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Effects of rikkunshito supplementation on resistance to oxidative stress and lifespan in mice

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Aim: Caloric restriction (CR), which limits the caloric intake to 60–70% of ad libitum (AL) amounts in various experimental animals, delays aging and extends the lifespan. We previously showed that neuropeptide Y (NPY), an appetite-stimulating peptide, is essential for the anti-oxidative and life-extending effects of CR. Here, we investigated whether a Japanese traditional herbal medicine, rikkunshito (RKT), which induces NPY activation, has CR-like life-extending effects.

Methods: First, we evaluated the life-extending activity of RKT by examining the effect of long-term RKT administration on wild-type and NPY knockout mice. Furthermore, we tested whether RKT enhances CR-mediated beneficial effects under AL conditions with a normal diet and under mild CR conditions with a high-fat diet. We then used 3-nitropropionic acid or doxorubicin to induce oxidative stress, and analyzed the differences in survival rate, weight loss, gene expression and cellular oxidative damage among groups.

Results: RKT administration did not extend the lifespan of wild-type or NPY knockout mice. In the oxidative stress models, RKT treatment upregulated anti-oxidative gene expression in the liver. Furthermore, RKT administration reduced the oxidative damage in the liver compared to the CR conditions alone. However, on induction of oxidative stress by 3-nitropropionic acid or doxorubicin, RKT administration did not affect the survival rate.

Conclusions: These results show that RKT administration only partially mimics the effects of CR at the cellular level, but not at the organismal level to increase the lifespan of mice. **Geriatr Gerontol Int 2019; ••: ••–••.**

Keywords: calorie restriction, ghrelin, longevity, metabolism, oxidative stress.

Introduction

In 1935, McCay *et al*. investigated caloric restriction (CR) in rats and reported that CR extended their lifespans.[1] Although the precise mechanisms of how CR contributes to anti-aging are unknown, many have suggested a link between the suppression of insulin signaling and its associated metabolic adaptations.[2–4] CR reduces serum insulin concentration and suppresses the insulin–insulin-like growth factor 1 (IGF-1) pathway, which is important for longevity regulation.[5] Furthermore, CR promotes expression of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc-1a*) gene, which is important for mitochondrial function. *Pgc-1a* increases glucose uptake, fatty acid oxidation and mitochondrial biosynthesis in the liver, fat and muscle tissue, thereby enhancing mitochondrial function and anti-oxidant defense, and reducing damage from oxidative stress.[6,7]

CR also activates Nrf2, a transcription factor that binds to the anti-oxidant response element, which in turn promotes anti-oxidant defense and suppresses cancer development.[8] The nuclear translocation of Nrf2 leads to the expression of genes involved in anti-oxidant defense, including glutathione S-transferase M3 (*Gstm3*) and heme oxygenase 1 (*Hmox1*).[9,10] We have reported the potential roles of these genes on longevity.[11] *Pgc-1a* has important roles in the regulation of lipid metabolism and oxidative stress resistance, because Nrf2 activity is regulated by *Pgc-1a*.[12] Furthermore, we previously reported that CR increased *Pgc-1a* expression and activation by an insulin/IGF-1 signal-dependent mechanism, which might be linked to the central upregulation of neuropeptide Y (NPY).[7]

These days, it is important to identify calorie restriction mimics, or substances that can induce CR-like anti-aging beneficial effects without changing actual food intake.[13,14] We reported a link between CR and the increased expression of NPY, which has appetite-stimulating effects that are controlled by leptin signaling.[15] CR elevates NPY expression by reducing plasma leptin concentrations.[16,17] Furthermore, even under leptin-deficient conditions, CR increased ghrelin signaling and enhanced NPY expression.[7] We previously reported that increased oxidative stress resistance or lifespan extension by CR was not observed in NPY knockout (KO) mice.[18] This study suggested that an agent, which induces the expression of NPY, might be a therapeutic CR mimic.[19]

On the basis of this hypothesis, we focused on rikkunshito (RKT), a Japanese herbal medicine with appetite-stimulating effects, in the current study. RKT is a herbal medicine comprised of several herbs including *Atractylodes lancea* rhizome, ginseng, pinellia tuber, *Poria sclerotium*, jujube, citrus unshiu peel, *Glycyrrhiza* and ginger. RKT elevated the plasma ghrelin level in humans and mice.[20] Furthermore, ghrelin activated NPY neurons to promote feeding behavior.[21] Therefore, we used RKT as a candidate CR mimetic to activate NPY by ghrelin and its receptor signaling.

A recent mouse study involving *klotho* mice, senescence-accelerated mice prone 8 and outbred Institute of Cancer Research mice showed that RKT administration extended their lifespan.[22] Here, we investigated whether these RKT-mediated life-extending effects are dependent on NPY signals, which are important for oxidative stress resistance observed in CR animals. We found RKT supplementation induced higher anti-oxidative gene expression with reduction of oxidative damage in the liver. However, in our experimental design, RKT supplementation did not increase the survival of wild-type (WT), NPY KO mice and mild CR mice, respectively.

Methods

Animals

All animal experiment protocols were approved by the Committee on the Ethics of Animal Experiments of the Waseda University and Nagasaki University. In experiment 1, male NPY KO mice (129S-*Npy*^{tm1Rpa}/J) and female NPY WT mice (129S6/SvEvTac) obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and Taconic Farms (Germantown, NY, USA) were used to generate NPY KO (NPY−/−) mice and WT (NPY+/+) mice. They were bred in a barrier facility at the Center for Frontier Life Sciences at Nagasaki University. Mice were housed in cages in a barrier facility (temperature 22–25°C; 12-h light/dark cycle) under specific pathogen-free conditions for the duration of the study. Male mice were fed ad libitum (AL) with CRF-1 (Oriental Yeast, Tokyo, Japan). Then, at the age of 82–89 weeks, mice were divided into two diet groups: AL and AL + RKT, namely, with or without 2% (w/w) RKT supplementation (n = 19–20), to test the effect of RKT on aged mice. RKT was provided by TSUMURA & CO. (Tokyo, Japan) as a dried powder extract and mixed with CRF-1. After 12 weeks, plasma was collected and biochemically analyzed (n = 6–8), as described below. After 14 weeks, several mice were euthanized, and white and brown adipose tissues were dissected for analysis of basal biochemical and metabolic data. A preliminary experiment for the detection of RKT's effects on the ghrelin–NPY pathway is shown in Appendix S1 and Figure S1.

To minimize genetic variation, we used inbred C57BL/6 mice (Charles River Laboratories Japan, Yokohama, Japan), instead of 129S mixed background WT mice or other outbred mice, in the following experiments. Furthermore, we used young mice, because RKT supplementation started at middle age had no life-extending effects in experiment 1.

In experiment 2, we used 14–16-week-old male and female C57BL/6 mice. Mice were housed in cages in a barrier facility (temperature 22–25°C; 12-h light/dark cycle) under specific pathogen-free conditions for the duration of the study. The mice were fed CRF-1 during a 7-day habituation period. Mice were then assigned to a free-feeding control group (AL) consisting of 18 mice (10 males, 8 females) and a free-feeding RKT treatment group (AL + RKT) consisting of 19 mice (10 males, 9 females). The AL group was fed CRF-1, and the AL + RKT group was fed CRF-1 and 2% RKT (w/w) pelleted with the feed. Both groups were fed ad libitum over the course of the experiment. Tap water was selected as the drinking water for mice. Mice were physically observed each day, food intake levels were measured twice a week and the animals were weighed once a week. Oxidative stress was applied by drug administration at 14 weeks post-RKT treatment, as described below.

In experiment 3, we used 16–18-week-old male and female C57BL/6 mice (Charles River Laboratories Japan). The mice were fed CRF-1 during a 6-day habituation period. Mice were then assigned into three groups: a free-feeding no-RKT control group (AL) consisting of seven mice (0 males, 7 females), an alternate day-fed no-RKT group (CR) consisting of 13 mice (6 males, 7 females) and an alternate day-fed RKT-treatment group (CR + RKT) consisting of 14 mice (7 males, 7 females). The AL group was fed a high-fat diet, HFD-60 (Oriental Yeast), ad libitum in which fat content accounted for approximately 60% of the calories. For the CR group, mice were fed HFD-60 every other day. The CR + RKT group was fed HFD-60 with 2% RKT every other day. Mouse behavior was observed every day, the food intake was measured every 2 days. Mouse weight was measured every week. A total of 15 weeks after RKT administration, oxidative stress was induced by compound treatment, as described below.

Oxidative stress induction and sample collection

In experiment 2, 75 mg/kg bodyweight (BW) of 3-nitropropionic acid (3NPA) was injected intraperitoneally twice a week to induce oxidative stress. A total of 30 min after the second dose of 3NPA, male mice were euthanized by cardiac exsanguination under 2.5% isoflurane inhalational anesthesia. Then, the liver was harvested, frozen in liquid nitrogen and stored at −80°C. For females, the dosage was increased to 150 mg/kg BW after 5 weeks of 3NPA administration. Weight, dietary intake and animal behavior were monitored until death, which was set at 20% weight loss and previously described criteria.[18] The time of death was recorded.

In experiment 3, 6 mg/kg BW of doxorubicin (DXR), was injected intraperitoneally twice a week, instead of 3NPA to induce oxidative stress by another molecular mechanisms. One day after the seventh dose of DXR, males from the CR and CR + RKT groups were euthanized by cardiac exsanguination under 2.5% isoflurane inhalational anesthesia. The heart and liver were harvested, frozen in liquid nitrogen, and stored at −80°C. Females from the AL, CR and CR + RKT groups were administered DXR until euthanasia end-points were reached: 20% weight loss, behavioral changes and reduction of body temperature. Weight, dietary intake and animal behavior were monitored, and the time of death was recorded.

Measurements of plasma parameters

In experiment 1, plasma parameters were measured using the kits described below in accordance with the manufacturers' protocols. Plasma acyl ghrelin was measured using the Active ghrelin ELISA kit (LSI Medience Corporation, Tokyo, Japan). IGF-I was measured using the Mouse/Rat IGF-1 immunoassay (R&D Systems, Minneapolis, MN, USA). Adiponectin was measured using the Adiponectin ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan).

In experiment 2, to measure plasma glucose levels, 2 μL of plasma collected during organ sample harvesting was analyzed with the Glucose CII Test Wako Kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) according to the kit instructions. Absorbance at 545 nm was measured using the ChroMate Model 4300 Microplate Reader (Awareness Technology, Palm City, FL, USA).

In experiment 3, after 12 weeks of RKT administration, blood samples from each group were harvested and inactivated with HCl. Plasma acyl ghrelin levels were measured with the Active

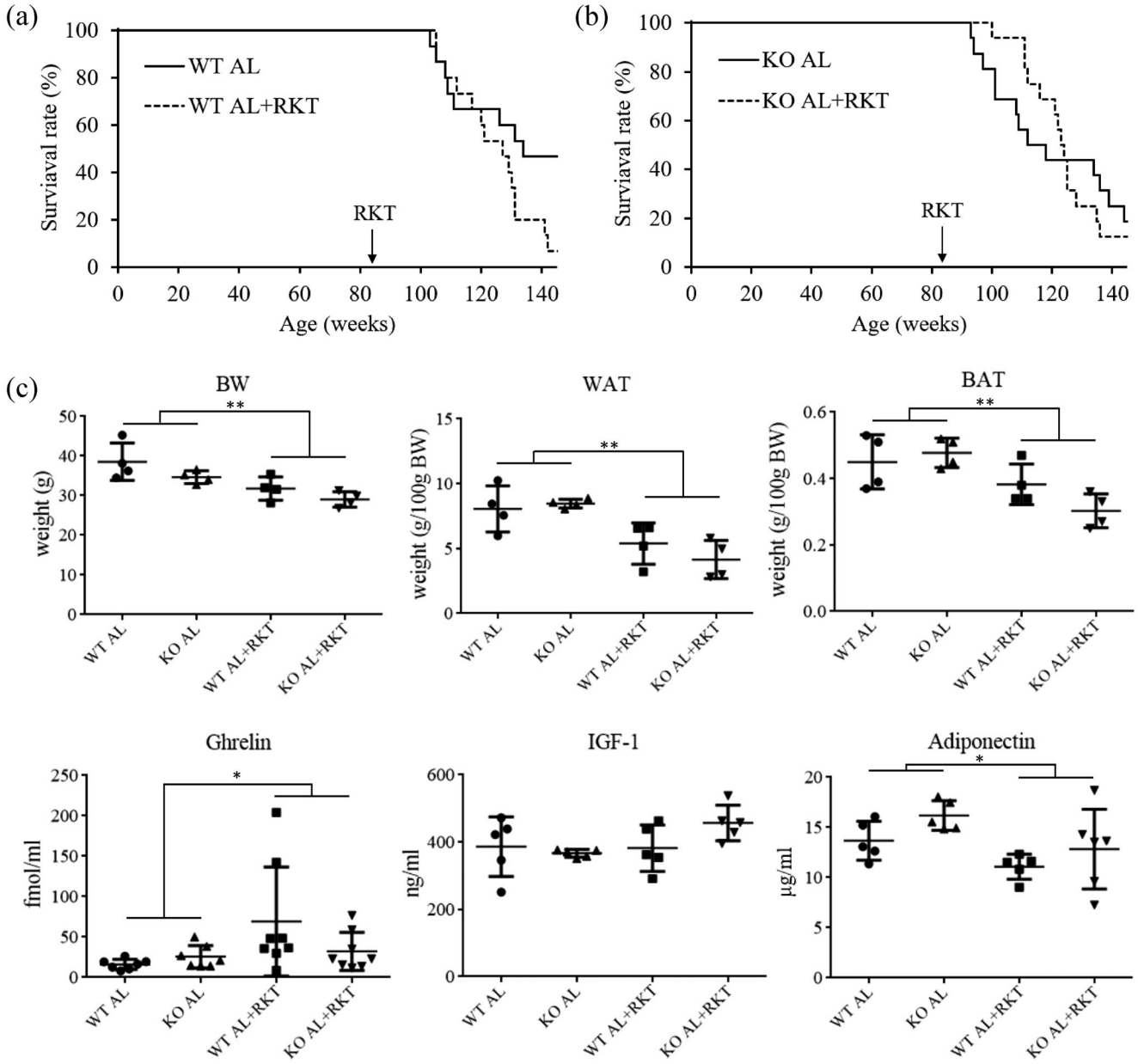


Figure 1 Lifespan, body and adipose tissue weight, and blood parameters of wild-type (WT) and neuropeptide Y (NPY) knockout (KO) mice under long-term rikkunshito (RKT) treatment started from middle age in experiment 1. (a) Survival rate of male WT mice with or without RKT treatment. Solid line, WT ad libitum (AL); dashed line, WT AL + RKT. RKT treatment did not significantly extend the lifespan of WT mice, as assessed by the log–rank test (n = 15). (b) Survival rate of male NPY KO mice with or without RKT treatment. Solid line, KO AL; dashed line, KO AL + RKT. RKT treatment did not significantly extend the lifespan of NPY KO mice, as assessed by the log–rank test (n = 16). (c) Bodyweight (BW), weights of white adipose tissue (WAT) and brown adipose tissue (BAT), and plasma concentrations of ghrelin, IGF-1 and adiponectin were measured at 12–14 weeks after RKT administration (male, n = 4–8). Data are shown as bar charts where scatter plots and bars represent the mean ± SD. Genotype and RKT administration were used as factors in a two-factor ANOVA. RKT administration significantly reduced BW, WAT, BAT and adiponectin levels, and significantly increased ghrelin levels. There were no genotype effects or interactions between the two factors. Statistically significant differences are annotated using an asterisk (*P < 0.05; **P < 0.01).

Ghrelin ELISA Kit (LSI Medience Corporation) using 25 µL of plasma in accordance with the kit's instructions.

The detailed descriptions for the methods of real-time polymerase chain reaction and western blot analysis are shown in Appendix S1.

Statistical analysis

Data were analyzed using IBM SPSS Statistic 22 (IBM, Armonk, NY, USA) by one-way analysis of variance (ANOVA), repeated measures ANOVA, Student's t-test and two-factor ANOVA based on the

administration of 3NPA and RKT. Survival analysis was carried out using the log–rank test based on Kaplan–Meier analysis. Tukey's test was used for post-hoc tests. The graphs show the mean and its standard deviation. Statistical significance was set at a *P*-value of <0.05. Statistically significant differences are annotated using an asterisk (*$P < 0.05$; **$P < 0.01$) for the *t*-test, and separate letters for ANOVA.

Results

Lifespan and changes in body and adipose tissue weight in experiment 1

In experiment 1, we tested the effect of long-term RKT treatment on lifespan under free-feeding conditions. As shown in Figure 1, RKT treatment that started from middle age did not significantly extend the lifespan of WT or NPY KO mice compared with the control diet-fed group (Fig. 1a,b).

A total of 14 weeks of RKT administration and genotype were used as factors in two-factor ANOVA. RKT administration significantly reduced BW, white adipose tissue weight and brown adipose tissue weight ($P < 0.01$; Fig. 1c). However, no genotype effects or interactions between the two factors.

Plasma parameters in experiment 1

In experiment 1, plasma samples were taken 12 weeks after RKT treatment was started in middle age, and the level of acyl ghrelin, IGF-1 and adiponectin were measured as plasma biomarkers of potential CR effects. RKT administration significantly increased ghrelin levels ($P < 0.05$; Fig. 1c). RKT administration did not affect IGF-1 levels; however, RKT administration significantly reduced adiponectin levels ($P < 0.05$; Fig. 1c). There were no genotype effects or interactions between the two factors.

Changes in weight, food intake and plasma glucose level in experiment 2

In experiment 2, there was no significant weight difference between males in the AL and AL + RKT groups (Fig. 2a left). There was no significant weight difference between females in the AL and AL + RKT groups (Fig. 2a right). Note that repeated measures ANOVA was used for the statistical analysis of BW in experiment 2. The food intake amount did not differ between groups in male (left) or female (right) mice (Fig. 2b). RKT administration did not affect weight and did not stimulate food intake.

Plasma samples were collected from the AL and AL + RKT groups after the second dose of 3NPA or saline solution, and the plasma glucose concentration (mg/dL) was measured. 3NPA and RKT administration were used as factors in a two-factor ANOVA, which showed an upregulation effect for 3NPA, but not for RKT or for interactions between the two factors (Fig. 2c).

Survival and rate of weight loss after 3NPA treatment in experiment 2

After 3NPA treatment, the AL and AL + RKT groups had a mean lifespan ± standard deviation of 23.5 ± 10.8 days and 25.0 ± 13.3 days, respectively (Fig. 2d), which were not statistically different by Kaplan–Meier survival analysis. Under AL, RKT administration did not suppress weight loss or increase the survival rate. The rate of weight loss was calculated from the weight before 3NPA administration and 2 weeks after 3NPA administration. Although the AL and AL + RKT groups did not have a significant difference in weight, RKT administration suppressed weight loss (Fig. 2e).

Analysis of gene expression in experiment 2

In experiment 2, liver samples from the AL and AL + RKT groups after the second dose of 3NPA were analyzed by real-time polymerase chain reaction to determine the expression of genes related to oxidative stress resistance, lipid metabolism, cell cycle and mitochondrial function. All results were standardized to the expression level of *18S rRNA*. The two 3NPA administered groups were analyzed using Student's *t*-test.

RKT administration significantly increased *Gstm3* expression (Fig. 3a). Furthermore, *Hmox1* expression was significantly increased on RKT administration (Fig. 3b). However, expression of the longevity gene, *Sirt1*, was not upregulated by RKT administration (Fig. 3c). Analysis of the expression of genes related to cell survival showed *p21* expression induced by oxidative stress was significantly higher in the AL + RKT group than in the AL group (Fig. 3d). RKT did not affect the expression of the β-oxidation gene, *Cpt1a* (Fig. 3e). However, expression of the mitochondrial function gene, *Pgc-1a*, was significantly increased with RKT administration (Fig. 3f).

Analysis of protein levels in experiment 2

In experiment 2, western blot analysis was carried out to examine the levels of 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (HNE) in liver samples harvested after the second dose of 3NPA. 3-NT was used as a protein oxidation stress marker, and HNE was used as a lipid oxidation stress marker. Data from the 3NPA-treated AL and AL + RKT groups are shown in Figure 3g and h. We found that RKT-administered groups had lower levels of 3-NT (Fig. 3g). No significant difference was detected in HNE levels, whereas a slight reduction in HNE levels was observed for the RKT administered groups (Fig. 3h).

Results of experiment 3

Figure 4 shows the results of experiment 3 (a detailed description of experiment 3 is shown in Appendix S1). In brief, no weight difference was observed with RKT administration in both males (Fig. 4a left) and females groups (Fig. 4a right). Food intake for the CR and CR + RKT groups corresponded to mild CR conditions (Fig. 4b right). The plasma ghrelin concentration was significantly higher in the CR + RKT group compared with other groups ($P < 0.05$; Fig. 4c left and right). The CR and CR + RKT groups had a significantly higher survival rate than the AL group ($P < 0.01$), whereas no significant difference in survival rate was found between the CR and CR + RKT groups (Fig. 4d). No significant difference in weight loss was observed between the CR and CR + RKT groups 1 week after DXR administration (Fig. 4e). However, the levels of 3-NT and HNE were significantly lower in the CR + RKT group compared with the CR group ($P < 0.05$; Fig. 4f,g).

Discussion

In experiment 1, we fed mice with RKT starting in middle age and analyzed its effects on lifespan extension in normal aging mice. We expected the effects of RKT to be blunted only in NPY KO mice. When we fed NPY KO mice with RKT, there was no significant effect on lifespan; however, WT mice fed RKT also showed no significant effect on lifespan. As expected, RKT increased

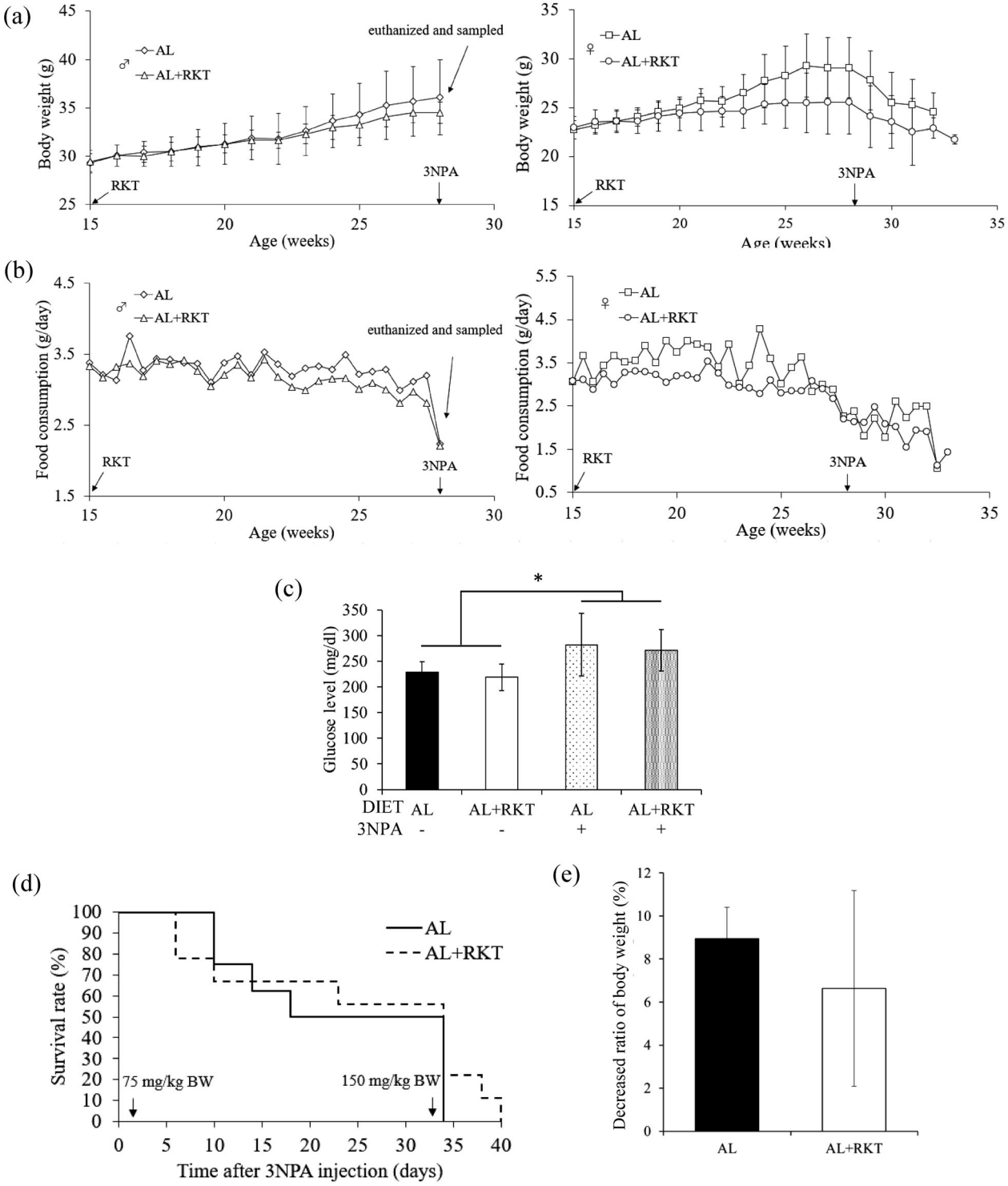


Figure 2 Changes in bodyweight (BW), food intake, rate of weight loss, survival and plasma parameters in experiment 2. (a) BW and (b) food intake of the ad libitum (AL) and the AL + rikkunshito (RKT) groups (n = 8–10). Data of male mice are shown on the left and for female mice on the right. ◇, AL male; △, AL + RKT male; □, AL female; ◦, AL + RKT female. Male mice were sampled after the second dose of 3-nitropropionic acid (3NPA). RKT did not cause a significant difference in BW when analyzed by repeated measures ANOVA. (c) Plasma glucose levels of the four groups corresponding to AL, AL + RKT (with and without 3NPA; male, n = 5). Data represent the mean ± SD. 3NPA and RKT administration were used as factors in a two-factor ANOVA. Significant effect of 3NPA is shown by an asterisk (*P < 0.05), but no significant RKT effect or interaction between the two factors was observed. (d) The survival rate after 3NPA administration (female, n = 8–10). Solid line, AL; dashed line, AL + RKT. No significant difference was observed between the AL and AL + RKT groups. (e) Rate of weight loss 2 weeks after 3NPA administration (female, n = 5–6). Data represent the mean ± SD. RKT administration did not cause a significant difference in either parameter, but was found to suppress weight loss slightly.

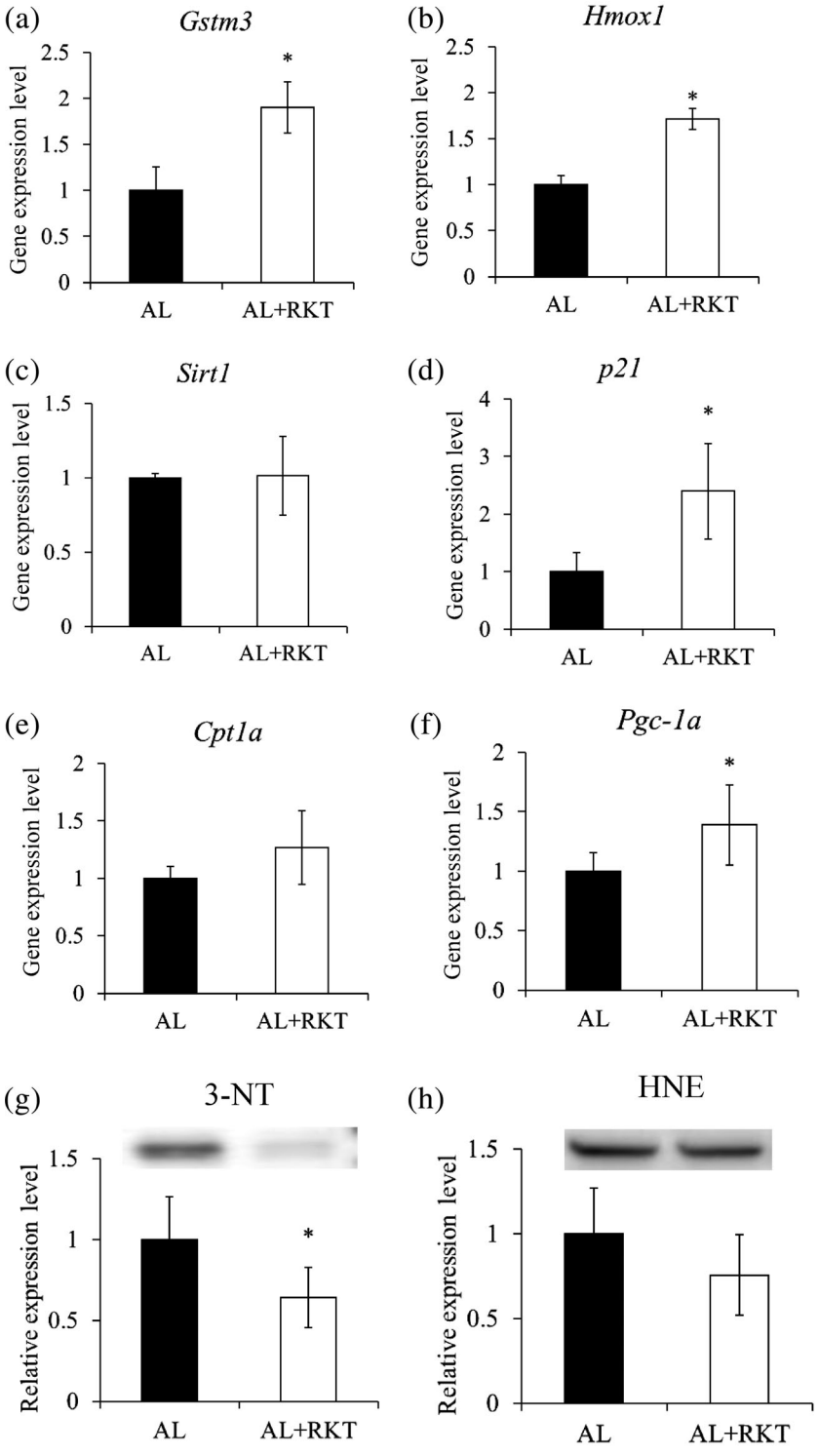


Figure 3 Gene expression and oxidative stress marker levels in the liver in experiment 2. (a–f) The expression of *Gstm3*, *Hmox1*, *Sirt1*, *p21*, *Cpt1a* and *Pgc-1a* in liver samples from 3-nitropropionic acid (3NPA)-injected mice treated with or without RKT (male, $n = 4–5$). All results were standardized according to *18S rRNA* expression levels. Data represent the mean ± SD. Student's *t*-test was carried out and statistically significant differences are annotated using an asterisk (*$P < 0.05$). (g) 3-Nitrotyrosine (3-NT) and (h) 4-hydroxynonenal (HNE) levels were analyzed using liver samples harvested after 3NPA treatment, and the Student's *t*-test was carried out (male, $n = 5$). Data represent the mean ± SD. Statistically significant differences are shown by an asterisk (*$P < 0.05$). The western blot shown at the top is representative of these experiments (male, $n = 5$).

plasma ghrelin concentrations similar to that in CR animals. However, IGF-I concentrations were not decreased and adiponectin concentrations were not increased, similar to that in CR animals with RKT supplementation.

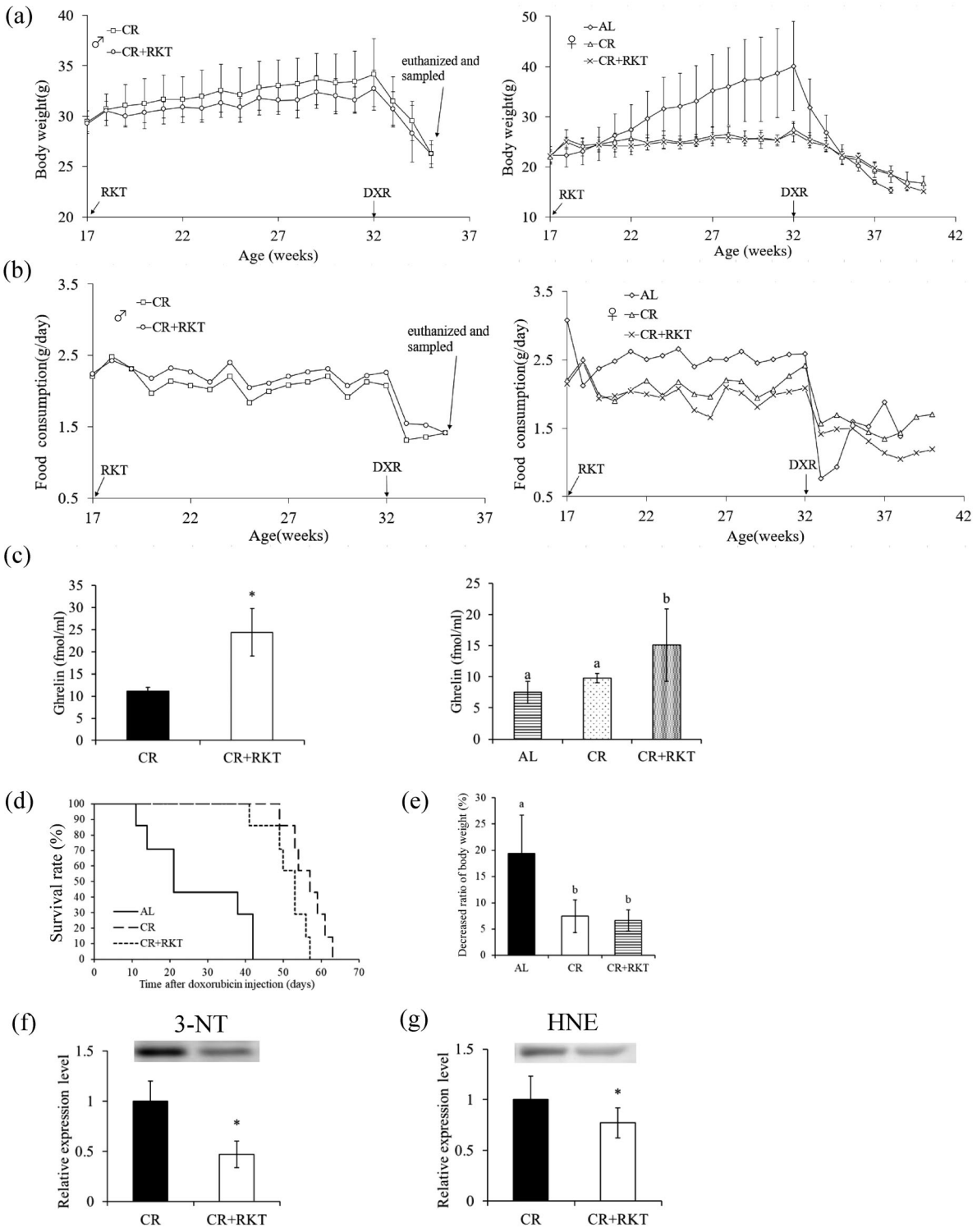
Compared with data showing that RKT treatment through drinking water extended the lifespan of senescence-accelerated mice prone 8 and Institute of Cancer Research mice, the RKT dose in our model was two- to fourfold higher.[22] This indicated that a high concentration of RKT might have a negative effect on lifespan extension, or the timing and method of RKT supplementation is important to extend the lifespan, unless its effect is strain-specific.

To investigate whether RKT treatment can mimic other CR effects, such as anti-oxidative capacity, we induced oxidative stress by using 3NPA. Oxidative stress induced by 3NPA perturbs mitochondrial functions and induces neurodegeneration.[23] NPY has neuroprotective effects against chemically induced toxicity.[24] CR also has a similar neuroprotective effect.[25,26] We reported that

these protective effects were related to the NPY upregulation by CR.[7,18] Therefore, we investigated protection against 3NPA in mice fed diets with or without RKT.

We analyzed the long-term effects of 3NPA by survival analysis, whereas the short-term effects (acute responses) were tested at 30 min after 3NPA injection. We previously carried out gene expression analyses at 0, 15, 30, 60 or 240 min after 3NPA injection (also in our unpublished data).[27] On the basis of that study, we analyzed the samples at 30 min after 3NPA treatment to determine acute responses to 3NPA. Furthermore, in experiment 3, we used another type of oxidative stress inducer, DXR, to confirm stress resistance.

In experiment 2, we analyzed CR-mimicking effects of RKT to examine its efficacy in normal AL feeding. BW was not



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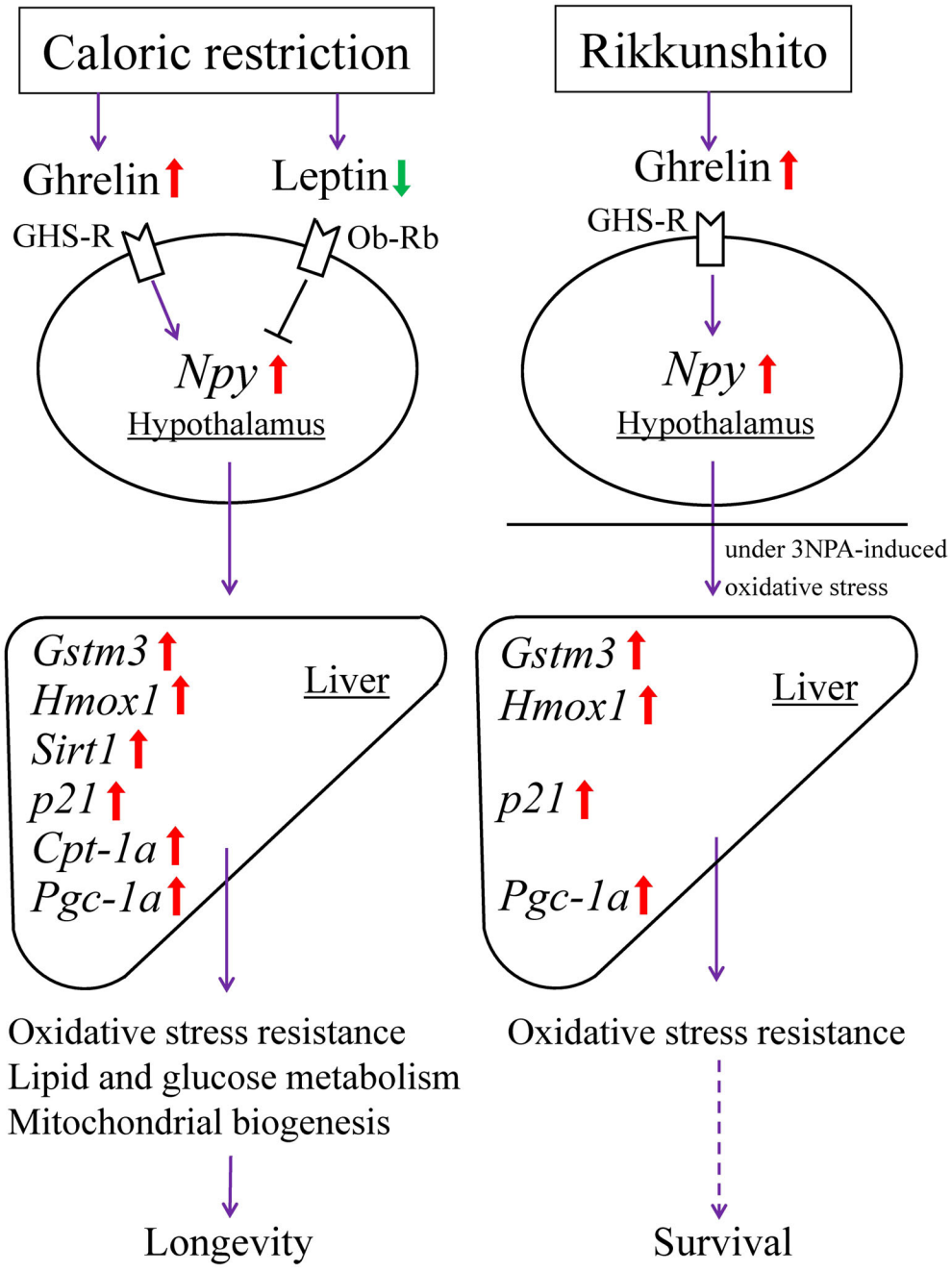

Figure 5 A hypothetical and schematic representation of the comparison between calorie restriction-mediated signals and rikkunshito-mediated signals. We previously reported that caloric restriction upregulated NPY by increasing plasma ghrelin and decreasing plasma leptin concentrations. NPY is important for oxidative stress resistance, lipid and glucose metabolismadaptation, and mitochondrial biogenesis in the liver. In the present study, rikkunshito activated the ghrelin–neuropeptide Y pathway and the expression of oxidative stress resistance-related genes, but not metabolism-related genes in the livers of 3-nitropropionic acid (3NPA)-induced oxidative stress model mice. However, these changes did not increase the survival rate. *Cpt1a*, carnitine palmitoyltransferase 1α; GHS-R, growth hormone secretagogues receptor (ghrelin receptor); *Gstm3*, glutathione S-transferase M3; *Hmox1*, heme oxygenase 1; *Npy*, neuropeptide Y; Ob-Rb, long form of the leptin receptor; *Pgc-1a*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *Sirt1*, sirtuin 1.

Figure 4 Changes in bodyweight (BW), food intake, plasma ghrelin levels, survival, rate of weight loss and oxidative stress marker levels in liver samples in experiment 3. (a) BW and (b) food intake for the ad libitum (AL), caloric restriction (CR) and CR + rikkunshito (RKT) groups (n = 6–7). Data of male mice are shown on the left, and on the right for female mice. □, CR male; ○, CR + RKT male; ◇, AL female; △, CR female; ×, CR + RKT female. The CR group showed a significant decrease in BW compared with the AL group ($P < 0.05$), but no significant difference was observed with or without RKT administration. (c) Plasma ghrelin concentration of males (left) and females (right; n = 5–7). Data represent the mean ± SD. Data were analyzed by Student's t-test and one-way ANOVA. Statistically significant differences ($P < 0.05$) are shown using an asterisk (*) for the t-test and separate letters for ANOVA. The CR + RKT group had a significantly higher plasma ghrelin concentration than the other group ($P < 0.05$). (d) Survival on doxorubicin administration (female, n = 7). Solid line, AL; long dashed line, CR; dashed line, CR + RKT. The two CR groups had a significantly higher rate of survival than the AL group ($P < 0.01$), but no significant difference observed between the CR and CR + RKT groups. (e) The rate of weight loss at 1 week after DXR administration (female, n = 6–7). Data represent the mean ± SD. Statistically significant differences ($P < 0.01$) are shown using separate letters for ANOVA. The two CR groups had a significantly lower rate of weight loss compared with the AL group ($P < 0.01$), but no significant difference observed between the CR and CR + RKT groups. (f) 3-Nitrotyrosine (3-NT) and (g) 4-hydroxynonenal (HNE) levels were analyzed using liver samples harvested after doxorubicin treatment, and a Student's t-test was carried out (male, n = 5). Data represent the mean ± SD. Statistically significant differences are shown by an asterisk (*$P < 0.05$). The western blot shown at the top is representative of these experiments (male, n = 5).

significantly increased by RKT administration when analyzed by repeated measures ANOVA. These data show that RKT promotes appetite stimulation during conditions that induce a reduced appetite, such as during chemotherapy treatment, but RKT might not promote appetite under normal conditions. [28] Indeed, it was reported that RKT administration to a normal-appetite mouse did not significantly increase its BW.[20]

We also expected that RKT would increase anti-oxidative functions and extend lifespan on 3NPA administration, but the extension of mean lifespan in the RKT group was not statistically significant. The oxidative stress-induced upregulation of gluconeogenesis was shown in 3NPA-treated male mice; however, there was no diet effect or interaction between diet and 3NPA regarding the plasma glucose levels. However, the gene expression of gluconeogenic enzyme regulator, *Pgc-1a*, was increased in the 3NPA-administered AL + RKT group when compared with that of the AL group. Given this, RKT did not increase lifespan under the severe oxidative stress conditions of this experiment. However, the expression of the *Gstm3* and *Hmox1* genes were higher in the 3NPA-administered AL + RKT group compared with that of the AL group. Because these anti-oxidant response element-containing gene expressions were regulated by Nrf2 transcriptional activity, even under 3NPA-treated conditions, RKT supplementation might activate Nrf2 activity.[29] Moreover, RKT administration significantly decreased 3-NT levels in the livers of 3NPA treated mice. The expression of *p21* after oxidative stress induction, was significantly higher in the AL + RKT group compared with the AL group. We reported that under low oxidative stress conditions, CR suppressed the expression of *p21*, whereas its expression was increased under high oxidative stress conditions.[27] *Cpt1a* gene expression was not significantly increased by RKT treatment. This suggests that mitochondrial functions might not be activated by RKT treatment, although *Pgc-1a* gene expression was significantly increased by RKT treatment. Together with the results of plasma glucose levels, RKT might not activate CR-mediated lipid and glucose metabolism changes. Although RKT did not increase the expression of *Cpt1a* after 3NPA treatment, RKT stimulated *Pgc-1a* activation, which has an important role in protection against 3NPA-induced oxidative damage.[30] Finally, *Sirt1* gene expression was not significantly upregulated by RKT in the livers of oxidative stress-induced mice. However, a previous report showed that RKT-induced cAMP–cAMP response element binding protein (cAMP–CREB) pathway regulation was important for hypothalamic SIRT1 activation to induce life-extending effects and suppress myocardial abnormalities.[22] Therefore, the activation of SIRT1 by RKT and the induced beneficial effects might be tissue-specific.

In experiment 3, we investigated RKT supplementation of a high-fat diet with feeding every other day for CR, to determine whether RKT enhanced the CR beneficial effects under metabolic stress conditions in combination with DXR-induced oxidative stress. Mice that underwent mild CR along with RKT administration showed greater protection against oxidative stress compared with the AL groups. However, there was no extension of survival on DXR-induced oxidative stress when compared with the CR and CR + RKT groups. RKT enhanced plasma ghrelin levels, even under CR conditions, and there was no further oxidative stress resistance in CR without RKT. This indicated that ghrelin signal enhancement when a mild CR and RKT are combined was insufficient to enhance the beneficial effects of CR against oxidative stress.

Because we used different strains and sexes of mice in different experimental procedures, the interpretation of our results is complicated. However, RKT supplementation had no life-extending effects in middle-aged male mice both in the presence or absence of NPY (experiment 1), and no increased survival rate was observed in young inbred female mice in the 3NPA-induced oxidative stress model (experiment 2) or in young inbred female mice in the DXR-induced oxidative stress under high-fat diet-induced metabolic stress model (experiment 3). The present result would imply that *Pgc-1a* affected on Nrf2 activation for anti-oxidant stimulation with target gene upregulation (*Gstm3 and Hmox1*), not energy metabolism-related genes expression (*Sirt1 and Cpt1a*), by RKT supplementation in young inbred male mice (Fig. 5). To further elucidate our results, the effects of RKT should be investigated under AL conditions and various levels of CR.

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Disclosure statement

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website:

Appendix S1 Supporting methods and results.

Figure S1 Rikkunshito (RKT) supplementation activates the ghrelin–neuropeptide Y (NPY) pathway. (a) A significant increase in plasma ghrelin levels was observed in the ad libitum (AL) + RKT group compared with AL groups ($P < 0.05$, $n = 5$). (b) A significant increase in *Npy* gene expression was observed in the brains of the AL + RKT group compared with the AL group ($P < 0.05$, $n = 3$). Statistically significant differences ($P < 0.05$) are shown using an asterisk (*) for the Student's *t*-test.

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