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Short communication

Increased hypothalamic neuropeptide Y concentration or hyperphagia in streptozotocin-diabetic rats are not mediated by glucocorticoids

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Abstract

Hypothalamic neuropeptide Y containing neurones are overactive and may mediate hyperphagia in insulin-deficient diabetic rats, but the factors stimulating them remain uncertain. To determine the possible role of glucocorticoids, we investigated the effects of the glucocorticoid receptor blocker mifepristone (RU486) on food intake and regional hypothalamic neuropeptide Y concentrations in streptozotocin-diabetic rats. RU486 (30 mg/kg) or corn oil vehicle control was given orally for 3 weeks to diabetic rats. Food intake and neuropeptide Y levels in the hypothalamic arcuate and paraventricular nuclei were increased in untreated diabetic rat groups ($P < 0.01$), and though RU486 did increase plasma corticosterone levels ($P < 0.01$) it did not have any effect on either feeding or neuropeptide Y levels (P = NS). These negative findings suggest that glucocorticoids may not be responsible for increasing hypothalamic neuropeptide Y or for hyperphagia in insulin-deficient diabetes. © 1997 Elsevier Science B.V.

Keywords: Diabetes; Hypothalamus; Neuropeptide Y; RU486

1. Introduction

Insulin-dependent diabetes is characterised by marked hyperphagia, reduced thermogenesis and impaired secretion of most pituitary hormones (Frankish et al., 1995). As the hypothalamus appears to be important in regulating food intake and energy balance, these energetic and neuroendocrine disturbances of diabetes may be mediated by changes in specific hypothalamic neurones and neurotransmitters.

Neuropeptide Y, a 36-amino-acid peptide which is related to pancreatic polypeptide, is concentrated in appetite-regulating areas of the hypothalamus. It is synthesised in neurones of the arcuate nucleus which project to end mainly in the paraventricular nucleus and dorsomedial nuclei (Chronwall et al., 1985). Neuropeptide Y injected into the paraventricular nuclei or other hypothalamic sites induces intense carbohydrate-preferring hyperphagia, with a fall in sympathetically-mediated brown adipose tissue activity, and its long term administration leads to obesity (Stanley et al., 1986); indeed, it is the most potent appetite stimulant known (Dryden et al., 1994 for review).

There is convincing evidence that the neuropeptide Y neurones of the arcuato–paraventricular pathway are stimulated under conditions of negative energy balance, notably insulin-deficient diabetes and starvation, where neuropeptide Y levels and neuropeptide Y mRNA levels and neuropeptide Y secretion within the paraventricular nucleus are increased (Williams et al., 1989; Beck et al., 1990; White et al., 1990; Frankish et al., 1995; Sahu et al., 1995). It has therefore been suggested that hypothalamic neuropeptide Y neurones act homeostatically to defend body weight, by stimulating food intake and reducing energy expenditure, under conditions when the body's energy stores are under threat. Other central actions of exogenous neuropeptide Y include effects on pituitary hormone secretion, including increased ACTH and corticosterone release, possible by increasing corticotrophin-releasing factor release (Haas and George, 1987), all of which mimic changes found in diabetes (Frankish et al., 1993). Increased activity of the arcuate nucleus neuropeptide Y neurones may therefore be responsible for hyperphagia and for some of the major neuroendocrine alterations in diabetes.

Factors that regulate the arcuate neuropeptide Y neurones may include leptin and insulin (inhibitory) and glucocorticoids (stimulatory) (Dryden et al., 1994), but those

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that stimulate these neurones in diabetes are unknown. Adrenal steroids may modulate the activity of hypothalamic neuropeptide Y neurones through type II glucocorticoid receptors, which are found on neuropeptide Y-containing neurones in the arcuate nucleus and other hypothalamic areas (Hisano et al., 1988), and glucocorticoid treatment increases neuropeptide Y synthesis (Wilding et al., 1993). Moreover, glucocorticoids are apparently required for neuropeptide Y to be able to stimulate feeding and for the obesity syndrome produced by neuropeptide Y, as these effects can be prevented by adrenalectomy and restored by glucocorticoid replacement (Stanley et al., 1989; Dryden et al., 1994; Sainsbury et al., 1997). Strack et al. (1995) suggested that the increased glucocorticoid and reduced insulin levels in starvation and diabetes could explain much of the variance in body weight and the increase in neuropeptide Y mRNA levels in the arcuate nucleus.

This study aimed to investigate if glucocorticoids could modulate the hyperphagia and the increased hypothalamic neuropeptide Y neuronal activity associated with diabetes. This hypothesis was tested using the glucocorticoid receptor antagonist RU486 (mifepristone). RU486 inhibits the development of obesity and increases brown adipose tissue activity in fatty rats probably via enhanced release of corticotrophin-releasing factor (Langley and York, 1990; York, 1993); in normal rats, it also prevents neuropeptide Y-induced hyperphagia (Tempel and Leibowitz, 1993). RU486 was used at the dose (30 mg/kg/day) previously shown to prevent hyperphagia and obesity in *fa/fa* Zucker rats (Langley and York, 1990). We believe that this is an appropriate dose to employ, as neuropeptide Y is also thought to underlie hyperphagia in the fatty rat, which shows comparable increases in neuropeptide Y messenger RNA, neuropeptide Y levels (Dryden et al., 1994 for review) and neuropeptide Y secretion (Dryden et al., 1995) as those seen in streptozotocin-diabetic rats.

2. Methods

2.1. Animals and experimental protocol

Male Wistar rats (Charles River UK, Margate, Kent) were kept on a 12 h light–dark cycle (09.00–21.00 h) with free access to chow and water.

Under brief halothane anaesthesia, two groups of rats ($n = 8$/group) were injected with streptozotocin (55 mg/kg) into a tail vein to induce diabetes, which was confirmed by tail-prick blood glucose levels consistently > 18 mM. Blood glucose levels were monitored every 2–3 days using an electrochemical meter and glucose oxidase test strips (Exactech; Medisense, Birmingham).

Starting three days after induction of diabetes RU486 (Roussel-UCLAF; 30 mg/kg/day) emulsified in corn oil was administered by gavage to one of the diabetic groups (Langley and York, 1990). Non-diabetic controls ($n = 8$) and untreated diabetics received corn oil alone. Three weeks after induction of diabetes the animals were killed by CO_2 inhalation.

2.2. Hypothalamic microdissection

The brain was removed rapidly and eight selected hypothalamic regions were microdissected from fresh brain slices using a vibrating microtome (Williams et al., 1989). Pooled tissue from each area was boiled for 10 min in 0.1 M HCl to extract the peptide and sonicated to disperse the tissue and the extracts were frozen at $-40°C$. The areas studied were the medial preoptic area, lateral preoptic area, anterior hypothalamic area, lateral hypothalamic area, paraventricular nucleus, ventromedial hypothalamus, dorsomedial hypothalamus and the arcuate nucleus.

2.3. Assays

Neuropeptide Y concentrations in hypothalamic tissue were measured by radioimmunoassay (RIA) employing ^{125}I-labelled neuropeptide Y (Amersham, Bucks), porcine neuropeptide Y as standard (Bachem, Essex) and an neuropeptide Y antiserum which we raised in a rabbit against porcine neuropeptide Y. Cross-reactivity with other related peptides was $< 1\%$, the assay sensitivity was < 1 fmol/ml and the intra-assay coefficient of variation was 3%. Protein content of the samples were measured by a modified Lowry technique, and the neuropeptide Y levels expressed as fmol/μg protein.

Insulin and corticosterone levels were measured using commercial RIA kits (Pharmacia Upjohn, St. Albans, and DPC, Caernarfon) with intra-assay coefficients of variation of $< 6\%$ and 5%. Plasma glucose was determined using a glucose–oxidase based autoanalyser.

2.4. Statistical analyses

Data are shown as means $\pm$ S.E.M. Food intake and plasma data were compared using a one-way analysis of variance (ANOVA) coupled to post-hoc Bonferroni modified *t*-tests. Neuropeptide Y levels were analysed using a repeated measure two-way ANOVA coupled to post-hoc Bonferroni modified *t*-tests. As this analysis revealed a significant effect, differences between groups in individual nuclei were further compared using Student's *t*-test for unpaired data. A significance level of $P < 0.05$ chosen for all data.

3. Results

3.1. Food intake, body weight and metabolic data

These data are shown in Table 1. All diabetic rats were consistently hyperglycaemic (blood glucose levels > 24

mmol/l), and terminal plasma glucose levels were increased in both diabetic groups (both $P < 0.01$). In the untreated diabetic rats, food intake was increased by 72% compared with nondiabetic controls ($P < 0.001$). RU486 did not alter hyperphagia, compared with untreated diabetics (P = NS).

Compared with the non-diabetic controls, untreated and RU486-treated diabetic rats both gained weight more slowly (by 13% and 8% respectively; $P < 0.05$) and had increased water intake (both eight-fold; $P < 0.001$). There were no significant differences in these parameters between the RU486-treated and untreated diabetic groups.

Plasma insulin levels were significantly reduced by 50% in the untreated diabetic rats and by 46% in the RU486-treated diabetic rats compared with the non-diabetic controls (both $P < 0.05$). Plasma corticosterone levels were increased in the RU486-treated diabetic rats (by 227% $P < 0.01$) compared with the non-diabetic controls. Corticosterone concentrations were also raised in the untreated diabetic rats compared with controls, although this difference did not reach statistical significance (P = NS).

3.2. Neuropeptide Y concentrations

Neuropeptide Y levels in the eight hypothalamic regions are shown in Fig. 1. Two-way analysis of variance (repeated measures) revealed a significant effect attributable to group; $F[2,198] = 3.5$ ($P < 0.03$) and region; $F[7,198] = 172$ ($P < 0.001$). Neuropeptide Y levels were significantly raised in untreated diabetics and RU486-treated diabetics compared with the nondiabetics, in the arcuate nucleus (by 125% and 102%, respectively; both $P < 0.01$), paraventricular nucleus (by 30% and 18%, respectively; both $P < 0.05$) and lateral hypothalamic area (by 80% and 70%, respectively; both $P < 0.05$). However, there were no significant differences between the untreated diabetic and RU486-treated diabetic groups in these or any other areas (P = NS). There were no significant changes from controls in neuropeptide Y levels in any other region in either treated or untreated diabetic groups.

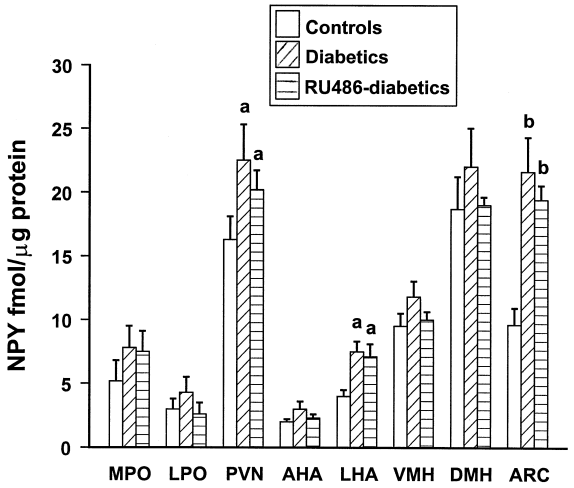

Fig. 1. Hypothalamic neuropeptide Y levels (fmol/μg protein) in 8 selected hypothalamic nuclei in the three groups of rats. Error bars are S.E.M. Key: MPO, medial preoptic area; LPO, lateral preoptic area; PVN, paraventricular nucleus; AHA, anterior hypothalamic area; LHA, lateral hypothalamic area; VMH, ventromedial nucleus, DMH, dorsomedial nucleus; ARC, arcuate nucleus. Statistical significance of differences between groups; [a]$P < 0.05$, [b]$P < 0.01$ vs nondiabetic controls.

Table 1
Food and water intake, body weight, plasma concentrations of glucose, insulin and corticosterone

	Controls	Diabetics	RU-486-diabetics
n	8	8	8
Initial body weight (g)	208 ± 3	214 ± 3	213 ± 3
Weight gain (g)	60 ± 5	35 ± 4[b]	45 ± 3[b]
Food intake (g/day)	23 ± 1	39.6 ± 4[b]	37.4 ± 1[b]
Water intake (ml/day)	20.5 ± 1	186.9 ± 6[b]	165.2 ± 16[b]
Glucose (mmol/l)	7.8 ± 0.2	30 ± 1.5[a]	28.4 ± 1.4[a]
Insulin (mu/l)	20.7 ± 2.3	10.2 ± 1.1[a]	11.1 ± 2.3[a]
Corticosterone (ng/ml)	56 ± 23	100 ± 34	183 ± 34[b]

Statistical significance of differences vs controls: [a]$P < 0.05$, [b]$P < 0.01$.

4. Discussion

The neuropeptide Y-containing neurones of the arcuato–paraventricular projection appear to play an important role in controlling energy balance, and it is clearly important to identify the factors which regulate their activity. Insulin-deficient diabetes provides an interesting model, in which overactivity of the neuropeptide Y neurones has been demonstrated and could explain the marked hyperphagia and aspects of hypothalamo–pituitary dysfunction in this condition.

Glucocorticoids are thought to stimulate the arcuate neuropeptide Y neurones and also induce hyperphagia. The arcuate neuropeptide Y neurones and the hypothalamo–pituitary–adrenocortical axis are intimately linked at several levels. These neurones express type II glucocorticoid receptors (Hisano et al., 1988) and glucocorticoids (notably dexamethasone) stimulate hypothalamic neuropeptide Y expression and peptide levels both in vitro (Corder et al., 1988) and in vivo (Wilding et al., 1993), as well as the expression by neuropeptide Y neurones of neuropeptide YY_1 receptors (Larsen et al., 1994). The comprehensive studies by Strack et al. (1995) of diabetic and/or adrenalectomised rats also suggest that corticosterone and insulin are important regulators (stimulatory and inhibitory, respectively) of the neuropeptide Y neurones, but do not prove any direct modulatory action of these agents. Corticosteroids also powerfully influence feeding behaviour and energy balance, including the effects of neuropeptide Y. Adrenalectomy prevents the obesity from

developing in various genetically obese and hypothalamic-lesioned rodents, which may have increased sensitivity to glucocorticoids and in which corticosterone is elevated, and this effect is reversed by glucocorticoid replacement (York, 1993). Similarly, neuropeptide Y-induced hyperphagia and obesity are inhibited by adrenalectomy (Stanley et al., 1989; Sainsbury et al., 1997). It is not known whether these effects reflect direct; actions of corticosterone or are mediated by corticotrophin-releasing factor neurones which are thought to inhibit them.

In this study, we used the anti-glucocorticoid agent RU486 to investigate whether glucocorticoids may modulate the increased in neuropeptide Y levels associated with streptozotocin-diabetes. In common with previous studies, untreated diabetic rats were hyperphagic and neuropeptide Y levels were increased in the arcuate nucleus, as well as in the paraventricular nucleus where neuropeptide Y is released and exerts its hyperphagic effects (White et al., 1990; McKibbin et al., 1991; Frankish et al., 1993).

RU486 treatment (30 mg/kg/day) increased plasma corticosterone levels comparable with data reported in other studies (Pesonen et al., 1991, 1992). This presumably indicates that RU486 exerted at least some actions at the hypothalamo–pituitary level, i.e. interruption of the negative feedback inhibition by corticosterone. However, RU486 did not attenuate the hyperphagia of diabetes, and neither did it significantly reduce neuropeptide Y levels in any region. Neuropeptide Y levels in these regions have shown robust changes that relate to food intake in various experimental settings, including fasting and diabetes (Beck et al., 1990; Dryden et al., 1994; Frankish et al., 1995; Strack et al., 1995). However, the present data argue against glucocorticoids being an important stimulator of the neuropeptide Y neurones and of food intake in diabetes. We acknowledge that effects on other aspects of neuropeptide Y neuronal activity (e.g. neuropeptide Y synthesis and its release in the paraventricular nucleus) were not studied and therefore cannot be excluded. In addition to its glucocorticoid receptor antagonist activity, RU486 is also an anti-progestogen. Such activity could influence our findings, although we used male rats to minimise possible interference from progesterone.

It is also possible that the dosage used (30 mg/kg) was inadequate in diabetic rats, perhaps because of impaired intestinal absorption or loss of a permissive action of insulin. The same dosage decreased food intake and body weight in adult obese Zucker rats (Langley and York, 1990), although a lower dose (20 mg/kg) failed to affect neuropeptide Y mRNA levels in young obese Zuckers (Pesonen et al., 1991). Glucocorticoid status may also be an important determinant, as Akabayashi et al. (1994) have recently demonstrated that corticosterone only has a significant effect on the activity of neuropeptide Y neurones after adrenalectomy and not under normal conditions. This may be particularly relevant to the states of altered energy balance such as in diabetes and particularly in the Zucker rat, in which glucocorticoid levels are raised. RU486 failed to reduce neuropeptide Y in these and other studies (Pesonen et al., 1991), and did not alter corticosterone levels in the fatty Zucker rat (Pesonen et al., 1991, 1992). In the case of the fatty Zucker rat at least, these findings may be a result of the altered corticotrophin-releasing factor activity, which may obscure RU486-induced changes (Bchini-Hooft Van Huijsduijnen et al., 1993). The lack of clear changes in corticosterone levels in conditions such as diabetes, where corticosterone levels are raised, could be due to an increase in its secretion through a stress response, especially considering the high variance in the levels in the two diabetic groups.

Other potential regulators of the hypothalamic neuropeptide Y neurones in diabetes are insulin and leptin. Normalisation of plasma insulin in diabetic rats reduces hypothalamic neuropeptide Y and neuropeptide Y mRNA levels and food intake to near control levels (McKibbin et al., 1991; Sipols et al., 1995; Frankish et al., 1995). Leptin is a strong candidate and is known to reduce neuropeptide Y levels in conjunction with a decrease in food intake and increased BAT activity in normal rats (Stephens et al., 1995; Wang et al., 1997; Dryden and Williams, 1997). Loss of the inhibitory leptin signal (as in the *ob/ob* mouse) or failure of the central nervous system to perceive it due to mutations in the leptin receptor (*db/db* mouse and *fa/fa* Zucker rat) lead to hyperphagia, reduced brown adipose tissue thermogenesis and weight gain, and may also be responsible for the increased neuropeptide Y neuronal activity in these models. Leptin levels fall in insulin-deficient diabetes, and this may similarly lead to enhanced neuropeptide Y neuronal activity in these animals.

We conclude that glucocorticoids are unlikely to be important in the increased activity of the hypothalamic neuropeptide Y neurones and hyperphagia in insulin-deficient diabetes.

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