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Caloric restriction decreases ER stress in liver and adipose tissue in *ob*/*ob* mice

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ABSTRACT

Endoplasmic reticulum (ER) stress plays a crucial role in the development of insulin resistance and diabetes. Although caloric restriction (CR) improves obesity-related disorders, the effects of CR on ER stress in obesity remain unknown. To investigate how CR affects ER stress in obesity, *ob*/*ob* mice were assigned to either *ad libitum* (AL) (*ob*-AL) or CR (*ob*-CR) feeding (2 g food/day) for 1–4 weeks. The body weight (BW) of *ob*-CR mice decreased to the level of lean AL-fed littermates (*lean*-AL) within 2 weeks. BW of *lean*-AL and *ob*-CR mice was less than that of *ob*-AL mice. The *ob*-CR mice showed improved glucose tolerance and hepatic insulin action compared with *ob*-AL mice. Levels of ER stress markers such as phosphorylated PKR-like ER kinase (PERK) and eukaryotic translation initiation factor 2α and the mRNA expression of activating transcription factor 4 were significantly higher in the liver and epididymal fat from *ob*-AL mice compared with *lean*-AL mice. CR for 2 and 4 weeks significantly reduced all of these markers to less than 35% and 50%, respectively, of the levels in *ob*-AL mice. CR also significantly reduced the phosphorylation of insulin receptor substrate (IRS)-1 and c-Jun NH_2-terminal kinase (JNK) in *ob*/*ob* mice. The CR-mediated decrease in PERK phosphorylation was similar to that induced by 4-phenyl butyric acid, which reduces ER stress *in vivo*. In conclusion, CR reduced ER stress and improved hepatic insulin action by suppressing JNK-mediated IRS-1 serine-phosphorylation in *ob*/*ob* mice.

1. Introduction

Obesity is a major public health problem worldwide, and is associated with insulin resistance, hypertension, and impaired glucose and lipid metabolism. These abnormalities often coincide, leading to the "metabolic syndrome" and diabetes. Excessive caloric intake contributes to the development of obesity [1]. Lifestyle modification with caloric restriction (CR) is a key component of the treatment of obesity and diabetes. CR is also the only intervention known to extend the lifespan in a range of organisms, including mammals, and improves obesity-related disorders through defined and undefined mechanisms [2,3]. Indeed, a reduction in food intake in obese patients elicits body weight (BW) loss and improves metabolic parameters [1–3].

Endoplasmic reticulum (ER) stress is evident in obese animals and humans [4–7] and is thought to play a crucial role in the development of insulin resistance and the pathogenesis of diabetes [4,5,8–10]. The ER is an intracellular organelle that coordinates the synthesis, folding and trafficking of proteins. During stress, unfolded and misfolded proteins accumulate in the ER and initiate an adaptive response known as the unfolded protein response (UPR) via three ER transmembrane proteins, PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor (ATF) 6 [8–10].

ER stress is evident in the liver and adipose tissues of obese mice [4–8] and activates c-Jun NH_2-terminal kinase (JNK), which inhibits insulin signaling via serine-phosphorylation of insulin receptor substrate-1 (IRS-1). By contrast, chemical chaperones such as 4-phenyl butyric acid (PBA) reduced ER stress, the UPR and JNK activation, and hence improved insulin sensitivity in obese animals [5,8]. During ER stress, activated PERK phosphorylates eukaryotic translation initiation factor (eIF) 2α, thereby inhibiting 80S ribosome assembly and protein synthesis, and consequently decreasing the functional demand on the ER. Phosphorylation of eIF2α causes a general decrease in translation, although some proteins such as ATF4 are translated more efficiently [10] while activated IRE1 induces the cleavage of the X-box-binding protein 1 (XBP1) mRNA, generating a spliced variant (XBP1s).

Although ER stress is upregulated in obese animals, the effects of CR on ER stress have not been elucidated. Nutrient starvation, in addition to nutrient excess, could lead to ER stress [8–11]. Since, during CR, various nutrients and energy are restricted due to limited intake, CR procedure might induce ER stress. Thus, the effects of CR on ER stress and UPR are largely unknown and should be determined. In addition, understanding the CR-mediated changes

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in ER function might reveal important information for the clinical use of CR. Therefore, the purpose of this study was to evaluate the effects of CR on ER stress in key metabolic tissues in obese animals.

2. Materials and methods

2.1. Animals and CR procedure

All mice used in the present study were purchased from Charles River Japan, Inc. Mice were fed a standard chow (3.9% fat, 54.7% carbohydrate, 18.8% protein by calories; Nosan Corporation, Yokohama, Japan) *ad libitum* (AL) for 1 week. They were then divided to the experimental groups and housed individually. All procedures were approved by the Animal Care and Use Committee of Kumamoto University. Three experiments, Studies 1–3, were performed.

In Study 1, 5-week-old male C57BL/6J (*lean*) and *ob*/*ob* mice were used. The obese mice were randomized to receive either CR (*ob*-CR) or AL (*ob*-AL) feeding (n = 24/group). To reduce food intake and BW, the *ob*-CR mice were given a reduced amount of regular chow (2 g food/day), as previously described [12]. This intervention maintained the BW of *ob*/*ob* mice comparable to that of AL-fed C57BL/6 mice and further extended the lifespan of *ob*/*ob* mice [12]. *Ob*-AL and *lean*-AL mice were given free access to the food.

Studies 2 and 3 were designed to investigate the effects of CR on ER stress and UPR. In Study 2, we compared the effects of CR and PBA administration on ER stress in *ob*/*ob* mice. Mice were fed a regular chow containing PBA (1 g/kg/day) (*ob*-PBA) [5] or vehicle (0.9% saline) either AL (*ob*-AL) or under CR conditions (2 g food/day, *ob*-CR) for 4 weeks (n = 5/group). All mice were given 0.5 g of the respective diet containing PBA or vehicle from 9:00 to 12:00. The *ob*-PBA and *ob*-AL mice were allowed to free access to food while food was restricted in *ob*-CR mice as described in Study 1.

In Study 3, 6-week-old male KK and KK-Ay mice were given free (KK-AL and Ay-AL, respectively) or restricted (KK-CR and Ay-CR, respectively) access to food for 2 weeks (n = 5/group) as described in Study 1.

Metabolic parameters, and glucose and insulin tolerance tests (GTT and ITT) were evaluated as previously described [13,14]. Plasma and tissue triglyceride (TG) levels were measured using a kit from Wako Pure Chemical (Osaka, Japan).

2.2. Western blot analysis

Anesthetized mice were intraperitoneally injected with saline or regular insulin (2 U/kg BW). The liver and adipose tissues were dissected 15 min after the injection and immediately frozen in liquid nitrogen. Western blotting was performed as described previously [13,14] using antibodies purchased from Cell Signaling Technology, except for anti-ATF4 antibody, which was purchased from Santa Cruz Biotechnology Inc.

2.3. Quantitative real-time RT-PCR (qRT-PCR) analysis

qRT-PCR was performed as previously reported [14] using specific primer sets (Supplementary Table S1). The relative expression of mRNA was calculated for each gene using 18S ribosomal RNA (18S) as an internal control.

2.4. Data analysis

All data are presented as means ± standard deviation (SD) and were analyzed using Student's t-test or one-way analysis of variance as appropriate. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of CR on body weight and glucose metabolism

Food intake was significantly higher in *ob*-AL mice than in *lean*-AL mice throughout the study, while *ob*-CR consumed less food than *lean*-AL (Fig. 1A). The CR procedure used in this study reduced the BW of the *ob*/*ob* mice during the first 2 weeks of feeding (Fig. 1B). The BW of *ob*-CR mice was significantly lower than that of *ob*-AL mice at 1 week ($p < 0.05$), and was similar to that of *lean*-AL mice at 2 weeks. After 2 weeks, the growth curves for *ob*-CR and *lean*-AL were comparable, and the BW of *ob*-AL mice was significantly greater than that of *ob*-CR and *lean*-AL mice.

After 4 weeks of feeding, fasting glucose, insulin and serum TG levels, and homeostasis model assessment of insulin resistance (HOMA-IR) were significantly lower in *ob*-CR mice than in *ob*-AL mice (Table 1). Glucose tolerance was markedly improved in *ob*-CR mice compared with that in *ob*-AL mice, and no difference between *ob*-CR and *lean*-AL mice was observed (Fig. 1C), suggesting that CR strongly improved glucose tolerance in *ob*/*ob* mice. However, CR did not significantly improve systemic insulin sensitivity (Fig. 1D) because insulin resistance was evident in *ob*-CR and *ob*-AL mice as compared with *lean*-AL mice. These results indicate differential effects of CR on glucose tolerance and insulin sensitivity in *ob*/*ob* mice.

3.2. CR reduced fat accumulation in ob/ob mice

To quantitatively investigate fat accumulation in insulin-sensitive tissues and the effects of CR, we measured TG content in the liver and quadriceps skeletal muscle. TG content in these tissues was significantly lower in *ob*-CR mice than in *ob*-AL mice (Supplementary Table S2), but was significantly greater than that in lean-AL mice (data not shown). Despite the similar BW, the increased tissue TG content in *ob*-CR mice may be due to the deficit in leptin action in these mice [15,16].

3.3. CR for 2 and 4 weeks, but not 1 week, reduced hepatic ER stress in ob/ob mice

We investigated how CR affects hepatic ER stress in *ob*/*ob* mice. Increased phosphorylation of PERK and eIF2α were evident in the liver of *ob*-AL mice compared with *lean*-AL mice after 4 weeks of treatment, and were reduced in *ob*-CR mice by 59% and 51% , respectively ($p < 0.01$)(Fig. 2A, B). Next, we investigated the effects of CR for 1 and 2 weeks. CR for 2 weeks significantly reduced the phosphorylation of PERK and eIF2α by 40% and 36%, respectively (both, $p < 0.05$) compared with *ob*-AL mice. On the other hand, CR for 1 week did not affect the phosphorylation of PERK (Supplementary Fig. S1A and B). These results suggest that CR for 2–4 weeks could reduce hepatic ER stress in *ob*/*ob* mice.

To confirm the CR-mediated changes in PERK and eIF2α phosphorylation, we determined the mRNA and protein expression of ATF4, a UPR gene downstream of eIF2α. ATF4 protein and mRNA levels were significantly higher in *ob*-AL mice than in *lean*-AL mice, and were reduced by 4 weeks of CR (Fig. 2A–C). qRT-PCR analyses revealed significant increases in the mRNA expression of glucose-regulated protein 78 kDa (GRP78) and XBP1s, but not of C/EBP homologous protein (CHOP), in the liver from *ob*-AL mice compared with those from *lean*-AL mice. After 4 weeks of treatment, the mRNA expression of GRP78 and XBP1s was significantly reduced in *ob*-CR mice compared with that in *ob*-AL mice (Fig. 2C). Interestingly, CHOP mRNA expression was decreased in *ob*-CR mice compared with both *ob*-AL and *lean*-AL mice (Fig. 2C). Similar data on the mRNA expression of ATF4 and GRP78 were obtained in mice treated for 2 weeks (data not shown).

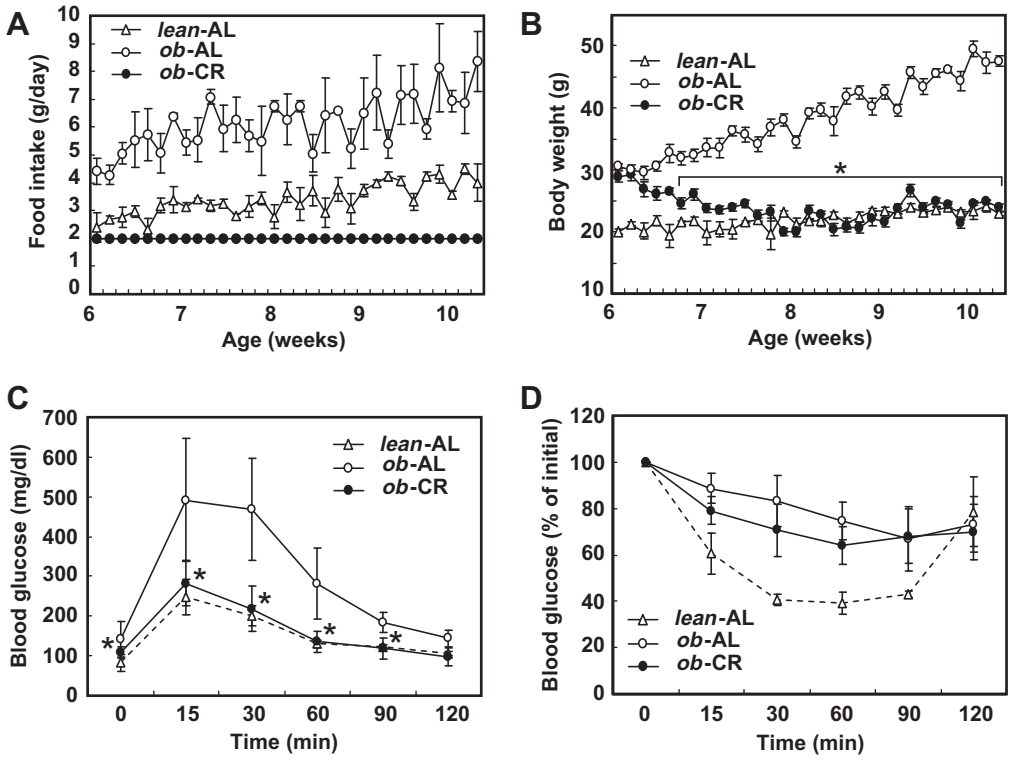

Fig. 1. Effects of caloric restriction (CR) on food intake (A), body weight (B), glucose tolerance (C) and insulin tolerance (D) in *ob/ob* mice. Data are means ± SD (n = 7–9/group). $^{*}p < 0.05$ vs *ob*-AL mice. Open circles: *ob*-AL; closed circles: *ob*-CR; open triangles: *lean*-AL.

Table 1
Metabolic parameters of *ob*-AL, *ob*-CR and *lean*-AL after 4 weeks of treatment.

	lean-AL	*ob*-AL	*ob*-CR
Body weight (g)	22.9 ± 2.4	41.6 ± 2.3*	21.4 ± 1.0#
Fasting blood glucose (mg/dL)	82.3 ± 14.8	142.3 ± 25.5*	109.0 ± 18.9*#
Fasting insulin (ng/mL)	1.1 ± 0.1	5.3 ± 0.9*	2.8 ± 0.8*#
HOMA-IR	4.9 ± 0.1	42.8 ± 10.5*	18.1 ± 7.2*#
Serum TG (mg/dL)	28.3 ± 3.8	74.0 ± 9.7*	32.1 ± 14.7#

Data are means ± SD (n = 7–9/group). $^{*}p < 0.05$ vs *lean*-AL mice; $^{\#}p < 0.05$ vs *ob*-AL mice. HOMA-IR: homeostasis model assessment insulin resistance; TG: triglyceride; *lean*-AL: *ad libitum*-fed C57BL/6J mice; *ob*-AL: *ad libitum*-fed *ob/ob* mice; *ob*-CR: caloric-restricted *ob/ob* mice.

3.4. CR for 2 and 4 weeks, but not for 1 week, reduced ER stress in epididymal fat tissue in ob/ob mice

Next, we investigated the effects of CR on ER stress in adipose tissue. Increased phosphorylation of PERK and eIF2α were evident in the adipose tissue of *ob*-AL mice compared with *lean*-AL mice, both of which were clearly reduced in *ob*-CR mice treated for 4 weeks (by 77% and 65%, respectively, versus *ob*-AL mice; both, $p < 0.01$) (Fig. 3A, B). CR for 2 weeks elicited weaker, but statistically significant ($p < 0.05$) decreases in the phosphorylation of PERK and eIF2α of 35% and 42%, respectively. However, CR for 1 week did not reduce the phosphorylation of PERK in adipose tissue (Supplementary Fig. S1C and D).

ATF4 mRNA and protein expression (Fig. 3C, A, B, respectively) was significantly higher in *ob*-AL mice than in *lean*-AL mice, and was significantly reduced in *ob*-CR mice treated for 4 (Fig. 3A–C) and 2 weeks (data not shown). The inhibitory effect of CR for 4 weeks on ER stress in adipose tissue was confirmed by qRT-PCR of GRP78 and CHOP (Fig. 3C).

3.5. PBA and CR for 4 weeks reduced hepatic ER stress in ob/ob mice

Next, we investigated the effect of the chemical chaperone PBA on hepatic ER stress and compared its effects with those of CR for 4 weeks. Oral administration of PBA did not affect food intake, and the BW of *ob*-PBA mice was similar to that of vehicle-treated *ob*-AL mice (data not shown), as previously reported [5]. However, PBA significantly reduced the phosphorylation of PERK and eIF2α and the protein expression of ATF4 (Supplementary Fig. S2A and B). Although the effects on PERK were similar, the effects of CR on eIF2α and ATF4 were significantly greater than those induced by PBA ($p < 0.05$).

3.6. Effects of CR on hepatic insulin signaling in ob/ob mice

We next investigated the effects of CR on hepatic insulin signaling in terms of Akt (Ser-473) phosphorylation, a sensitive marker for insulin action. In the absence of exogenous insulin stimulation, which reflects insulin action induced by endogenously secreted insulin after an overnight fast, Akt phosphorylation was significantly reduced in *ob*-AL mice compared with *lean*-AL mice (Fig. 4A, B). CR significantly increased Akt phosphorylation by 156% compared with *ob*-AL mice. Furthermore, exogenously administered insulin strongly enhanced Akt phosphorylation in *lean*-AL mice. In *ob*-AL mice, insulin-stimulated Akt phosphorylation was 41% lower than that in *lean*-AL mice, and was partially but significantly increased by CR by 47% ($p < 0.01$) compared with that in *ob*-AL mice. These results suggest that CR improved hepatic insulin signaling in basal and insulin-stimulated states in *ob/ob* mice.

3.7. Effects of CR on hepatic JNK signaling in ob/ob mice

Expression of markers for insulin resistance associated with ER stress, including JNK phosphorylation and Ser-307 phosphorylation

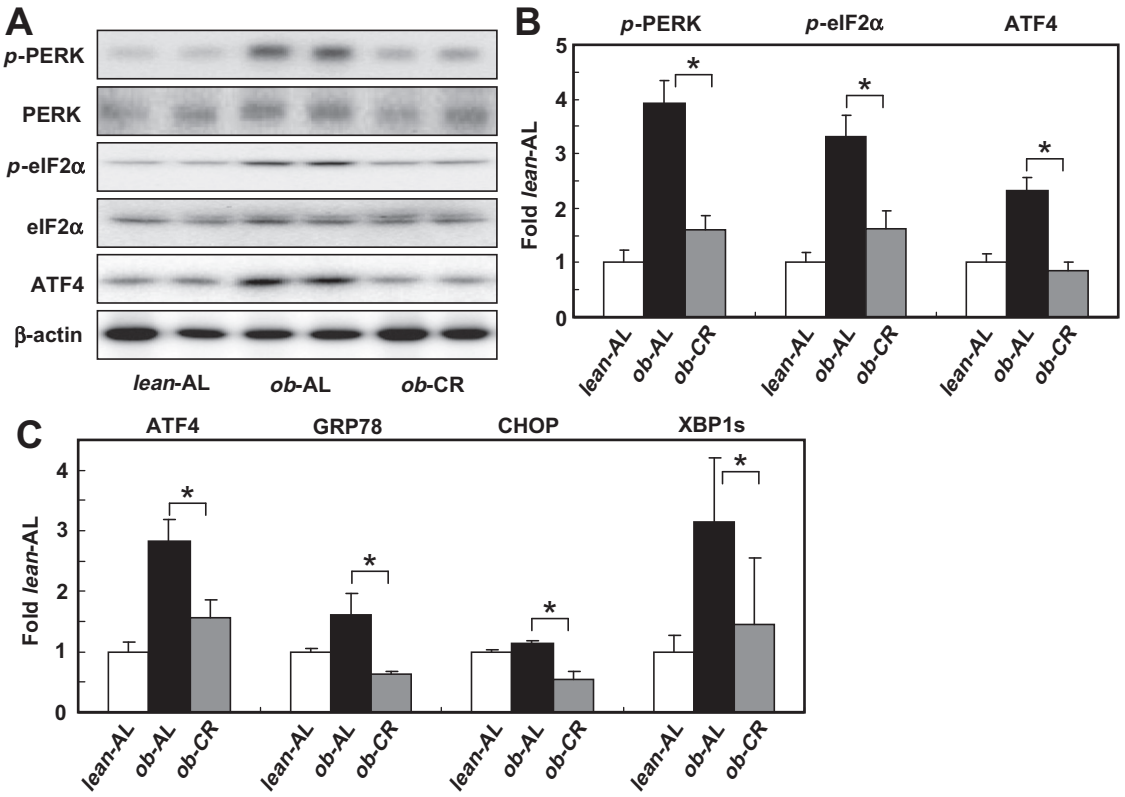

Fig. 2. Effects of CR on hepatic ER stress markers in *ob*/*ob* mice. (A) Representative Western blots for phosphorylated PERK (p-PERK) and eIF2α (p-eIF2α), and the protein expression of PERK, eIF2α, ATF4 and β-actin after 4 weeks of treatment. (B) Quantification of p-PERK, p-eIF2α and ATF4 protein expression normalized for total protein levels and β-actin. (C) qRT-PCR analyses of ATF4, GRP78, CHOP and XBP1s mRNA expression. $^{*}p < 0.05$ vs *ob*-AL mice.

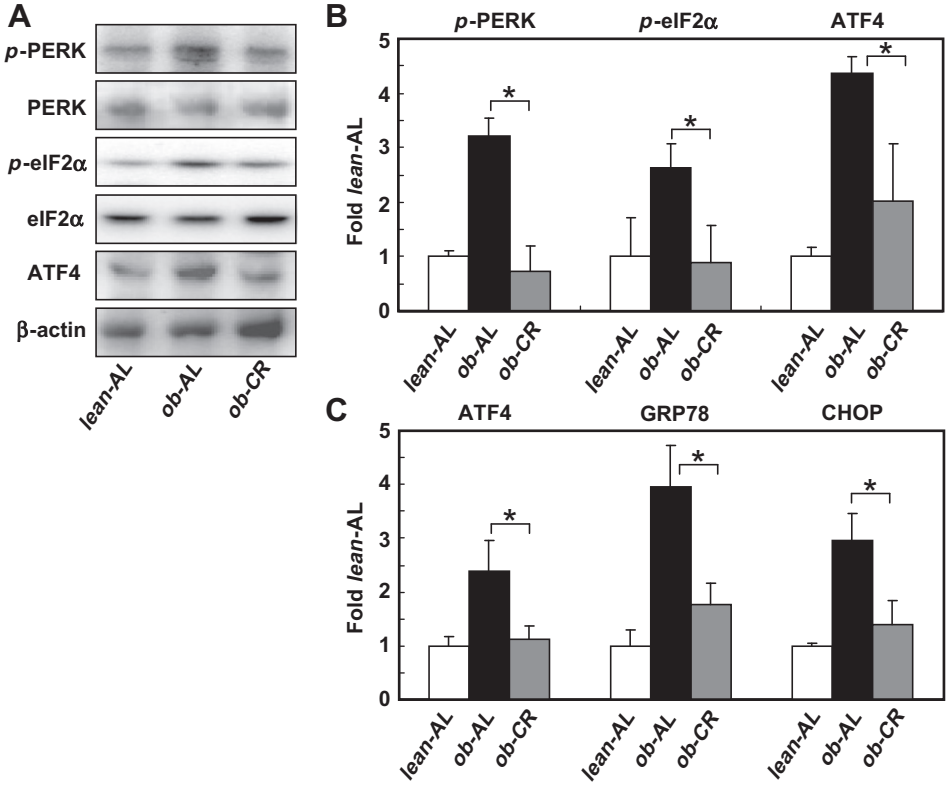

Fig. 3. Effects of CR on ER stress in adipose tissue. (A) Representative western blots for phosphorylated and total protein expression of PERK, eIF2α, ATF4 and β-actin in epididymal fat after 4 weeks of treatment. (B) Quantification of p-PERK, p-eIF2α and ATF4 protein levels. (C) qRT-PCR analyses for ATF4, GRP78 and CHOP mRNA expression. $^{*}p < 0.05$ vs *ob*-AL mice.

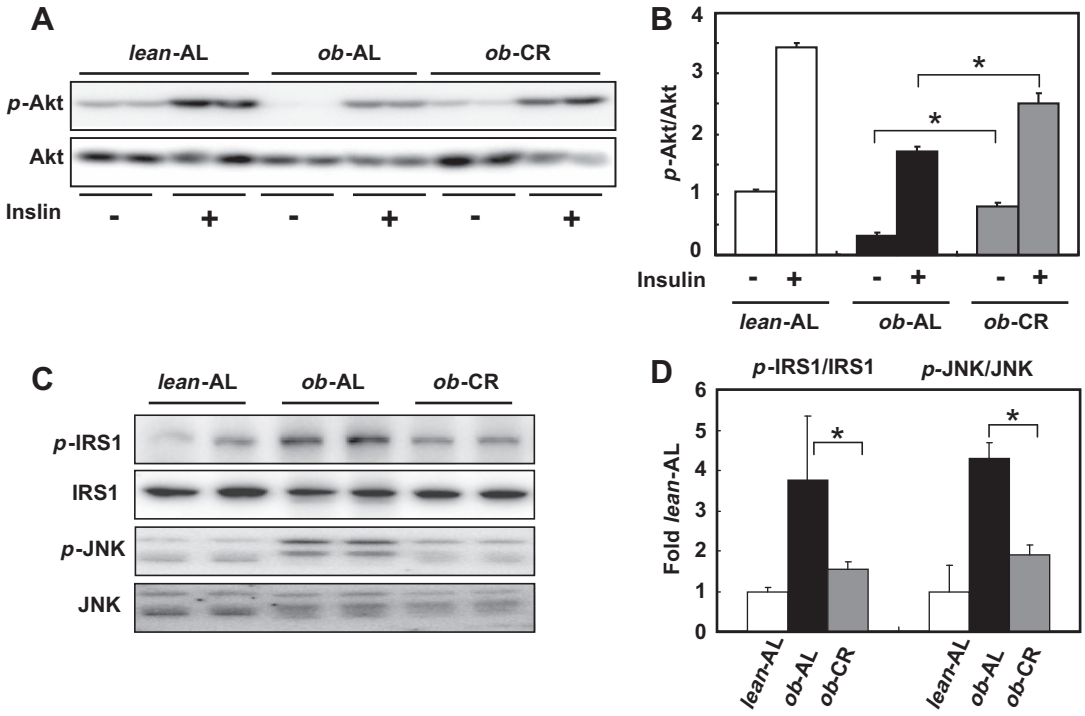

Fig. 4. Effects of CR on hepatic insulin signaling and JNK activation in *ob*/*ob* mice. Mice were fasted overnight and injected with insulin or 0.9% saline. (A) Representative western blots for insulin-induced phosphorylated (p-Akt) and total Akt protein expression. (B) Quantification of Akt phosphorylation. (C) Respresentative Western blots for phosphorylation of IRS-1 at serine residue (Ser-307) and JNK (Thr-183/Thr-185). (D) Quantification of IRS-1 and JNK phosphorylation. $^{*}p < 0.05$ vs *ob*-AL mice.

of IRS-1, which is mediated by JNK activation, were investigated by western blotting. The phosphorylation of these molecules were significantly increased in *ob*-AL mice compared with *lean*-AL mice (Fig. 4C, D). CR significantly reduced the phosphorylation of both molecules by 41% and 45%, respectively, versus *ob*-AL mice (both, $p < 0.05$).

3.8. CR reduced hepatic ER stress in KK and KK-Ay mice

To extend our findings in CR-induced reduction of hepatic ER stress in *ob*/*ob* mice, similar experiments were performed in another model of obesity, namely KK and KK-Ay mice. As expected, CR for 2 weeks significantly reduced the phosphorylation of PERK and eIF2α and ATF4 protein expression in the liver of these animals (Supplementary Fig. S3A and B).

4. Discussion

In the present study, we demonstrated, for the first time, that CR for 2–4 weeks reduced ER stress in the liver and adipose tissue in a leptin-deficient model of obesity (*ob*/*ob* mice), and in other obese animals. The CR-mediated reduction in ER stress in *ob*/*ob* mice was accompanied by significant weight-loss, reduced hepatic TG content, and improved glucose and lipid metabolism. Although systemic insulin sensitivity was not significantly improved, hepatic insulin signaling was significantly increased by CR. Importantly, JNK and IRS-1 serine phosphorylation were both reduced by CR. Because these two effects are characteristic changes induced in part by IRE1 activation during obesity-related ER stress [4,5,7,8], the reduced phosphorylation of both molecules suggest that CR suppresses IRE1 activity. During CR-mediated weight-loss, *ob*/*ob* mice may lose fat tissue and skeletal muscle mass [16], an important determinant for insulin-induced glucose clearance. This may partially explain the differential effects of CR on glucose tolerance and insulin sensitivity. It is unsurprising that not all of the characteristic phenotypes of *ob*/*ob* mice can be normalized by CR alone and that leptin administration may be required to improve all of the metabolic disorders in *ob*/*ob* mice [15,17,18]. The CR-induced improvements in metabolic status observed in our study are consistent with those reported in other studies showing that CR in *ob*/*ob* or *db*/*db* mice partially improved glucose and lipid metabolism [17–19].

To date, only one clinical study of obese non-diabetic subjects has demonstrated that weight-loss by gastric bypass surgery reduced ER stress in liver and fat tissues, and improved metabolic parameters [7]. In the study, the authors showed reductions in several ER stress markers at 1 year after the surgery. Surgery is an effective approach for achieving weight-loss in obese patients and the effects of the surgery were achieved by reduced food intake and suppressed intestinal absorption and activation of the sympathetic nervous system [20]. Because of limitations in human studies, the short-term effects of surgery were not evaluated. By contrast, we investigated the effects of food restriction alone and the short-term effects of CR on ER stress markers, and demonstrated that CR reduced ER stress within 2–4 weeks. Our findings are consistent with their report because most of the observations on ER stress markers, weight loss, and glucose and lipid metabolism were similar [7]. Importantly, CR for 1 week did not significantly reduced PERK phosphorylation in *ob*/*ob* mice, suggesting that weight loss or reduced tissue TG content are involved in CR-mediated regulation of ER stress.

We also observed parallel changes in PERK, eIF2α and ATF4, as well as other UPR markers, in most cases investigated, suggesting that CR primarily reduces the load for protein folding in the ER in obese animals, and thus reduces the activity of PERK followed by regulation of the eIF2α–ATF4 axis.

The exact cause of ER stress in obesity is still unknown [8–10]. In the present study, although food intake and the nutrient influx to organs significantly declined after the initiation of CR, CR for 1 week failed to reduce ER stress, indicating that the reduction in

nutrient influx to liver or adipose tissues could not fully explain the CR-mediated reduction in ER stress. These results also suggest that a period of reduced food intake exceeding 1 week is needed to significantly reduce ER stress in *ob*/*ob* mice.

The CR-mediated reductions in ER stress, BW and tissue TG content were greater after 4 weeks than after 2 weeks, suggesting time-dependent effects of CR on these parameters. Furthermore, excess nutrient supply and tissue lipid accumulation may cause these metabolic disorders. The effects of further reductions of food intake and other CR methods, such as intermittent fasting [21], on ER stress in the liver and adipose tissues as well as in other important tissues including the heart, brain and kidney in obese animals should be addressed in future studies.

Our results, showing that CR for 4 weeks reduced ER stress in *ob*/*ob* mice and previous observations in obese subjects [7], suggest that the effects of CR on ER stress in obesity are independent of the leptin signal because the circulating leptin concentrations decrease during/after weight loss in obese subjects, and *ob*/*ob* mice have an absolute deficit in leptin signaling. Furthermore, high concentrations of leptin in obese animals, except for *ob*/*ob* mice, are associated with increased ER stress in liver and fat tissues and the recently reported finding that the administration of PBA, but not leptin, reduced hypothalamic ER stress [22], suggest that leptin may not directly reduce ER stress. Because we did not analyze the effects of leptin in this study, this should be addressed in future studies.

Although the CR-mediated improvement in hepatic insulin resistance was closely associated with reductions in visceral fat and liver TG content [23,24], other possible mechanisms for this improvement might include reductions in oxidative stress, production of proinflammatory cytokines [1–3] and ER stress. Indeed, ATF4 was recently reported to be involved in the regulation of obesity, glucose metabolism and energy expenditure [25–27]. We have demonstrated that CR effectively reduces obesity-related upregulation of ATF4, suggesting a possible novel pathway for the prevention and treatment of obesity-related disorders.

In conclusion, in this study we explored the effects of CR on ER stress in obese animal models. We found that CR reduced ER stress in the liver and fat tissue of several animal models of obesity. Our results may provide further insight into the regulation of ER stress in obesity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.120.

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