analytical Sciences) was administered as a bolus (10 μCi) at 75 minutes to measure skeletal muscle glucose uptake. Insulin-stimulated hepatic glucose production (HGP) was estimated by subtracting the glucose infusion rate (GIR) from the whole-body glucose uptake rate. After the hyperinsulinemic-euglycemic clamp, the mice were injected with avertin intravenously, and blood was collected from the orbital plexus. The masses of the epididymal fat and the soleus and gastrocnemius muscles were determined, and the tissues were stored at −80°C. Plasma glucose levels were measured using an Analox glucose analyzer (UK), and plasma insulin concentrations were measured using an enzyme-linked immunosorbent assay (Merck Millipore, USA). Plasma free fatty acid (Wako Diagnostics, USA), triglyceride (Sigma-Aldrich, USA) and total cholesterol (Asan Pharmaceutical, South Korea) levels were measured using enzymatic colorimetric methods.

In Vivo Insulin Signaling

Overnight-fasted mice were injected intraperitoneally with insulin (1.5 U/kg) or an equal volume of saline. After 10 minutes, the animals were anesthetized with avertin, and blood was collected from the orbital plexus. The soleus and gastrocnemius muscles were quickly dissected and frozen in liquid nitrogen for analysis.

Western Blotting

Antibodies against Akt, AS160, glycogen synthase kinase 3β (GSK-3β), JNK, ERK, p38, and their phosphorylated forms were obtained from Cell Signaling Technology (USA). Antibodies against 4-hydroxy-2-nonenal (4-HNE) and acetylated-SOD2 were obtained from Abcam (UK), antibodies against SOD2, clusterin, p53, and glyceraldehyde 3-phosphate dehydrogenase were obtained from Santa Cruz Biotechnology (USA), antibodies against Prx2 and nitrotyrosine (NT) were obtained from Ab Frontier (South Korea), and antibodies against heme oxygenase 1 (HO-1) were obtained from Enzo Life Sciences (USA). Western blotting was performed as described previously (22). Briefly, skeletal muscles tissues (25 mg) were homogenized in lysis buffer, and the extracted proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The resolved proteins were then transferred to 0.45-μm polyvinylidene fluoride membranes (Merck Millipore), which were blocked with a solution containing 5% skim milk before being incubated with the glyceraldehyde 3-phosphate dehydrogenase antibody (1:1,000 dilution) and the remaining primary antibodies (1:1,000 dilution) for 1 hour at room temperature and overnight at 4°C, respectively. Specific antibody binding was detected using horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence detection regent (Merck Millipore), and the signals were recorded and quantified using a LAS-3000 image analyzer and Multi Gauge 3.0 software (Fujifilm, Japan).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed as described previously (22). Briefly, 25-mg skeletal muscles tissues were homogenized in TRI reagent (Sigma–Aldrich), and the RNAs was reverse transcribed into cDNA using a reverse transcription kit (Applied Biosystems, USA). qRT-PCR was performed using a Real-Time PCR 7500 System and Power SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions. β-actin as a reference gene for sample normalization was validated through its Ct levels. Each reaction mixture was incubated at 95°C for 10 minutes and amplified over 45 cycles of 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 35 seconds. The primer sequences were designed using the Primer Express Program (Applied Biosystems). The following primers were used: β-actin (121 bp: forward, 5′-TGGACAGTGAGGCAAGGATAG-3′; reverse, 5′-TACTGCCCTGGCTCCTAGCA-3′), fructose 1,6 bisphosphatase (F 1,6-BP; 125 bp: forward, 5′-AGCCTTCTGAGAAGGATG CTC-3′; reverse, 5′-GTCCAGCATGAAGCAGTTGAC-3′), glucose 6-phosphatase (G6Pase; 72 bp: forward, 5′-CTCTTG CTATCTTTCGAGGAAA-3′; reverse, 5′-CCAACCACAAGATG

ACGTTC-3′), interleukin 1β (IL-1β; 71 bp: forward, 5′-GCCCAT CCTCTGTGACTCA-3′; reverse, 5′-AGTGCAGCTGTCTAAT GGGA-3′), IL-6 (71 bp: forward, 5′-GTCGGAGGCTTAATTACAC ATG-3′; reverse, 5′-TCAGAATTGCCATTGCACA-3′), phosphoenolpyruvate carboxykinase (PEPCK; 100 bp: forward, 5′-GAACTGACAGACTCGCCCTAT-3′; reverse, 5′-ACTTGAT GAACTCCCCATCTC-3′), Prx2 (100 bp: forward, 5′-AAATGA TGAGGGCATTGCTT-3′; reverse, 5′-TACAGAGCGTCCCAC AGGTA-3′), and tumor necrosis factor α (TNF-α; 71 bp: forward, 5′-GCAGAGAGGAGGTTGACTTTC-3′; reverse, 5′-GCAGAGA GGAGGTTGACTTTC-3′).

Grip Strength and Rotarod Tests

The inverted-cling grip test was used to assess limb strength (23). A mouse was placed on a square screen covered with a 5-mm wire mesh, and the screen was rotated 180 degrees over 1–2 seconds. The mouse was inverted above soft surface and the amount of time for which mouse was suspended before falling was recorded. Two examiners performed this experiment; one recorded the suspension time, and the other handled the mouse. Three trials were performed, and the mice were allowed at least 1 h of rest between trials. The average score of the last two trials was calculated to determine final score. An accelerating rotarod test was performed to assess motor coordination and walking speed (24,25). One day before the experiment, the mice were trained on an apparatus with a test protocol. Immediately before the test session, the mice were allowed an acclimation period, during which the speed of the rotarod was held constant at 4 rpm. During the test sessions, the rotation speed was increased continuously acceleration from 4 to 40 rpm over 5 minutes. Three trials were performed with at least a 1-hour intertrial interval, and the last two trials were averaged to determine the final score.

Statistical Analysis

The results are expressed as mean ± standard error. Differences among four groups were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's post hoc test with age, insulin, and genotype as main factors. Differences between two groups were analyzed using Student's *t* test. Pearson's correlation coefficient was calculated to determine the relationship between p53 expression and skeletal muscle glucose uptake. A *p* value less than .05 was considered significant.

Results

Prx2 Gene Ablation Has no Effect on Muscle Mass, Fat Mass, or Plasma Lipid Levels

In a previous study, Prx3 KO mice exhibited increased adipose tissue mass and total cholesterol levels (18). To determine whether genetic ablation of Prx2 also affects obesity parameters, we measured the muscle mass, fat mass, and lipid profiles of wild-type and Prx2 KO mice. Body weight and muscle mass were similar between wild-type and Prx2 KO mice in the young group, and increased significantly with aging in both groups of mice ($p < .0001$ for age in body weight and muscle mass, two-way ANOVA). However, no differences were observed between the two genotypes in old mice (Figure 1A and B). Fat mass was not significantly different between wild-type and Prx2 KO mice in the young or old group and increased with aging ($p < .01$ for age, two-way ANOVA; Figure 1C). Plasma free fatty acid, triglyceride, and total cholesterol levels were not significantly different between wild-type and Prx2 KO mice in the young or old group. Plasma triglyceride and total cholesterol levels increased with aging ($p < .001$ for age in triglycerides and total cholesterol, two-way ANOVA; Figure 1D–F). These results suggest that a genetic defect in Prx2 does not significantly affect fat mass, muscle mass, or plasma lipid profiles in either young or old mice.

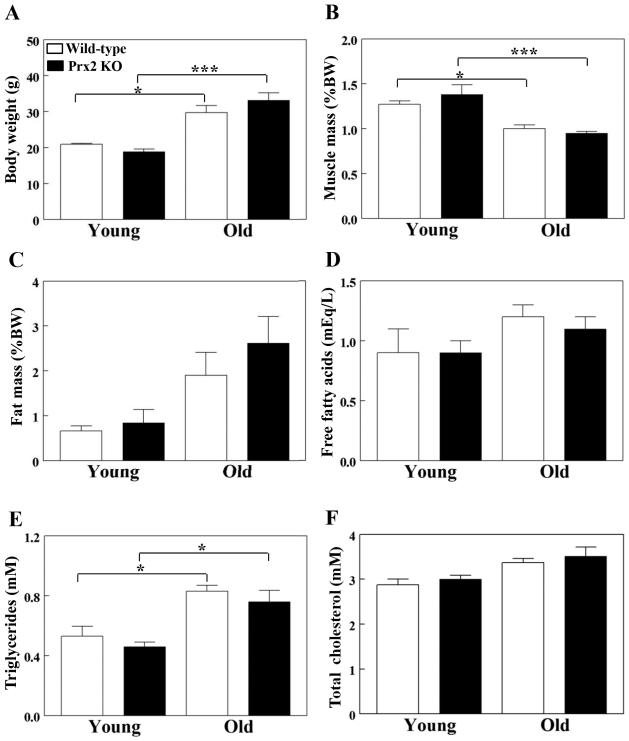

Figure 1. Body weight, muscle mass, fat mass, and plasma lipid levels in 2-month-old (young) and 24-month-old (old) mice. (**A**) Body weight. (**B**) Soleus and gastrocnemius muscle masses in both legs. (**C**) Epididymal fat pad mass. (**D**) Plasma free fatty acid, (**E**) triglyceride, and (**F**) total cholesterol levels. Skeletal muscle and fat masses were calculated as percentages of body weight. The results are presented as the mean ± *SE* for five to eight experimental cases per group. Data were analyzed using two-way analysis of variance followed by Tukey's post hoc test with age and genotype as main factors; body weight ($p < .0001$ for age), muscle mass ($p < .0001$ for age), fat mass ($p < .01$ for age), and plasma triglyceride ($p < .001$ for age) and cholesterol ($p < .001$ for age). *$p < .05$ and ***$p < .001$. Prx2 KO = Peroxiredoxin 2 knockout.

Insulin Resistance Is Increased in Old Prx2 KO Mice

A hyperinsulinemic-euglycemic clamp study was performed to determine whether genetic deficiency of Prx2 affected insulin sensitivity. Before the clamp study, basal plasma glucose and insulin levels were measured after overnight fasting (Supplementary Table 1). Basal levels of fasting plasma glucose were not altered by Prx2 gene ablation or aging. Plasma levels of insulin increased significantly with aging in Prx2 KO mice (108 ± 4 and 125 ± 6 pM in young and old, respectively) but not in wild-type mice (101 ± 2 and 114 ± 4 pM in young and old, respectively; $p < .05$ for genotypes and age, two-way ANOVA). During the hyperinsulinemic-euglycemic clamp study, plasma glucose levels were maintained at approximately 6.5 mM and plasma insulin levels increased to 400–600 pM. There was no significant difference in plasma glucose or insulin levels among the groups during the clamp study (Supplementary Table 1).

The GIR required to maintain a constant glucose level decreased with aging in both genotypes ($p < .0001$ for age, two-way ANOVA). The GIR was not significantly different between wild-type and Prx2 KO mice in the young group but was lower in Prx2 KO mice than

in wild-type mice in the old group ($p < .05$ for genotype, two-way ANOVA) (Figure 2A). Whole-body glucose turnover also decreased with aging in both wild-type and Prx2 KO mice ($p < .0001$ for age, two-way ANOVA). While whole-body glucose turnover was similar between the genotypes in the young group, it was significantly lower in Prx2 KO than in wild-type mice in the old group ($p < 0.05$ for genotype and $p = 0.05$ for the interaction between age and genotype, two-way ANOVA; Figure 2B). Nevertheless, during the clamp study, HGP was similar between wild-type and Prx2 KO mice in the young and old groups, suggesting that aging did not significantly affect HGP in either genotype (Figure 2C). Consistent with these results, the mRNA levels of the enzymes involved in gluconeogenesis, that is, G6Pase, PEPCK, and F1,6-BP, were similar between wild-type and Prx2 KO mice in both the young and old groups (Supplementary Figure 1). Similar to whole-body glucose turnover, skeletal muscle glucose uptake was lower in old Prx2 KO mice than in old wild-type mice but did not differ between the two genotypes in the young group ($p < .05$ for genotype, two-way ANOVA). Glucose uptake in skeletal muscle decreased with aging in both wild-type and Prx2 KO mice ($p < .0001$ for age, two-way ANOVA; Figure 2D). Glucose uptake in adipose tissue also decreased with aging ($p < .05$ for age, two-way ANOVA) but was not different between wild-type and Prx2 KO mice in the young or old groups (Figure 2E). TNF-α and IL-6 mRNA levels in adipose tissue increased with aging ($p < .05$ for age, two-way ANOVA), but there was no difference in these levels between the two genotypes (Supplementary Figure 2). These results suggest that Prx2 deficiency aggravates insulin resistance in old mice by reducing insulin sensitivity in skeletal muscle.

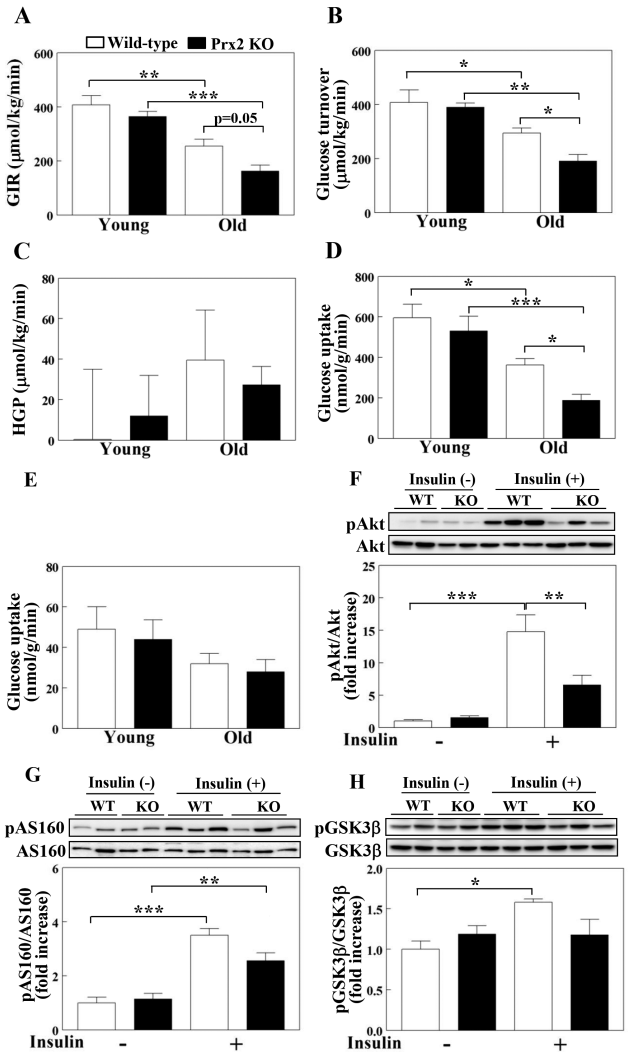

Figure 2. Glucose metabolism as assessed by the hyperinsulinemic-euglycemic clamp test (**A–E**) and insulin signaling pathways in skeletal muscle (**F–H**). (**A**) The glucose infusion rate (GIR), (**B**) whole-body glucose turnover, (**C**) hepatic glucose production (HGP) and glucose uptake in (**D**) soleus muscle and (**E**) adipose tissue in 2-month-old (young) and 24-month-old (old) mice. The results are presented as the mean ± *SE* for five to eight experimental cases per group. Data were analyzed using two-way analysis of variance followed by Tukey's post hoc test with age and genotype as main factors; GIR ($p < .0001$ for age and $p < .05$ for genotype), whole-body glucose turnover ($p < .0001$ for age, $p < .05$ for genotype, and $p = .05$ for the interaction between age and genotype) and glucose uptake in soleus muscle ($p < .0001$ for age and $p < .05$ for genotype) and adipose tissue ($p < .05$ for age). *$p < .05$, **$p < .01$, and ***$p < .001$. The phosphorylation levels of (**F**) Akt, (**G**) AS160, and (**H**) glycogen synthase kinase 3β (GSK3β) were measured in the soleus muscle of 24-month-old mice using western blotting. The results are presented as the mean ± *SE* ($n = 5$ for wild-type; $n = 6$ for Prx2 KO). Data were analyzed using two-way analysis of variance followed by Tukey's post hoc test with insulin and genotype as main factors; pAkt ($p < .0001$ for insulin, $p < .05$ for genotype, and $p = .01$ for the interaction between insulin and genotype), pAS160 ($p < .0001$ for insulin and $p < .05$ for interaction between insulin and genotype), and pGSK3β ($p = .05$ for insulin and $p < .05$ for interaction between insulin and genotype). *$p < .05$, **$p < .01$, and ***$p < .001$. Prx2 KO = Peroxiredoxin 2 knockout; WT = Wild-type.

Insulin resistance develops through the suppression of intracellular insulin signaling pathways, including Akt/AS160/GSK3β. Therefore, we measured the levels of pAkt, pAS160, and pGSK3β to determine if the insulin-induced activation of intracellular signaling molecules is suppressed in the skeletal muscle of old Prx2 KO mice. Without insulin, pAkt, pAS160, and pGSK3β levels were similar between wild-type and Prx2 KO mice. pAkt levels were significantly increased by insulin in wild-type mice but not in Prx2 KO mice, and they were significantly lower in Prx2 KO mice than in wild-type mice ($p < .0001$ for insulin, $p < .05$ for genotype, and $p = .01$ for the interaction between insulin and genotype). Insulin significantly increased pAS160 levels in both wild-type and Prx2 KO mice ($p < .0001$ for insulin, two-way ANOVA). There was a significant interaction between insulin and genotype in pAS160 levels ($p < .05$, two-way ANOVA). pGSK3 levels were significantly increased by insulin in wild-type mice but not in Prx2 KO mice ($p = .05$ for insulin and $p < .05$ for the interaction between insulin and genotype, two-way ANOVA; Figure 2F–H). These results suggest that Prx2 deficiency aggravates skeletal muscle insulin resistance in old mice by suppressing intracellular signaling pathways.

Oxidative Stress and Inflammation Are Increased in Old Prx2 KO Mice

To determine whether aging affected Prx2 expression in skeletal muscle, we analyzed Prx2 mRNA and protein levels in the gastrocnemius muscles of wild-type mice. Although there was no significant difference in Prx2 mRNA levels between young and old mice, Prx2 protein levels were significantly lower in old mice than in young, suggesting that Prx2 protein levels decrease with aging (Figure 3A and B).

Next, we measured the levels of oxidative stress markers, antioxidants, and cytokines to determine whether genetic ablation of Prx2 affected oxidative stress and inflammation in young and old mice. The protein levels of 4-HNE, NT, HO-1, clusterin, SOD2, and Ac-SOD2 and the mRNA levels of TNF-α and IL-1β were similar between wild-type and Prx2 KO mice in the young group

(Supplementary Figure 3). However, in the old group, the levels of 4-HNE, NT, HO-1, SOD2, and acetylated SOD were significantly higher in the skeletal muscle of Prx2 KO mice than in that of wild-type mice (Figure 3C–H). Moreover, plasma TNF-α levels and IL-1β mRNA levels in skeletal muscle were significantly higher in Prx2 KO mice than in wild-type mice. TNF-α mRNA levels in skeletal muscle were also higher in Prx2 KO mice, which did not reach statistical significance (p = .06; Figure 3I–K). These results suggest that Prx2 deficiency increases oxidative stress and inflammation in the skeletal muscle of old mice but not that of young mice.

p53 Levels Are Upregulated in the Skeletal Muscle of Old Prx2 KO Mice

Aging increases oxidative stress and inflammation, factors that might be involved in the aggravation of insulin resistance in old Prx2 KO mice. Since the insulin resistance induced by oxidative stress and inflammation is mediated by the activation of mitogen-activated protein kinase (MAPK) subfamily members, we measured the protein levels of pJNK, p-p38, and pERK. pJNK levels were lower in Prx2 KO mice than in wild-type mice in the young group (p < .0001 for genotype, two-way ANOVA) but were not altered by aging in either wild-type or Prx2 KO mice (Figure 4A). The levels of p-p38 and pERK were not significantly affected by Prx2 gene ablation or aging (Figure 4B and C). These results suggest that these MAPK subfamilies are not involved in the increase in insulin resistance in old Prx2 KO mice.

Aging increases p53 levels, and the association between insulin resistance and p53 levels has been reported previously (26). We also observed that p53 protein levels were upregulated in old mice in both genotypes (p < .0001 for age, two-way ANOVA). Although the expression levels of p53 were similar between wild-type and Prx2 KO mice in the young group, aging upregulated p53 levels to a significantly greater extent in Prx2 KO mice than in wild-type mice (p < .0001 for genotype and p < .01 for the interaction between age and genotype, two-way ANOVA; Figure 4D). p53 protein levels were negatively correlated with skeletal muscle glucose uptake in the combined old Prx2 KO and wild-type group (Figure 4E). These

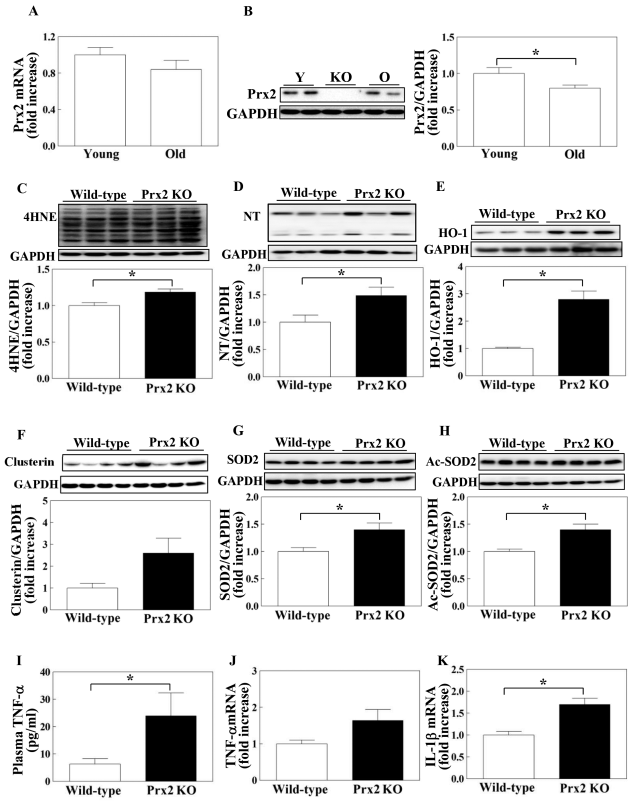

Figure 3. The expression levels of Prx2, oxidative stress markers, antioxidants, and cytokines in skeletal muscle. (**A**) The mRNA and (**B**) protein levels of Prx2 were measured in 2-month-old (young) and 24-month-old (old) wild-type mice. The protein levels of (**C**) 4-hydroxynonenal (HNE), (**D**) nitrotyrosine (NT), (**E**) heme oxygenase 1 (HO-1), (**F**) clusterin, (**G**) superoxide dismutase 2 (SOD2), and (**H**) acetylated-SOD2 (Ac-SOD2) were measured in old wild-type and Prx2 KO mice. (**I**) The plasma levels of tumor necrosis factor α (TNF-α) and the mRNA levels of (**J**) TNF-α and (K) interleukin 1β (IL-1β) were measured in old wild-type and Prx2 KO mice. mRNA levels were measured using quantitative polymerase chain reaction, and tissue protein levels were measured using western blotting. The results are presented as the mean ± *SE* for six to eight experimental cases per group. Statistical analysis was performed using Student's *t* test. *p < 0.05 and **p < 0.01. O = Old; Prx2 KO = Peroxiredoxin 2 knockout; Y = Young.

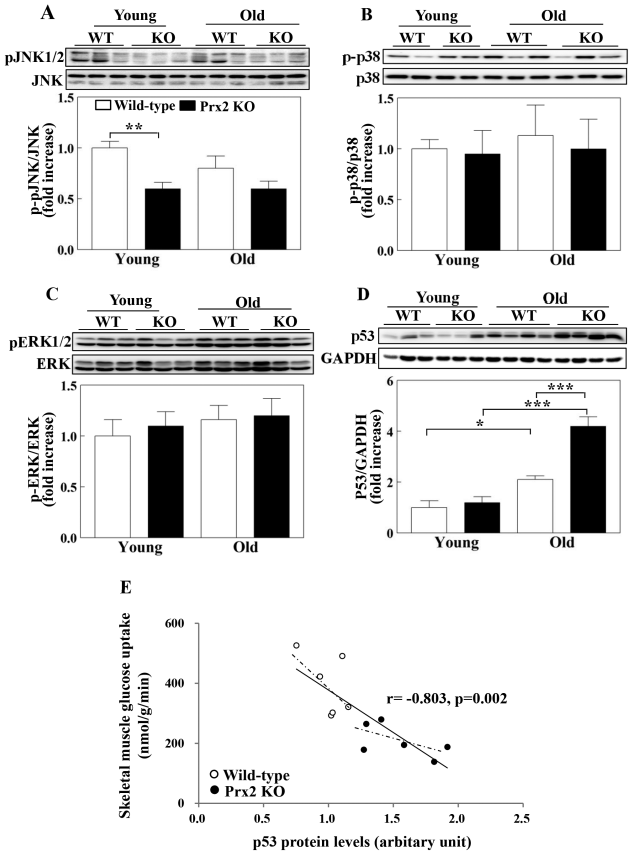

Figure 4. The expression levels of phosphorylated mitogen-activated protein kinase (MAPK) subfamily members and p53 in skeletal muscle, and the correlation between p53 expression and insulin sensitivity in 2-month-old (young) and 24-month-old (old) mice. The levels of phosphorylated (**A**) c-Jun N-terminal kinase (JNK), (**B**) p38, and (**C**) extracellular signal-regulated kinase (ERK) and (**D**) p53 protein. The results are presented as the mean ± *SE* for six to eight experimental cases per group. Data were analyzed using two-way analysis of variance followed by Tukey's post hoc test with age and genotype as main factors; pJNK (p < .0001 for genotype) and p53 (p < .0001 for age, p < .0001 for genotype and p < .01 for the interaction between age and genotype). *p < .05, **p < .01, and ***p < .001. (**E**) The correlations between p53 levels and glucose uptake in the skeletal muscle of 24-month-old wild-type and Prx2 KO mice. The experiments in **E** include six mice in each group. Prx2 KO = Peroxiredoxin 2 knockout; WT = Wild-type.

results suggest that p53 is associated with the aggravation of insulin resistance in old Prx2 KO mice.

Skeletal Muscle Strength Is Reduced in Old Prx2 KO Mice

Skeletal muscle function is closely associated with oxidative stress and insulin resistance (11). Since Prx2 ablation increased oxidative stress and insulin resistance in old mice, we theorized that Prx2 deficiency might also accelerate reduction in skeletal muscle function with aging. Age-associated changes in the muscle function in mice can be assessed with grip strength and rotarod tests (25). In inverted-cling grip test, the length of time spent suspended on a grid wire before falling in old mice was significantly shorter in old Prx2 KO mice than in old wild-type mice (Figure 5A). However, rotarod speed before falling was similar between wild-type and Prx2 KO mice (Figure 5B). These results suggest that Prx2 deficiency reduces grip strength in old mice.

Discussion

The present study demonstrates that Prx2 deficiency aggravates aging-induced insulin resistance via the suppression of intracellular insulin signaling pathways in skeletal muscle. The augmentation of oxidative stress and inflammation with aging may mediate these effects in Prx2 KO mice. The upregulation of p53 with aging in Prx2 KO mice is also closely associated with insulin resistance in skeletal muscle. Skeletal muscle strength is also diminished in old Prx2 KO mice. Thus, Prx2 has a protective effect against increases in insulin resistance and declines in muscle strength with aging.

The relationship between Prxs and insulin resistance has been explored previously (23,25–29). The plasma levels of Prx1, 2, 4, and 6 are higher in diabetic patients than control subjects; however, patients with poorly controlled diabetes exhibit lower levels of Prx2, 4, and 6 than patients with well-controlled patients (27). In animal experiments, overexpression of Prx3 and 4 improves insulin resistance (19,20), whereas genetic ablation of Prx3 and 6 induces insulin resistance (18,21). Prx1 exerts effects on insulin resistance that contrast with those of Prx3, 4, and 6. Specifically, Prx1 deficiency paradoxically ameliorates palmitate-induced insulin resistance in HepG2 cells (28). Here, we demonstrated for the first time that Prx2 has a protective effect against insulin resistance in mice. Consistent with our finding, plasma Prx2 levels were negatively correlated with plasma glucose and hemoglobin A1C levels in human subjects (27).

In this study, we simultaneously measured insulin sensitivity in the whole body and peripheral tissues, and we found that skeletal muscle is the primary tissue in responsible for insulin resistance in Prx2 KO mice. These results are supported by the finding that the insulin-induced activation of intracellular signaling molecules was also suppressed in the skeletal muscle of Prx2 KO mice. However, the liver and adipose tissue are not involved in insulin resistance in Prx2 KO mice, a finding inconsistent with those of previous studies. The protective effects of Prx3 and 4 against insulin resistance are mainly mediated by reductions in lipid accumulation and oxidative stress in the liver and adipose tissues (19,20). Prx6 deficiency has adverse effects on a variety of tissues, such as skeletal muscle, the liver, and the pancreas by suppressing skeletal muscle insulin signaling, increasing hepatic lipid accumulation, and reducing insulin secretion, respectively (21). The differential effects of Prx isoforms on tissue insulin resistance may be attributed to difference in their tissue expression levels because Prx3 and 4 are expressed at higher levels in the liver (19,29), and Prx6 is highly expressed in skeletal muscle and the pancreas (21,30). The physiological effects of Prx6 on phospholipase A2 activity may be another mechanism affecting insulin sensitivity (31). Since these previous studies did not measure insulin sensitivity in peripheral tissues, our study aimed to provide more direct and concrete evidence supporting the effects of Prx2 on insulin sensitivity in vivo using the hyperinsulinemic-euglycemic clamp.

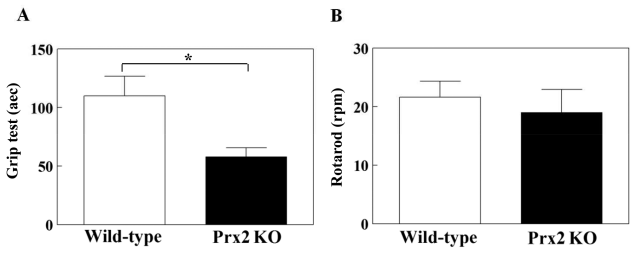

Figure 5. Inverted-cling grip and rotarod test results for 24-month-old peroxiredoxin 2 knockout (Prx2 KO) and wild-type mice. (**A**) Inverted-cling grip and (**B**) rotarod test results. The results are presented as the means ± *SE* ($n = 5$ for wild-type; $n = 8$ for Prx2 KO). Statistical analysis was performed using Student's *t* test. $^*p < 0.05$.

An important finding of this study is that Prx2 deficiency significantly increases oxidative stress and insulin resistance in old mice but not in young mice, suggesting that the accelerated accumulation of oxidative stress with age worsens insulin resistance in Prx2-deficient mice. It appears that Prx2 has a protective effect against aging-induced insulin resistance by suppressing oxidative stress. Inflammatory cytokines such as TNF-α are produced in myocytes and macrophages in skeletal muscle, and the levels of these cytokines are higher in frail elderly people than in healthy young men (32,33). It seems that an increased level of oxidative stress in old Prx2 KO mice is responsible for elevating the cytokine levels; the inflammatory cytokines partly contribute to the aggravation of insulin resistance by suppressing insulin signaling pathways (34).

Since Prx2 gene is absent in whole body, Prx2 deficiency in other tissue may affect insulin resistance in skeletal muscle in this study. Since Prx2 plays an important physiological role in endothelial function by reducing oxidative stress, it is possible that Prx2 deficiency induces endothelial dysfunction, which also contributes to the aggravation of insulin resistance in skeletal muscle in old Prx2 KO mice (35).

JNK activation by oxidative stress is known to suppress insulin signaling pathways and thus lead to insulin resistance (10); therefore, we assumed that JNK levels would be higher in the skeletal muscle of Prx2 KO mice. Moreover, JNK activation was reported previously in the skin and aortas of Prx2 KO mice (14,36). However, in contrast to our assumption, pJNK levels were paradoxically lower in the skeletal muscle of Prx2 KO mice than in that of wild-type mice in both the young and old groups. Direct or indirect interactions between Prx2 and JNK may be involved in the suppression of JNK activity in Prx2 KO mice. Recent studies support these interactions; Prx2 activates JNK in cancer cells, and JNK activates Prx2 in the neurons of Drosophila (37,38). Nevertheless, our study revealed that JNK activity was not only lower in Prx2 KO mice but also not significantly altered with aging. This suggests that JNK is not responsible for increasing insulin resistance in old Prx2 KO mice. Among the MAPK subfamily members, p38 and ERK are also associated with oxidative stress and insulin resistance, but we did not observe their activation with aging or by Prx2 deficiency. Therefore, the MAPK subfamily is not involved in aggravating insulin resistance in old Prx2 KO mice.

Interestingly, we observed the upregulation of p53 in the skeletal muscle of old mice, which was further enhanced by Prx2 deficiency.

It was reported that oxidative stress contributes to increased p53 expression in aged skeletal muscle (39,40), and recent evidence demonstrates that p53 is involved in regulating glucose metabolism in cancer cells, as well as normal cells and tissues (41). The codon 72 polymorphism of p53 is linked to insulin resistance and type 2 diabetes mellitus (42). The upregulation of p53 in adipose tissue induces insulin resistance, whereas the inhibition of p53 activity improves insulin resistance (26). Ceramides induce p53 activation and reduce Akt phosphorylation in myoblasts, and exercise training improves oxidative stress and insulin resistance, changes accompanied by reduction in p53 levels in the skeletal muscle of Goto-Kakizaki rats (43,44). In the present study, p53 expression was negatively correlated with insulin sensitivity in skeletal muscle, suggesting that p53 activation may contribute to the aggravation of insulin resistance in the skeletal muscle of old Prx2 KO mice. The mechanisms underlying p53-induced insulin resistance may be mediated indirectly by inflammation or directly by reductions in the expression and/or activity of insulin signaling molecules (26,45). This notion could be confirmed by further studies demonstrating alterations in insulin sensitivity by the direct modulation of p53 activity in skeletal muscle.

Oxidative stress is a well-known causative factor for skeletal muscle loss (46). However, despite increases in oxidative stress, muscle mass was not significantly affected by Prx2 deficiency, whereas inverted grip strength was significantly reduced. In mice, muscle mass is not always correlated with muscle performance. Mice fed an antioxidant-deficient diet for 7 months exhibited enhanced oxidative stress and reduced muscle strength but no changes in muscle mass (47). Therefore, it seems that Prx2 deficiency increases oxidative stress with aging, leading to reductions in muscle strength but not in muscle mass. However, motor coordination and walking speed were not affected by Prx2 deficiency. Age-associated changes in muscle function in mice exhibit reduction in both grip strength and motor coordination (25). We do not have an explanation for this discrepancy; discrepancies between the results of modalities testing skeletal muscle functions have been found in previous studies. Reduced grip strength but normal rotarod test results were observed in SOD1-mutant transgenic mice (12), while contrasting results pertaining to grip strength and motor coordination were reported in a mouse model of partial trisomy and monosomy 21 (48). Nevertheless, our results suggest that Prx2 has a protective effect against reductions in muscle function. Consistent with our data, Prx3 deficiency resulted in decreased muscle contractile force by increasing oxidative stress and suppressing mitochondrial biogenesis (49,50). Not only muscle mass but also muscle function is closely associated with insulin sensitivity in skeletal muscle. Therefore, it is possible that reduced muscle strength partly contributes to the aggravation of insulin resistance in Prx2 KO mice.

In conclusion, Prx2 has a protective effect against aging-induced reductions in insulin sensitivity and muscle strength by suppressing oxidative stress. Prx2 may be a target in the treatment of aging-associated diseases.

Supplementary Material

Supplementary data is available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

None reported.

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