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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).