EXPERIMENTAL STUDY

Plasma calcitonin gene-related peptide is increased prior to obesity, and sensory nerve desensitization by capsaicin improves oral glucose tolerance in obese Zucker rats

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Abstract

Objective: It has earlier been demonstrated that capsaicin-induced desensitization improves insulin sensitivity in normal rats. However, whether increased capsaicin-sensitive nerve activity precedes the onset of insulin resistance in diet-induced obesity – and therefore might be involved in the pathophysiology – is not known. Further, it is of relevance to investigate whether capsaicin desensitization improves glycaemic control even in obese individuals and we therefore chose the obese Zucker rats to test this.

Design and methods: Plasma levels of calcitonin gene-related peptide (CGRP; a marker of sensory nerve activity) was assessed in 8-week-old Zucker rats. To investigate whether capsaicin desensitization (100 mg/kg at 9 weeks of age) would also ameliorate glycaemia in this non-diabetic model, we assessed oral glucose tolerance at 7 weeks after capsaicin.

Results: It was found that plasma CGRP levels were elevated in obese Zucker rats prior to the onset of obesity (16.1 ± 3.4 pmol/l in pre-obese Zucker rats vs 6.9 ± 1.1 pmol/l in lean littermates; $P = 0.015$) despite similar body weights. Furthermore, capsaicin desensitization reduced both fasting blood glucose (4.3 ± 0.2 mmol/l vs 5.1 ± 0.2 mmol/l in controls; $P = 0.050$) as well as the mean blood glucose level during an oral glucose tolerance test (OGTT) (6.8 ± 0.3 mmol/l vs 8.6 ± 0.5 mmol/l in control obese rats; $P = 0.024$) whereas the plasma insulin levels during the OGTT were unchanged. However this did not lead to an improvement in insulin resistance or to a reduction of tissue triglyceride accumulation in muscle or liver.

Conclusion: We concluded that capsaicin-induced sensory nerve desensitization improves glucose tolerance in Zucker rats. Since, in this study, plasma CGRP levels, a marker of sensory nerve activity, were increased in the pre-obese rats, our data support the hypothesis that increased activity of sensory nerves precedes the development of obesity and insulin resistance in Zucker rats.

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Introduction

Type 2 diabetes is common among subjects with obesity (1) and is associated with impairment of both insulin secretion and action (2). However, the detailed mechanism underlying the development of diabetes in obesity has not been established to date. We recently proposed that increased activity of sensory nerves is important in modulating the early development of insulin resistance, impaired glucose tolerance and type 2 diabetes mellitus. This hypothesis was based upon results from Zucker diabetic fatty (ZDF) rats (3, 4) in which glycaemia was markedly improved by capsaicin-mediated desensitization. Capsaicin selectively

binds to small unmyelinated sensory nerves and reversibly inactivates these nerves when given to adult rodents. The beneficial effects of capsaicin in ZDF rats could possibly be due to reduced levels of calcitonin gene-related peptide (CGRP), since this neuropeptide is released from sensory nerves upon stimulation and has been shown *in vitro* to induce insulin resistance (5, 6) and inhibit insulin secretion (7, 8). However, whether increased activity of sensory nerves precedes the onset of diabetes and whether sensory desensitization improves glycaemia in prediabetic rats and thereby prevents the development of diabetes is not known. The aim of the present study was therefore to assess plasma CGRP levels in young pre-obese Zucker rat as well as

assessing the influence of capsaicin-mediated desensitization on glucose tolerance in this model of obesity and glucose intolerance (9, 10). Finally, we also assessed the levels of intramuscular and intrahepatic levels of triglycerides, glycogen and cholesterol after capsaicin treatment of these rats, because pathologic accumulation of lipids in peripheral tissue such as muscle (11) and liver (12) is believed to contribute to the development of insulin resistance, as lipid accumulation in the pancreas (13) is believed to contribute to impaired insulin secretion which are both seen in type 2 diabetes.

Materials and methods

Laboratory animals

Male obese Zucker rats (10 lean and 30 obese) were purchased from Charles River Lab. Inc., Sulzfeld, Germany and kept at Novo Nordisk A/S under ambient controlled conditions with a constant temperature ($20 \pm 2^\circ\text{C}$) and a fixed daylight cycle (lights off at 1800 h), with up to five rats per cage with free access to tap water and Altromin chow (Brogaarden Aps., Gentofte, Denmark). Plasma for determination of CGRP was obtained from 8-week-old rats. At the age of 9 weeks the rats were dosed with either capsaicin or vehicle (control) and the rats were followed for 49 days. The lean rats served as normal controls and were dosed with vehicle. Body weight was measured from before capsaicin (-7 days) until the end of the study (49 days). Food intake was measured once weekly for the last 3 weeks (days 35, 42 and 49). Oral glucose tolerance was determined 49 days after capsaicin treatment, the rats were killed the next day and tissue was stored for further analysis of tissue content of lipids and glycogen. Principles of laboratory animal care were followed (EU directive no. 86/609) and the type of experiment was approved by the Danish animal experiment inspectorate.

Capsaicin-mediated desensitization

Capsaicin (Fluka Chemica, Buchs, Switzerland) was dissolved in vehicle (10% ethanol and 10% cremophore (BASF, Ludwigshafen, Germany) in isotonic saline). The obese rats were randomly divided into two groups and were given either capsaicin (100 mg/kg) or vehicle s.c. in the scruff of the neck during general anaesthesia. Because of the large dose of capsaicin, the procedure was divided over 3 days (20, 30 and 50 mg/kg per day). The rats were anaesthetized by injection of 0.2 ml/100 g 1:1 Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml; Janssen Pharmaceutica, Beerse, Belgium)–Dormicum (1 ml; midazolam 5 mg/ml, sodium chloride 5 mg/ml, acidic hydrochloride 25% and sodium hydroxide 4 mg/ml; Hoffmann-La Roche AG, Basle, Switzerland) and supplemented by 0.1 ml/100 g at the sign of reduced anaesthesia (reduced muscle relaxation of the tail). The rats

were kept anaesthetized up to 4 h until the signs of capsaicin-mediated respiratory depression were absent. During the procedure the rats were kept on a heating blanket in a heated chamber (24°C) for the first hour after they woke up. The rats were given analgesic treatment (1×0.06 mg/rat buprenorphine (Anorfin; A/S GEA, Hvidovre, Denmark and 1×5 mg/kg carprofen (Rimadyl; Pfizer Animal Health A/S, Ballerup, Denmark)) and supportive therapy (saline and oral glucose) during the capsaicin treatment and for 2 days after treatment.

Fasting plasma CGRP

The rats were deprived of food overnight (18 h) but had free access to water. They were then anaesthetized in carbon dioxide and bled from the retro-orbital plexus. Sampled blood was mixed with EDTA/aprotinin and spun (10 000 r.p.m. for 5 min at 4°C) and then 100 μl plasma aliquots for the determination of plasma CGRP were stored at -20°C until analysis. The CGRP of the plasma samples was eluted by using a C-18 reverse phase cartridge (Sep-Pack; Waters, Milford, MA, USA). The content of CGRP in the samples was determined by a rat CGRP enzyme immunometric assay (SPLbio, Paris, France).

Oral glucose tolerance test (OGTT)

The rats were deprived of food for 12 h (during the light phase, 0600–1800 h) and then basal fasting blood glucose and fasting plasma insulin were determined. The rats were then given an oral glucose bolus of 2 g/kg by gavage, and the blood glucose and plasma insulin levels were determined at 30, 60 and 120 min following the start of the test. All samples were obtained from the tail-tip capillary of unrestrained, conscious rats. The rats had been accustomed to the personnel and the tail bleeding procedure before the experiment and no disturbance of normal behaviour was observed. Tail-tip blood for the assessment of blood glucose (10 μl) was sampled in heparinized 10 μl glass capillary tubes and immediately diluted in 500 μl EBIO buffer solution (Eppendorf, Hamburg, Germany) and kept on ice until analysis by the immobilized glucose oxidase method (EBIO Plus auto analyser; Eppendorf). Approximately 70 μl tail-tip blood was collected in heparinized 100 μl glass capillary tubes containing heparin and kept on ice until centrifugation (8000 r.p.m. for 6 min at 4°C) when 15 μl plasma was transferred to cooled Micronic racks (Micronic B.V., Lelystad, The Netherlands) containing 60 μl bovine calf serum, and stored at -20°C until analysis for plasma insulin by an in-house ELISA method. In brief, the assay for determination of insulin in rat and mouse serum or plasma was a two-site immunoassay using two polyclonal guinea pig antibodies raised against rat insulin (GP114 and GP116). Peroxidase was measured using 3,3',5,5'-tetramethylbenzidine peroxidase as substrate. Purified rat insulin (Novo

Nordisk batch 220891) was used as calibrator. The coefficient of variation was determined to be less than 10%.

Analysis of tissue cholesterol, triglycerides and glycogen

The rats were anaesthetized in 0.2 ml/100 g 1:1 Hypnorm—Dormicum as before, decapitated and bled. The abdominal cavity was opened and a piece of the abdominal musculature and the liver were quickly isolated, frozen in liquid nitrogen and stored at -80°C until analysis. Defrosted tissue (50 mg) was added to 2 ml 0.15 M sodium acetate, containing 0.75% Triton X-100 and placed in a bath of boiling water for a total of 2–5 min, and homogenized for the first 15–30 s. After centrifugation, free glucose, triglyceride, cholesterol and glycerol were determined on a Cobas Mira analyser, using commercially available enzymatic assay kits from Hoffman-La Roche, Basel, Switzerland (unimate glucose enzymatic hexokinase/glucose-6-phosphatdehydrogenase endpoint (HK) method, Triglyceride enzymatic endpoint method, where triglyceride was converted to glycerol and fatty acids by lipase and glycerol was enzymatically converted to quinoeimine dye, and cholesterol enzymatic endpoint method with cholesterol esterase, c.oxidase and peroxidase). Glycogen was enzymatically converted to glucose using amyloglucosidase and total glucose was determined. The interassay coefficients of variation for glycogen, triglyceride and cholesterol were less than 10%.

Statistical analysis

The data were analysed by comparing the obese rats with the lean littermate rats, or by comparing the capsaicin-treated obese rats with the obese control rats. In both cases, the two-tailed Student's *t*-test for the comparison of group means was used except for the insulin and food intake values. Since the variances in the insulin and food intake means were dissimilar in the different groups, the Mann–Whitney U test was used to analyse these data. The area under the curves (AUC) of the blood glucose and plasma insulin profiles were calculated by the trapezoidal method. Insulin secretion during the OGTT was indirectly estimated as Δ plasma insulin (30 min) divided by the 30-min blood glucose level. Insulin sensitivity during the OGTT was indirectly estimated as the inverse product of $\text{AUC}_{0-120\text{ min}}$ plasma insulin $\times$ $\text{AUC}_{0-120\text{ min}}$ blood glucose. Data are shown as means $\pm$ S.E.M. $P < 0.05$ was regarded as statistically significant.

Results

Plasma CGRP, body weight and food intake

Body weight was the same in the all rats until 9 weeks of age (Fig. 1). Nevertheless, plasma CGRP levels were elevated in pre-obese rats vs their lean littermates

Figure 1 The effect of capsaicin treatment on body weight in male obese Zucker rats. Individual body weights were assessed throughout the study. At the age of 8 weeks, plasma CGRP was assessed and at the age of 9 weeks (time 0) obese Zucker rats received 100 mg/kg capsaicin s.c. (trt. = treated) or vehicle s.c. and lean littermates received vehicle s.c. An OGTT was performed 7 weeks after capsaicin at the age of 16 weeks. Tissue was preserved on the following day for lipid and glycogen content analysis. Data are presented as means $\pm$ S.E.M.

already at week 8, i.e. before the appearance of obesity (16.1 ± 3.4 pM in obese vs 6.9 ± 1.1 pM in lean littermate rats; $P = 0.015$; Fig. 2). Thereafter, body weight gain was higher in the obese Zucker rats compared with their lean littermates. The obese rats were significantly heavier at the end of the study (505 ± 9 g for obese rats vs 363 ± 10 g for the lean littermate rats; $P < 0.0005$). The mean daily food intake was lower in lean than in obese rats (29.7 ± 0.4 g/rat per day in lean rats; $P = 0.016$). Capsaicin, however, did not significantly alter the food intake of the obese rats (43 ± 6 g/rat per day in obese capsaicin-treated rats vs

Figure 2 The level of plasma CGRP in male pre-obese Zucker rats. Plasma CGRP measured following an 18-h fast during carbon dioxide anaesthesia in pre-obese and lean Zucker rats at the age of 8 weeks and prior to treatment with capsaicin and prior to the onset of severe obesity (body weights similar to lean littermates). Data are presented as means $\pm$ S.E.M. * $P < 0.05$

35±2 g/rat per day in obese vehicle-treated rats; not significant (NS)).

Capsaicin-mediated desensitization

The obese rats were desensitized with capsaicin at week 9. The prolonged anaesthetic procedure resulted in some mortality in all groups: lean rats had a mortality rate of 20%, the vehicle-treated obese rats 60% and the capsaicin-treated rats showed a further increase in mortality to 70%. All surviving capsaicin-treated rats responded negatively to topical corneal application of capsaicin (negative eye-wipe response), confirming effective capsaicin-mediated desensitization, and were included in the experiment. One of each of the vehicle-treated obese and lean rats were similarly tested, and were found to have positive eye-wipe response confirming normal capsaicin sensitivity. Based on this finding, all surviving control rats were included in the experiment.

Figure 3 The oral glucose tolerance and glucose-stimulated insulin secretion *in vivo* 7 weeks after capsaicin treatment. Oral glucose (2 g/kg) was given by gavage at time 0 in 12-h fasted Zucker rats. (a) The blood glucose profiles and (b) the plasma insulin levels were measured at time 30, 60 and 120 min after glucose in vehicle-treated lean rats, vehicle-treated obese rats and in capsaicin-treated obese rats. Data are presented as means±S.E.M. ****P* < 0.0005, lean vs obese Zucker rats; §*P* < 0.001, capsaicin-treated vs control Zucker rats.

Oral glucose tolerance

At the age of 16 weeks, both fasting blood glucose (*P* < 0.0005; Fig. 3) and mean blood glucose during the OGTT (*P* < 0.0005) were lower in lean rats vs the obese rats. Similarly, fasting insulin (*P* < 0.001) and mean insulin (*P* < 0.001) levels during the OGTT were lower in lean compared with obese rats (Fig. 3). At the same time, fasting blood glucose (4.3±0.2 mM after capsaicin vs 5.1±0.2 mM in obese control rats; *P* = 0.050; Fig. 3) as well as mean glucose during the OGTT were reduced after capsaicin treatment in obese Zucker rats (6.8±0.3 mM after capsaicin vs 8.6±0.5 mM in obese control rats; *P* = 0.024). In contrast, plasma insulin levels were not significantly affected by capsaicin treatment in obese Zucker rats in either the fasting insulin (1345±89 pM after capsaicin vs 1712±520 pM in control obese rats; NS; Fig. 3) or the mean plasma insulin level during the OGTT (2033±235 pM after capsaicin vs 2465±651 pM in the obese control rats; NS). The insulin response to the oral glucose in relation to the glucose levels was also not significantly affected by capsaicin (NS) as estimated indirectly. Furthermore, the indirect method of estimating insulin sensitivity showed that lean rats were significantly more insulin sensitive than obese rats and also that capsaicin did not significantly reduce insulin resistance in the obese rats (NS) in this study.

Liver and muscle levels of triglycerides, glycogen and cholesterol (Table 1)

At the end of the study, levels of glycogen, triglycerides and cholesterol in the muscles and in the liver were generally elevated in the obese Zucker rats as compared with their lean littermates. Following the capsaicin treatment in obese Zucker rats, the levels of liver and muscle triglycerides and glycogen were unaffected compared with the obese control rats whereas total liver cholesterol was significantly elevated (*P* = 0.021).

Discussion

The main finding in this study was that capsaicin-induced sensory nerve desensitization improved glucose tolerance in Zucker rats. This study further showed that plasma CGRP levels, a marker of sensory nerve activity, were increased in pre-obese rats, and our data thus support the hypothesis that increased activity of sensory nerves precedes the development of obesity in Zucker rats.

Plasma CGRP

The observed rise in plasma levels of the sensory neuropeptide CGRP in pre-obese Zucker rats, compared with lean individuals, occurred prior to the onset of obesity.

Table 1 Characteristics of tissue levels of triglycerides, cholesterol and glycogen in the liver and in muscle. Data are represented as means $\pm$ S.E.M.

	Obese, vehicle ($\mu\text{mol/g}$ tissue)	Obese, capsaicin ($\mu\text{mol/g}$ tissue)	Lean, vehicle ($\mu\text{mol/g}$ tissue)
Muscle triglycerides	26.1 $\pm$ 4.8	29.2 $\pm$ 6.8	13.8 $\pm$ 1.4*
Muscle cholesterol	5.6 $\pm$ 1.4	5.0 $\pm$ 1.3	4.2 $\pm$ 1.4
Muscle glycogen	27.7 $\pm$ 3.5	25.7 $\pm$ 6.1	18.5 $\pm$ 4.1
Liver triglycerides	31.5 $\pm$ 4.3	40.9 $\pm$ 3.3	5.6 $\pm$ 1.5***
Liver cholesterol	4.0 $\pm$ 0.6	6.1 $\pm$ 0.4§	2.9 $\pm$ 0.4*
Liver glycogen	360.4 $\pm$ 16.6	313.5 $\pm$ 28.9	243.8 $\pm$ 43.3

* $P < 0.05$, *** $P < 0.0005$, lean vs obese Zucker rats; § $P < 0.001$, capsaicin-treated vs control obese Zucker rats.

Previous studies have reported increased CGRP levels in established obesity in Zucker rats (14) as well as in obese humans (15). We used capsaicin to examine whether sensory nerve activity contributes to the development of obesity-induced insulin resistance in Zucker rats. Capsaicin has been used as a tool for examining the role of sensory nerves in various disease states (16).

Food intake and body weight

The obese rats in this experiment consumed more food than the lean rats and, consequently, became obese in agreement with the literature (17). Further, as expected (17), the obese rats in this study had elevated blood glucose and were glucose intolerant because of increased insulin levels as compared with the lean littermates, suggesting both hyperinsulinaemia and insulin resistance. Neither body weight nor food intake was changed by capsaicin in this experiment, suggesting that glucose homeostasis was affected directly. The findings that body weight was unchanged is in contrast to data from normal rats, where body weight generally decreases after capsaicin desensitization (18) and is associated with reduced fat deposition (19, 20). The reason for this could be that obese Zucker rats – which are functionally leptin deficient – have an increased drive for food through pathways other than the sensory nerves and that this therefore overrides the effect of capsaicin desensitization as compared with normal rats. Taken together, our data suggested that energy expenditure was not altered following capsaicin in the obese Zucker rats. However, a direct measurement of energy expenditure as well as body composition was not assessed in this experiment and should be performed in future studies.

Glucose homeostasis

The main finding in this study was that oral glucose tolerance was significantly improved following capsaicin treatment in the fatty Zucker rat. In addition, 12-h fasting blood glucose was significantly reduced by capsaicin treatment. This is in agreement with earlier reports where reduction of fasting blood glucose was also seen following capsaicin treatment of normal

rodents (21) and of ZDF rats (3) and therefore seems to be a consistent phenomenon. The magnitude of the reduction though seemed to depend on the level of glycaemia before treatment. The improvement of oral glucose tolerance, however, did not seem to be associated with significant improvements of either insulin secretion or improvement of overall insulin sensitivity. Regional improvement of insulin sensitivity of, for instance, the liver could be speculated to occur after desensitization but this was not assessed in this experiment and therefore remains to be established. Another explanation could be that capsaicin is not able to reverse insulin resistance in 9-week-old obese Zucker rats. Finally, it could be speculated that gastric emptying could be delayed following capsaicin but this also remains to be studied.

Lipids

The accumulation of lipids and glycogen was found to be elevated in Zucker rats as compared with lean rats. Since there is a known relationship between intracellular triglycerides and insulin resistance (11) and since we did not detect any improvement of whole body insulin sensitivity following capsaicin, it was not surprising to observe that the tissue content of triglycerides and glycogen was not altered in this experiment. Plasma lipids were not assessed in this study. However, we have previously found that the levels of plasma cholesterol were reduced following capsaicin treatment in the closely related ZDF rat (3). Further, other groups have found that dietary capsaicin to non-obese rats has been associated with decreased plasma lipids (22), possibly due to decreased absorption of lipids from the gastrointestinal tract (23), and it has also been associated with decreased hepatic lipid accumulation (24) but also with no effect on plasma lipids (25). This suggests that the effect of capsaicin on lipids can vary between different forms of administration and experimental models. One peculiar finding, however, was that capsaicin treatment gave rise to elevation of hepatic cholesterol levels in Zucker rats. Given that the whole body insulin sensitivity was not improved, we did not expect a decrease in hepatic cholesterol but, on the other hand, we did not expect

a rise either. The rise was unexpected since we have previously found that plasma cholesterol levels are reduced in ZDF rats (3) and because the level of hepatic cholesterol has previously been found to be unaltered after oral capsaicin (25). It could therefore be speculated that, in the hyperphagic obese Zucker rat, the supply of energy and thus cholesterol is generally in excess and the load of fat that needs to be cleared from the plasma is therefore constantly high. It is therefore possible that the elevated liver cholesterol levels could reflect an improved clearing of the plasma cholesterol and/or reduced biliary excretion. The role of systemic capsaicin desensitization in cholesterol metabolism should be investigated in detail.

Capsaicin desensitization

We used a dose of 100 mg/kg capsaicin, since this dose has previously been shown to effectively desensitize adult rats as reviewed by Holzer (6). We found that this dose effectively desensitizes sensory nerves in obese Zucker rats, but that capsaicin treatment in this experiment was associated with increased mortality. This was also observed in the vehicle-treated obese rats as well as in vehicle-treated lean rats however. Presently, we do not know why this increased mortality occurred. Some explanations can, however, be speculated upon. (1) Capsaicin is known to suppress respiratory function at the doses administered. This feature has limited its use in adult rats and most desensitizing studies are therefore performed in neonatal rats that can be desensitized by a single dose of 50 mg/kg s.c. capsaicin. However, since lean or obese Zucker rats cannot be distinguished until the age of 4–5 weeks unless genotyping is used, desensitization has to be performed in non-neonatal rats. The large dose could therefore have introduced respiratory depression. (2) Further, anaesthesia of obese individuals is known to lead to accumulation of anaesthetics in the fat depot, resulting in prolonged anaesthesia of unpredictable length. In this study we used an injection anaesthesia which we also found was related to increased sleeping times as compared with lean rats and it is possible that this could have contributed to the mortality observed. (3) Moreover, obese Zucker rats have defects in their thermoregulation and it is possible that the body core temperature became reduced during the anaesthetic procedure. These features, or a combination thereof, could have lead to increased mortality in this experiment. We therefore suspect that the observed mortality was rather associated with the anaesthetic procedure than with the capsaicin treatment itself. It is therefore recommended to decrease the time of anaesthesia, for instance by using inhalation anaesthetics during capsaicin treatment as well as to monitor core body temperature during and after capsaicin treatment in obese Zucker rats.

We conclude that capsaicin-induced sensory nerve desensitization improves glucose tolerance in Zucker rats. Since, in this study, plasma CGRP levels, a marker of sensory nerve activity, were increased in the pre-obese rats, our data support the hypothesis that increased activity of sensory nerves precedes the development of obesity and insulin resistance in Zucker rats.

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