Genetic ablation of C-reactive protein gene confers resistance to obesity and insulin resistance in rats

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Abstract

Aims/hypothesis Besides serving as a traditional inflammatory marker, C-reactive protein (CRP) is closely associated with the development of obesity, diabetes and cardiovascular diseases as a metabolic and inflammatory marker. We hypothesise that CRP protein directly participates in the regulation of energy and glucose metabolism rather than just being a surrogate marker, and that genetic deficiency of CRP will lead to resistance to obesity and insulin resistance.

Methods *Crp* gene deletion was achieved by transcription activator-like effector nuclease (TALEN) technology in rats. The *Crp* knockout animals were placed on either a standard chow diet or a high-fat diet. Phenotypic changes in body weight, glucose metabolism, insulin sensitivity, energy expenditure and inflammation condition were examined. The central impact of CRP deficiency on leptin and insulin hypothalamic signalling, as well as glucose homeostasis, were examined via intracerebral ventricular delivery of leptin and CRP plus glucose clamp studies in the wild-type and *Crp* knockout rats.

Results CRP deficiency led to a significant reduction in weight gain and food intake, elevated energy expenditure and improved insulin sensitivity after exposure to high-fat diet. Glucose clamp studies revealed enhanced hepatic insulin signalling and actions. Deficiency of CRP enhanced and prolonged the weight-reducing effect of central injected leptin and promoted the central and peripheral roles of leptin. By contrast, reinstatement of CRP into the hypothalamus of the knockout rats attenuated the effects of central leptin signalling on insulin sensitivity and peripheral glucose metabolism.

Conclusions/interpretation This study represents the first line of genetic evidence that CRP is not merely a surrogate blood marker for inflammation and metabolic syndromes but directly regulates energy balance, body weight, insulin sensitivity and glucose homeostasis through direct regulation of leptin's central effect and hypothalamic signalling.

Keywords Central nervous system · C-reactive protein · Insulin resistance · Leptin resistance

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Abbreviations

CNS	Central nervous system
CRP	C-reactive protein
EHC	Euglycaemic–hyperinsulinaemic clamp
GIR	Glucose infusion rate
GDR	Glucose disposal rate
hCRP	Human C-reactive protein
HFD	High-fat diet
HGP	Hepatic glucose production
ICV	Intracerebroventricular
InsR	Insulin receptor
KO	Knockout
LPS	Lipopolysaccharide
NCD	Normal chow diet
RER	$\dot{V}O_2 / \dot{V}CO_2$
STAT3	Signal transducer and activator of transcription 3

Research in context

What is already known about this subject?

- C-reactive protein (CRP) is associated with insulin resistance and metabolic diseases such as hepatic steatosis, cardiovascular disease and type 2 diabetes, as shown in numerous clinical studies
- Human C-reactive protein (hCRP) can block leptin functions in the central nervous system (CNS), and elevation of hCRP within the CNS has a negative effect on the functions of leptin

What is the key question?

- Does CRP functionally participate in the regulation of glucose homeostasis, inflammation and energy metabolism or is it merely a bystander under chronic inflammatory conditions?

What are the new findings?

- Systemic *Crp* knockout provided rats with resistance against high-fat diet-induced gain in body weight, insulin resistance and chronic inflammation, and also significantly promoted energy expenditure and insulin-regulated glucose metabolism
- CRP plays a direct role in the regulation of energy balance, glucose metabolism and insulin sensitivity by, at least in part, attenuating leptin's central and peripheral actions

How might this impact on clinical practice in the foreseeable future?

- Increasing blood CRP levels in people with obesity or type 2 diabetes may negate leptin's physiological functions. Thus, reduction of CRP-induced leptin resistance in peripheral circulation and the CNS may be a promising therapeutic strategy for the treatment of obesity and type 2 diabetes

TALEN Transcription activator-like effector nuclease
WT Wild-type

Introduction

Chronic low-grade inflammation is known to contribute to the occurrence and development of insulin resistance and type 2 diabetes [1]. A large number of inflammatory factors have been identified as predictors of insulin resistance, type 2 diabetes and other metabolic diseases [2, 3], among which C-reactive protein (CRP) is a reliable and widely used clinical biomarker [4, 5]. As a member of the pentraxin protein family, CRP is mainly synthesised and secreted by the liver and released into circulation in response to inflammation [6]. Numerous in vivo and in vitro studies have demonstrated that CRP is associated with insulin resistance and metabolic diseases such as obesity, hepatic steatosis, cardiovascular disease and type 2 diabetes [7–9]. Despite these strong association findings from clinical studies, the critical question remains unanswered: does CRP functionally participate in the regulation of glucose homeostasis, inflammation and energy metabolism or is it merely a surrogate bystander?

Leptin is an adipokine that is synthesised and released by adipose tissue and plays an indispensable role in the control of body weight and food intake [10]. Leptin resistance is an important feature in the development of obesity, insulin

resistance and type 2 diabetes in humans [11]. Early studies (including our own) have shed some light on the potential role of CRP in regulating leptin's functions [11]. For instance, human C-reactive protein (hCRP) can bind to leptin to block leptin signalling and impair its function, which in turn confers leptin resistance [12]. In *ob/ob* mice, subcutaneous administration of hCRP attenuated the effects of exogenous leptin on food intake and body weight [12]. Furthermore, elevation of hCRP within the central nervous system (CNS) could negate the actions of leptin on energy balance [13].

The physiological actions of both insulin and leptin can be negatively influenced by chronic inflammation, in which CRP is intimately involved; it is therefore critical to define the direct and precise impact of CRP on leptin-regulated energy balance and metabolism at the genetic level under in vivo conditions. Although studies on a CRP-targeting mouse model have revealed its protective role in acute inflammation [14], mouse CRP is not regarded as an ideal system for investigating the actions of hCRP, particularly for the study in metabolism and cardiovascular functions. Therefore, research on the metabolic roles of CRP has been hampered by the lack of appropriate CRP-deficient animal models. On this note, it is encouraging to see the report that manipulation of circulating rat CRP levels with antibodies has successfully simulated the actions of hCRP in the cardiovascular system [15], implying that rat CRP can function in a fashion similar to that of hCRP. Herein, we report the creation of a rat *Crp* gene-targeting

model to investigate the impact of CRP deficiency on energy balance, inflammation and insulin sensitivity. With this *Crp* knockout (KO) rat model, we would be able to define, at the genetic level, whether and how CRP directly regulates energy balance, insulin sensitivity and glucose homeostasis.

Methods

Animals *Crp* KO rats were generated from Cyagen Biosciences and then housed in the Animal Experimental Centre of Chongqing Medical University. *Crp* KO rats on a Sprague Dawley background were through transcription activator-like effector nuclease (TALEN) technology (CYAGEN, Guangzhou, China). Briefly, the analysis of rat *Crp* exonic and intronic sequences led to the design of the location of a gene deletion (exon 1). We used this to construct the TALEN plasmid (sequences for lead mRNAs: TALEN-1-L: TCACGATAAGCTTCTCTC, TALEN-1-R: TATAATAGCTCCTACCTT). Nuclear injection of the in vitro transcribed mRNA resulted in the birth of the F0 generation of positive rats, which were crossed with the wild-type (WT) to produce the heterozygotic F1 generation. Heterozygous F1 rats were mated with heterozygous F1 rats to obtain homozygous *Crp* KO rats or heterozygous F2 or WT littermates. The phenotype was confirmed through sequencing. Animals were housed in a temperature- and humidity-controlled facility with 12 h light/dark cycles with ad libitum access to water and food. All animals used were based on their corresponding genotypes, once their genotypes were identified, and they were randomly assigned to either the NCD- or HFD-fed groups, or randomly assigned to various treatment groups. To construct a high-fat diet (HFD)-induced insulin resistance model, eight-week-old male WT and *Crp* KO rats were fed either a normal chow diet (NCD) or an HFD (45% fat; Medicine, Jiangsu, China) for 12 weeks. For the age-related metabolic study, eight-week-old male WT and *Crp* KO rats were fed an HFD for 16 months. Animal experimental procedures were approved by the Animal Experimentation Ethics Committee, Chongqing Medical University.

Cannulation and indwelling catheters For the intracerebroventricular (ICV) study, 7 days prior to the in vivo experiments, rats were anaesthetised and fixed in a stereotaxic apparatus. Indwelling catheters were stereotactically implanted into the third cerebral ventricle (V3). The correct cannula placement into V3 was confirmed by testing the drinking response to angiotensin II as described previously [16]. For the euglycaemic–hyperinsulinaemic clamp (EHC) study, 7 days after stereotaxic surgery the rats were anaesthetised with intraperitoneal pentobarbital (50 mg/kg) and catheters were inserted into the right internal jugular vein and left carotid artery.

ICV infusion protocols To investigate the dose-dependent effects of ICV CRP and leptin infusion on glucose metabolism, different concentrations of CRP (22.5, 11.25 and 2.25 µg/h) and leptin (2.5, 0.5 µg/h and 15, 0.5 ng/h; R&D Systems, MN, USA) were continuously infused into the ICV area through the ICV catheter during the GTT. Blood glucose was measured at the indicated time points. For subsequent experiments we infused CRP (3.75 µg/h) and leptin (0.83 µg/h) into the ICV area during the EHC.

GTT, ITT and EHC GTTs and ITTs were conducted as reported previously [17]. For the EHC study, rats were restricted to ~230.12 kJ of food the day before the EHC to keep the identical nutritional status during the EHC. After a 12 h fast, EHCs were performed in conscious, unrestrained rats as described previously [18]. Briefly, a continuous infusion of HPLC-purified [^3H]glucose (PerkinElmer, MA, USA; 2.22×10^5 Bq bolus, 7.4×10^3 Bq/min) was initiated at 0 min and maintained throughout the experiment. The EHCs were started at time 90 min after [^3H]glucose infusion. Somatostatin ($3 \mu\text{g kg}^{-1} \text{min}^{-1}$) was infused intravenously together with insulin ($1.2 \text{ mU kg}^{-1} \text{min}^{-1}$) to inhibit endogenous insulin secretion. A 25% glucose solution was started at time 90 min and adjusted every 10 min to maintain blood glucose at basal levels. For ICV infusion protocols, at time –120 min, ICV infusions of PBS or recombinant rat leptin (0.83 µg/h, R&D Systems) and/or CRP (3.75 µg/h, R&D systems) were initiated and maintained until the end of the EHC. Blood samples were collected at the indicated time points for insulin, NEFA, and glucose-specific activity measurements. To assess insulin-mediated glucose uptake in fat and muscle tissues, a 2-deoxy-D [^3H]glucose (2-DG, Amersham, Los Angeles, CA, USA) was administered as a bolus (1.1×10^6 Bq) 45 min before the end of clamp experiments. Blood samples (50 µl) were taken at 5, 15, 30 and 45 min after the injection to determine the tracer disappearance curve. At the end of the EHC, the rats were anaesthetised and tissue samples were obtained and freeze-clamped in situ with aluminium tongs precooled in liquid nitrogen and stored at –80°C.

Measurement of hepatic triacylglycerol level Liver tissues were homogenised and centrifuged at 12,000 g at 4°C for 15 min. The supernatant was collected and the content of liver triacylglycerol was determined according to the instructions (Triglyceride Colorimetric Assay Kit, Cayman Chemical, USA).

Metabolic analyses and indirect calorimetry in conscious rats Body weight was measured daily in rats aged 8–20 weeks for the HFD-induced insulin resistance study and from age 6–18 months for the age-related metabolic study. Food intake, locomotor tolerance and rectal temperature were measured at the indicated time points and duration. Rectal temperature was measured with an electronic thermometer. For cold adaptation,

rats were placed at 4°C for 3 h, and the skin temperature was recorded using an infrared Thermoviewer (FOTRIC 220, FOTRIC, Shanghai, China). Energy expenditure was calculated by 24 h $\dot{V}O_2$ and $\dot{V}CO_2$, as described previously.

Long-term ICV leptin treatment To determine the dose-dependent effects of ICV leptin on food intake, eight-week-old male WT and *Crp* KO rats were fed an HFD for 12 weeks and were treated with ICV leptin injection twice daily for 6 days. To determine the dosage for ICV leptin injection, different doses of leptin (0.5, 2.5, 7.5 and 15 μ g) were injected into the ICV area of rats. Food intake was measured.

Immunohistochemistry Rat hypothalamic tissues were collected, fixed in 4% paraformaldehyde, and paraffin-embedded following ICV leptin treatment. After incubation with a primary antibody against signal transducer and activator of transcription 3 (STAT3) (#12640, 1:400, Cell Signaling Technology, Boston, MA, USA) or phospho-STAT3 (#9145, 1:400, Cell Signaling Technology, Boston, MA, USA) and a goat anti-rabbit secondary antibody detection kit (Zsbio, Beijing, China), the tissue sections were visualised with a confocal microscope (Nikon A1+R, Tokyo, Japan).

Analysis of mRNA and proteins Real-time quantitative PCR was performed as described previously using β -actin as an internal control gene [19]. Primer sequences are listed in electronic supplementary material (ESM) Table 1. Protein expression was analysed by western blot, as indicated previously [20]. Basic information on all the antibodies used in this study is listed in ESM Table 2. All antibodies were diluted with antibody diluent (Beyotime Biotechnology, Shanghai, China).

Animal PET and data acquisition Positron emission tomography (PET)–computed tomography (CT) scans were performed on rats on a Super Nova PET/CTS (PINGSENG Medical Technology, Kunshan, Jiangsu Province, China). Fat volume analysis is based on the grey scale value of fat (CT value) lower than other organs and muscles. Fat was screened and separated according to different threshold values of grey scale value. Cruiser software (version 1.5.0, PINGSENG Medical Technology) was used to capture the images, and the collection parameters were set as 60 kV tube pressure, 600 μ A tube flow, DSD (the vertical distance from the source focus to the detector) 510 mm and FOV (field of view) 110 mm. After image acquisition, recon software (recon 1.5.0, PINGSENG Medical Technology) was used for reconstruction. This process was carried out by professionals.

Statistical analysis Data are expressed as mean $\pm$ standard error of the mean. For comparing two groups, two-tailed unpaired Student's *t* test was used for unpaired data. The

analysis of the GTT and ITT at each time point was performed using Student's *t* test for paired data. We used WT littermates of the *Crp* KO rats for the GTT and ITT. Where necessary, variance analysis (ANOVA) was used, followed by Tukey's post hoc test for multiple comparisons of means. This information is provided in the legends of the respective figures. A *p* value <0.05 was considered to be statistically significant.

Results

The impact of CRP deficiency on energy balance To investigate the physiological effect of CRP deficiency on energy homeostasis, *Crp* KO rats were generated using TALEN technology (see [Methods](#)). CRP protein expression was not detectable in the livers of the *Crp* KO rats, and the circulating CRP levels were also nil compared with the expression in the WT littermates. Quantitative PCR analysis also revealed complete knockout of *Crp* expression in the fat, muscle, and pancreas (ESM Fig. 1a–c). While examining the growth of the *Crp* KO rats fed on NCD, we found no discernible difference in body weight, fat composition, and food intake (Fig. 1a–g). In addition, the rectal temperature (measured at either room temperature or 4°C) and thermal images showed no significant differences between the two genotypes (ESM Fig. 2a, b).

Under an HFD regimen, the body weight, volume of fat and energy balance phenotypes showed divergence between the mutant and WT rats. Accordingly, the body weight, fat mass and food intake of *Crp* KO rats were significantly reduced while the lean mass was not significantly changed (Fig. 1a–f) compared with WT rats. The mean daily food intake and cumulative food intake were also lower in the HFD-fed KO rats than in the WT controls (Fig. 1f, g). Interestingly, the *Crp* KO rats almost completely resisted the elevation of systolic and diastolic blood pressure induced by HFD (Fig. 1h, i); however, such phenotype appeared to accompany the attenuated weight gain of the *Crp* KO rats (ESM Fig. 3a, b). Whether this phenotype was only secondary to the differences in weight gain or a result of CRP's direct participation in the regulation of blood pressure remains to be investigated.

Upon further dissecting the impact of *Crp* deficiency on energy homeostasis under HFD conditions, we found that the *Crp* KO rats showed increased energy expenditure. Consistent with this observation, the rectal temperature was higher in the *Crp* KO rats (Fig. 2a), and thermal imaging analysis showed that the *Crp* KO rats had a higher temperature in interscapular brown adipose tissue than the WT rats (Fig. 2b). Further exposure to a cold environment at 4°C also revealed elevated temperature in the mutant rats (Fig. 2c). In a 24 h metabolic cage study, $\dot{V}O_2$ (Fig. 2d, e) and $\dot{V}CO_2$ (Fig. 2f) were increased relative to those in the HFD-fed WT rats. The

Fig. 1 *Crp* KO rats displayed reduced gain in body weight, fat mass, food intake and blood pressure. Eight-week-old male WT and *Crp* KO rats were fed an NCD or HFD for 12 weeks. The *Crp* KO protects the rats from the weight gain as evident in the body weight growth curve (a) and the cumulative body weight (b) during high-fat feeding. A micro-CT scan showed significant reduction in fat composition (c, d) and fat mass (but

not in lean mass) (e). Under the HFD regimen, *Crp* KO also led to decreased mean daily food intake (f) and cumulative food intake (g) as well as the systolic blood pressure (h) or diastolic blood pressure (i). Data are expressed as the mean $\pm$ SEM, * p <0.05, ** p <0.01 for *Crp* KO rats compared with WT rats (unpaired Student's *t* test was used for data). ATV, adipose tissue volume

HFD-fed KO rats also displayed a reduced respiratory exchange ratio (RER, $\dot{V}O_2 / \dot{V}CO_2$) (total or during the day, Fig. 2g, h), implying an increased tendency towards burning off fat. Interestingly, locomotor tolerance was higher in the NCD-fed KO rats than that in the WT rats, while there was no significant difference between the two groups of HFD-fed rats (Fig. 2i, j). Taken together, these results demonstrate that CRP deficiency promotes the host's resistance to obesity and development of high blood pressure, and gives rise to elevated energy expenditure and enhanced locomotor activity.

CRP deficiency ameliorates HFD-induced insulin resistance

Elevated circulating CRP levels have been clinically associated with insulin resistance and development of type 2 diabetes. Other than a modestly decreased level of fasting blood insulin and triacylglycerol, there was no discernible difference in a series of metabolic indications, such as fasting blood glucose, NEFA, and total cholesterol between the *Crp* KO and WT rats while they were maintained on NCD (ESM Table 3). Under the same condition, GTT and ITT in conscious and unrestrained rats also did not show differences between the two genotypes (Fig. 3a, b). However, when challenged with HFD, the *Crp* KO rats displayed better GTT and ITT curves than the WT rats (Fig. 3a, b). We then performed an EHC protocol to investigate the impact of *Crp* deletion on glucose metabolic

kinetics in the livers of both genotypes under both NCD and HFD conditions (Fig. 3c; basic blood metabolic parameters during the EHC provided in ESM Table 4). Under both the NCD and HFD regimens, the glucose infusion rate (GIR) (Fig. 3d, e) and glucose disposal rate (GDR) (Fig. 3f) were greater, while hepatic glucose production (HGP) was lower (Fig. 3g, h), in the *Crp* KO rats than those in the WT rats, indicating the enhancement of global insulin sensitivity in the mutant rats. In addition, glucose uptake was markedly higher in the skeletal muscle tissues (Fig. 3i), but not in the adipose tissues (Fig. 3j), of the HFD-fed *Crp* KO rats when compared with those in the WT rats. Consistent with these results from the clamp studies in vivo, quantitative PCR and western blot analysis showed that the mRNA and protein expression of hepatic PEPCK was significantly decreased, and insulin-stimulated phosphorylation of hepatic InsR and Akt was more pronounced, in the *Crp* KO rats than those in the WT controls (Fig. 3k, l). We also measured the expression of PEPCK in the fat tissue, which is involved in gluconeogenesis, under HFD conditions and found no significant difference between the WT and the KO rats (ESM Fig. 4a). To confirm whether *Crp* KO has an impact on lipid accumulation in the liver, we examined the triacylglycerol content in liver. The levels of triacylglycerol in the liver were significantly lower in HFD-fed *Crp* KO rats than in the rats of the HFD-fed WT group (ESM Fig. 4b). In addition, we performed hepatic section

Fig. 2 *Crp* KO rats displayed elevated energy expenditure. Under high-fat feeding, the KO rats displayed increased rectal temperature (a). When placed at 4°C, the thermal images (b) and rectal temperature (c) all indicated elevated heat generation in the KO rats. In metabolic cage studies, the 24 h $\dot{V}O_2$ (d, e) and 24 h $\dot{V}CO_2$ (f) were all significantly increased, while the RER ratios (g, h) decreased, in the KO rats while fed with HFD.

staining and found lipid droplets were numerous and augmentative in the livers of the HFD group. However, lipid droplets obviously decreased in HFD-fed *Crp* KO rats (ESM Fig. 4c). Importantly, measurement of cytokines revealed that *Crp* KO rats completely resisted HFD-induced elevation of circulating TNF- α levels (ESM Fig. 5), indicating that CRP plays an intimate role in promoting inflammatory response. Taken together, these observations provide strong genetic evidence that CRP is directly involved in the regulation of hepatic and skeletal muscular insulin sensitivity as well as chronic inflammation, rather than just being a surrogate circulating marker of these two physiological events.

The impact of CRP deficiency on the central action of leptin in rats Some of the metabolic and energy balance phenotypes described above echo some of our previous findings that showed that hCRP could interact with human leptin and neutralise the function of leptin [12]. To explore the hypothesis that such leptin-associated mechanisms underlie the observed phenotypes, we performed ICV infusion of leptin

In the locomotor test, both the running distance (i) and running time (j) were higher in $\dot{V}O_2$ the KO rats than those in the WT controls fed NCD. Data are expressed as the mean $\pm$ SEM ($n=8-10$ /group). * $p<0.05$, ** $p<0.01$ vs NCD-WT or HFD-WT as shown (unpaired Student's *t* test was used for data)

in unrestrained rats, during which we evaluated glucose metabolism and insulin sensitivity with EHC studies using a tracer dilution methodology (Fig. 4a). In *Crp* KO rats maintained on an NCD diet, infusion of ICV leptin resulted in significantly higher GIR than that in the WT rats (Fig. 4b, c). Systemic CRP deficiency also significantly promoted leptin-initiated inhibition of HGP (Fig. 4d, e) as well as elevation of GDR (Fig. 4f). In addition, *Crp* KO also promoted central leptin-mediated glucose uptake in skeletal muscle but not in adipose tissue (Fig. 4g, h). Similar findings were discovered when the protocols were performed on the rats maintained on HFD. Compared with HFD-fed WT rats, ICV delivery of leptin in HFD-fed *Crp* KO rats resulted in a marked increase of GIR (Fig. 5a, b) and GDR (Fig. 5e) as well as a reduction in HGP by 46% (Fig. 5c, d). Again, centrally delivered leptin generated more pronounced glucose uptake in the skeletal muscle, but not the adipose tissue, in the mutant rats than in the WT control rats (Fig. 5f, g). The signalling pathways leading to the activation of hypothalamic STAT3 and Akt are critical for leptin-mediated energy metabolism

Fig. 3 *Crp* KO rats are resistant to HFD-induced insulin resistance. Eight-week-old male WT and *Crp* KO rats were fed an NCD or HFD for 12 weeks. The GTT (**a**) and ITT (**b**) curves are shown; ** $p < 0.01$ vs HFD-WT at specific time points of test, as shown. Illustration of the experimental procedure and EHC protocol (**c**). *Crp* deficiency enhanced insulin sensitivity as evident from the elevated GIR shown in the GIR time course (**d**), the cumulative GIR (**e**) and the GDR (**f**), in both NCD- and HFD-fed animals. In the EHC protocol, *Crp* KO also led to

pronounced suppression of HGP (**g**, **h**), and further enhanced glucose uptake in the skeletal muscle tissue (**i**), but not in the adipose tissue (**j**). Determination of hepatic expression of *Pepck* mRNA (**k**). Western blot assays of hepatic expression of PEPCK protein, insulin-stimulated phosphorylation of InsR and Akt and densitometric quantification (**l**). Data are expressed as the mean $\pm$ SEM ($n = 8-10$ /group). In (**e-l**) $^{\dagger}p < 0.05$, $^{\ddagger}p < 0.01$ vs NCD-WT; $^{*}p < 0.05$, $^{**}p < 0.01$ vs HFD-WT (**a**, **b**: paired t test, statistical tests are paired at each time point; **e-l** unpaired Student's t test)

[21–23]. Indeed, western blot analysis revealed that the phosphorylation of hypothalamic STAT3 and Akt was significantly elevated in the HFD-fed *Crp* KO rats compared with the

HFD-fed WT rats (Fig. 5b). Combined together, these results strongly support the concept that systemic CRP deficiency promoted the effect of central leptin on hepatic and skeletal

Fig. 4 The effect of central leptin injection on glucose metabolism in normal diet-fed WT and *Crp* KO rats. Eight-week-old male WT and *Crp* KO rats were fed NCD for 12 weeks. The experimental procedure and clamp protocol (**a**). *Crp* deficiency elevated leptin-stimulated GIR as evident in the GIR time course (**b**) during the EHC and the cumulative GIR (**c**). *Crp* KO further decreased HGP (**d**, **e**), while increasing GDR (**f**).

Crp deficiency enhanced leptin-stimulated glucose uptake in the skeletal muscle tissue (**g**), but not in the adipose tissue (**h**). Data are expressed as the mean $\pm$ SEM ($n=8/\text{group}$). * $p<0.05$, ** $p<0.01$ vs NCD-*Crp* KO-Leptin or NCD-WT-PBS, as shown (two-way ANOVA); PBS was used as a control of leptin

muscle glucose metabolism, and enhanced central leptin-stimulated STAT3/Akt signalling, particularly under HFD-induced obesity and insulin resistance conditions.

Blockade of the effects of CNS leptin action by restoration CRP level in the CNS

The findings thus far have shown that CRP deficiency enhanced the functions of hypothalamic leptin. We hypothesised that the restoration of CRP level in the CNS would neutralise leptin signalling and its physiological effects. To test this hypothesis, CRP and/or leptin were infused into the ICV region of NCD-fed *Crp* KO or WT rats, and an EHC protocol was performed to assess the changes in hepatic glucose kinetics in vivo (Fig. 6a). ICV infusion of leptin alone in the *Crp* KO rats predictably elevated peripheral GIR and GDR by 12% and 6%, respectively, relative to the ICV infusion of PBS in the KO or WT rats (Fig. 6b, c). Following the re-introduction of CRP through ICV infusion in *Crp* KO rats, GIR and GDR were significantly attenuated by 40% and 15%, respectively, compared with the parallel vehicle infusion alone in the KO rats, re-affirming the regulation of glucose metabolism by CRP. Importantly, ICV co-infusion of CRP and leptin overcame the inhibitory effects of the re-introduction of CRP on GIR and GDR (Fig. 6b, c). In a parallel analysis, ICV restoration of rat

CRP in *Crp* KO rats stimulated HGP, relative to ICV delivery of PBS (Fig. 6d, e), while at the same time, it decreased glucose uptake in skeletal muscle (but not in the adipose tissue) (Fig. 6f, g). Importantly, in both cases, ICV co-infusion of both CRP and leptin completely blunted the effect of CRP restoration (Fig. 6b–f). Central delivery of rat CRP also blocked leptin signalling in the hypothalamus of *Crp* KO rats. ICV infusion of CRP alone already significantly inhibited Akt phosphorylation in the hypothalamus compared with ICV infusion of the vehicle in the *Crp* KO rats (Fig. 6h). Although ICV infusion of leptin alone predictably increased Akt phosphorylation, co-infusion of CRP with leptin in the *Crp* KO rats markedly inhibited leptin-stimulated Akt phosphorylation (Fig. 6h). Thus, restoration of CRP within the CNS disrupted glucose homeostasis and promoted hepatic and skeletal muscle insulin resistance. These deleterious effects could be overcome by co-infusion with leptin, supporting the notion that CRP-initiated regulation of metabolism is mediated at least in part through blocking leptin's actions.

Effect of continuous ICV injection of leptin on energy homeostasis in HFD-fed rats

The findings described above prompted us to further interrogate the theory that the presence of CRP constitutes part of the mechanisms underlying leptin

Fig. 5 The effect of central leptin infusion on glucose metabolism in HFD-fed WT and *Crp* KO rats. Eight-week-old male WT and *Crp* KO rats were fed HFD for 12 weeks. The experimental procedure and clamp protocol were the same as shown in Fig. 4a. *Crp* deficiency elevated leptin-stimulated GIR as evident in the GIR time course (a) during the EHC and the cumulative GIR (b). *Crp* KO further decreased HGP (c, d), while increasing GDR (e). *Crp* deficiency enhanced leptin-stimulated

glucose uptake in the skeletal muscle tissue (f), but not in the adipose tissue (g). The effects of ICV leptin on STAT3 and Akt phosphorylation in hypothalamus from NCD- and HFD-fed WT and *Crp* KO rats (h). Data are expressed as the mean $\pm$ SEM ($n=6-8$ /group). * $p<0.05$, ** $p<0.01$ vs HFD-*Crp* KO-PBS or HFD-WT-PBS, as shown (a–g, two-way ANOVA; h, unpaired Student's *t* test); PBS was used as a control of leptin

resistance, and that, if so, CRP deficiency would allow a persistent and prolonged weight-reducing effect following exogenous leptin injection. To test this concept, we examined the food intake and body weight following a standard consecutive central injection of leptin under the HFD regimen. Such a protocol would typically generate an immediate drop in food intake in the WT animals, followed by a rapid rebound in food consumption, which was also evident in our experiment (Fig. 7a). ICV injection of leptin twice daily for 6 days in HFD-fed *Crp* KO rats resulted in a more pronounced reduction in food intake from day 1 to day 4, relative to that in the WT rats (Fig. 7a). Although food intake also began to show a modest increase in the KO rats starting from day 4, the anorexic effect was still sharply lower than that in the WT controls (Fig. 7a). Similar patterns of changes were also observed in body weight from day 2 to day 6 (Fig. 7b).

Consistent with these physiological observations, quantitative PCR analysis revealed a sharp increase in the hypothalamic expression of *Pomc* in the *Crp* KO rats following ICV leptin treatment (Fig. 7c); however, hypothalamic expression of *Agrp* and *Npy* was not significantly different between the two genotypes. In addition, western blot analysis showed that

consecutive ICV leptin injection persistently elevated STAT3 phosphorylation in the hypothalamus of the *Crp* KO rats relative to that in the control rats (Fig. 7d), a result also confirmed by immunohistochemical staining of phosphorylated STAT3 (Fig. 7e). Thus, *Crp* deficiency profoundly ameliorated leptin resistance, and allowed prolonged and sustained anorexic and weight-reducing effects. Elevated blood CRP is directly involved in leptin resistance as well as diminished insulin sensitivity.

The effect of *Crp* deletion on body weight and insulin sensitivity during ageing The incidence of metabolic syndromes increases with ageing, both of which have been shown to be positively associated with blood CRP levels [24]. By inference, *Crp* deficiency is expected to have long-lasting metabolic benefits, particularly during ageing. WT and *Crp* KO rats were placed under NCD or HFD diet regimens for 18 months. From 8 months to 18 months, the gain in body weight induced by HFD was persistently lower (Fig. 8a, b), while rectal temperature was higher (Fig. 8c), throughout the observed ageing period in the *Crp* KO than in the WT rats. Interestingly, during the same process, HFD-induced increase

Fig. 6 The effect of CRP re-constitution in the hypothalamus of the *Crp* KO rats on leptin-regulated glucose metabolism in vivo. The experimental procedure and clamp protocol: eight-week-old WT and *Crp* KO rats were fed an NCD for 12 weeks. An ICV catheter was implanted on rats on day 1. The internal jugular vein and carotid artery cannulations were performed on day 7, and the EHC and ICV leptin and/or ICV CRP were performed on day 10, as indicated in the [Methods](#) (a). Leptin-stimulated cumulative GIR (b) and GDR (c) were increased in the CRP-deficient rats, while co-infusion with rat CRP blunted leptin's effect. Similarly,

CNS infusion of leptin led to suppression in HGP (d, e) in the *Crp* KO rats but not in the WT rats, which was neutralised by the CNS co-infusion of CRP. CNS infusion of leptin stimulated glucose uptake in skeletal muscle tissue (f), but not in the adipose tissue (g) in the *Crp* KO rats. Determination of Akt phosphorylation in the hypothalamus (h). Data are expressed as the mean $\pm$ SEM ($n=8$ /group). * $p<0.05$, ** $p<0.01$ vs *Crp* KO-PBS, *Crp* KO-Leptin, *Crp* KO-CRP or *Crp* KO-Leptin-CRP, as shown (a–g, two-way ANOVA; h, unpaired Student's *t* test)

of systolic and diastolic blood pressure remained attenuated (Fig. 8d, e) in the *Crp* KO rats. In glucose metabolism, GTT and ITT tests showed that the improvement in glucose metabolism and insulin sensitivity lasted for at least 12 months, as indicated by the improved profiles in the *Crp* KO rats (Fig. 8f, g at 8 months of age, and h, i at 12 months of age). Combined together, these findings unequivocally demonstrate that CRP has a long-term, persistent regulatory impact on body weight energy expenditure and glucose metabolism, as well as blood pressure.

Discussion

Previous genetic studies have revealed no null mutations and very few variations in the amino acid sequences of CRP [25–27], which implies that CRP has important physiological functions that are critical to the survival and well-being of hosts. Even though mouse CRP is not necessarily equivalent

to hCRP in terms of functions, *Crp* KO in mice demonstrated that circulating CRP protects the host from pulmonary damage following microbial infection [28, 29]. Indeed, we also found that *Crp* KO rats were much more susceptible to inflammatory cell infiltration and lung damage than the WT controls following the acute treatment of lipopolysaccharide (LPS) (data not shown), which indicates that CRP is not merely a marker of acute inflammation [30–32].

CRP in mice is not an acute phase reactant and shows a very mild response to LPS stimulation [33–36]. Although *Crp* KO mice have been developed [37–39], they have limited use in the study of CRP functions in metabolic and cardiovascular diseases. For example, *Crp* deletion in mice did not affect the development of atherosclerotic plaques. In fact, these pitfalls in developing a mouse model of *Crp* deletion propelled the development of transgenic mice harbouring the hCRP gene (CRP) for investigating autoimmune and cardiovascular diseases [36, 40, 41]. Interestingly, interference of rat CRP functions by exogenous injection of specific antibodies did

Fig. 7 The effects of repeated ICV leptin treatment on energy homeostasis in HFD-fed *Crp* KO rats. Eight-week-old male WT and *Crp* KO rats were fed an HFD for 12 weeks and were treated with ICV leptin twice daily for 6 days, as indicated in the [Methods](#). Daily leptin injection led to greater and prolonged reduction in food intake (**a**) and body weight (**b**) in the KO rats than in the WT controls. Determination of the expression levels of *Pomc*, *AgRP*, and *Npy* mRNA in the hypothalamus (**c**). Determination of the level of STAT3 phosphorylation in the hypothalamus by western blot assays (**d**). Immunohistochemical staining for

STAT3 phosphorylation in the hypothalamus (**e**); original magnification, $\times 100$ or enlarged $\times 200$ (enlargement shown by box), scale bars 200 μm , or 100 μm in enlarged images. 3V, third ventricle and ARC, arcuate nucleus. Data are expressed as the mean $\pm$ SEM ($n=4-5$ /group for the western blot and immunostaining experiments). * $p<0.05$, ** $p<0.01$ vs HFD-WT, WT-PBS or WT, $\dagger\dagger p<0.01$ vs WT-Leptin. (**c**, two-way ANOVA; **a-b**, **d** and **e**, unpaired Student's *t* test); PBS was used as a control of leptin

decrease atherosclerotic development [42], implying that rats are a better model of human physiology than mice in this regard [43]. However, it is difficult to use this approach to stably affect CRP expression to allow the study of metabolic phenotypes. Therefore, the development of *Crp* KO rats can serve as a genetic model for studying the physiology of CRP, especially in relation to metabolic diseases, insulin resistance and energy balance.

Abundant clinical studies in the past decade have demonstrated strong positive correlations between blood CRP levels and adiposity, insulin resistance and type 2 diabetes [27, 44]. However, the question of whether CRP actively participates in the regulation of metabolism still requires definitive genetic proof. The results presented in this study demonstrate that systemic *Crp* deficiency provided the rats with resistance against HFD-induced gain in body weight, insulin resistance

Fig. 8 Effect of *Crp* deficiency on body weight, insulin sensitivity and other metabolic variables during ageing. In a long-term study (particularly on HFD for 72 weeks), the KO rats consistently gained less weight than their WT controls as reflected in the body weight curve (**a**) and cumulative body weight (**b**). The rectal temperature (**c**) was also higher in the HFD-fed KO rats than in the controls throughout the observation; meanwhile, the systolic (**d**) and diastolic (**e**) blood pressure readings were also

significantly lower in the KO animals than in the controls. In the GTTs performed at 8 months (**f**) or 12 months (**g**), or the parallel ITTs (**h**; 8 months) and (**i**; 12 months), the HFD-fed KO animals displayed significantly enhanced GTT and ITT profiles. Data are expressed as the mean $\pm$ SEM ($n=8-10$ /group). * $p<0.05$, ** $p<0.01$ vs HFD-WT (**a-e**, unpaired Student's *t* test; **f-i**, paired *t* test, statistical tests are paired at each time point)

and chronic inflammation, and also significantly promoted energy expenditure and insulin-regulated glucose metabolism. These phenotypes mirrored CRP elevation in human obesity and type 2 diabetes, and fully support the hypothesis that CRP is a direct regulator of metabolism rather than just a metabolic disease marker.

The metabolic phenotypes that result from systemic CRP deficiency are mediated, at least in part, through the regulation of the physiological functions of leptin that are indispensable for controlling a series of physiological functions, such as energy balance, and glucose and fat metabolism [45, 46]. Previous research has shown that hCRP can interact with human leptin and negatively impact the physiological functions of human leptin in the CNS of *ob/ob* mice [13, 47]. However, these studies were carried out in a heterologous system and the duration of observation was short, and as a result, it was difficult to assess the effects of hCRP on leptin signalling in the CNS or in the periphery. The genetic model of *Crp* KO rats allowed us to investigate the metabolic function of CRP over an extended period, including the ageing process. For example, repeated ICV injection of leptin in the *Crp* KO rats resulted in sustained weight loss and reduction of food intake, which was in sharp

contrast to the diminution of leptin's anorexic actions as well as sharp rebound of body weight in the WT rats. Importantly, reinstatement of CRP in the hypothalamus of *Crp* KO rats negated the effects of leptin on energy balance as well as glucose metabolism, and essentially re-instated the phenotypes of CRP-induced leptin resistance. The lack of CRP expression further promoted leptin-stimulated STAT3 phosphorylation and the expression of pro-opiomelanocortin (POMC), an anorexic peptide, in the hypothalamus. Combined together, these results provide strong genetic evidence that CRP directly participates in the regulation of energy balance by negatively influencing leptin's actions.

Largely mediated via its central receptors at the hypothalamus, leptin's important roles in regulating hepatic glucose fluxes, insulin sensitivity and lipid metabolism have been well documented [48, 49]. By inference, the negative impact of CRP on the functions of leptin should counter its actions in metabolism. Indeed, following the central delivery of leptin, glucose clamp analysis showed that *Crp* deficiency led to an increase in GIR, reduced HGP and elevated glucose uptake in the muscles. Consistent with these observations, hepatic insulin signalling was also significantly enhanced. Chronic

inflammatory condition, a contributing factor to insulin resistance [50–52], was significantly ameliorated in the *Crp* KO rats, as indicated by the drop in blood TNF- α levels. Earlier studies have shown that rat CRP could promote inflammation by directly binding to Fc γ R on macrophages, thus inhibiting the production of IL-10 while increasing the synthesis of TNF- α and IL-12 [53–55]. We also noted that the protective effects on metabolic phenotypes was long term, indicating that the regulation of CRP on body weight and insulin sensitivity can last through the ageing process. However, this protective effect did diminish, although it was still statistically significant, as the KO animals aged beyond 12 months. This is reminiscent of the *Il6* KO model in which the mice were protected from the metabolic syndromes at a young age, but this effect did not last during the ageing process [56, 57]. It is likely that as the animal becomes older, the build-up of fat and the changes of various hormones (e.g. the decline of testosterone), will also play prominent roles in the regulation of metabolism. Thus, a rise in blood CRP levels will be detrimental to insulin sensitivity and glucose homeostasis, as a result of the combined effects on leptin signalling and inflammation, which eventually leads to type 2 diabetes.

In summary, we have for the first time generated a rat *Crp* KO model to provide genetic evidence that CRP directly regulates energy balance, chronic inflammation, blood pressure, glucose metabolism and insulin sensitivity. Some of these pathophysiological effects were achieved, at least in part, through controlling inflammation and negating leptin's physiological functions. These findings unequivocally support the concept that elevation of blood CRP levels in people with obesity and type 2 diabetes is not merely a surrogate marker. Reduction of circulating CRP levels or CRP-induced leptin resistance may serve as a promising therapeutic strategy for the treatment of obesity and type 2 diabetes.

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Data availability Data related to this manuscript are available on request from the authors.

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