HHS Public Access

Author manuscript

Metabolism. Author manuscript; available in PMC 2023 November 01.

Published in final edited form as:

Metabolism. 2018 June ; 83: 92–101. doi:10.1016/j.metabol.2018.01.012.

Caloric restriction improves glucose homeostasis, yet increases cardiometabolic risk in caveolin-1-deficient mice

Korapat Mayurasakorn, MD[1], **Nurul Hasanah**[1,2], **Tsuyoshi Homma, PhD**[1], **Mika Homma**[1], **Isis Katayama Rangel, PhD**[1], **Amanda E. Garza, PhD**[1], **Jose R. Romero, PhD**[1], **Gail K. Adler, MD, PhD**[1], **Gordon H. Williams, MD**[1], **Luminita H. Pojoga, PhD**[1,*]

[1]Division of Endocrinology, Diabetes and Hypertension, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA

[2]Universiti Teknologi MARA, Kuala Lumpur, Malaysia

Abstract

Background and Purpose—The plasma membrane protein caveolin-1 (CAV-1) has been shown to be involved in modulating glucose homeostasis and the actions of the renin-angiotensin-aldosterone system (RAAS). Caloric restriction (CR) is widely accepted as an effective therapeutic approach to improve insulin sensitivity and reduce the severity of diabetes. Recent data indicate that polymorphisms of the CAV-1 gene are strongly associated with insulin resistance, hypertension and metabolic abnormalities in non-obese individuals. Therefore, we sought to determine whether CR improves the metabolic and cardiovascular (CV) risk factors in the lean CAV-1 KO mice.

Materials/Methods—Twelve- to fourteen-week-old CAV-1 knockout (KO) and genetically matched wild-type (WT) male mice were randomized by genotype to one of two dietary regimens: ad libitum (*ad lib*) food intake or 40% CR for 4 weeks. Three weeks following the onset of dietary restriction, all groups were assessed for insulin sensitivity. At the end of the study, all groups were assessed for fasting glucose, insulin, HOMA-IR, lipids, corticosterone levels and blood pressure (BP). Aldosterone secretion was determined from acutely isolated Zona Glomerulosa cells.

Results—We confirmed that the CAV-1 KO mice on the *ad lib* diet display a phenotype consistent with the cardiometabolic syndrome, as shown by higher systolic BP (SBP), plasma glucose, HOMA-IR and aldosterone levels despite lower body weight compared with WT mice on the *ad lib* diet. CAV-1 KO mice maintained their body weight on the *ad lib* diet, but

Corresponding Author: Luminita H. Pojoga, PhD, Division of Endocrinology, Diabetes and Hypertension, Brigham and Women's Hospital, 221 Longwood Ave, Boston, MA 02115. Ph. 617-278-0415; Fx. 617-732-5764, lpojoga@partners.org.

Author contributions

KM, NH, TH, MH, AEG and IKR: data acquisition and analysis; KM, NH, JRR, GHW, and LHP: drafting of the manuscript; GKA, GHW, and LHP: study concept and design; KM and LHP: statistical analysis. All authors contributed to manuscript review and revision.

DISCLOSURES

There is no conflict of interest.

had substantially greater weight loss with CR, as compared to caloric restricted WT mice. CR-mediated changes in weight were associated with dramatic improvements in glucose and insulin tolerance in both genotypes. These responses to CR, however, were more robust in CAV-1KO vs. WT mice and were accompanied by reductions in plasma glucose, insulin and HOMA-IR in CAV-1KO but not WT mice. Surprisingly, in the CAV-1 KO, but not in WT mice, CR was associated with increased SBP and aldosterone levels, suggesting that in CAV-1 KO mice CR induced an increase in some CV risk factors.

Conclusions—CR improved the metabolic phenotype in CAV-1 KO mice by increasing insulin sensitivity; nevertheless, this intervention also increased CV risk by inappropriate adaptive responses in the RAAS and BP.

Keywords

Caloric restriction; insulin resistance; caveolin; cardiometabolic dysfunction; the metabolic syndrome; aldosterone

INTRODUCTION

Caloric restriction (CR) is widely recognized as a fundamental component of the prevention and treatment of diabetes, insulin resistance and the metabolic syndrome [1] especially in the obese population[2–4]. CR improves HOMA-IR, hepatic and adipocyte insulin signaling, and blood pressure (BP) [2, 4–6]. Several predisposing factors for cardiometabolic disturbances - driven by genetics and environment - have been explored extensively in the last decade[7–9].

Insulin resistance, diabetes and the metabolic syndrome also occur in non-obese individuals, who constitute up to 20–60% of patients with type 2 diabetes in many populations (e.g. in northern European[10] and Asian countries[11]) where obesity is not “epidemic”. This “metabolically unhealthy non-obese (MUN)” phenotype can present with similar metabolic abnormalities as seen in obese individuals. For example, studies in individuals with type 2 diabetes (T2DM) have demonstrated that MUN individuals have the same cardiovascular (CV) risks as do obese patients[9, 10, 12, 13]. However, the factors underlying MUN are less clear than for those who are obese.

Our group has shown that polymorphisms of the caveolin 1 (CAV-1) gene were strongly associated with insulin resistance in hypertensive individuals and that CAV-1 KO mice also display abnormal metabolic trends[8], suggesting that the CAV-1 KO mouse model may be translationally relevant to humans who carry polymorphic variants of the CAV-1 gene. Evidence demonstrates co-aggregation and co-heritability between insulin resistance and hypertension, suggesting a close-linked genetic susceptibility of the two conditions[14]. Together with obesity and/or dyslipidemia, these pro-atherogenic risk factors are often classified as the metabolic syndrome (MetS). Recently, we also found that MetS risk is modified by an interaction between the CAV-1 genotype and body mass index (BMI), whereby the minor allele carrier status strongly predicted MetS and diabetes in non-obese but not in obese individuals and was associated *in silico* with decreased CAV-1 expression[15]. Interestingly, the CAV-1 KO mouse is also lean.

The CAV-1 protein is a key component of caveolae, important microdomains on the surface of the plasma membrane of most cells. Highly abundant in adipose and vascular tissues, CAV-1 has been shown to play an intricate role in cholesterol transport/efflux and in the regulation of signaling pathways critical for glucose and BP homeostasis[8, 16–20].

Effects of CR on insulin resistant individuals with a lean phenotype are largely unknown. Therefore, understanding the CR-mediated changes in the CAV-1 KO mouse model might reveal important information for the clinical use of CR in non-obese diabetic and/or insulin-resistant humans carrying CAV-1 gene polymorphisms. We hypothesized that CR would improve all of the components of the metabolic syndrome in the lean, CAV-1 KO mouse.

2. MATERIALS AND METHODS

2.1 Animals and CR procedure

Details for the animals used in this study are provided in the Supplementary Data. The experimental design is indicated in Fig. 1. Day 0 was defined as the day when animals of both genotypes were randomized to *ad lib* or CR. Animals were housed in individual cages on day −5, with *ad lib* access to both water and chow. After acclimation, twenty-four hour urine was collected on day −3 and BP was measured on day −2. Food was provided twice/d and food intake of individual animals was measured daily to establish basal food intake. On day 0 mice from each genotype were randomized into two subgroups: *ad lib* and 40% CR (i.e. 60% of *ad lib* intake), for a total of four genotype-diet groups (n=12/group): WT-*ad lib*, CAV-1 KO-*ad lib* (CAV-*ad lib*), WT-40% CR (WT-CR) and CAV-1 KO-40% CR (CAV-CR). CR mice were given a diet corresponding to approximately 60% of the amount of food consumed by mice of the same genotype fed an *ad lib* diet. Body weight and food intake were measured periodically at 4 PM. Mice remained on *ad lib* or CR diets for 4 wk. On day 26, mice were transferred to individual metabolic cages for 24-hr urine collection, and the assessment of food and water intake. The next day, BP was measured. At completion of the experiment, mice were euthanized under deep anesthesia with isoflurane, the abdominal and thoracic cavities were opened, blood samples were collected from the abdominal aorta in BD microtainer tubes (EDTA) and the kidneys and adrenal glands were rapidly harvested. Kidney samples were snap frozen and stored at −80°C until processed for transcript assessment. Adrenal glands were immediately processed for acute stimulation studies. Blood samples were separated and plasma kept at −80°C until assayed.

2.2 Insulin measurements and HOMA-IR calculation

Plasma samples collected at day 28 were stored at −80°C until assayed. Measurements were made by enzyme-immunolinked assay, using the Mouse Ultrasensitive Insulin kit (Alpco Diagnostics, Salem, NH), as previously described[8]. Homeostasis model assessment-insulin resistance (HOMA-IR) was calculated to assess changes in insulin resistance (fasting insulin (mU/L) × fasting glucose (mg/dL) / 405) [21].

2.3 Fasting glucose and glycemic response to intraperitoneal glucose tolerance test (GTT)

Glucose metabolism was assessed on day 0 and day 21 of the experiment. Mice were fasted for 15–18 hours (overnight), and the next morning baseline body weight (BW) was recorded.

Mice were challenged with intraperitoneal (ip) glucose (1.5 mg/g BW) consistent with our previous studies in rodents[18]. At 0, 15, 30, 60, 90 and 120 min, blood was sampled from the tail for glucose determinations with a glucometer (AlphaTrak 2, Abbott, IL) At the end of the GTT, mice were returned to cages and allowed access to food and water. Areas under the curves (AUC) were calculated by using the trapezoid method [22].

2.4 Insulin tolerance test (ITT)

On day 24, after a 4h fast, mice were administered an ip injection of 0.75 IU/kg BW recombinant human insulin (Humulin R, 100 U/mL, Eli Lilly, Indianapolis, IN). The dose of insulin given to each mouse was consistent with other published studies[17, 23]. At 0, 15, 30, 60, 90 and 120 min blood glucose was sampled from the tail as described above. Inverse areas under the curves (Inverse-AUC) were calculated by subtracting a rectangular area between the highest and lowest glucose values on y-axis from areas calculated by the conventional trapezoid method.

2.5 Blood pressure (BP) measurements

Systolic (SBP) and diastolic BP (DBP) as well as heart rate (HR) were measured noninvasively in conscious mice on days −2 and 26, by tail-cuff plethysmography (BP analyzer, Kent Scientific, CT) as previously described[18, 24]. Conscious mice were warmed at 30°C for 10 min and allowed to quietly rest before BP measurements. BP measurements were taken in the morning in a quiet room, and mice were kept calm and handled by the same person. Mice were acclimatized to the tail cuff BP measurement procedure at least once/week prior to the measurements. The rate pressure product (RPP), an indicator of myocardial workload, was calculated by multiplying SBP by HR. The pulse pressure (PP) [25] was calculated as the difference between SBP and DBP.

2.6 Aldosterone, plasma renin activity (PRA), corticosterone and urine Na^+ measurements

Plasma aldosterone and corticosterone levels were determined by RIA as per manufacturer's instructions (Coat-A-Count, Siemens, Los Angeles, CA). The PRA determination involved an initial incubation of plasma to generate angiotensin I, followed by quantification by RIA using a solid-phase RIA kit (DiaSorin, Stillwater, MN), as previously described [26]. Na^+ concentration was assessed in urine samples collected on day 26, by an enzymatic colorimetric test (Stanbio, Boerne, TX).

2.7 Lipid profile assessment

Blood samples for lipid profiles including total cholesterol, triglyceride, HDL and LDL cholesterol and non-esterified fatty acids (NEFA) were assayed by an enzymatic colorimetric test which had been standardized by human sera or plasma prior to lipid measurements to have < 5% coefficient variation (Wako Chemicals, Richmond, VA)[27, 28].

2.8 Expression profiles in the kidney

Quantitative real-time polymerase chain reaction was performed as previously described[29]. Briefly, total mRNA was extracted using the RNeasy mini kit (Qiagen, CA), and the complimentary DNA (cDNA) was synthesized with the First strand cDNA Synthesis

Kit (GE Healthcare Life Sciences, Pittsburgh, PA) with random hexamer primers. The ABI PRISM 7000 Sequence Detection System real-time quantitative PCR (Applied Biosystems, Foster City, CA) was used to perform the real-time PCR using TaqMan Gene Expression Assays for mouse serum and glucocorticoid-regulated kinase 1 (SGK1) and α-endothelial sodium channel-α (αENaC). Reactions were analyzed with the ABI software using the ΔΔCt method. Target gene expression was normalized to 18S rRNA levels.

2.9 *Ex vivo* adrenal cell studies

Zona Glomerulosa (ZG) cells were obtained from freshly-isolated adrenals after animals were sacrificed as previously detailed[30, 31]. Briefly, adrenals were dissected and the capsular (glomerulosa) portion was isolated from the decapsulated (fasciculata-reticularis) portion. The ZG cell suspension was incubated at 37°C for 50 min in Krebs–Ringer bicarbonate buffer containing 2 mg/mL of glucose, 4% bovine serum albumin, and 3.7 mmol/L of K^+ (KRBGA), supplemented with collagenase (3.7 mg/mL) and DNAse (0.05 mg/mL). The cells were centrifuged at 700 g, 4°C for 10 min, the supernatant was discarded, and the pellet washed twice and resuspended in KRBGA. For each experiment, 35–45 × 10^3 cells/0.5 mL of KRBGA were incubated alone (basal) or in the presence of agonist (ACTH [10^{-10} M, GeneraMedix, Liberty Corner, NJ] or Ang II [10^{-9}, 10^{-8}, 10^{-7} M, #9525, Sigma, St. Louis, MO]) for 1 hr at 37°C under a 5% CO_2/95% O_2 atmosphere. At the end of the incubation, aldosterone release was determined in duplicate as described above. Data were normalized to the number of cells in each incubate and then reported as fold increase over basal level aldosterone release.

2.10 Statistical analyses

Data are presented as mean ± SEM. Student's *t*-test for paired data was used for comparison of two means before and after treatment. One-Way ANOVA, followed by post hoc Bonferroni correction to account for multiple comparisons, was used to compare between treatment groups. Two-way ANOVA was used for the *ex vivo* adrenal studies. Statistical significance was determined as $p < 0.05$. All statistical analyses were carried out by using GraphPad (GraphPad Inc., CA).

3. RESULTS

3.1 Baseline characteristics

Table 1 shows baseline characteristics of the CAV-1 KO vs. WT animals. At the same age CAV-1 KO mice were significantly lighter than their WT counterparts ($p<0.001$, Table 1). The average food and water intakes were not significantly different in CAV-1 KO vs. WT mice. As compared to the WT, CAV-1 KO mice exhibited, at baseline, significantly higher levels of fasting blood glucose, insulin resistance, SBP and pulse pressure ($p<0.05$, Table 1), consistent with a MetS phenotype in these animals. Importantly, CAV-1 KO vs. WT also displayed a dysfunctional renin-angiotensin-aldosterone-system (RAAS), as shown by higher aldosterone levels, lower PRA levels and higher aldosterone/PRA and aldosterone/corticosterone ratios ($p< 0.05$, Table 1).

3.2 CAV-1 KO mice were leaner and displayed delayed weight gain

Over 4 weeks, the growth curves for the animals on *ad lib* diet were similar (slopes: CAV-*ad lib* vs. WT-*ad lib*; 0.06±0.02 vs. 0.08±0.02 g/day, p=0.34); however, the CAV-*ad lib* mice weighed at least 10% less than the WT-*ad lib* mice at each time point (p<0.001, Fig. 2A). Consistent with this observation, the total weight gain over 4 weeks was significantly less in the CAV-*ad lib* than in the WT-*ad lib* mice, (Fig. 2C, p<0.05). These data are in agreement with the previously reported resistance to diet-induced weight gain in the CAV-1 KO[16]. Interestingly, both genotypes showed a significant trend for weight loss over the 4 weeks of CR diet, which was more pronounced in the CAV-CR compared to the WT-CR mice (slopes: −0.23±0.02 vs. −0.09±0.01 g/day, p<0.001) (Fig. 2B). CR effectively-blunted the BW gain in both genotypes starting on the first week of feeding, reaching significance (p<0.05) on day 6 for the CAV-CR vs. CAV-*ad lib*, and on day 9 for the WT-CR vs. WT-*ad lib* groups. At the end of the 4-wk protocol the BW in CAV-CR mice was almost 30% less than in their WT counterparts (p <0.001) (Fig. 2 B). Indeed, the total weight loss over 4 weeks of CR was significantly higher in the CAV-CR (6.4±0.6 g, 23.2±2.0%) than in the WT-CR mice (2.2±0.8 g, 6.6±2.5%) (Fig. 2C, p<0.05). These differences could not be attributed to differences in absolute food intake between genotypes on either diet (Fig. 2D), but rather to the impact of CR on cumulative feed efficiency (change in BW relative to food intake over the duration of the study) (mg/g food, CAV-CR: −74.8±7.9 vs. WT-CR: −24.3±9.8, p < 0.001). In addition, the food consumption normalized by BW was higher in CAV-*ad lib* vs. WT-*ad lib* (g food/100g BW, 16.5±1.0 vs. 12.3±0.7, p<0.05) but this difference was not significant between genotypes on the CR diet (12.8±0.7 vs. 10.5±0.2).

3.3 CR induced stronger improvements in glucose homeostasis in CAV-1 KO vs. WT mice

3.3.1 Effect of CR on fasting glucose, insulin and HOMA-IR levels—Fasting glucose, insulin and HOMA-IR levels in the *ad lib* groups (Table 2) were not significantly different from those recorded at baseline (Table 1). As shown in Table 2, fasting glucose, fasting insulin and HOMA-IR levels in CAV-*ad lib* mice were significantly higher than those in WT-*ad lib* animals (p<0.01), consistent with the results obtained at baseline (Table 1). Interestingly, fasting glucose, insulin and HOMA-IR levels in WT-CR were not significantly different from those in WT-*ad lib* mice; however, CR significantly improved these parameters in the CAV-1 deficient animals (p<0.01), decreasing them to levels indistinguishable from those observed in the WT (Table 2).

3.3.2 Effect of CR on the response to GTT—We examined whether CR was associated with improved glucose tolerance in the lean, CAV-1 deficient mice. In animals maintained on the *ad lib* diet (dark symbols in Fig. 3A–B), the time to reach maximum glucose concentration (Tmax) during GTT was 30 min for both the WT and the CAV-1 deficient animals. However, by the 90 min and 120 min time points, glucose levels dropped to only 85±9.1% and 73.5±6.7% in the CAV-*ad lib*, respectively (Fig. 3B), as compared to 63±2.8% and 56±2.8%, respectively. in the WT-*ad lib* group (Fig. 3A, p=0.03 for either time point). Indeed, the calculated AUC for the CAV-*ad lib* animals was also significantly greater than the corresponding one for the WT-*ad lib* (Fig. 3C). Compared to animals on *ad lib* diet, WT-CR and CAV-CR mice (open symbols in Fig. 3A–B) displayed improved glucose tolerance, as shown by the decrease in Tmax from 30 min to 15 min in both genotypes, as

well as by the significant drops in circulating glucose levels at t=30, 60, 90 and 120 min. This CR-induced improvement was also reflected in the calculated AUC: this parameter was 18% lower in the WT-CR vs. WT-*ad lib* ($p<0.01$), and 36% lower in the CAV-CR vs. CAV-*ad lib* ($p<0.01$) (Fig. 3C).

3.3.3 Effect of CR on the response to ITT—Because CR led to improvements in both fasting insulinemia and glucose tolerance responses, we next sought to determine if CR induced any changes in insulin responsiveness in the lean CAV-1 KO mice. Glucose profiles from the ITT were normalized to fasting glucose values (Fig. 3D). To quantify the differences in insulin sensitivity amongst experimental groups, inverse AUC values for each of the glucose curves from ITT were determined with higher values reflecting more insulin sensitivity (Fig. 3E). Glucose levels at t=0 were significantly higher in the CAV-*ad lib* than in their WT counterparts (138±6 vs. 100±7 mg/dL, $p<0.001$), consistent with the results above. The inverse AUC in the CAV-*ad lib* was also less than in the WT-*ad lib* animals (Fig. 3E), although this difference did not reach significance. Compared to the *ad lib* groups, CAV-CR and WT-CR mice displayed increased insulin-induced glucose uptake (insulin sensitivity) (Fig. 3D–E), with inverse AUC levels that were 55% higher in the WT-CR vs. WT-*ad lib* ($p<0.01$), and 151% higher in the CAV-CR vs. CAV-*ad lib* ($p<0.01$).

3.4 The effect of CR on lipid and leptin levels was similar in CAV-1 KO and WT mice

CR impacted not only upon total caloric intake but also upon individual fat intake, thus potentially affecting circulating lipid homeostasis. Indeed, as compared to WT-*ad lib*, WT-CR mice displayed an expected marked decrease ($p<0.0001$) in triglyceride levels (Fig. 4A); CAV-CR animals also showed a similar and strong reduction in circulating triglycerides ($p=0.0003$), with levels that were significantly lower in the CAV-CR mice vs. WT-CR (Fig. 4A). Total cholesterol levels were also decreased in the WT-CR and CAV-CR groups; however, this effect reached significance in the CAV-CR vs. CAV-*ad lib* ($p=0.04$) but not in the WT-CR vs. WT-*ad lib* ($p=0.15$). Interestingly, HDL, LDL and NEFA levels (not shown) were not significantly altered in either WT-CR or CAV-CR as compared to their *ad lib* counterparts. Compared to the *ad lib* diet, CR was associated with a nonsignificant trend for reduced leptin levels in both genotypes (WT-*ad lib*: 4.3±1.7 ng/ml; WT-CR: 2.1±0.7 ng/ml; CAV-*ad lib*: 3.6±0.5 ng/ml; CAV-CR: 1.3±0.3 ng/ml, n=3–4 mice/group), although values were only available from a few mice.

3.5 Differential effect of CR on blood pressure levels in CAV-1 KO and WT mice

SBP, pulse pressure and rate pressure product levels after the *ad lib* diet (Fig. 5) were not significantly different from the values obtained at baseline in the same groups (Table 1). CAV-*ad lib* animals displayed significantly higher SBP, pulse pressure and rate pressure product levels, as compared to WT-*ad lib* mice, in agreement with our previous reports (Fig. 5A–C)[18, 24, 32]. Interestingly, as compared to CAV-*ad lib* animals, CAV-CR mice displayed significantly elevated SBP, as well as pulse pressure and rate pressure product levels, an effect that was absent in the WT (Fig. 5A–C). These differences could not be attributed to differences in Na^+ intake between groups, since the CR diet was specifically designed to provide the same amounts of Na^+ and K^+ over 24h, as the *ad lib* diet. Indeed, 24h Na^+ excretion was not significantly different between the four study groups (Fig. 5D).

3.6 CR induced dissimilar effects on RAAS in CAV-1 KO and WT mice

To assess a possible cause for the detrimental effects of CR on BP hemodynamics, we assessed components of the RAAS in the four experimental groups. Plasma aldosterone levels were significantly higher in CAV-*ad lib* vs. WT-*ad lib* (104.6±9.2 vs.70.5±7.2 ng/dL, p=0.009) (Fig. 6A). Although aldosterone levels seemed slightly elevated in the WT-CR (85.8±15.9 ng/dL) as compared to the WT-*ad lib* group, this trend did not reach significance. However, CAV-CR mice had markedly elevated aldosterone levels (172.5±28.9 ng/dL), which were significantly higher not only than those in CAV-*ad lib* ($p<0.05$), but also as compared to the levels in the WT-CR group ($p<0.005$, Fig. 6A). PRA levels were significantly lower in the CAV-*ad lib* than in the WT-*ad lib* mice (Fig. 6B) but were not modified by CR. Consequently, aldosterone/PRA ratios were significantly higher in CAV-1 KO as compared to their WT counterparts, irrespective of the diet ($p<0.05$, Fig. 6C). Thus, our data suggest that the abnormal aldosterone production in the caloric restricted CAV-1 deficient animals is independent of PRA levels.

3.7 Differential effect of CR on adrenal function in CAV-1 KO and WT mice

We next assessed cortical adrenal responsiveness to typical secretagogues (AngII, K^+, ACTH) in isolated ZG cells[30] from the four experimental groups (Fig. 6D). The two way ANOVA analysis demonstrated that – as expected – the overall effect of the three secretagogues in ZG cells was significant [p-value (AngII) = 0.01; p-value (K^+) = 0.001; p-value (ACTH) < 0.0001]. There was no significant interaction between the effect of the secretagogues and the animal groups. As compared to the WT, the CAV-1 KO trended towards higher levels of aldosterone secretion at baseline and in response to the secretagogues. These trends did not reach significance in the CAV-*ad lib* vs. WT-*ad lib*; however, the CAV-CR displayed significantly elevated aldosterone levels at baseline, as well as increased adrenal responsiveness to the three secretagogues, as compared to the WT-CR ($p<0.05$, Fig. 6D).

To exclude an ACTH-mediated rise in circulating aldosterone levels in the CAV-*ad lib* and CAV-CR groups, we assessed circulating corticosterone levels in the four study groups. As compared to *ad lib*, CR induced significantly elevated corticosterone levels irrespective of genotype ($p<0.05$, Fig. 6E), consistent with an increased level of stress in the caloric restricted animals[33]. These data are also consistent with an increase in physical activity observed by us in both strains within the 1st week after CR, as previously reported for other strains[34]. Circulating aldosterone/corticosterone ratios were significantly elevated in CAV-1 KO vs. WT mice, irrespective of the diet they were on. Interestingly, CR induced a marked reduction in this ratio in the WT ($p<0.05$) but not the CAV-1 KO mice (Fig. 6F), suggesting an inability of the CAV-1 KO mice to downregulate their aldosterone production in the face of elevated ACTH (corticosterone) levels.

3.8 CR had a stronger effect on renal aldosterone targets in CAV-1 KO and WT mice

To determine the aldosterone effect at the tissue level, we assessed the transcript expression in the four experimental groups, for two typical downstream targets of aldosterone in the kidney: the serum and glucocorticoid-regulated kinase 1 (SGK1) and the epithelial Na^+ channel (ENaC) (Fig. 7). Both SGK1 and ENaC levels were significantly higher in kidney

tissues from the CAV-*ad lib* vs. WT-*ad lib* (Fig. 7A–B). CR induced significant increases in SGK1 and ENaC in the WT and even more so in the CAV-1 deficient animals, with CAV-CR mice displaying ~10× increases in expression as compared to the WT-*ad lib* – consistent with increased renal activation of the aldosterone pathway in these animals.

DISCUSSION

The present study was designed to test the hypothesis that CR can improve insulin resistance and cardiometabolic abnormalities in the CAV-1 KO mice, which display an insulin-resistant, albeit lean phenotype, similar to humans that carry polymorphisms in the CAV-1 gene[8, 15]. Our results herein demonstrate that the CR regimen has similar directional effects in WT and CAV-1 KO mice: an increase in stress hormone, i.e. corticosterone, levels, paralleled by reductions in BW, insulin resistance (as assessed by ipGTT and ipITT), triglyceride and total cholesterol levels, as well as in circulating leptin levels. Interestingly, the effects on BW and insulin sensitivity appeared greater in the CAV-1 KO compared to the WT, despite a similar effect on corticosterone levels. In CAV-1 KO (but not in WT mice), CR led to marked reductions in fasting glucose, insulin and HOMA-IR levels. However, in contrast to its beneficial effects on most metabolic parameters, CR induced – in the CAV-1 KO – a primary hyperaldosteronism state. This was documented by a marked increase in circulating aldosterone levels paralleled by higher aldosterone production by ZG cells and increased aldosterone/renin ratio, as well as elevated renal SGK1 and ENaC expression in the CAV-CR animals; consequently, systolic blood pressure, the rate pressure product and the pulse pressure were also increased. In contrast to these consequences of CR in CAV-1 KO mice, CR had no significant effect on circulating aldosterone, ZG responsiveness or hemodynamics in WT animals.

In agreement with previous studies, CAV-1 KO exhibited small weight for age[18, 35]. The mechanisms responsible for this phenomenon remain unclear. Recently, Asterholm et. al.[35] suggested that this may likely be due to “metabolic inflexibility” and reliance on carbohydrate intake in the CAV-1 KO, which prevent effective adaptation to changing nutrient availability. Our findings, showing that caloric restricted CAV-1 KO mice have poor cumulative feed efficiency as compared to WT, support this contention. Likewise, due to poor adaption to altered metabolic conditions following CR, CAV-1 KO mice lost substantially more BW than WT mice.

Our group documented that CAV-1 gene variants associate with reduced insulin sensitivity in hypertensive populations[8] as well as with increased risk of MetS in non-obese individuals and decreased CAV-1 expression levels[15]. Similarly, CAV-1 deficient mice recapitulate this phenotype: higher BP, hyperglycemia, higher insulin and HOMA-IR levels, and impaired glucose tolerance, in accordance with our previous reports[18, 36]. The results herein expand on our prior findings in mice, showing that CR normalizes the glucose responses to ipGTT and ipITT in both the WT (as previously reported by us[37] in the rat) and CAV-1 KO; however, fasting glucose, insulin and HOMA were only improved in the CAV-CR mice. We postulate that the normalized fasting glucose and glucose responses in the caloric restricted CAV-1 KO may be mediated through mechanisms related to improved hepatic glucose homeostasis and insulin sensitivity.

CR is known to improve lipid profiles[38]. As previously reported by us[37] and others, circulating triglyceride and total cholesterol levels were reduced in the WT-CR animals. A similar response was observed in the CAV-CR, yet the improvement in triglycerides was significantly greater in the CAV-1 KO than in the WT, but no differences in total cholesterol were observed between genotypes – suggesting that the improvement in glucose homeostasis in CAV-1 KO mice may mediate the changes in triglycerides, but not the changes in total cholesterol, by uncertain mechanisms.

Determinations of components of RAAS could show mechanistic changes after CR, affecting blood pressure and fluid volume. Numerous studies showed that aldosterone and PRA levels decreased during high salt intake[24, 39] and/or dietary weight loss[40]. However, in the current study aldosterone, PRA and BP levels were not changed after CR in the WT, but were altered in the CAV-1 KO. Intriguingly, the CAV-1 KO on an *ad lib* diet appears to display a state of mild hyperaldosteronism that is exacerbated by CR---a condition that is not present in the WT mice. This conclusion is supported by the higher circulating aldosterone, higher aldosterone/renin ratio and greater aldosterone output from stimulated ZG cells in the CAV-CR mice. In agreement with our recent findings in rats, CR also induced an increase in the renal expression for two typical aldosterone targets in the kidney, SGK1 and ENaC [37], yet this increase was greater in the CAV-1 KO, consistent with an activation of the aldosterone/MR pathway in this model. Nutritional deprivation has been shown to lead to increases in systemic stress hormones[33] such as ACTH, which could lead to adrenal hyperresponsiveness and exaggerated production of corticosteroids, as we have previously confirmed in a rat model of CR[37]. In the current study, CR increased plasma corticosterone levels similarly in WT and CAV-1 KO mice. Interestingly, the aldosterone/corticosterone ratio was suppressed in the WT-CR animals, indicating a negative feedback regulation of aldosterone production in the face of increased ACTH. However, this ratio was increased in the CAV-1 KO mice, and failed to downregulate in response to CR – potentially suggesting an impaired ability in these animals to effectively modulate aldosterone secretion in response to stress. There was also a logical secondary cardiovascular readout---the increased BP. These results differ from data in obese humans demonstrating that CR caused dramatic declines in both PRA and aldosterone levels and consequently reduced blood pressure[40, 41]. However, as stated above, polymorphic variants of the CAV-1 gene were associated, in humans, with the metabolic syndrome, and this association was stronger in lean, than in obese individuals[15]. Interestingly, our group has previously demonstrated that CAV-1 gene variation in humans associates with decreased CAV-1 expression, and that CAV-1 deficiency in mice and in humans may activate aldosterone/MR signaling on several pathways of glycemia and dyslipidemia[36]. Unfortunately, there are no published data regarding CR in lean humans who carry the CAV-1 risk allele.

The increases in aldosterone and BP levels after the 4-wk diet in CAV-CR may have long term deleterious effects on cardiovascular function via multiple mechanisms such as inducing expression of pro-inflammatory cytokines and endothelial inflammation [42, 43]. Our recently published reports provide the likely mechanisms responsible for the increased aldosterone. These mechanisms include the interaction of CAV-1, striatin and the mineralocorticoid receptor (MR); MR’s role in mediating an ultra-short feedback loop

in the glomerulosa cell and the aldosterone response to stress. In brief, previous data from our group suggest the existence of a CAV-1, striatin and MR complex, which is disrupted in the absence of CAV-1[44]. We have also shown that striatin is a critical mediator of the MR rapid/nongenomic effects[44, 45]. Thus, in the absence of CAV-1, we speculate that the rapid MR-mediated signaling is at best blunted, if not altogether absent. Such a rapid MR-mediated event may be the ultra-short feedback loop (likely mediated by striatin) recently identified by us in the adrenal ZG cells, whereby MR activation reduces aldosterone secretion in response to secretagogues[46]. Thus, in the absence of CAV-1, the disrupted MR-striatin complex may lead to a compromised ultra-short feedback loop mechanism, and therefore to increased aldosterone secretion from the ZG. Indeed, the current and previous[47, 48] studies document that CAV-1 KO mice display higher circulating aldosterone levels despite suppressed PRA. In our study CR is stressful to the mice as evidenced by the rise in corticosterone levels, presumably secondary to an increase in ACTH. Acutely, ACTH will also increase aldosterone. This rise aldosterone would be dampened in the WT animals because of the intact MR mediated, ultra-short feedback loop. However, in the CAV-1 KO, where the ultra-short feedback loop would be inoperable, the rise in aldosterone would not be blunted. Further studies are needed to test this and other molecular mechanisms.

There are several potential limitations of the present study. First, the use of single time points in the determination of hormone levels due to the small blood volume in mice limited the conclusions that could be drawn since most of these hormones are subject to diurnal variations especially during GTT and ITT. In addition, for the same reason (limited blood volume) we do not have information on insulin levels during the tolerance tests. Further, our study does not provide data on neither hepatic glucose production, nor on insulin-mediated glucose uptake; therefore, our results cannot be interpreted in terms of the effect of CR on hepatic versus peripheral insulin sensitivity. Moreover, long-term studies may yield different outcomes as compared to the results from our intermediate-term study reported herein. Another limitation pertains to several discrepancies between our recent results in rats[37] and the current report in mice: while we observed many similarities between the wild type CR mice and rats, the WT-CR mice did not display the changes in BP, Na excretion and ZG responsiveness that we observed in the rat model. These discrepancies cannot be attributed to differences in diet or protocol between the two studies, but may be due to selective, alternate response mechanisms in the two species. In addition, CAV-1 KO is a known regulator of insulin signaling and receptors specifically in adipose tissues. Future studies of these pathways should increase our understanding of the effect of CR on this phenotype and address the possible risk of cardiovascular injury in human carriers of the CAV-1 risk allele from altered aldosterone and other endocrine hormones[17, 33]. The negative impact of CR on aldosterone and hemodynamics could be secondary to increased stress since the CAV-1 KO mice were smaller at the start of the study and lost more weight. However, if that was the case, one would have observed greater corticosterone levels and higher PRA levels, neither of which was present.

Conclusions

CR is recommended in the management and treatment of various diseases particularly cardiometabolic syndrome. In CAV-1 KO mice, which are lean but also display characteristics of the cardiometabolic syndrome, CR produced the expected improvement in insulin resistance and glucose homeostasis. However, an unanticipated increase in cardiovascular risk was also uncovered: CR induced a likely pathophysiologic primary-type hyperaldosteronism state in the CAV-1 KO that was not observed in the WT. Thus, potentially, a note of caution should be raised for humans carrying the CAV-1 risk allele for the metabolic syndrome; such individuals may exhibit an increased rather than decreased overall cardiovascular risk in response to CR, secondary to inappropriately increased aldosterone production [17, 33].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Anis Amalina Abdul Hamid, Tham Yao and Paul Loutraris for technical assistance.

FUNDING SOURCES

This work was conducted with support from the following grants from the National Institutes of Health K24 HL103845 (GKA), R01 HL087060-01 (GKA), R01 HL104032 (LHP), R01 HL-69208 (GHW), T32 HL007609 (GHW), R01 HL11476 (GHW), as well as from the American Heart Association: 14GRNT20500000 (LHP) and from CAPES: process # 6752-14-4 (IKR).

ABBREVIATIONS

CAV-1	caveolin-1
HOMA	homeostasis model assessment
Ip-GTT	intraperitoneal glucose tolerance test
IP-ITT	intraperitoneal insulin tolerance test
IR	insulin resistance
ad lib	ad libitum group
CR	caloric restricted group
BP	blood pressure
WT	wild type

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HIGHLIGHTS

- Caloric restriction induced the expected improvements in glucose and lipid homeostasis in the lean, CAV-1 KO mice.
- Unexpectedly, caloric restriction increased the cardiovascular risk in these animals.
- The proposed underlying mechanism is a primary type hyperaldosteronism state in the CAV-1 deficient mice.
- Caution should be exercised before advocating caloric restrictive diets in humans that display lower levels of CAV-1 expression (such as those carrying CAV-1 gene variants).

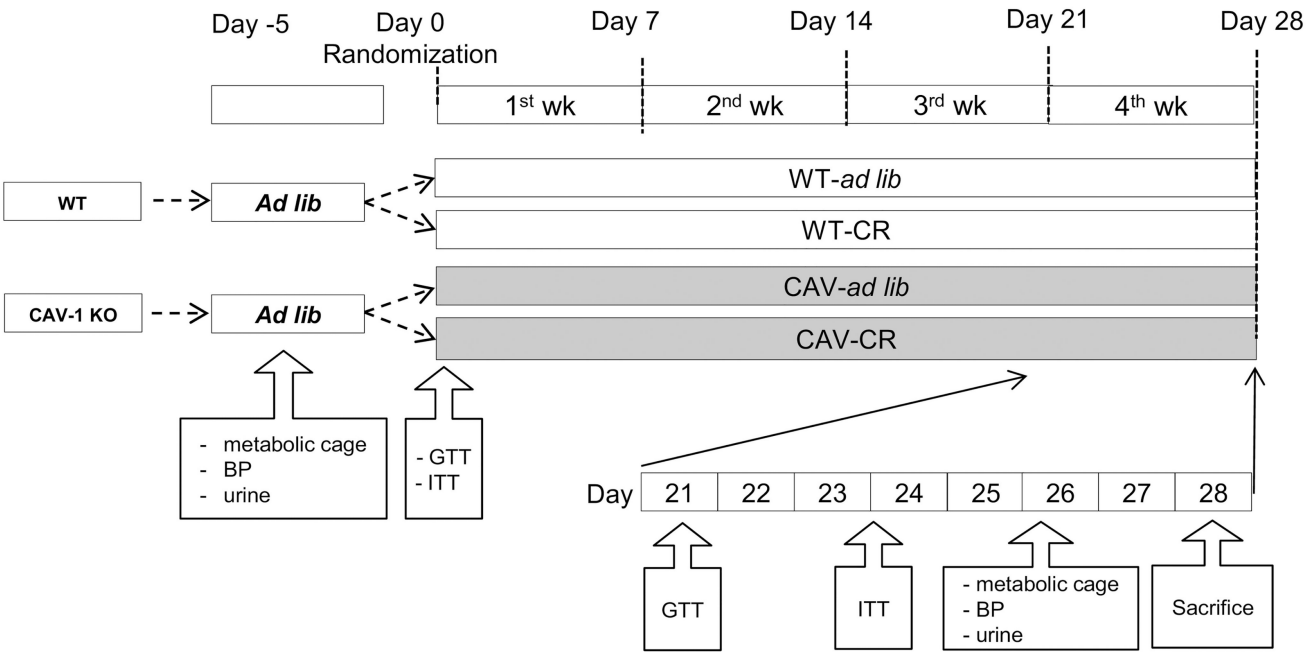

Figure 1. Experimental design

On day −5 mice of both genotypes were acclimatized in an individual cage with free access to ad lib diet and water. On day 0, WT and CAV-1 KO mice were randomized into ad lib (control for each genotype) or CR groups for 4 wk. The timing for performance of the GTT, ITT, BP measurement, urine collection and sacrifice are indicated.

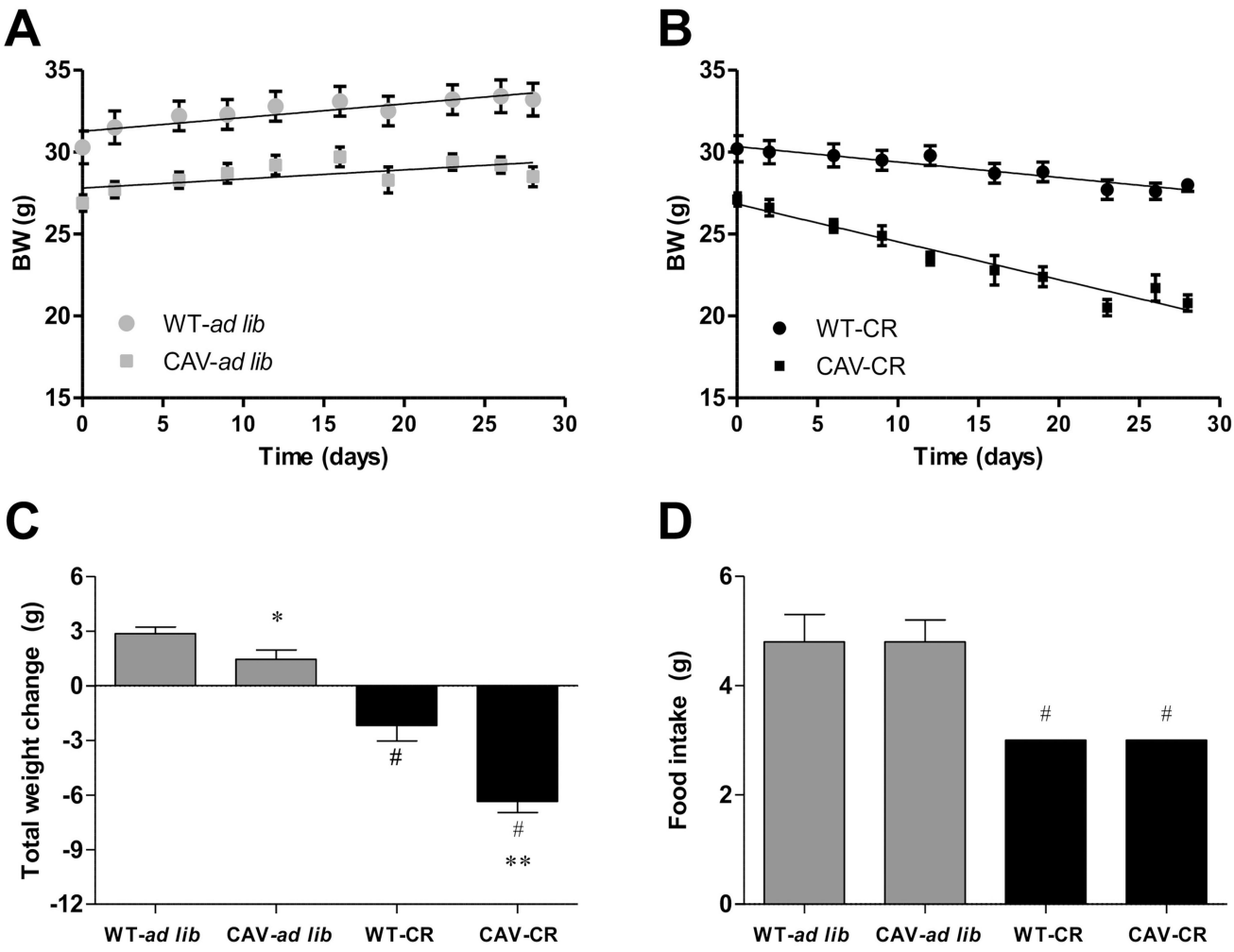

Figure 2. Body weights and food consumption in CAV-1 KO vs. WT mice

(A) Growth curves for WT and CAV-1 KO mice over 4 weeks of *ad lib* diet. (B) Growth curves for WT and CAV-1 KO mice over 4 weeks of CR diet. (C) Absolute body weight changes (g) after 4 weeks of *ad lib* or CR diet. (D) Food intake in WT and CAV-1 KO mice after 4 weeks of *ad lib* or CR diet. *$p < 0.05$ and ** $p < 0.001$ vs. WT on the same diet, $^{\#}$ $p < 0.001$ vs. the same genotype on *ad lib* diet.

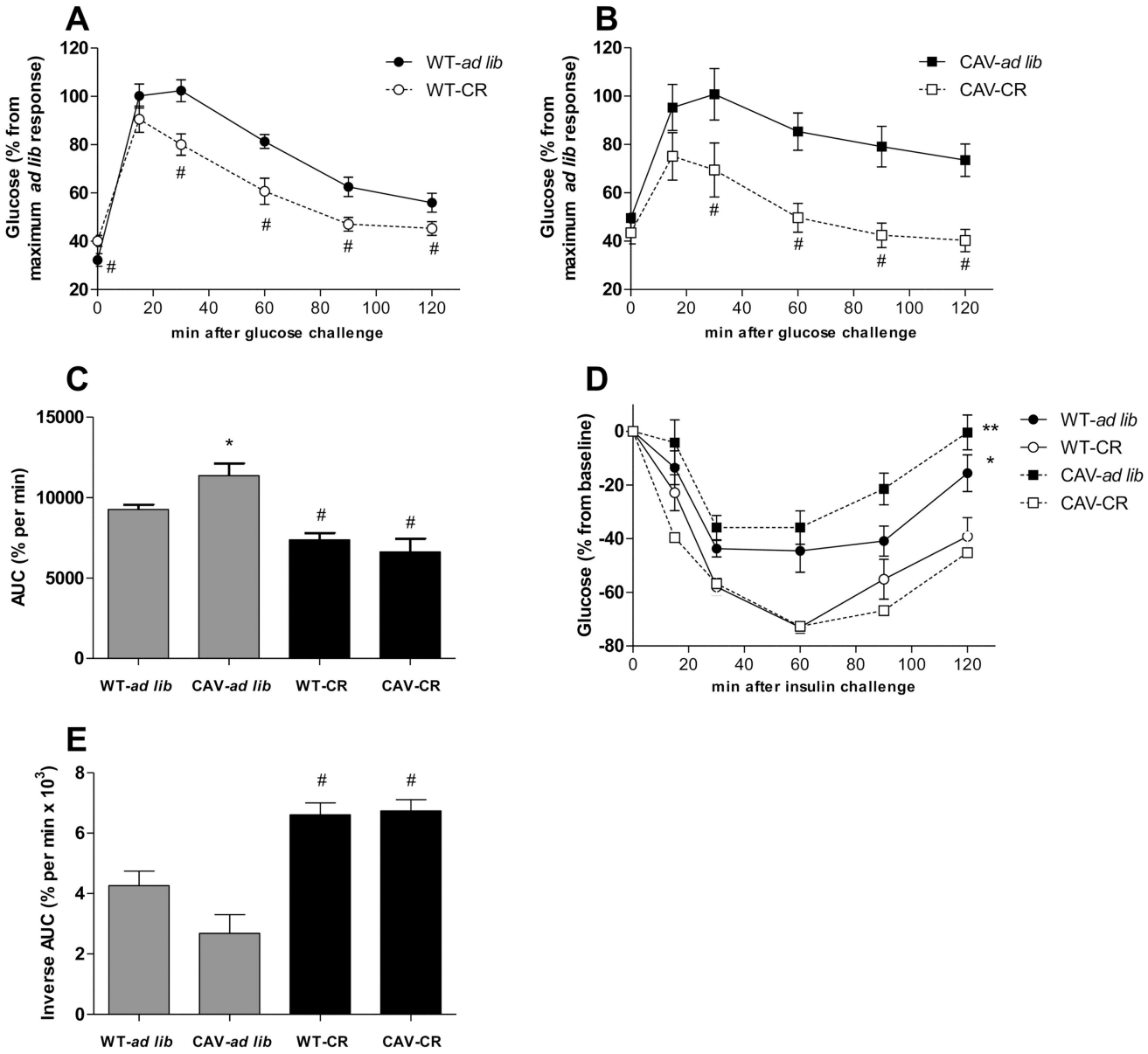

Figure 3. Effect of CR on insulin resistance in WT and CAV-1 KO mice

(A)–(B) glucose excursion during the ip-GTT in WT (A) and CAV-1 KO mice (B) (data expressed as % of the highest glucose levels in the *ad lib* diet group); (C) area under the curve (AUC) during the GTT; (D) glucose levels during the ip-ITT (data expressed as % of fasting glucose); (E) inverse area under the curve (AUC) during the ITT. Data were analyzed using an analysis of variance (ANOVA) of the GTT and ITT profiles assessing the interaction between exposure group (*ad lib* vs. CR) and time during the test. *$p < 0.01$ vs. WT on the same diet, $^{\#}$ $p < 0.01$ vs. the same genotype on *ad lib* diet.

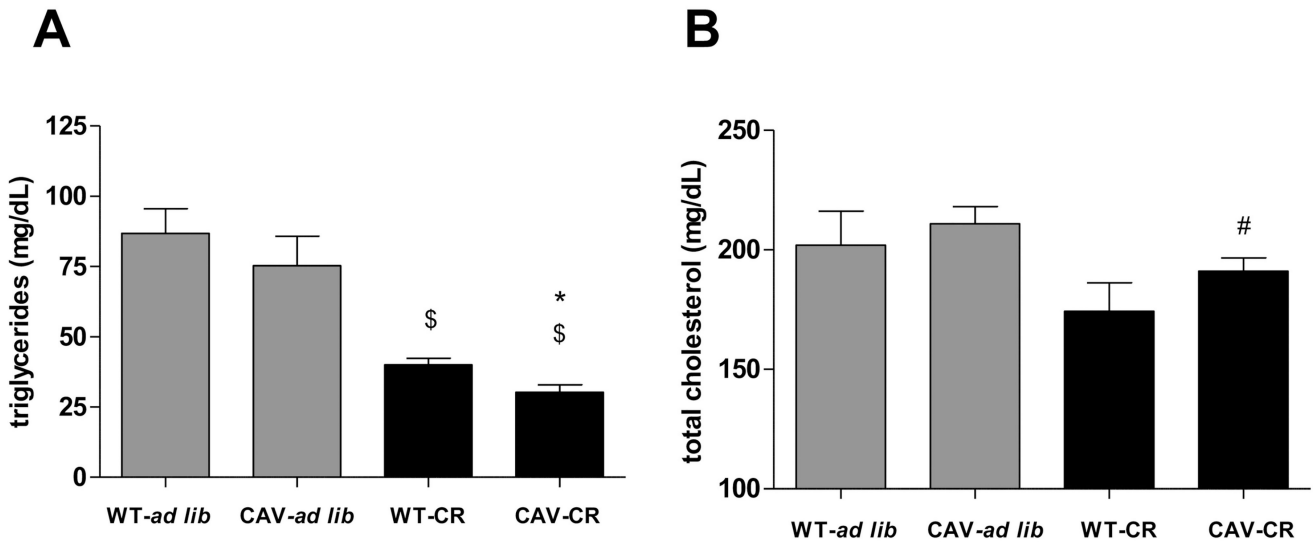

Figure 4. Effect of CR on the lipid profile in WT and CAV-1 KO

Triglycerides (A) and total cholesterol (B) levels were measured in all groups after 10 hour fasting. *$p < 0.05$ vs. WT on the same diet, $^{\$}p<0.001$ and $^{\#}p < 0.05$ vs. the same genotype on *ad lib* diet.

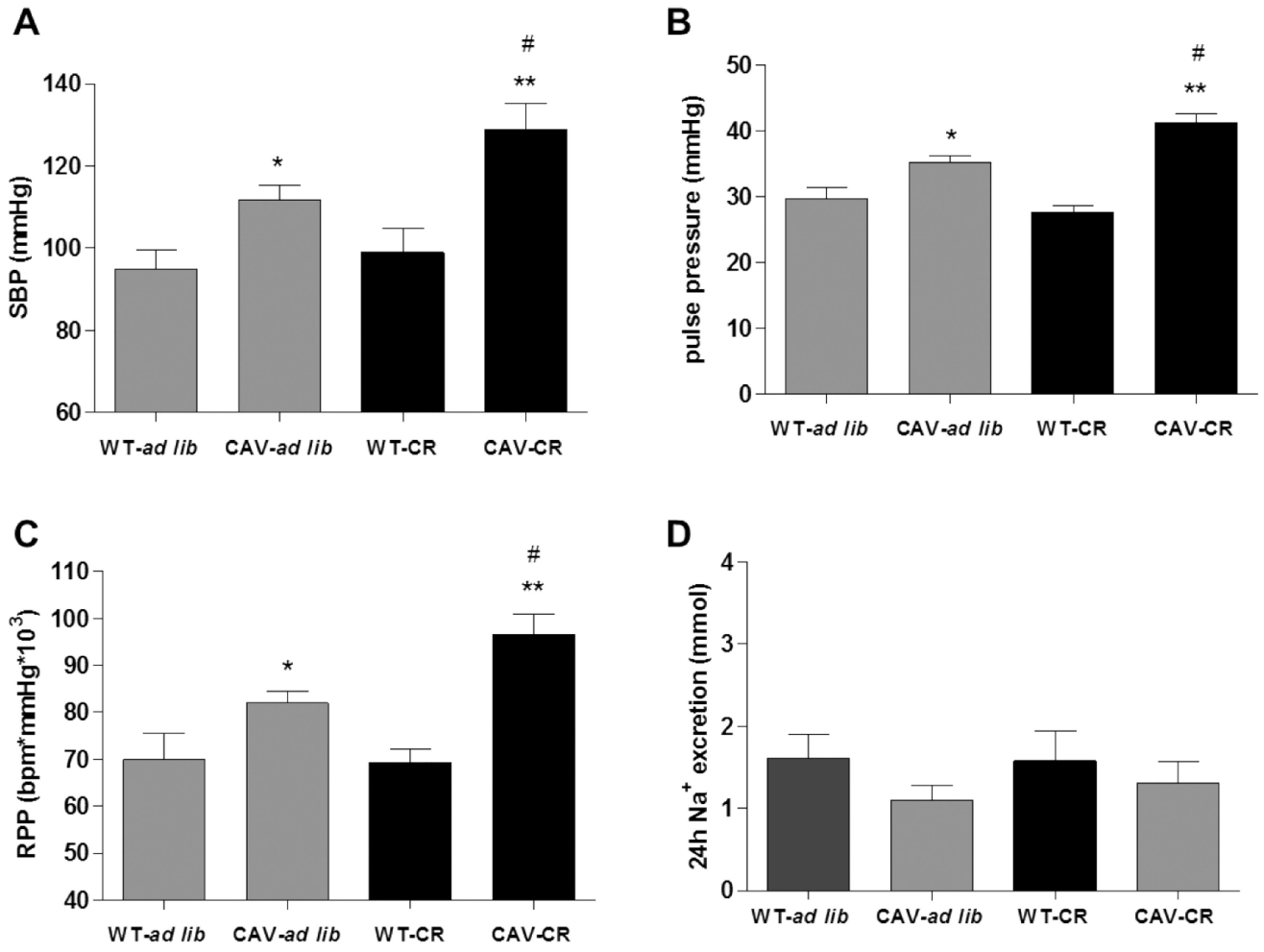

Figure 5. Effect of CR on hemodynamic parameters and Na^+ excretion

SBP levels (A), PP (B), RPP (C) and 24 hr urinary Na^+ levels (D) in WT and CAV-1 KO maintained on *ad lib* vs. CR diet. *$p < 0.05$, ** $p < 0.005$ vs. WT on the same diet; $^{\#}$ $p < 0.05$ vs. the same genotype on *ad lib* diet.

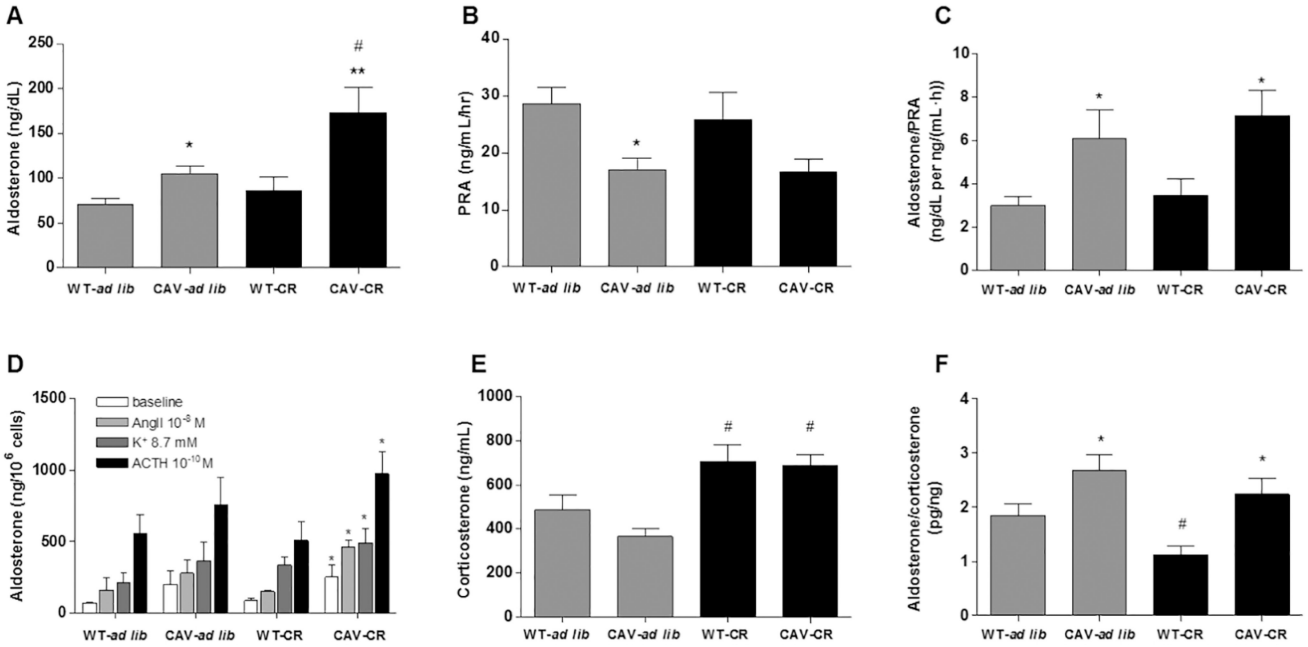

Figure 6. Effect of CR on RAAS and adrenal function

Circulating aldosterone (A), PRA (B), aldosterone to PRA ratio (C), corticosterone (E), and aldosterone to corticosterone ratio (F) in WT and CAV-1 KO maintained on *ad lib* vs. CR diet. Aldosterone release in response to 10^{-8} M Ang II, 8.7 mM K^+ and 10^{-10} M ACTH (D) was measured in Zona Glomerulosa cells acutely isolated from mice in the four experimental groups. *$p < 0.05$, ** $p < 0.005$ vs. WT on the same diet; $^{\#}$ $p < 0.05$ vs. the same genotype on *ad lib* diet.

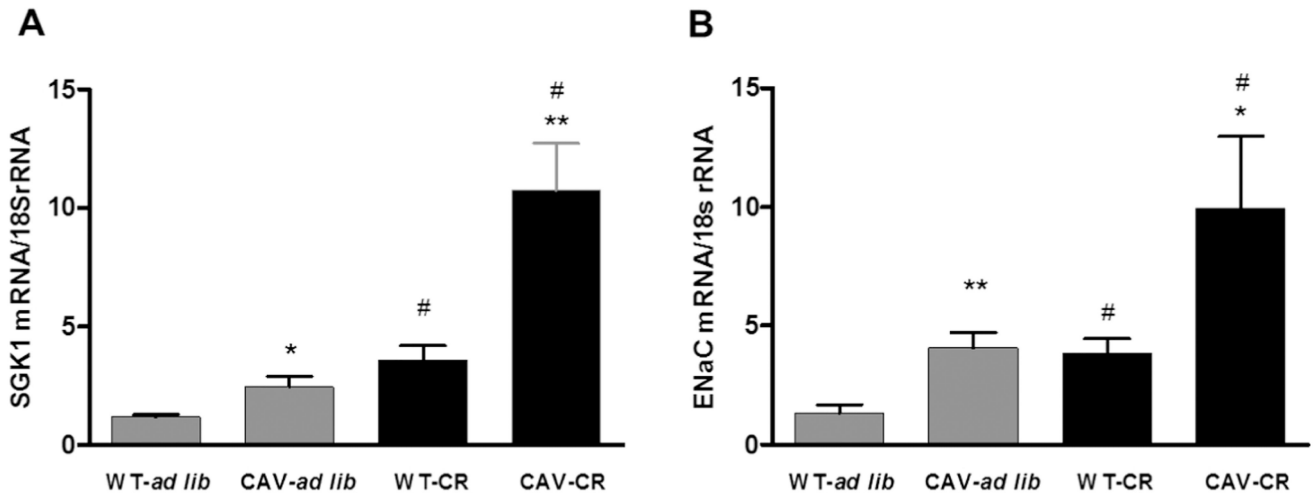

Figure 7. Effect of CR on renal SGK1 (A) and ENaC (B) expression in CAV-1 KO and WT mice

ENaC and SGK1 transcript levels were assessed in kidney tissues from WT ad lib, WT-CR, CAV-1 KO ad lib and CAV-1 KO CR mice. *$p < 0.05$, ** $p < 0.005$ vs. WT on the same diet; # $p < 0.05$ vs. the same genotype on *ad lib* diet.

Table 1

Baseline characteristics of WT vs. CAV-1 KO mice

	WT B6129SF2/J	Cav-1 KO Cav1tm1Mls/J	p-value
Physical Characteristics			
BW (g)	30.3±0.7	27.0±0.3	< 0.0001
FI (g)	5.0±0.18	4.7±0.14	NS
WI (ml)	6.7±0.86	8.4±0.71	NS
Sodium Intake (mEq/d))	3.4±0.2	3.5±0.2	NS
Hemodynamic parameters			
DBP (mmHg)	65.8±5.4	75.7±4.1	NS
SBP (mmHg)	94.8±5.8	112.3±4.0	<0.05
Pulse Pressure (mmHg)	29±1.3	37±1.3	<0.05
Heart Rate (bpm)	749±11	704±22	NS
RPP (bpm*mmHg*10^3)	69.2±4.7	77.6±2.5	NS
Blood parameters			
Glucose (mg/dL)	115.5±4.9	134.5±5.1	<0.05
Insulin (μU/mL)	6.34±0.3	9.51±1.4	0.057
HOMA-IR index	1.76±0.29	3.11±0.53	<0.05
Corticosterone (ng/mL)	485.9±66.0	364.8±38.9	NS
Aldosterone (ng/dL)	77.2±9.2	110.6±10.6	<0.05
PRA (ng/hr)	28.6±2.9	18.8±2.6	<0.05
Aldo/PRA (ng/dL per ng/(mL·h))	3.0±0.4	6.1±1.3	<0.05
Aldo/corticosterone (pg/ng)	1.9±0.22	3.1±0.31	<0.05
Urinary Sodium (mEq/d)	1.9±0.4	1.1±0.2	0.07

Values are mean±SEM., n=12–24 in each group FI; food intake, WI; water intake, PRA; plasma renin activities. BW, FI, WI and BP were assessed before (baseline) experiment. Other parameters were assessed at completion of the study.

Measured at day −7 to day 0: BW, DBP, SBP

Measured at the end of experiment: FI, WI, blood parameters

Table 2

Effect of CR on fasting glucose, insulin and HOMA-IR levels.

	WT-*ad lib*	**CAV-*ad lib***	**WT-CR**	**CAV-CR**
Fasting glucose (mg/dL)	110±8.7	132±4.9*	121±4.8	104±12.3#
Fasting insulin (μU/mL)	5.5±0.34	8.9±1.13*	5.4±0.89	4.2±0.56#
HOMA-IR index	1.52±0.19	2.88±0.41*	1.77±0.30	1.23±0.19#

* $p < 0.01$ vs. WT on the same diet,

# $p < 0.01$ vs. the same genotype on *ad lib diet*.