Decreased glucose tolerance and plasma adiponectin:resistin ratio in a mouse model of post-traumatic stress disorder

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Abstract

Aims/hypothesis Obesity and type 2 diabetes are among the most serious health pathologies worldwide. Stress has been proposed as a factor contributing to the development of these health risk factors; however, the underlying mechanisms that link stress to obesity and diabetes need to be further clarified. Here, we study in mice how chronic stress affects dietary consumption and how that relationship contributes to obesity and diabetes.

Methods C57BL/6J mice were subjected to chronic variable stress (CVS) for 15 days and subsequently fed with a

standard chow or high-fat diet. Food intake, body weight, respiratory quotient, energy expenditure and spontaneous physical activity were measured with a customised calorimetric system and body composition was measured with nuclear magnetic resonance. A glucose tolerance test was also applied and blood glucose levels were measured with a glucometer. Plasma levels of adiponectin and resistin were measured using Lincoplex kits.

Results Mice under CVS and fed with a high-fat diet showed impaired glucose tolerance associated with low plasma adiponectin:resistin ratios.

Conclusions/interpretation This study demonstrates, in a novel mouse model, how post-traumatic stress disorder enhances vulnerability for impaired glucose metabolism in an energy-rich environment and proposes a potential adipokine-based mechanism.

Keywords Adiponectin · Glucose tolerance · High-fat diet · Mouse · Post-traumatic stress disorder · Resistin

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Abbreviations

CVS Chronic variable stress
GTT Glucose tolerance test
HPA Hypothalamic–pituitary–adrenal
PTSD Post-traumatic stress disorder
SPA Spontaneous physical activity

Introduction

Obesity and type 2 diabetes are worldwide health threats. Of the myriad factors thought to be responsible for these metabolic disorders in our society, chronic stress is among the most prevalent [1]. Stress-induced hypercortisolism,

upregulation of the hypothalamic–pituitary–adrenal (HPA) axis and downregulation of the sympathetic nervous system have each been proposed to contribute to the prevalence of these pathologies [2, 3], although the mechanism(s) by which stress leads to obesity and insulin resistance has yet to be determined.

Post-traumatic stress disorder (PTSD), a specific type of anxiety disorder, has an estimated lifetime prevalence of 8–26% in the general population [4–6] and 15–31% in war veterans [7–12]. PTSD is a risk factor for diabetes [13, 14], and the prevalence of diabetes is significantly higher in patients with PTSD than in age-matched males in the general population [15]. Consistent with this, veterans of the Vietnam War with PTSD have higher plasma lipid levels than controls [16]. PTSD is also a risk factor for diabetes. In the USA, 76% of male veterans with PTSD are overweight or obese, compared with 65% in the US population [17].

With an increasing number of veterans now returning from combat operations, understanding the relationship between mental health and obesity has become a major priority, i.e. whereas the correlation between PTSD and obesity/type 2 diabetes is clear, the mechanisms underlying this association are not. That said, it is reasonable to hypothesise that neuroendocrinological changes induced by stress exposure, specifically activation of the HPA axis and/or the sympathetic nervous system, may contribute to the prevalence of reduced insulin sensitivity [2, 3] and type 2 diabetes [18, 19].

Stress is associated with changes in feeding behaviour and food preference; specifically, exposure to stress leads to higher fat and sugar intake [20–23]. Compounding the problem, consumption of a high-fat diet exacerbates the stress-induced HPA-axis response, including elevation of glucocorticoids [24]. Finally, numerous studies suggest that chronic activation of the stress system, in the presence of a fat-enriched diet, results in increased visceral adiposity and insulin resistance reminiscent of Cushing's syndrome [3, 25–28].

To begin to determine the direction of causality, we generated a mouse model of PTSD. We hypothesised that mice that had undergone chronic stress would be more prone to increased consumption of a high-fat diet and the development of obesity and diabetes. We used exposure to a non-habituation chronic stress regimen (chronic variable stress [CVS]) and assessed the development of obesity and insulin resistance in mice subsequently exposed to a hyperenergetic or control environment. Mice exposed to CVS and fed a high-fat diet, but not mice fed a standard chow diet, had impaired glucose tolerance associated with low plasma adiponectin: resistin ratios and showed higher expression of genetic markers of adipogenesis, adipocyte differentiation, hypoxia and inflammation in retroperitoneal adipose tissue. To our knowledge, this is the first demonstration of prejudicial effects on glucose metabolism effected by a fat-enriched diet in a mouse model of chronic stress.

Methods

Animals C57BL/6J male mice (12 weeks old, Jackson laboratories, Bar Harbor, ME, USA) were singly housed under a 12 h light–dark cycle (lights on at 06:00 hours) with ad libitum access to low-fat chow (LM485 Harlan-Tekland Laboratory Diets, Madison, WI, USA) or a 40% high-fat diet (D03082706 Research Diets, New Brunswick, NJ, USA) and tap water. Mice were singly housed in order to make precise longitudinal measurements of food intake and energy expenditure feasible. All animal protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Energy balance and body composition measurements Mice were placed for 4 days for adaptation in a customised indirect calorimetry system (TSE Systems, Bad Homburg, Germany), with a flow rate of 0.5 l/min as previously described by Nogueiras et al. [29]. Measurements of food intake, spontaneous physical activity (SPA), respiratory quotient and energy expenditure were taken at baseline while mice were maintained on chow, 4–6 days after exposure to CVS and after 1 month on a chow or high-fat diet. All energy-balance variables were monitored simultaneously online [30], food intake was recorded every 30 min and SPA, respiratory quotient and energy expenditure were recorded every 45 min.

Body composition measurements were taken using nuclear magnetic resonance (Whole Body Composition Analyzer; Echo NRI, Houston, TX, USA) at the beginning of the study, after 15 days exposure to CVS and after 1 month of the low-fat or high-fat diet.

CVS Mice were subjected to a series of randomly alternating stressors (administered twice daily) over a period of 15 days [31, 32]. Individual timing of the stressors was selected on the basis of the stress duration required to provoke an adrenocortical response [33] without endangering the animals' well-being. The alternating stressor paradigm is designed to prohibit adaptation to any individual stressor. Stressors employed were: (1) 1 h in individual cages (minus bedding) in a cold room (4°C); (2) 20 min warm swim (31–33°C); (3) 1 h in individual cages on a shaker, causing vibration stress; (4) 30 min in a hypoxia chamber (8% oxygen/92% nitrogen, vol./vol.); and (5) overnight in large-cage housing.

Glucose tolerance test (GTT) Mice were fasted overnight and then injected intraperitoneally with 2 g glucose/kg body weight (50% wt/vol. D-glucose [Sigma, St Louis, MO, USA] in 0.9% wt/vol. NaCl). Tail blood glucose levels [mmol/l] were measured with a glucometer (TheraSense Freestyle) before (0 min) and at 15, 30, 45, 60, and 120 min after injection.

Plasma analysis Mice were killed by decapitation at the end of the study and blood was collected and immediately chilled on ice. After 15 min of centrifugation at 3,000g and 4°C, plasma was stored at −80°C. Plasma analysis was also done in samples from tail bleeding after CVS and consumption of the diet for 1 month (Fig. 1). NEFA levels were measured in duplicate using a commercially available enzymatic assay kit (Autokit NEFA C, Wako, Neuss, Germany), triacylglycerol levels were determined in duplicate using Infinity Triglyceride reagent b (Thermo Electron, Pittsburgh, PA, USA) and plasma glucose was measured in duplicate using a commercial kit based on the glucose oxidase method (Biomerieux, Marcy l'Etoile, France). Plasma adiponectin, resistin, insulin and leptin levels were determined with a commercially available Multiplex Lumines xMAP assay (Millipore, Billerica, MA, USA) and mouse Lincoplex kits (Linco Research, St Charles, MO, USA). Intra- and inter-assay coefficients of variation were: <5% and <12%, respectively, for adiponectin; <4.5% and <10.3%, respectively, for resistin; 2.7–5.8% and 3.8–10.8%, respectively, for insulin; and <7% and <23%, respectively, for leptin. The panel of T₃, T₄ and thyroid stimulating hormone (TSH) were measured using 3 Plex rat thyroid assays (Linco Research, St Charles, MO, USA) and the intra- and inter-assay coefficients of variation were <10% and <5%, respectively. Plasma corticosterone levels were measured in duplicate using a ¹²⁵I radioimmunoassay kit from MP Biomedicals (Orangeburg, NY, USA). Intra- and inter-assay coefficients of variation for corticosterone were <10%. Plasma glycerol levels were measured in duplicate using a commercially available enzymatic assay kit from Sigma. All assays were performed according to the manufacturers' instructions.

Low-density array The expression of 43 genes implicated in carbohydrate, lipid, protein and steroid metabolism, inflammation, cell regulation, proliferation, differentiation, adhesion and migration was analysed in retroperitoneal white adipose tissue samples by real-time PCR using TaqMan Low Density

Arrays (Applied Biosystems, Foster City, CA, USA). Relative expression was measured using the 7900HT TaqMan Fast Real-Time PCR System (Applied Biosystems). PCR reactions were performed in 2 µl wells, with each of the 384 wells of the reaction card loaded with the specific primers and probes. The sequences of the primers and probes used were designed and validated by Applied Biosystems and were taken from the Assay-on-Demand mouse library. The specific identification numbers of the Taqman probes (Applied Biosystems) can be obtained from the authors. The relative expression level of each gene was normalised by the geometric average of 18S expression.

Morphometric analysis of adipocyte cell size Adipocyte cell size was determined by examining haematoxylin/eosin-stained histological sections of inguinal white adipose tissue. Morphometric analysis was performed by measuring 20 cells per mouse, and by quantifying average cell sizes with the ImageJ software (National Institutes of Health, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>).

Adrenal weight Mice were killed at the end of the study and the left adrenals were removed and immediately weighed.

Statistical analyses Quantitative data are presented as mean ± SEM. Values were analysed for statistically significant differences applying one and two-way ANOVA and Tukey's post hoc tests or two-tailed unpaired *t* tests. *p* < 0.05 was considered significant (GraphPad Prism, GraphPad Software, La Jolla, CA, USA; SigmaStat, Systat Software, San Jose, CA, USA).

Results

Exposure to CVS transiently decreases lean mass and SPA C57BL/6 male mice were exposed to CVS for 15 days,

Fig. 1 Experimental protocol. Body composition and energy balance variables were measured in C57BL/6 male mice before and after exposure to CVS for 15 days, and after chronic exposure to chow or a 40% high-fat diet for 1 month. HFD, high-fat diet

and body composition and energy balance were analysed. Subsequently, mice from the CVS group and a non-stressed control group were each distributed into two subgroups maintained on low-fat chow or a 40% high-fat diet for 1 month. Body composition and energy balance were then redetermined (Fig. 1). After 15 days' exposure to CVS, mice had significantly lower body weight (Fig. 2a; 26.5 ± 0.3 g, $n=16$, $p<0.001$) and lean mass (Fig. 2c; 21.7 ± 0.3 g, $n=16$, $p<0.001$) compared with unstressed controls ($n=15$; 27.9 ± 0.3 g and 23.1 ± 0.2 g, respectively). Fat mass (Fig. 2b) and food intake normalised per g body weight were unaltered (Fig. 2d). The respiratory quotient was significantly decreased during the first dark phase 0.77 ± 0.01 in the CVS mice ($n=15$, $p<0.01$) vs 0.82 ± 0.01 in the control mice ($n=15$), but no differences were found at later points (Fig. 2e). There were no significant differences between the groups in energy expenditure levels ($n=15$; Fig. 2f).

The 4 day total SPA levels were significantly decreased in the CVS mice ($75,308 \pm 2,909$ events, $n=16$, $p<0.001$)

compared with the control group ($108,090 \pm 4,966$ events, $n=15$; Fig. 3).

Plasma measurements were taken the day after the CVS period. Fasting plasma glucose levels were significantly lower in the CVS mice (7.2 ± 0.25 mmol/l, $n=15$, $p<0.001$) compared with the control mice (8.9 ± 0.32 mmol/l, $n=15$). Plasma triacylglycerols were significantly lower in the CVS mice (0.5771 ± 0.02304 nmol/l, $n=15$, $p<0.05$) compared with the control mice (0.6662 ± 0.02784 nmol/l, $n=14$). Plasma corticosterone was already normalised by the end of the stress exposure, as there were no significant differences between the groups at that time (control mice 455.5 ± 17.40 nmol/l, CVS mice 575.3 ± 30.67 nmol/l, $n=14$ –15).

Previous CVS exposure does not affect obesity onset with a high-fat diet As expected, consumption of a 40% high-fat diet for 1 month increased body weight in the control (32.45 ± 1.27 g, $p<0.01$, $n=8$) and in the CVS mice (31.28 ± 1.05 g, $p<0.01$, $n=8$), as well as fat mass (control mice 6.16 ± 1 g, CVS mice 5.33 ± 0.74 g, $p<0.01$, $n=8$), compared with mice

Fig. 2 Effects of CVS on body composition in C57BL/6 male mice. Mice subjected to CVS (black bars, $n=16$) had significantly lower body weight (a) and lean mass (c) compared with the control mice (white bars, $n=15$) with no changes in fat mass (b). There was no change in food intake between the CVS mice (grey symbols) and the control mice (white symbols) (d). The respiratory quotient was transiently decreased in the CVS mice compared with the controls during the first dark phase (e). There was no change in energy expenditure in CVS mice compared with the control mice (f). Two-tailed unpaired t test and two-way ANOVA ** $p<0.01$, *** $p<0.001$

Fig. 3 Effects of CVS on SPA in C57BL/6 male mice. Mice subjected to CVS (black bars/grey symbols, $n=16$) had significantly lower 4 day SPA compared with the control mice (white bars/white symbols, $n=15$). (a) SPA over the time during the 4 day assessment time. (b) Total SPA. Two-tailed unpaired t test *** $p<0.001$

consuming low-fat chow (control mice body weight 27.37 ± 0.62 g and fat mass 1.21 ± 0.11 g, CVS mice body weight 27.26 ± 0.47 g and fat mass 1.06 ± 0.13 g, $n=7-8$). However, there were no significant differences in body weight gain (Fig. 4a) or fat mass between the CVS mice ($n=8$) and the control mice ($n=8$) on either diet (data not shown). The percentage of lean mass gain was significantly higher in the CVS mice consuming chow ($11.13 \pm 1.05\%$, $n=8$, $p<0.01$) and the high-fat diet ($7.86 \pm 1.9\%$, $n=8$, $p<0.05$) compared with the control mice consuming chow ($2.74 \pm 1.12\%$, $n=7$) and the high-fat diet ($2.54 \pm 1.2\%$, $n=8$; Fig. 4b). The respiratory quotient was significantly decreased in the CVS mice (0.77 ± 0.01 , $n=15$, $p<0.01$) compared with the control mice (0.82 ± 0.01 , $n=15$) consuming chow, whereas respiratory quotient was unchanged in mice on the high-fat diet (Fig. 4c). There were no differences in food intake, energy expenditure and SPA among the groups ($n=7-8$; data not shown).

Chronic high-fat diet consumption impairs glucose metabolism in male mice previously exposed to CVS CVS mice consuming the high-fat diet had significantly lower plasma adiponectin ($2,308 \pm 172$ pg/ml, $n=8$, $p<0.05$) and higher resistin ($5,093 \pm 687$ pg/ml, $n=8$, $p<0.05$) compared with the control mice ($2,853 \pm 151$ pg/ml and $2,816 \pm 388$ pg/ml, respectively, $n=8$), (Fig. 5d,e). Interestingly, these differences in adiponectin and resistin preceded a significantly

lower glucose tolerance observed in mice previously exposed to CVS ($n=7$, two-way ANOVA, Tukey's post hoc test, $p<0.05$), compared with the control mice ($n=8$; Fig. 5f). These differences depended on the combination of CVS exposure and subsequent exposure to the high-fat diet. There were no differences in adiponectin (Fig. 5a), resistin (Fig. 5b) or glucose tolerance (Fig. 5c) in mice consuming chow. At this stage of the study and therefore preceding the differences in glucose tolerance, there were no significant differences between the groups in fasting plasma insulin levels with the chow (control mice 208.1 ± 14.34 pmol/l, CVS mice 224.1 ± 20.27 pmol/l, $n=7-8$) or the high-fat diet (control mice 310 ± 39.01 pmol/l, CVS mice 299.9 ± 45.68 pmol/l, $n=7-8$).

The analysis of relevant genes in white adipose tissue from CVS mice on the high-fat diet using low-density array ($n=4$, one-way ANOVA, Tukey's post hoc test) showed significantly higher expression of CCAAT/enhancer binding protein (C/EBP), beta (Cebpb; $p<0.01$; Fig. 6a), early growth response 2 transcription factor (*Egr2*; $p<0.05$; Fig. 6b), hypoxia-inducible factor 1 alpha (*Hif1a*; $p<0.05$; Fig. 6c) and chemokine (C-C motif) ligand 2 (*Ccl2*; $p<0.05$; Fig. 6d) compared with the control/high-fat diet mice ($n=4$). The expression of these genes indicates higher adipogenesis and adipocyte differentiation, hypoxia and inflammation, respectively, in accordance with the lower glucose tolerance observed in the CVS/high-fat diet mice.

Fig. 4 Effects of chronic consumption of a high-fat diet after exposure to CVS on body composition in C57BL/6 male mice. Mice subjected to CVS and fed with chow or a high-fat diet (black bars, $n=8$) had a significantly higher percentage of lean mass gain compared with the control mice (white bars, $n=7-8$) (b) with no statistically significant changes in body weight gain (a). Mice subjected to CVS

and fed with chow ($n=8$) had a significantly lower 6 day average respiratory quotient compared with the control mice ($n=7$), with no changes between the groups fed the high-fat diet ($n=8$) (c). Two-tailed unpaired t test and one-way ANOVA; Tukey's post hoc test * $p<0.05$. Ctrl, control; HFD, high-fat diet

Fig. 5 Effects of high-fat diet after exposure to CVS on plasma adiponectin and resistin and on glucose tolerance in C57BL/6 male mice. Mice subjected to CVS and fed with the high-fat diet (black bars/circles, $n=7-8$) had significantly lower plasma adiponectin levels (**d**), higher plasma resistin levels (**e**) and significantly lower glucose tolerance (**f**) compared with the control/high-fat diet mice (white bars/

circles, $n=8$). Mice fed chow did not differ in relation to CVS in plasma adiponectin, resistin and glucose tolerance (chow-fed control mice, white bars/circles, $n=7$; chow-fed CVS mice, black bars/circles, $n=7-8$) (**a-c**). Two-tailed unpaired t test and two-way ANOVA, Tukey's post hoc test $*p<0.05$

Mice fed chow did not differ in relation to CVS mice in the expression of the factors mentioned above ($n=4$; Fig. 6a–d). Mice subjected to CVS and fed with the high-fat diet ($n=3$) showed higher lipogenesis as indicated by the significantly higher expression of glycerol kinase (*Gyk*) compared with CVS/chow mice ($n=4$, $p<0.05$), but not when compared with control/high-fat diet mice ($n=4$). Other specific gene targets involved in carbohydrate metabolism (*Pdk2*, *Pdk4*, *Slc2a1*, *Slc2a4*), fatty acid synthesis (*Pparg*, *Acaca*, *Fasn*, *Mlxipl*, *Scd1*, *Scd2*), fatty acid transport (*Cd36*, *Fabp4*, *Fabp5*, *Slc27a1*), lipoprotein metabolism (*Abca1*, *Apoe*, *Cd68*, *Ldlr*, *Lrp1*, *Scarb1*), lipogenesis (*Srebf1*), lipolysis (*Lipe*, *Lpl*), inflammation (*Il10*, *Itgax*, *Tnf*), protein metabolism (*Agt*, *Ppib*), cytokine signalling (*Adipoq*, *Atg4c*, *Lep*, *Retn*), cell differentiation (*Dlk1*, *Vegfa*), cell adhesion/migration (*Emr1*), steroid metabolism (*Abcg1*, *Hmgcr*) and regulation of proliferation (*Insig1*) were not expressed at different levels in the CVS and the control groups.

CVS mice consuming the high-fat diet had significantly lower plasma NEFA (0.07714 ± 0.006 mmol/l, $n=7$, $p<0.05$) compared with the control mice (0.09625 ± 0.005 mmol/l, $n=8$; Fig. 6b) and lower plasma glycerol (739.1 ± 27.67 μ mol/l, $n=7$, $p<0.05$) compared with the control mice (901.2 ± 59.58 μ mol/l, $n=8$; Fig. 6d). There were no differences in plasma NEFA (control 0.0675 ± 0.01013 mmol/l vs CVS 0.0750 ± 0.005345 mmol/l, $n=8$) and glycerol (control 527.6 ± 69.86 μ mol/l vs. CVS 483.7 ± 41.65 μ mol/l, $n=7-8$) between the groups consuming chow

(Fig. 6a,c). There were no significant differences between the groups in fasting plasma insulin levels under chow (control mice 912.8 ± 200.9 pmol/l, CVS mice 637.5 ± 112.9 pmol/l, $n=6-8$) or with the high-fat diet (control mice $3,077\pm390.3$ pmol/l, CVS mice $4,815\pm1,656$ pmol/l, $n=7$; Fig. 6e, f). There were no differences in plasma leptin levels with the chow (control mice 3.201 ± 1.047 μ g/l, CVS mice 2.730 ± 0.3694 μ g/l, $n=7-8$) or the high-fat diet (control mice 39.19 ± 6.552 μ g/l, CVS mice 47.52 ± 10.01 μ g/l, $n=6-7$; Fig. 6g, h). Similarly, no significant differences were found: in plasma corticosterone levels between the groups with chow (control mice 222.8 ± 26.19 nmol/l, CVS mice 190.9 ± 51.33 nmol/l, $n=7-8$) or the high-fat diet (control mice 227.4 ± 27.91 nmol/l, CVS mice 206.7 ± 22.88 nmol/l, $n=7-8$); in plasma thyroid-stimulating hormone with chow (control mice 0.0009156 ± 0.0001525 mg/l, CVS mice 0.001080 ± 0.0003844 mg/l, $n=7-8$) or the high-fat diet (control mice 0.0004667 ± 0.0001080 mg/l, CVS mice 0.0005617 ± 0.0001139 mg/l, $n=7-8$); or in total plasma T_4 levels with chow (control mice $6,837\pm1,428$ nmol/l, CVS mice $5,187\pm468.7$ nmol/l, $n=6-8$) or the high-fat diet (control mice $4,537\pm1,178$ nmol/l, CVS mice $4,413\pm644.6$ nmol/l, $n=6-7$). Total plasma T_3 levels were below the lower limit of detection in most of the samples.

As expected, the control ($5,341\pm559.2$ μ m², $n=5$, $p<0.01$) as well as the CVS ($6,241\pm423.1$ μ m², $n=4$, $p<0.001$) mice fed a high-fat diet had significantly increased adipocyte size compared with the chow-fed groups (control mice $1,711$

Fig. 6 Effects of CVS followed by chronic exposure to high-fat diet on plasma NEFA, glycerol, insulin and leptin in C57BL/6 male mice. Mice subjected to CVS and fed with the high-fat diet (black bars, $n=7$) had significantly lower plasma NEFA (**b**) and glycerol levels (**d**) compared with the control/high-fat diet mice (white bars, $n=8$). Mice fed chow did not differ in relation to CVS in plasma NEFA and glycerol ($n=8$) (**a**, **c**). Mice fed chow or high-fat diet did not differ in relation to CVS in plasma insulin and leptin ($n=6-8$) (**e-h**). Two-tailed unpaired t test * $p<0.05$

$\pm 275.4 \mu\text{m}^2$, CVS mice $930.7 \pm 132.4 \mu\text{m}^2$, $n=2-4$). There were no significant differences between the groups regarding CVS status (Fig. 7f).

CVS mice fed a high-fat diet actually had a significantly lower adrenal weight relative to total body weight ($0.005688 \pm 0.0002537\%$, $n=5$, $p<0.01$) when compared with CVS chow-fed mice ($0.009121 \pm 0.0007406\%$, $n=7$) at the time they were killed. There were no significant differences among the groups in relation to chow consumption, CVS status or when total adrenal weights were compared (data not shown).

Discussion

Chronic stress elicits neurochemical, neuroanatomical and cellular changes that may have serious health consequences.

For instance, chronic stress has been proposed to participate in the aetiology and progression of neurological disorders such as depression, anxiety and PTSD [34]. PTSD in particular is highly correlated with the incidence of obesity [17]. However, the molecular mechanisms underpinning PTSD-induced metabolic disorders are unknown.

Here, we characterise a mouse model in order to study the long-term effects of a discrete period of stress exposure on glucose homeostasis. Mice that were chronically stressed and subsequently fed a high-fat diet had impaired glucose tolerance as well as decreased white adipose tissue insulin sensitivity, as indicated by gene markers for adipogenesis, adipocyte differentiation, hypoxia and inflammation, compared with non-stressed mice fed the same diet, suggesting that prior stress exposure has long-term consequences for metabolic regulation. Interestingly, CVS mice fed the high-fat diet had lower circulating adiponectin and higher circulating resistin levels, suggesting that stress-induced imbalances in these two adipokines may contribute to the impairment of glucose metabolism.

The application of CVS to male mice resulted in lower plasma triacylglycerol levels, body weight, lean mass and SPA, consistent with previous reports regarding several different stressors [2, 35, 36]. The decrease in body weight in male mice seems to be mainly related to a parallel decrease in lean mass. This muscle wasting could be a consequence of the higher levels of corticosterone caused by the application of the stress, as documented in Cushing's disease and in rodents undergoing different stressors [2, 37–39]; however, we did not find sustained differences in plasma corticosterone levels weeks, or even after the sustained stress exposure phase. This finding is consistent with our hypothesis that a metabolic impairment associated with PTSD is a late-onset consequence of re-programming rather than a direct and immediate result of higher HPA-axis activity. The decreased body weight was not accompanied by a decrease in adipose mass.

In addition to stress, another factor predisposing to the development of obesity and insulin resistance is exposure to a hyperenergetic environment typical of many western societies. Importantly, diet-induced obese mice are the most commonly used model for the study of polygenic obesity, as these mice share numerous features of obese patients. Although the effects of a high-fat diet in combination with a history of CVS are unknown, it is well established that chronic-restraint stress in rodents has a synergistic action on the HPA axis that translates into altered lipid metabolism and can lead to insulin resistance [24, 40]. Increased activity of the HPA axis with higher levels of circulating glucocorticoids opposes insulin action [41, 42], thereby potentially predisposing the organism to insulin resistance and its consequences. An association between the HPA axis

Fig. 7 Effects of CVS followed by chronic exposure to the high-fat diet on gene expression in white adipose tissue of C57BL/6 male mice. Mice subjected to CVS and fed with the high-fat diet (dark grey bars, $n=4$) show higher adipogenesis and adipocyte differentiation, hypoxia and inflammation as indicated by the significantly higher expression of CCAAT/enhancer binding protein (C/EBP), β (*Cebpb*) (a), early growth response 2 transcription factor (*Egr2*) (b) hypoxia-inducible factor 1 α (*Hif1a*), (c) and chemokine (C-C motif) ligand 2 (*Ccl2*) (d) compared with the control/high-fat diet mice (light grey

bars, $n=4$). Mice fed chow did not differ in relation to CVS in the expression of the genes mentioned above (white and black bars, $n=4$) (a–c). Mice subjected to CVS and fed with the high-fat diet (dark grey bars, $n=3$) showed signs of increased lipogenesis as indicated by the significantly higher expression of glycerol kinase (*Gyk*) (e) and adipocyte size (f) compared with CVS/chow mice (black bars, $n=4$ –5) but not when compared with control/high-fat diet mice (light grey bars, $n=4$). One-way ANOVA, Tukey's post hoc test $*p<0.05$, $**p<0.01$, $***p<0.001$

and energy balance has, for example, been observed in obese humans in whom dexamethasone, a synthetic glucocorticoid, and insulin synergistically activate lipoprotein lipase in adipose tissue, thereby facilitating fat deposition [43]. Therefore, we expected that consumption of a high-fat diet after exposure to chronic stress would promote the development of obesity and diabetes. Unexpectedly, however, our results indicate that the risk for the development of obesity does not differ from that in non-stressed mice fed the high-fat diet at the time they were killed.

Intriguingly, we discovered that insulin sensitivity is impaired in CVS/high-fat-fed mice. They were glucose intolerant and, consistent with that response, had lower adiponectin and higher resistin levels in the plasma. Adiponectin increases while resistin decreases [44–47] insulin sensitivity, consistent with the conclusion that the impaired glucose metabolism in CVS mice fed with a high-fat diet may be mediated by these endocrine pathways, rather than directly resulting from increased HPA-axis activity. Another finding consistent with this hypothesis is that impaired glucose metabolism was apparent in mice consuming the high-fat diet but not in those fed low-fat chow (in which adiponectin and resistin levels were normal), in spite of both groups receiving CVS. We therefore propose that the levels of adiponectin and resistin

are relevant for the development of insulin resistance in this case and are not secondary to increased plasma glucocorticoids, as previously implied [44–47].

Glucocorticoids have been found to inhibit adiponectin in vitro and in vivo, and adiponectin levels are decreased in Cushing's syndrome [48, 49]. Furthermore, glucocorticoids also stimulate resistin secretion [50]. As the impairment in glucose tolerance was present only in those CVS mice consuming the high-fat diet and not in CVS mice consuming chow, it is possible that glucocorticoids decreased adiponectin and increased resistin levels at the time of stress exposure. CVS mice subsequently fed with chow may have been able to recover to baseline levels of both hormones, while those on the high-fat diet may not.

White adipose tissue showed higher expression of genes that participate in the key processes of adipogenesis (*Cebpb*), adipocyte differentiation (*Egr2*), hypoxia (*Hif1a*) and inflammation (*Ccl2*) compared with the control/high-fat-fed mice, indicating impaired insulin sensitivity and perhaps an early state of developing obesity in the CVS/high-fat diet mice. In addition, plasma NEFA and glycerol levels were significantly lower, an indication of higher lipogenesis and fat synthesis. In addition, the CVS/high-fat diet mice showed higher expression of the glycerol kinase gene and adipocyte size, as indicators of lipogenesis, compared with the CVS/chow group. However, neither

glycerol kinase expression nor adipocyte size were significantly different from the control/high-fat-fed mice. As with adiponectin and resistin, these differences in gene expression profiles, NEFA and glycerol levels between CVS and control mice were only observed following chronic exposure to a high-fat diet. Therefore, our findings might indicate that chronic exposure to a hyperenergetic environment post-CVS may enhance or maintain stress-induced metabolic damage.

In summary, our study demonstrates that exposure to a high-fat diet after a period of CVS can impair glucose tolerance without affecting overall body composition at the time. These findings are consistent with clinical observations of veterans returning from exposure to severe stress into a hyperenergetic and sedentary environment. Therefore, this new animal model may offer unique insights into the molecular mechanisms leading to PTSD-induced metabolic disorders.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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