CHAPTER 6

NEONATAL CAPSAICIN TREATMENT INCREASES LEPTIN SENSITIVITY AND IMPROVES ENDOCRINE PROFILES RELEVANT TO GLUCOSE HOMEOSTASIS IN RATS

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ABSTRACT

Several mechanisms involved in ingestive behavior and neuroendocrine activity rely on vagal afferent neuronal signaling. This idea is seemingly contradictive with observations that vagal afferent neuronal ablation by neonatal capsaicin (CAP) treatment has a remarkably small effect on long-term regulation of energy balance and fuel homeostasis. The current study addressed the hypothesis that humoral endocrine factors and/or their sensitivities compensate for the loss of vagal afferent information, particularly when subjects face disturbances in ambient fuel levels. Therefore, male adult rats neonatally treated with CAP or with the vehicle (VEH) underwent IVGTTs during which blood fuel levels, and circulating adipocyte, pancreatic, and adrenal hormones were assessed. CAP rats displayed similar hyperglycaemia as VEH rats, but with markedly reduced plasma insulin and corticosterone responses. These results indicate that CAP rats have increased insulin sensitivity during hyperglycemic episodes, and lower plasma levels of corticosterone in CAP rats relative to VEH rats could underlie this effect. After the IVGTT, CAP rats had increased plasma adiponectin and reduced plasma resistin levels, and these alterations in adipocyte hormones might be relevant for post-ingestive metabolic (e.g., fatty acid oxidation) processes. In a second experiment, anorexigenic efficacies of cholecystokinin and leptin were assessed. While VEH rats, but not CAP rats, responded with reduced food intake to i.p. injected cholecystokinin, only CAP rats responded to i.v. infused leptin with a reduction in food intake. It is concluded that changes in HPA axis activity and leptin signaling could underlie compensations in fuel handling and energy balance following CAP treatment.

1 INTRODUCTION

Energy homeostasis is maintained by a large array of biochemical and physiological mechanisms that help to ensure the constancy of the internal environment under varying nutritional conditions and energy demands. An important component of the underlying regulatory processes consists of peripheral information regarding the energetic status which is conveyed via

vagal neuronal afferents to the CNS. In turn, these signals are relayed in CNS neuronal networks where they play a role in the sensation of hunger and satiety as well as in the regulation of neuroendocrine control of fuel homeostasis (39).

With the advent of capsaicin (CAP) -a pungent ingredient of red peppers which selectively destroys primary C-afferents and small myelinated Aδ-afferents (for review see (19, 37))- a non-invasive tool was provided to study the role of vagal primary afferents in the regulation of energy balance. CAP-treated animals have disturbances in short-term satiety signaling (submitted), and do not respond to cholecystokinin (CCK) with a reduction in food intake (29, 30, 33). However, CAP-treated rats have similar daily food intake (submitted), similar or even lower body weight (9) and improved glucose homeostatic control compared to controls. Furthermore, deafferentated animals are more resistant to ageing-associated obesity (26) and have a long-term decrease in white adipose tissue mass (9). Finally, CAP treatment results in increased whole body insulin sensitivity (22), and a lower degree of ageing-associated insulin resistance (26). These observations indicate that CAP-treated animals are able, or even have improved capability, to maintain body and fuel homeostasis, despite the fact that they lack seemingly important information transmitted via vagal afferents to the CNS. To date, the underlying mechanisms are poorly understood.

Another class of peripheral factors highly relevant to the regulation of ingestive behavior and fuel homeostasis consists of endocrine/hormonal compounds which are released into the blood stream, and affect enzymatic/endocrine processes and metabolic fluxes in various peripheral organs and tissues (41). In addition, most of these factors can enter the CNS where they alter the activity of neuronal circuitry involved in ingestive behavior, neuroendocrine outflow and metabolism (41). One hypothesis pertinent to the observations that CAP-treated rats are able to maintain body and fuel homeostasis might be that vagal afferent ablation is compensated by these redundant endocrine factors involved in the regulation of fuel homeostasis and ingestive behavior. To investigate this hypothesis, the concentration of blood fuels (i.e., plasma glucose and free fatty acids) and circulating hormones involved in blood glucose regulation and ingestive

behavior (i.e., insulin, leptin, adiponectin, resistin and corticosterone) were investigated in overnight fasted rats that were neonatally treated with CAP or with the vehicle (VEH). In addition, the changes in these blood parameters were assessed during and after an intravenous glucose tolerance test (IVGTT). In a second experiment, anorexigenic efficacies of CCK, leptin, and the synthetic melanocortin (MC) receptor-agonist, melanotan II (13), were assessed in CAP and VEH rats. The latter study was performed since the MC4 receptor is implicated in the leptin signaling cascade (31).

2 MATERIALS AND METHODS

2.1 Animals and housing

Twenty-eight male Wistar rats from the breeding facility of our University were used and housed in climate-controlled rooms (22 °C ± 2) under a 12h: 12h light-dark cycle (lights on at 8:00 am). Food and water were ad libitum available, unless mentioned otherwise. All experiments were checked and approved by the Local Ethics Committee of our University.

2.2 Capsaicin treatment

Rats were treated neonatally with CAP (8-methyl-N-vallinyl 6 nonenamide, 50 mg/kg; Sigma Chemical, The Netherlands) at the age of day 2 (n=14) by subcutaneous (s.c.) injection. This was done under 100% O_2 conditions to avoid hypoxia. CAP was dissolved in vehicle consisting of 10% ethanol (10%) and 5% cremophore-0.9% sodium chloride solution (90%). As a control, VEH solution was injected s.c. (n =14). Each animal was given the same volume of 50 µl based on an average weight of the pups of 8 grams. At injection, both groups did not differ significantly in body weight (CAP 8.84 g ± 0.20; VEH 8.32 g ± 0.23). CAP-treated and VEH-treated pups grew up separately –to avoid selective mother care- in litters of 5-9 pups, in the proportion of 5-7 male on 2 females (untreated). After weaning at the age of 23 days, rats were individually housed in clear Plexiglas cages (25 × 25 × 30 cm) with a bedding of sawdust. Following treatments, body weights were assessed at days 34, 58, and thereafter every 10 days until experiments. An eye wipe response (0.1% capsaicin solution) was done at the age of 3 months in order to test the

effectiveness of the CAP treatment. As opposed to the VEH controls, none of the neonatally CAP-treated animals responded to the test and all animals were therefore included in the experiment.

2.3 Surgery

After the eye-wipe test, 16 animals were implanted with double heart catheters in the left and right jugular veins according to techniques described by Steffens (35). An additional 12 animals were provided with heart catheters only in the right jugular vein according to the same techniques. Surgery was performed under anaesthesia with isoflurane/N_2O/O_2. Fynadine (0.01 ml/ 100g body weight) was given s.c. as post-surgical analgesia. Animals had at least 2 weeks of recovery before the start of experiments.

2.4 Intravenous glucose tolerance test (IVGTT)

Overnight food-deprived CAP (n=8) and VEH-treated rats were subjected to an IVGTT, which was performed in the light period between 12:00 am and 1:00 pm. At least half an hour before the start of the IVGTT, rats were connected with their indwelling cannulae to blood sampling (right jugular catheter) and infusion (left jugular catheter) tubing. These tubes extended out of the rats' cages, which allowed stress-blood sampling and/or intravenous infusion. After taking two basal blood samples at t = -11 and t = -1 min, a glucose solution (15% dissolved in sterile demineralized water) was infused over a 30-min. period at a rate of 15 mg per minute (450 mg total). Additional samples were taken at t = 1, 3, 5, 10, 15, 20, 25, 30, 40, 50 minutes in order to assess blood glucose and plasma insulin. In general, samples consisted of 0.2 ml whole blood for assessment of blood glucose (50 µl) and plasma insulin (50 µl) levels. At t=-11, t=30, and t=50, an additional 0.2 ml of blood was taken for determination of plasma levels of adiponectin (3 µl), leptin (30µl), resistin (30 µl), corticosterone (10 µl), and free fatty acids (FFAs, 10 µl). Blood and plasma samples were stored at – 20 °C until analysis. Blood glucose levels were measured by the ferricyanide method of Hoffman, plasma levels of insulin, adiponectin, leptin, resistin and corticosterone were measured by commercial radioimmunoassay kits (Linco Research, Nucli lab, The Netherlands), and

plasma levels of FFAs were assessed with a NEFA C enzymatic kit (WAKO Chemicals GmbH, Germany).

2.5 Anorexigenic efficacies of CCK, leptin and melanotan-II

In another group of CAP (n=4-6) and VEH (n=4-6)-treated rats, the anorexigenic efficacies of CCK, leptin, and the synthetic melanocortin 3/4 receptor agonist, melanotan-II were assessed. Therefore, rats' food hoppers were removed from their home cages 2 hours before lights off. In a counterbalanced design, and with 5 days elapsing between successive experiments, rats were i.v. infused between 30 and 15 minutes before lights off solutions containing leptin (70 μg/250 μl saline, Calbiochem, Germany), melanotan-II (50 μg/250 μl saline, Sigma Chemical, the Netherlands), or with saline (250 μl) only. After all treatments, food hoppers were returned to the cages at lights off, and cumulative food intake was assessed at 1, 2, and 4 hours in the dark phase. In other tests, but under similar experimental conditions, these animals were i.p. injected with saline (250 μl) or with saline containing CCK (4 μg/kg Sigma Chemical, the Netherlands) just before the dark phase. Because vagal afferent ablation is known to impair peripheral actions of CCK on ingestive behavior (29, 30, 33), this latter comparison was performed as a positive control for CAP treatment. Seven animals of each group were decapitated (non-fasted) at the end of the experiment and weights of fat pads (retroperitoneal and epididymal fat) and liver as well as basal plasma leptin levels were assessed.

2.6 Statistical analysis

Analysis of variance (ANOVA) with repeated measurements was performed for statistical evaluation with time (sampling points) as within subject factor and group (CAP or VEH) as between subject factor. Post-hoc pair wise comparisons (LSD-test) were done based on estimated marginal means. Statistical testing was performed from sampling point -11 or -1 minute till sampling point 30 min. at the end of the glucose infusion. One-sided student's t-test was used for unpaired observations. A value of $p \leq 0.05$ was considered significant for all tests.

3 RESULTS

Body weights of CAP and VEH rats are shown in figure 1. Although CAP rats appeared slightly lighter than VEH rats, there were no significant differences over time. In VEH and CAP rats, epididymal fat pad weights (8.9±0.8 g and 7.5±0.9 g, respectively), retroperitoneal fat pad weights (2.6±0.3 g and 2.0±0.5 g, respectively), liver weights (16.5±0.7 g and 15.5±1.4 g, respectively), and plasma leptin levels (3.95±0.79 ng/ml and 3.54±1.12 ng/ml, respectively) did not differ significantly.

3.1 Intravenous glucose tolerance test (IVGTT)

Figure 2 shows the changes in blood glucose and plasma insulin levels before, during, and after the 30-minute intravenous glucose infusion. ANOVA with repeated measurements revealed significant effects of time on plasma levels of insulin and glucose ($F_{8, 112}$ = 40.6, p< 0.001 and $F_{8, 80}$ = 66.7, p< 0.05 resp.). There was no significant time × group interaction for insulin ($F_{8, 112}$ = 1.7, p= 0.11) or glucose ($F_{8, 80}$ = 0.46, p= 0.88). There was a significant group effect on plasma insulin levels during glucose infusion ($F_{1, 14}$ = 4.9, p< 0.05), but blood glucose levels did not differ significantly between CAP and VEH ($F_{1,10}$ = 0.46, p= 0.51). This difference in insulin response was particularly clear at t= 1 minute, which is considered as the first-phase insulin response (CAP= 4.55 ± 0.43, VEH= 7.03 ± 0.71, p< 0.01).

Figure 3 shows changes in the plasma concentrations of the adipocyte hormones leptin, adiponectin, and resistin at t=-1, 30 and 50 min. At baseline (t=-1), none of the assessed levels of differed among CAP en VEH, and these levels were not different during glucose infusion either. However, after cessation of glucose infusion (t=50 min), the plasma adiponectin level of CAP rats was significantly higher (p<0.05) than that of VEH rats. In contrast, plasma resistin was lower at t=50 min in CAP rats relative to VEH controls (p<0.05). Plasma levels of leptin were not different in CAP and VEH rats ($F_{1, 13}$ = 2.12, p = 0.17). Figure 4 shows the changes observed in plasma concentrations of corticosterone and FFAs. In VEH controls, plasma levels of cortictsterone were increased as a result of glucose infusion, but this effect was not observed in CAP rats. Thus, plasma levels of corticosterone in CAP rats

were significantly lower ($p<0.01$) than in VEH rats at t=30 min. During glucose infusion, plasma levels of FFAs were reduced in both groups relative to baseline. After infusion, there was a partial rebound in VEH rats, but not in the CAP rats. Thus, plasma FFAs were significantly reduced at t=50 min ($p<0.05$) in CAP rats relative to VEH controls.

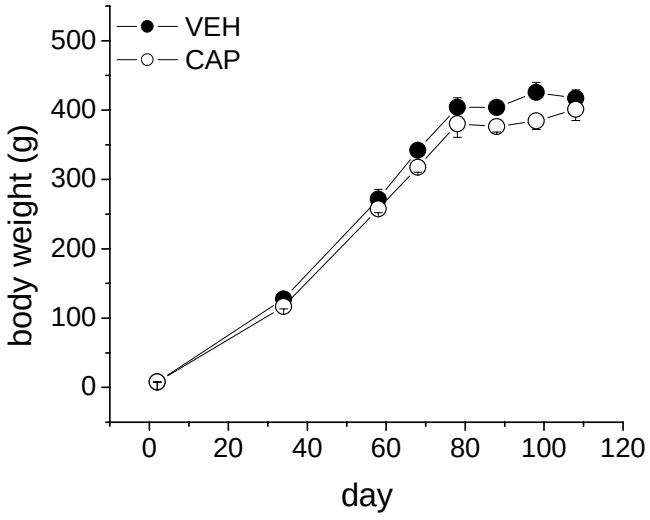

Figure 1
Effects of neonatal capsaicin (CAP) treatment and vehicle (VEH) treatment on body weight gain of male Wistar rat.

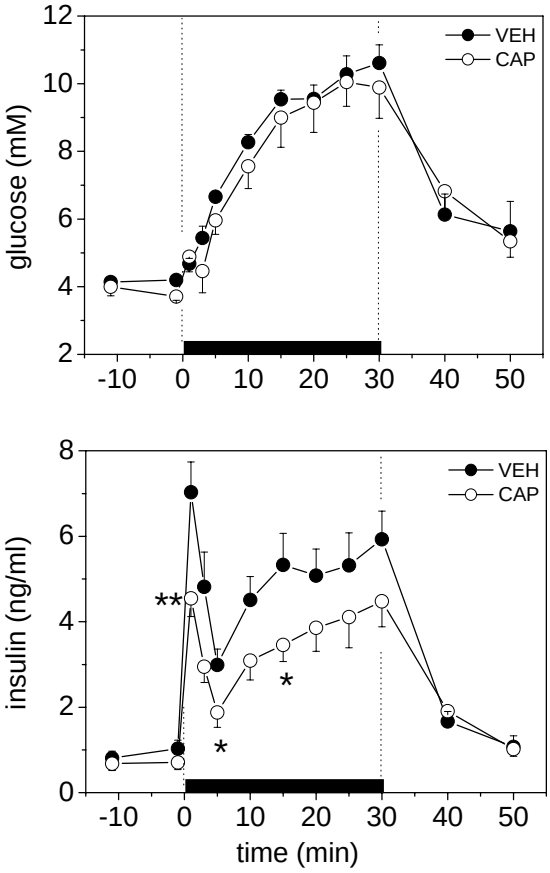

Figure 2
*Blood glucose and plasma insulin levels before, during, and after an IVGTT consisting of a 15% glucose infusion infused over 30 min. in adult male Wistar rats, which were neonatally treated with capsaicin (CAP) or vehicle (VEH). *, **; $p < 0.05$ and $p < 0.01$ respectively.*

3.2 Anorexigenic efficacies of CCK, leptin and melanotan-II

Figure 5 shows the effect of iv infusion of leptin and MTII relative to saline treatment, and of ip injection of CCK relative to saline treatment on food intake. CCK (4 μg/kg) caused a significant reduction in food intake relative to saline treatment in VEH controls ($p<0.05$) during the first hour of the dark phase, and a tendency to reduce food intake during the second hour. These effects were not observed in CAP rats. In contrast, i.v. leptin infusion (70 μg) appeared to be effective over the first 2 hours in the dark phase only in CAP

rats ($p<0.05$), but not in VEH rats. This effect was mostly due to the fact that the leptin-treated CAP rats did not have food intake during the second hour, whereas food intake over the first hour was similar as in VEH rats. Finally, iv infusion of MTII was equally effective in reducing food intake over the full 4-hour period in CAP and VEH rats. Interestingly, MTII was more effective to reduce food intake over the first hour in VEH rats than in CAP rats ($p<0.01$).

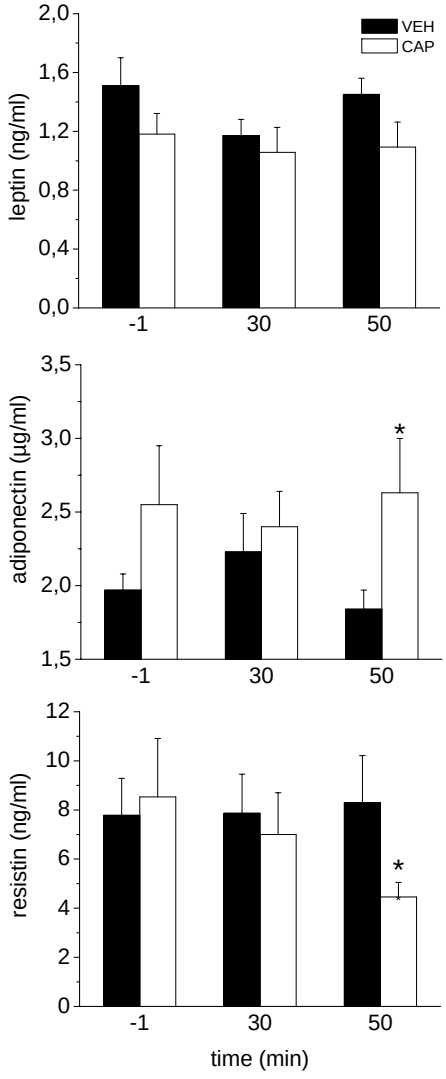

Figure 3
Circulating adipocyte factors leptin, adiponectin and resistin before, during, and after an IVGTT consisting of a 15% glucose infusion infused over 30 min. in adult male Wistar rats, which were neonatally treated with capsaicin (CAP) or vehicle (VEH)., $p < 0.05$.*

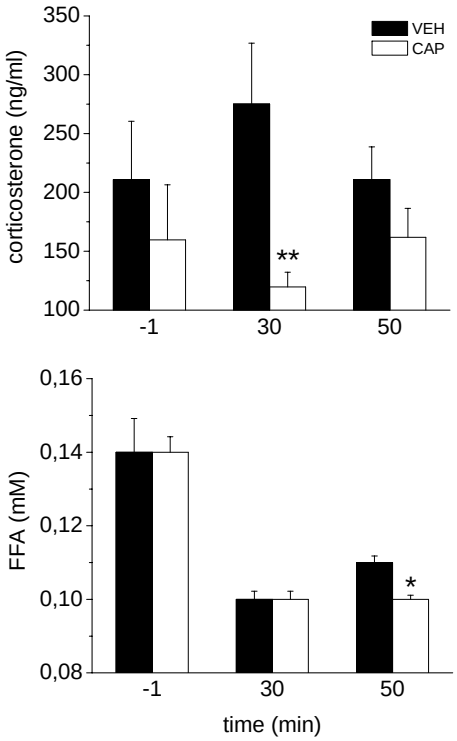

Figure 4
*Plasma corticosterone levels and free fatty acid (FFA) levels before, during, and after an IVGTT consisting of a 15% glucose infusion infused over 30 min. in adult male Wistar rats, which were neonatally treated with capsaicin (CAP) or vehicle (VEH). *, **; p < 0.05 and 0.01 respectively.*

4 DISCUSSION

Vagal afferent ablation in rats by neonatal capsaicin (CAP) treatment has been shown in other studies to be ineffective (van de Wall et al., submitted), or in some cases even preventive (26) of causing disturbances in energy balance and fuel homeostasis. Since vagal afferents are thought to serve important homeostatic functions (38), the present study was designed to investigate the hypothesis that neonatal CAP treatment results in compensatory adjustments by redundant endocrine factors involved in the regulation of energy balance and fuel homeostasis. Important for consideration of the data in the present study is that bodyweights of our CAP and VEH rats were not different, nor were there any overt differences in weights of organs and tissues relevant to

nutrient balance. Basal (i.e., non-fasted) plasma leptin levels did not differ between CAP and VEH animals either.

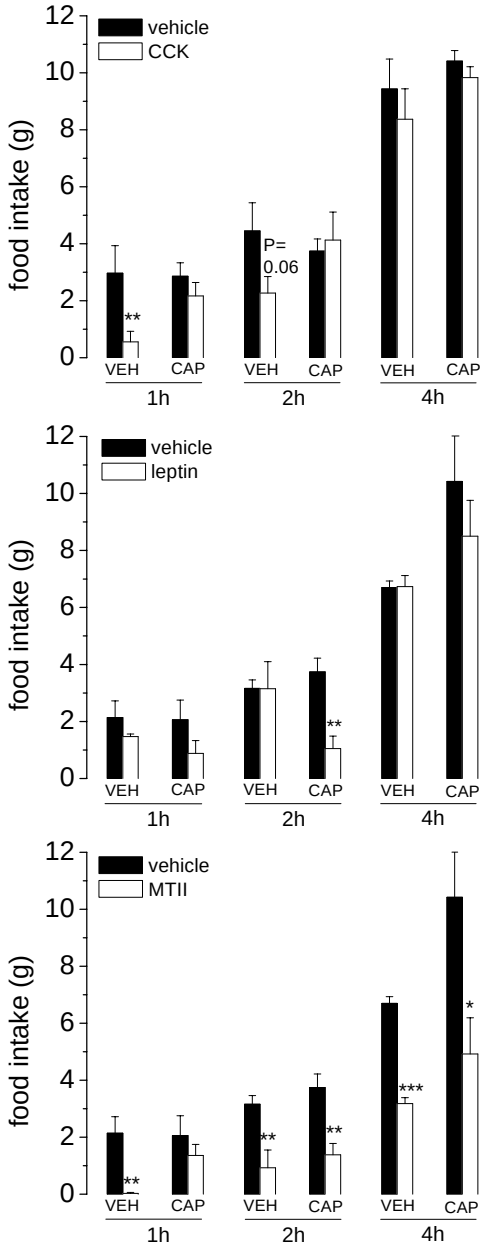

Figure 5
*Effects of cholecystokinin (CCK), leptin and melanotan-II (MTII) on food intake in adult male Wistar rats which were neonatally treated with capsaicin (CAP) or vehicle (VEH). *, **, ***; p < 0.05, 0.01 and 0.001 respectively.*

Consistent with a seemingly normal regulation of energy balance was the observation that CAP and VEH-treated rats had indistinguishable fasting levels of adipocyte (i.e., leptin, adiponectin, and resistin), pancreatic (i.e., insulin) and adrenal (i.e., corticosterone) hormones; i.e., all factors known to correlate strongly with changes in energy balance. A different picture emerged when viewing the data obtained with the IVGTT. Thus, whereas the IVGTT caused similar increments in blood glucose levels in CAP and VEH rats, the plasma insulin response was markedly reduced in CAP rats relative to that in VEH-treated controls. These data confirm our previous findings in non-fasted CAP and VEH rats challenged with different glucose loads, yielding similar dose-dependent elevations in blood glucose levels, but with much lower plasma insulin responses in CAP rats relative to those seen in VEH rats (van de Wall et al., submitted). While the reduced glucose-mediated insulin response in CAP rats might be the result of absence of tonic activation of vagal afferents by gut hormones (1, 32), one implication of these findings is that CAP rats are more insulin-sensitive than VEH controls. This idea is in agreement with the findings of Koopmans et al. (22), who observed increased whole body insulin action in CAP rats under euglycemic hyperinsulinemic clamp conditions.

Humoral factors that stimulate insulin-dependent glucose uptake are leptin and adiponectin (18) in a variety of tissues, whereas corticosterone (3), resistin (36) and FFAs (5, 6) have the opposite effects. Among these, only the plasma level of corticosterone was significantly different in CAP and VEH rats at the end of the IVGTT. More specifically, the IVGTT led to an increase in the plasma corticosterone level in the VEH rats, but this effect was not observed in CAP rats. Although we have not performed a full analysis of plasma corticisoterone levels over the course of the IVGTT, these data might suggest that this mechanism underlies the increased insulin sensitivity in CAP rats. A lower corticosterone response in CAP rats was previously observed by Koopmans et al. (22), and, together with the data in the present study, this reinforces a role for vagal afferents in the activation of the HPA axis during hyperglycaemia. It seems likely that vagal afferents normally convey stimulatory actions of gut hormones, such as CCK, on HPA axis activity (21). These effects might be amplified under hyperglycemic condition, analogous to the mechanism underlying stimulated insulin secretion (32). Such a

dependency on hyperglycaemia would be consistent with the finding in the present study that the difference in plasma corticosterone levels in CAP and VEH rats disappeared as rats regained normoglycaemia after the IVGTT.

After cessation of the IVGTT, blood glucose, plasma insulin and corticosterone levels returned to normal, but this was associated with significantly higher plasma adiponectin and lower resistin levels in CAP rats relative to VEH controls. It might be possible that the transiently different plasma corticosterone level in CAP and VEH rats contributed to these effects (12, 24) but additional or more important factors are not ruled out. Although it is unlikely that the changes in adiponectin and resistin contributed to the differences in glucose-to-insulin indexes during the preceding IVGTT, they might have a major impact on successive excursions of blood glucose, or on the metabolic consequences of these. In fact, the lower level of plasma FFA in CAP rats after the IVGTT might be a direct consequence of elevated plasma adiponectin levels and/or reduced plasma resistin levels in these animals. Indeed, adiponectin has been shown to increase oxidation of FFA in skeletal muscle (43) and to stimulate muscle fatty acid transporter(23). This would result in accelerated FFA clearance from the blood. A link between circulating FFAs and resistin is less clear, but correlation analysis in mice suggests an interaction between high circulating resistin levels with hyperlipidemia, as well as with obesity and insulin resistance (35). Our results are in agreement with Spiridonov (34) who also reports decreased FFA levels after neonatal treatment. Typically, higher levels of FFA are associated with disturbances in glucose homeostatic mechanisms (27) and this could mean that decreased levels of FFA contribute to the enhanced glucose disposal in CAP rats in following fuel excursions.

Despite the observed changes in adiponectin, resistin, and corticosterone responses, there was no effect of the IVGTT on the plasma levels of leptin, nor were there any differences between the plasma leptin levels of CAP and VEH rats at baseline. One idea that we addressed was the possibility that CAP treatment increases leptin signaling. Whereas injection of CCK, dosed to cause a reduction in food intake in VEH rats, did not have any effect in CAP rats in the present study (and confirming previous reports by (29, 30, 33), we observed that peripherally infused leptin caused a reduction in food

intake in the CAP rats, but failed to do so in VEH rats. These effects were particularly pronounced during the second hour in the dark phase; i.e., after the rats had eaten their first meals. Important for consideration of the effects of peripherally elevated levels of leptin is that these can be signaled directly in the CNS (i.e., through increased transport of leptin across the blood-brain barrier), and additionally via vagal afferent fibers (28, 42). Since CAP rats lack a substantial part of their vagal afferent innervation, yet have an increased sensitivity to leptin with respect to food intake modulation, it is likely that leptin's enhanced anorexigenic actions are mediated via interaction with CNS pathways. Actions of leptin on ingestive behavior are mediated through neural networks among which the brain melanocortin (MC) system might be most relevant (40). Since VEH rats responded slightly stronger to the anorexigenic actions of the brain-specific melanocortin receptor agonist, melanotan-II (14, 20), than CAP rats (presumably due to compensatory actions), the difference between leptin sensitivity in VEH and CAP rats is either located upstream from brain MC receptors, or requires changes in neuronal circuitry parallel to the brain MC system.

Provided that the augmented anorexigenic effects of leptin are coincided with amplified neuroendocrine and metabolic actions of leptin (41), this could possibly have contributed to the lower plasma levels of corticosterone (2) and resistin (4), and the elevated plasma level of adiponectin (10, 44) in CAP rats. One point of discussion is that capsaicin treatment delays the onset of type 2 diabetes mellitus in Zucker rats (17). This suggests that leptin would not be the (only) mediating factor in the effects of capsaicin treatment glucose homeostasis. However, results of al-Barazanji (2) suggest that leptin retains some efficacy in the obese Zucker rat, and that these are greatly amplified by removal of glucocorticoids. It is for example reported that adrenalectomy in Zuckers increases insulin sensitivity and metabolism, and reduces body weight gain (7, 11, 15). Thus, the exact mechanism behind the delay on the onset of type 2 diabetes mellitus in Zucker rats due to capsaicin treatment remains to be elucidated, but effects mediated via alterations in leptin signaling are anticipated.

Taken together, this study shows that CAP treatment results in endocrine, metabolic, and probably neuronal adjustments which serve to

maintain energy balance and fuel homeostasis in these animals. Absence of gut hormone signaling had primary effects on plasma corticosterone levels, which could have contributed to augmented insulin action during hyperglycaemia. Secondary effects on plasma adiponectin and resistin levels unlikely contributed to these effects, but could have major consequences on post-ingestive metabolism or successive fuel excursions. While these effects were associated with increased leptin sensitivity (with food intake suppression as read-out parameter), it remains to be investigated whether increased leptin signaling is a consequence or a cause of these effects. As such, these sort of interactions might have major implications for the aetiology of obesity and diabetes because these diseases are characterized by dysregulation of the hypothalamic-pituitary-adrenal axis (8), as well as of adipocyte hormone secretion and leptin signaling (16, 25).

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