Short-term caloric restriction in db/db mice improves myocardial function and increases high molecular weight (HMW) adiponectin

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Abstract

Background—Obesity and metabolic syndrome lead to the development of metabolic heart disease (MHD) that is characterized by left ventricular hypertrophy (LVH), diastolic dysfunction, and increased mitochondrial ROS. Caloric restriction (CR) is a nutritional intervention that protects against obesity, diabetes, and cardiovascular disease. Healthy adipose tissue is cardioprotective *via* releasing adipokines such as adiponectin. We tested the hypothesis that CR can ameliorate MHD and it is associated with improved adipose tissue function as reflected by increased circulating levels of high molecular weight (HMW) adiponectin and AMP-activated protein kinase (AMPK) in *db/db* mice.

Methods—Genetically obese *db/db* and lean *db/+* male mice were fed either *ad libitum* or subjected to 30% CR for 5 weeks. At the end of the study period, echocardiography was carried out to assess diastolic function. Blood, heart, and epididymal fat pads were harvested for mitochondrial study, ELISA, and Western blot analyses.

Results—CR reversed the development of LVH, prevented diastolic dysfunction, and decreased cardiac mitochondrial H_2O_2 in *db/db* (*vs. ad lib*) mice. These beneficial effects on the heart were associated with increased circulating level of HMW adiponectin. Furthermore, CR increased AMPK and eNOS activation in white adipose tissue of *db/db* mice, but not in the heart.

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Conclusions—These findings indicate that even short-term CR protects the heart from MHD. Whether the beneficial effects of CR on the heart could be related to the improved adipose tissue function warrants future investigation.

Keywords

caloric restriction; metabolic heart disease; AMPK; adipose tissue

Background

Obesity and metabolic syndrome are associated with metabolic heart disease (MHD) (2). The key features of MHD are left ventricular hypertrophy (LVH), diastolic dysfunction, and compromised mitochondrial function with impaired energetics and increased ROS production (6, 13). Over time, MHD can lead to clinical heart failure with a preserved ejection fraction (HFpEF) which is associated with serious consequences for morbidity and mortality. Caloric restriction (CR) is an effective dietary intervention that protects against obesity-related comorbidities such as diabetes and cardiovascular disease (7). It is not known whether CR can protect the development of obesity-related MHD. However, in rodents, CR ameliorates diastolic dysfunction induced by abdominal aortic constriction, ischemic/ reperfusion injury, and aging (7, 22). Accordingly, our first goal was to determine whether short-term (5 weeks) CR can ameliorate the development of MHD in genetically-obese *db/db* mice.

The mechanism by which CR exerts beneficial effects on the heart is not precisely known. Several possibilities have been suggested including reversal of insulin resistance, activation of myocardial AMPK and/or SIRT1 signaling, and increased circulating levels of the cardioprotecive adipokine adiponectin (8, 23). Recently, we observed that *roux-en-y* gastric bypass, an extreme form of CR, markedly increased adipose tissue AMPK activity and circulating levels of HMW adiponectin in obese patients (28). AMPK is a pivotal nutrient sensor with the ability to regulate whole-body metabolism (10); whereas adipose tissue exerts paracrine and endocrine actions on the heart by releasing a variety of adipokines. For example, circulating levels of adiponectin are decreased in obese humans and rodents, and a deficiency in adiponectin may contribute to the pathophysiology of several cardiovascular diseases including heart failure (19). Therefore, our second goal was to explore adipose tissue AMPK signaling and adiponectin production in *db/db* mice with CR intervention.

Methods

Animals and CR

10-week old male *db/db* and littermate lean *db/+* mice were purchased from Jackson Laboratory (Bar Harbor, ME). Upon arrival, mice had *ad libitum* access to water and chow diet and were on a 12: 12 h light/dark cycle. All 24 mice (12 *db/db* and 12 *db/+*) were singly housed and food intake was recorded daily for 3-4 days. Next, mice were randomized into the following treatment groups for 5 weeks: control (*ad libitum*; D12450B, Research Diets, New Brunswick, NJ), or 30% CR (D03020702B, Research Diets). The CR diet is a 30% reduction in carbohydrate intake, and designed to limit total energy consumption while

insuring adequate nutrition. The amount of food consumed by the *db/db* and the *db/+ ad libitum* groups was recorded daily, and 70% of that was given to the respective CR groups. Body weights were monitored weekly. At the whole-body level, CR prevented weight gain in *db/db* and caused weight loss in *db/+* mice. At the end of the study, hearts were harvested for isolation of mitochondria and the remaining was snap-frozen in liquid nitrogen and stored at −80°C for further use. Epididymal fat pads were excised, snap-frozen, and stored at −80°C. All animal protocols were approved by the Boston University Institutional Animal Care and Use Committee.

Measurements in blood

Mice were fasted overnight and blood samples were collected from the chest cavity when the hearts were excised. Blood glucose was measured using an ACCU-CHEK Aviva glucometer (Roche, Indianapolis, IN). Commercial kits were used to measure plasma levels of insulin (ALPCO, Salem, NH), HMW & total adiponectin (ALPCO), resistin (R&D Systems, Minneapolis, MN), and visfatin (BioVision).

Western blot analyses

Total proteins were isolated from the heart that had been homogenized in RIPA buffer (Boston BioProducts, Ashland, MA) supplemented with protease (Roche) and phosphatase (Sigma-Aldrich, St. Louis, MO) inhibitors. Protein lysates from epididymal fat were prepared in lysis buffer (Cell Signaling, Beverly, MA). Protein concentrations were determined using the bicinchoninic acid assay (Thermo Scientific, Rockford, IL). Ten micrograms of protein were separated by gel electrophoresis, transferred to a PVDF membrane (Millipore, Billerica, MA), and then incubated with primary antibodies as described previously (29). Phospho (Thr[172]) and total AMPK, phospho (Ser[1177]) and total eNOS were purchased from Cell Signaling Technology. Antibody for GAPDH was from Santa Cruz Biotechnology (Santa Cruz, CA). Proteins were visualized by enhanced chemiluminescence (Thermo Scientific), and quantified with Scion Image Software (NIH).

Mitochondrial ROS production

Mitochondria from the hearts were isolated as previously described (16). Briefly, hearts were minced in cold relaxation buffer, homogenized in buffer containing 5 mM HEPES, 1 mM EDTA, 0.25 M sucrose with pH 7.4. The resulting lysates were centrifuged at $500 \times g$ for 10 min, 4°C. The supernatant was centrifuged at $9,000 \times g$ for 15 min, 4°C. The pellet (mitochondria) was re-suspended in 100 μL buffer containing 125 mM KCl, 10 mM HEPES, 5 mM $MgCl_2$, 2 mM K_2HPO_4. Mitochondrial H_2O_2 production was measured using Amplex Ultra Red (Life Technologies, Grand Island, NY) as previously described (25).

Two-dimensional and M-mode echocardiography and Doppler echocardiography

LV dimensions and systolic function were measured in non-anesthetized mice using an Acuson Sequoia C-256 echocardiograph machine equipped with a 15 MHz linear transducer (model 15L8), as we have described previously (20). LV diastolic function was assessed by transmittal and tissue Doppler echocardiography in anesthetized mice as we have previously reported (21).

Statistics

Data are expressed as means ± SE. GraphPad Prism software (La Jolla, CA) was used for all analyses. The comparison was performed using Student's *t*-test, one-way, or two-way ANOVA as appropriate. Minimal level of significance was set at $p < 0.05$.

Results

Effects of CR on body weight and circulating parameters

5 week, 30% CR prevented weight gain in *db/db* and caused weight loss in *db/+* mice (Figure 1A). Compared to the *db/+* group, the *db/db* mice had higher fasting plasma glucose, which increased further with CR (Figure 1B). Similarly, the fasting insulin level was higher in *db/db vs. db/+* (Figure 1C).

CR prevents LVH and diastolic dysfunction in db/db mice

Left ventricle (LV) wall thickness was increased in *db/db* (*vs. db/+*) mice, and was reversed by 5-week CR (Figure 2A). Likewise, diastolic relaxation, as reflected by myocardial peak early diastolic velocity (Em), was decreased in *db/db* (vs. *db/+*) mice and corrected by CR (Figure 2B). LV end-diastolic dimension (EDD), systolic dimension (ESD), and fractional shortening (LVFS) were similar in *db/db* and *db/+ ad lib* mice, and were not affected by CR (Figure 2C-E).

Mitochondrial ROS production

Complex I and II substrate-driven mitochondria H_2O_2 production was measured in isolated hearts. In *db/db* mice, CR (vs. *ad lib*) decreased the rate of H_2O_2 generation by both complex I and II substrates (Figures 3A,B). In contrast, CR had no effect on mitochondrial H_2O_2 production in *db/+* mice (Figure 3C,D).

Effect of CR on adipokine production

In epididymal fat, a visceral depot in rodents, the mRNA levels of adiponectin, resistin, visfatin, and omentin were not significantly different in *db/db vs. db/+* mice on *ad lib* diet, nor were they affected by CR (data not shown). However, the plasma levels of total and HMW adiponectin were decreased in *db/db* (*vs. db/+*) mice (Figure 4A,B). CR increased HMW, but not total adiponectin production (Figure 4A,B). Plasma levels of resistin and visfatin were not different in *db/db* vs. *db/+* mice, and were not affected by CR (Figure 4C,D).

CR activates AMPK in white adipose tissue but not in the heart in db/db mice

AMPK phosphorylation at residue Thr[172] was measured as an indicator of its activity (11). In epididymal fat, AMPK phosphorylation was decreased in *db/db vs. db/+* mice, and was fully restored by CR (Figure 5A,B). Likewise, phosphorylation of an AMPK downstream target eNOS (Ser[1177]) was decreased in *db/db* (*vs. db/+*) on *ad lib* diet, and was restored by CR (Figure 5C). In contrast, cardiac AMPK phosphorylation was only slightly lower in *db/db vs. db/+* on *ad lib* diet, and was not affected by CR (Figure 5D,E).

Discussion

The main finding of this study is that in genetically obese *db/db* mice, CR for 5 weeks ameliorates the key features of MHD including LVH, diastolic dysfunction, and excess mitochondrial ROS. To the best of our knowledge, this is the first study to demonstrate the beneficial effects of short-term CR on obesity-related MHD in a mouse model. We also found that CR led to increased production of HMW adiponectin, which is associated with AMPK activation in white adipose tissue, but not in the heart.

CR prevents the development of MHD

The *db/db* mouse has been shown to develop LVH and both systolic and diastolic dysfunction (4). In this study, we found LVH and diastolic dysfunction in the *db/db* mice on *ad libitum* diet, whereas systolic function as reflected by ejection fraction, was normal. Differences in methodologies (1) and/or mice strains (27) could explain the discrepancy between our findings (normal systolic function) vs. others (systolic dysfunction). In support of our data, systolic function was preserved in diet-induced obese mice despite the presence of diastolic dysfunction (21, 25). Two limitations should be noted. First, the evaluation of LV diastolic function by tissue Doppler does not allow us to determine the relative effects of CR on relaxation versus a decrease in stiffness. Likewise, we were unable to confirm the echocardiographic assessment of LVH with a direct measurement of mass due to the need to snap freeze the tissue for optimal biochemical analysis.

Another important characteristic of MHD is mitochondrial dysfunction with impaired energetics and excess ROS (25, 26). Increased mitochondrial H_2O_2 production has been observed in obese *db/db* mice as well as mice with diet-induced obesity (5, 25), possibly as a consequence of increased fatty acid oxidation leading to electron leakage for the electron transport chain (5, 14). CR has been shown to decrease cardiac mitochondrial H_2O_2 generation in obese rats (18) and in lean aging mice (24). Our study extends these findings by demonstrating that CR also diminished cardiac mitochondrial H_2O_2 in *db/db* mice.

HMW Adiponectin

Adiponectin levels are known to decrease with obesity (15). Therefore, it was not surprising that plasma levels of adiponectin were lower in *db/db* (*vs. db/+*) mice. While CR had no effect on total adiponectin level in *db/db* mice, it increased the level of HMW adiponectin by 3 folds. Several isoforms of adiponectin are present in the plasma where the HMW form is considered most bioactive (15). Since adiponectin is cardioprotective, our data suggest that the beneficial effects of CR are mediated, in part, by increased HMW adiponectin. The mechanism responsible for the selective increase in HMW *vs.* total adiponectin is not clear. In a study with morbidly obese humans, we found that *roux-en-y* gastric bypass (an extreme form of CR) increased circulating HMW adiponectin 3 months post-operatively, although total adiponectin was not measured (28). In lean mice, 5 weeks of CR increased all three isoforms of adiponectin (23). Given that adiponectin is the most abundant adipokine secreted by white adipose tissue (17), our findings suggest that even short-term CR can improve adipose tissue health in *db/db* mice. In contrast to adiponectin, several other adipokines that are known to modulate cardiovascular function were unaffected by CR.

Role of AMPK

AMPK is a pivotal nutrient sensor with the ability to regulate whole-body metabolism (9) and it can be activated by adiponectin. Although cardiac AMPK has been shown to play a role in CR-mediated cardioprotection in several models (23), our data indicate that AMPK activity in the heart was only slightly decreased in *db/db* (*vs. db/+*) mice, and was not altered by CR. In obese humans, we demonstrated that adipose tissue AMPK activity is decreased in insulin resistant (*vs.* insulin sensitive) group (29); moreover, we found increased adipose tissue AMPK activity associated with higher circulating levels of HMW adiponectin following *roux-en-y* gastric bypass (28). In the present study, adipose tissue AMPK activity was markedly decreased in *db/db* (*vs. db/+*) mice, and was accompanied by a decrease in eNOS phosphorylation, a downstream target of AMPK. Conversely, CR increased both AMPK and eNOS phosphorylation/activation to levels comparable to non-obese *db/+* mice. Whether adipose tissue AMPK can mediate a crosstalk with the heart warrants further investigation, as our conditioned media study shows that knocking down AMPK in adipocytes has an impact on cardiomyocyte function (Xu et al., *unpublished*).

Other effects of CR

Increased ROS production contributes to mitochondrial dysfunction. In the hearts of mice with diet-induced obesity, mitochondrial ROS production was elevated, causing oxidative post-translational modifications of mitochondrial proteins and leading to impaired mitochondrial oxidative respiration and ATP production (3, 26). Interestingly, overexpression of catalase targeted to the mitochondria prevented the obesity-induced LVH, diastolic dysfunction, and it also decreased mitochondrial ROS level (26). Our finding here that CR decreases cardiac mitochondrial ROS generation in *db/db* mice indicates that CR is associated with improved mitochondrial function of the heart. It further raises the possibility that the cardiac benefits of CR are due, at least in part, to decreased mitochondrial ROS production. CR-mediated decrease in mitochondrial ROS could be a direct consequence of decreased cardiac fatty acid oxidation.

While CR prevented weight gain in *db/db* mice, whole-body insulin resistance persisted. It is likely that a longer duration of CR is required to reverse insulin resistance in *db/db* mice (12). Furthermore, we cannot exclude the possibility that there was tissue-specific reversal of the insulin resistance.

To summarize, we show for the first time that in genetically-obese mice, short-term CR prevents the development of MHD. These beneficial effects occur despite whole-body insulin resistance, and are associated with improved adipose tissue health as reflected by elevated circulating HMW adiponectin and increased AMPK activation. These findings raise an intriguing possibility that interventions that target adipose tissue may be of value in the treatment of MHD.

Acknowledgments

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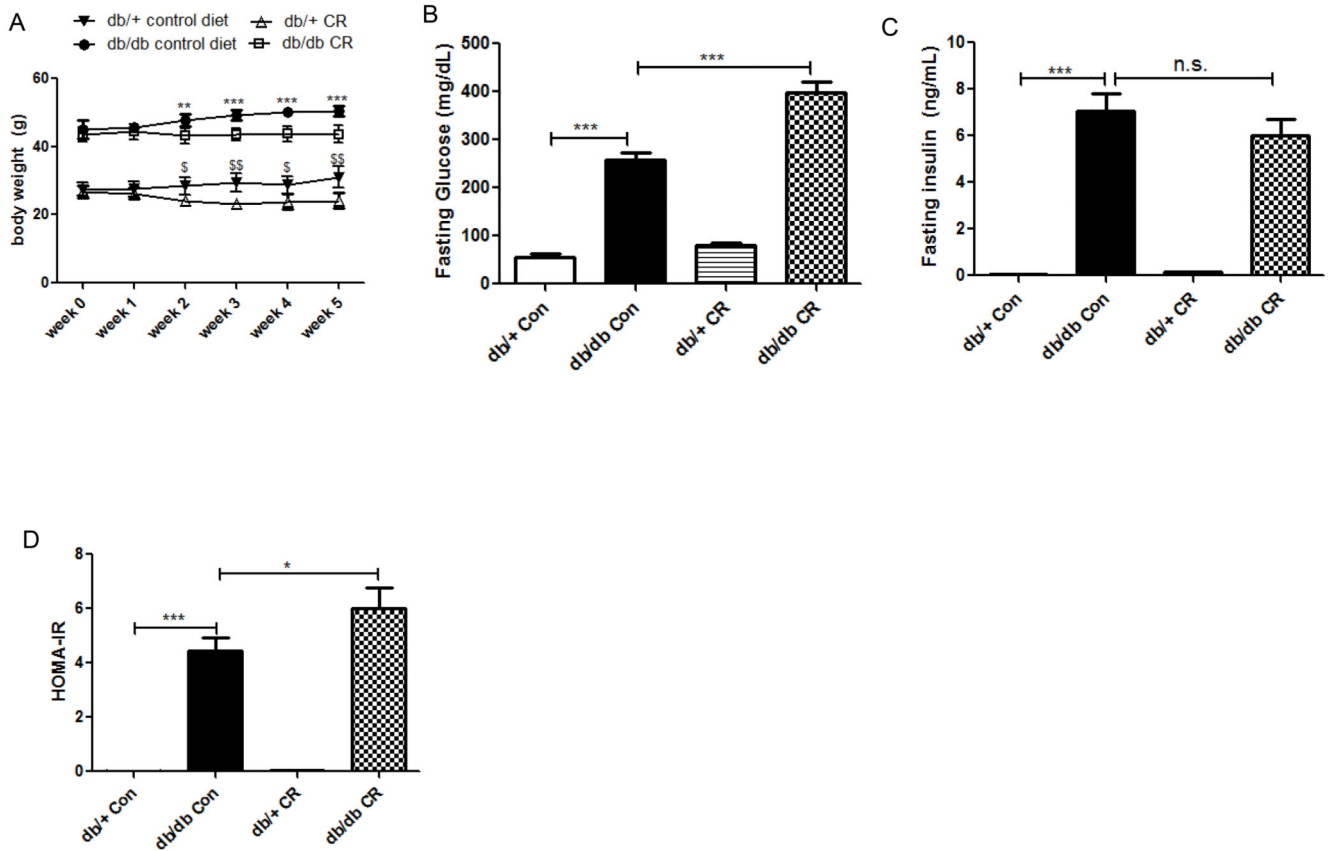
Figure 1.
Effects of 5-week CR on body weight, fasting blood glucose and insulin in *db/db* and *db/+* mice. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0001$ *vs. db/db* Con; $, $p < 0.05$, $$, $p < 0.01$ *vs. db/+* CR. Data are means + S.E.M. (n = 6/group).
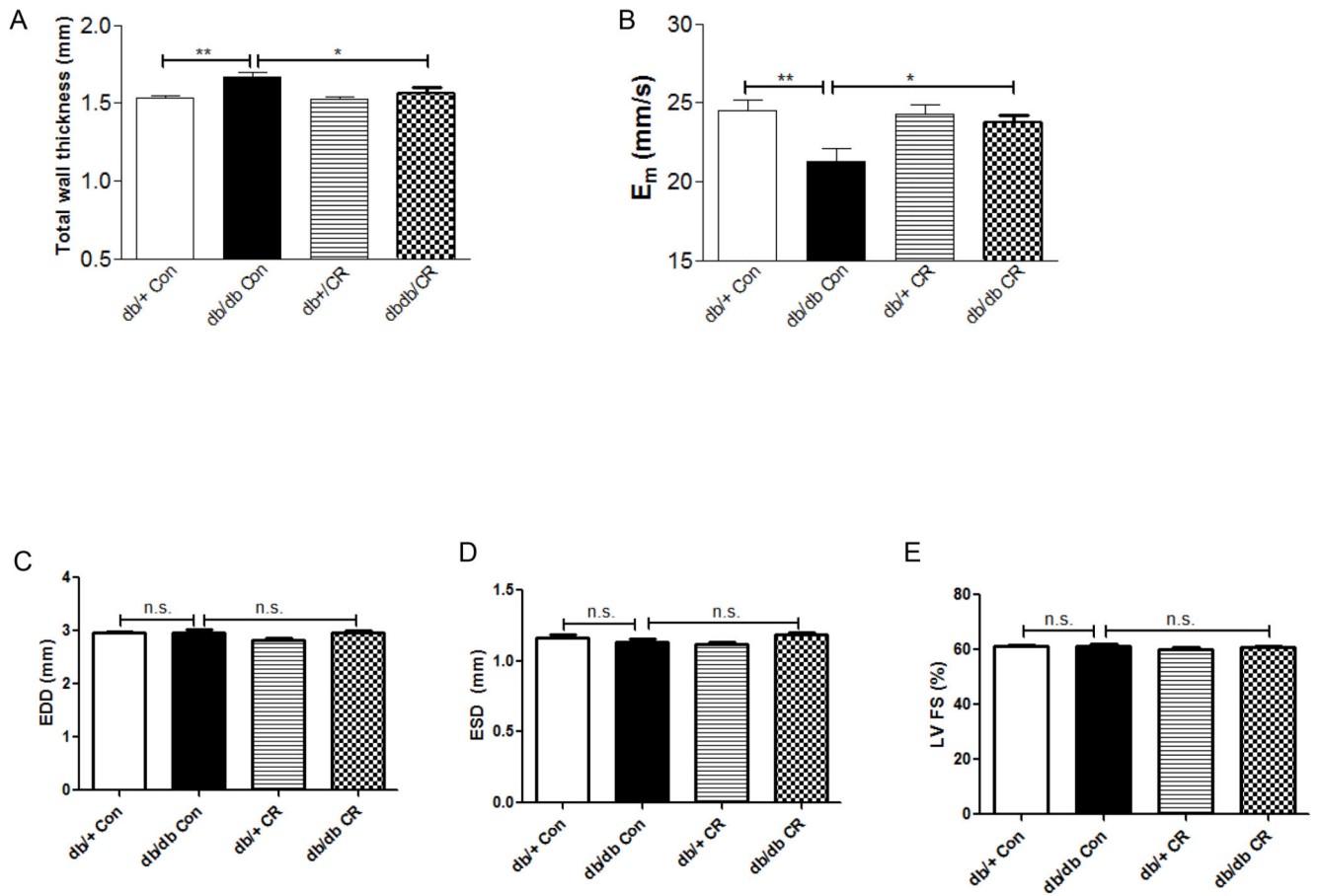
Figure 2.
Measurements of LV wall thickness (A), parameters related to diastolic (B) and systolic function (C-E) in *db/db* and *db/+* mice with CR. *, $p < 0.05$, **, $p < 0.01$ *vs. db/db* Con. Data are means + S.E.M. (n = 6/group).
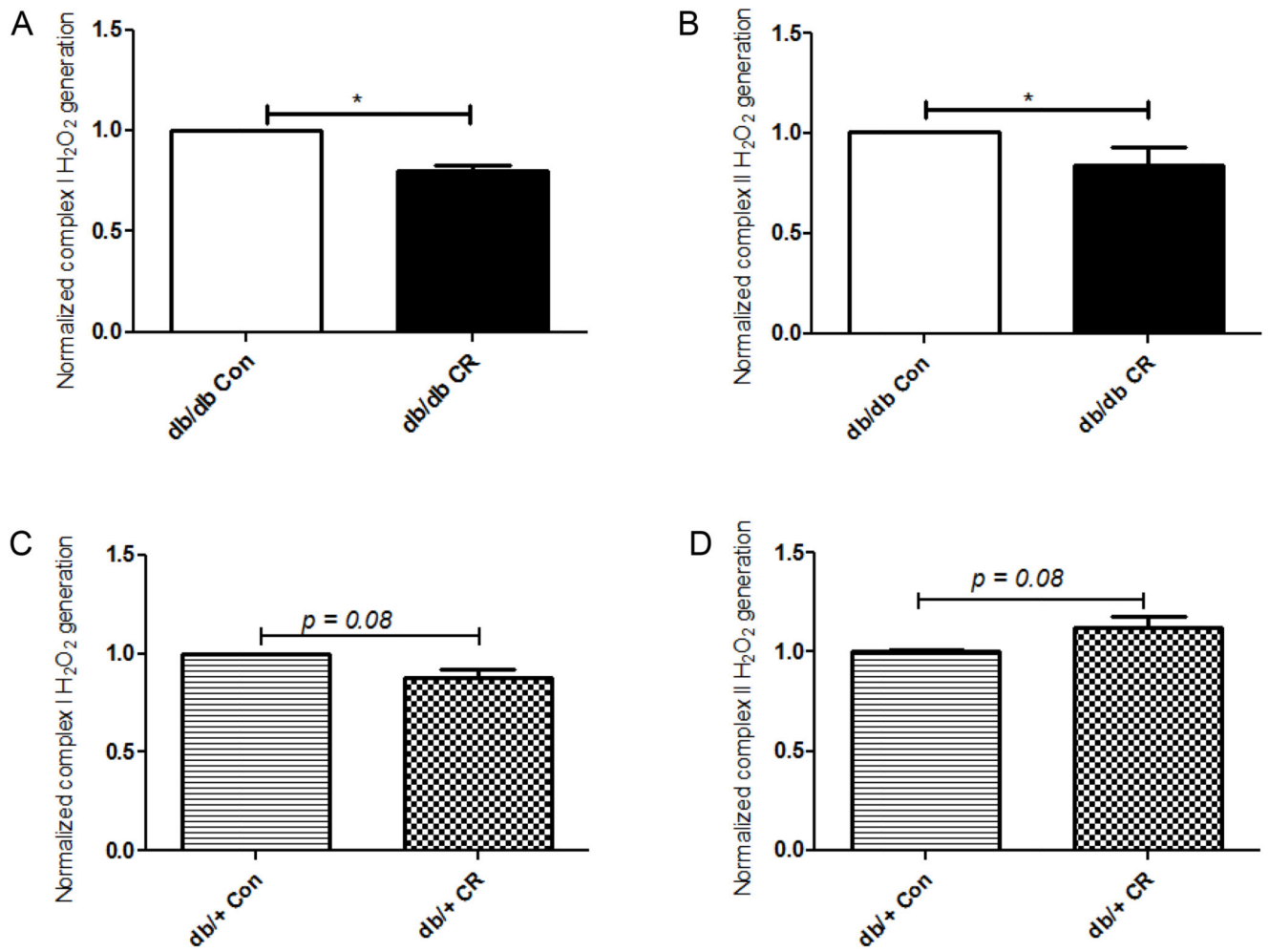
Figure 3.
Mitochondrial H_2O_2 production with a complex I substrate (A+C) and a complex II substrate (B+D) were measured in *db/db* and *db/+* mice. The amounts of H_2O_2 produced in *ad lib* (Con) groups were set to 1 for both genotypes. *, $p < 0.05$ *vs. db/db* Con. Data are means + S.E.M. (n = 6/group).
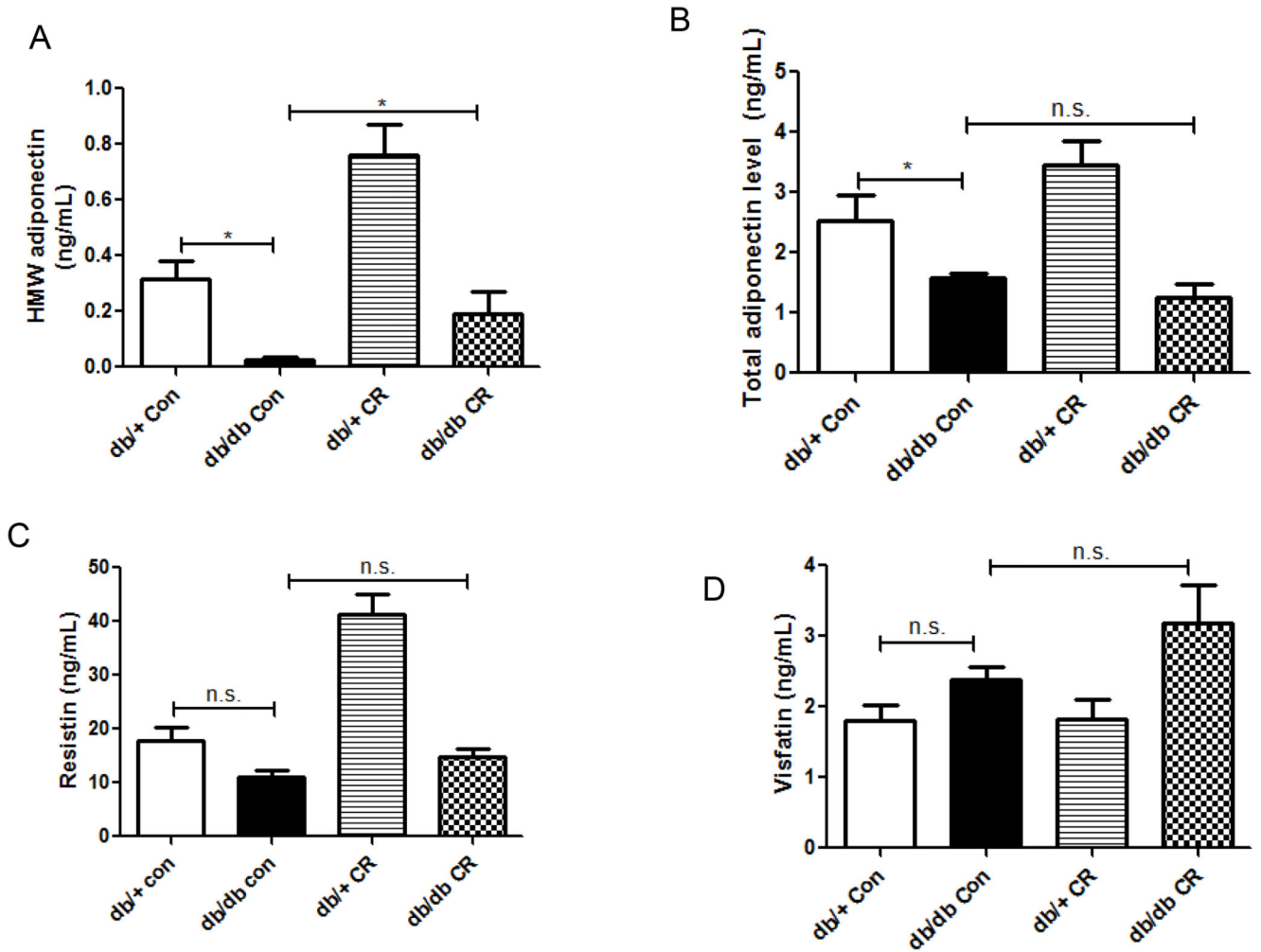
Figure 4.
Effects of CR on the circulating levels of adipokines. *, $p < 0.05$ *vs. db/db* Con. Data are means + S.E.M. (n = 6/group).
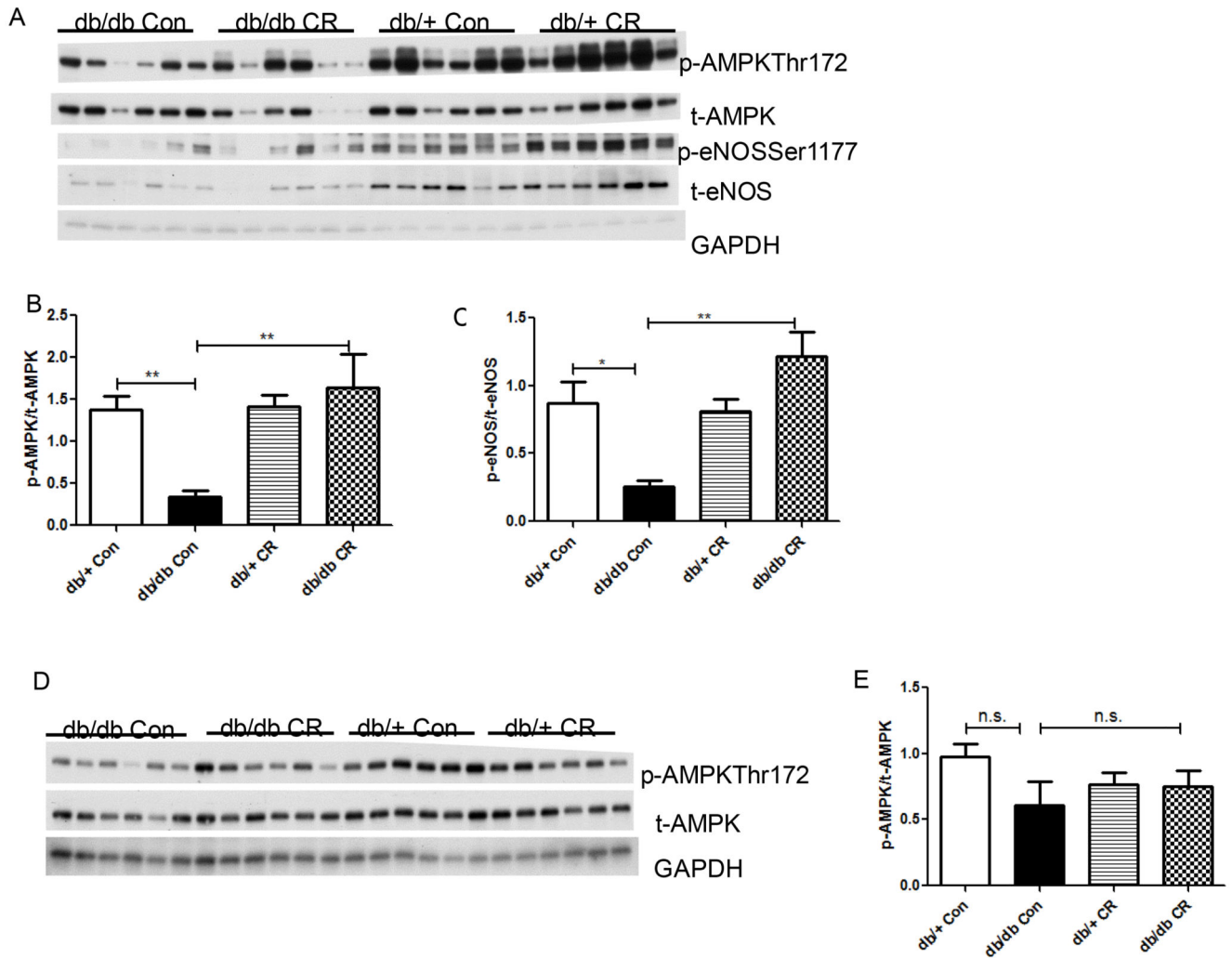
Figure 5.
Effects of CR on AMPK phosphorylation/activation in heart and epididymal fat. Relative phospho-protein levels were normalized against total AMPK and total eNOS, respectively. *, $p < 0.05$, **, $p < 0.01$ *vs. db/db* Con. Data are means + S.E.M. (n = 6/group).