Corticotropin-releasing factor and its receptors in the brain of rats with insulin and corticosterone deficits

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Abstract

The expression of genes encoding corticotropin-releasing factor (CRF) and its receptor type-1 (CRF₁R) and type-2 α (CRF₂R) has been studied in the brain of rats with streptozotocin (STZ)-induced diabetes and adrenalectomy (ADX). Diabetic rats had a lower body weight compared to control rats. Food and water intake were increased in diabetic rats and decreased in ADX animals. The plasma corticosterone levels measured at the nadir of the circadian rhythm were significantly higher in diabetic rats compared to non-diabetic animals. STZ-diabetic rats demonstrated an induction of expression of CRF mRNA in the magnocellular part of the paraventricular hypothalamic nucleus (PVN) and in the supraoptic nucleus (SON), whereas the CRF transcript in the parvocellular PVN was significantly lower in rats with insulin deficiency. ADX strongly triggered the expression of CRF mRNA in the parvocellular neurons of the PVN in both non-diabetic and diabetic rats, and it decreased magnocellular CRF mRNA in diabetic animals. The expression of the CRF₁R in the parvocellular and magnocellular PVN and in the SON was induced by diabetes and decreased after ADX. The levels of the CRF₂R mRNA in the ventromedial hypothalamic nucleus (VMH) were significantly lower in diabetic rats without any noticeable effects of ADX. The present results suggest opposite effects of insulin and corticosterone deficiency on the hypophysiotropic CRF and the CRF₁R mRNA contents, whereas the expression of CRF₂R was mostly related to insulin, but not to the corticosterone status.

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Introduction

Corticotropin-releasing factor (CRF) is a 41-amino acid peptide widely distributed throughout the brain and particularly concentrated in the medial parvocellular division of the hypothalamic paraventricular nucleus (PVN, Vale *et al.* 1981, Sawchenko & Swanson 1985). The CRF neurons of the parvocellular PVN are predominantly involved in the control of the hypothalamic–pituitary–adrenal (HPA) axis. Besides its stimulatory hypophysiotropic effects, CRF blunts energy balance through concomitantly reducing energy intake and augmenting energy expenditure (Richard *et al.* 2002). Central administration of CRF decreases food intake (FI) and food hoarding and increases thermogenesis (Rothwell 1990, Cabanac & Richard 1995, Buwalda *et al.* 1997).

Homeostasis-threatening (stressful) conditions strongly affect the expression of CRF in the PVN. The pattern of CRF activation in the PVN depends on the nature of the applied stresses. Psychological or ‘neurogenic’ stresses ultimately increase CRF contents in the parvocellular PVN, whereas some ‘systemic’ stresses, such as the osmotic challenge, may decrease CRF expression in the parvocellular PVN and increase CRF transcript levels in the magnocellular PVN (Imaki

et al. 1992, Sawchenko *et al.* 1996). The uncontrolled diabetes induced by streptozotocin (STZ) treatment can also be viewed as a model of chronic stress leading to increase in the HPA-axis basal activity, adrenal weight, plasma corticosterone levels, and urinary corticosterone excretion at the nadir of circadian activity (Dallman *et al.* 1994). In spite of a basal increase in the HPA-axis activity, STZ-treated rats demonstrate a reduced HPA-axis activation in response to administrations of CRF and adrenocorticotropin (ACTH), and a blunted response to the dexamethasone suppression test (Scribner *et al.* 1991, Chan *et al.* 2002). While the inhibitory and stimulatory effects of corticosterone and insulin respectively on the expression of PVN CRF have been acknowledged, the effect of STZ-induced diabetes on CRF expression is not entirely clear. Both, decrease and increase in CRF mRNA levels have been reported in the PVN after 4–8 days of STZ treatment (Sipols *et al.* 1995, Schwartz *et al.* 1997, Chan *et al.* 2001, 2005). It is noteworthy that the estimated CRF expressions in STZ-induced diabetes have been done so far without taking into account the subdivisions of the PVN. As mentioned above, activation of the different parts of the PVN may vary according to the type of stress and some stresses may even change the expression sites of CRF within the PVN subnuclei (Viau & Sawchenko 2002).

Table 1 Body weight, food intake, water intake, white fat tissue, and the plasma levels of glucose, insulin, and corticosterone

Group	Sham			ADX		ANOVA (P)		Corticosterone status (C)	Interaction (I×C)
	Non-diabetic (n=5)	Diabetic (n=6)		Non-diabetic (n=5)	Diabetic (n=7)	Insulin status (I)			
Body weight (BW; g)	341.9±2.4	208.8±5.4*		302.8±4.7†	256.6±7.5*†	<0.0001		0.4728	<0.0001
Body weight gain (g)	72.8±2.9	-53.1±4.0*		38.3±4.9†	-10.0±5.4*†	<0.0001		0.3702	<0.0001
Food intake (FI; g/day)	27.3±0.5	29.4±0.4		21.02±1.1	20.9±2.1	0.5299		<0.0001	0.4533
FI/BW (%)	8.1±0.1	14.1±0.4*		7.0±0.4	8.0±0.7†	<0.0001		<0.0001	<0.0001
WAT weight (g)	1.525±0.121	0.226±0.036*		0.924±0.089†	0.266±0.060*	<0.0001		0.0018	0.0006
WAT/BW (%)	0.446±0.036	0.108±0.017*		0.304±0.026†	0.101±0.021*	<0.0001		0.0077	0.0134
Water intake (ml/day)	62.4±8.8	376.7±18.6*		65.6±3.9	142.6±18.5*†	<0.0001		<0.0001	<0.0001
Plasma									
Glucose (mmol/l)	9.45±0.76	27.91±0.66*		7.66±0.29	23.92±1.86*	<0.0001		0.0346	0.3967
Insulin (mmol/l)	0.303±0.073	0.033±0.008*		0.192±0.046	0.020±0.005*	<0.0001		0.1179	0.2024
Corticosterone (μmol/l)	0.333±0.154	1.066±0.073*		0.011±0.011†	0.015±0.005†	0.0001		<0.0001	0.0001

*Significantly different from non-diabetic rats within the same corticosterone status, P<0.05. †Significantly different from sham-operated rats within the same insulin status, P<0.05.

The central effects of CRF are mediated through two distinct high-affinity membrane receptors referred to as CRF type-1 (CRF₁R, Perrin *et al.* 1993) and CRF type-2α (CRF₂R, Lovenberg *et al.* 1995) receptors. The CRF₁R is widely distributed throughout the brain, anterior, and intermediate pituitary. Within the PVN, CRF₁R mRNA is barely detectable under basal conditions, but can be readily induced by stressful stimuli (Rivest *et al.* 1995). The expression of the CRF₂R is restricted to the particular limbic regions and to the ventromedial hypothalamic nucleus (VMH). Numerous data argue for a role of the VMH in control of FI and insulin secretion (King *et al.* 1984, Dube *et al.* 1999). There is evidence that the CRF₂R is responsible for the anorectic effects of CRF (Vaughan *et al.* 1995). The CRF₂R is highly expressed in the VMH under basal conditions, but the hyperphagia in obesity or fasting led to significant decrease of CRF₂R levels in the VMH, which might reduce the anorectic effects of CRF in these animals (Richard *et al.* 1996, Timofeeva & Richard 1997). Alterations in the HPA-axis activity and FI in diabetic rats obviously imply both CRF₁R and CRF₂R, but so far, there has been no report demonstrating the effects of insulin deficiency and combined interaction between diabetes and adrenalectomy (ADX) on the expression of the CRF receptors within the brain.

The present study was designed to further elucidate the effects of corticosterone and insulin deficiency on the expression of CRF and CRF receptors in order to clarify the possible role of the central CRF system in the persisting hyperactivity of the HPA axis and altered FI in diabetes.

Materials and methods

Animals and treatments

Male Sprague–Dawley rats weighing 260–270 g were purchased from the Canadian Breeding Laboratories (St Constant, QC, Canada). All rats were cared for and handled according to the *Canadian Guide for the Care and Use of Laboratory Animals* and the present protocol was approved by our institutional animal care committee. Animals were housed individually in wire-bottom cages suspended above absorbent paper, in temperature-controlled (23±1 °C) and light-controlled (lights on between 0700 and 1900 h) environment. Animals were randomly assigned to four groups: sham-operated non-diabetic rats, sham-operated diabetic rats, ADX non-diabetic rats, and ADX diabetic animals. Bilateral removal of the adrenals was achieved through two small lateral skin incisions made under isoflurane anesthesia. Sham-operated animals were treated in the same way as ADX animals except that the adrenals were not excised. STZ (75 mg/kg) or vehicle (citrate buffer,

pH 4.5) was intraperitoneally injected to sham-operated or adrenalectomized rats immediately after surgery. After surgery, all rats were given 0.9% NaCl as a drinking fluid to prevent ADX-induced hyponatremia and to improve the glomerular filtration rate (DiTella *et al.* 1978, Will & Barnett 1983). STZ-ADX rats received 5% glucose and 0.9% NaCl as a drinking fluid for the first 24 h to prevent hypoglycemia and thereafter a solution containing solely 0.9% NaCl. Urine glucose was examined in the morning of the day following the injection of STZ with the reagent strips for urine analysis (Miles Canada Inc, Etobicoke, ON, Canada). The STZ-injected rats had more than 1–2% urinary glucose and had the symptoms of polydipsia and polyuria. All rats were fed *ad libitum* a stock diet (Agway Prolab, Rat/Mouse/Hamster 1000 Formula) for the duration of experiment. FI, body weight (BW), and water intake (WI) were measured daily. All rats were sacrificed in the morning 8 days after the ADX surgery and/or STZ administration.

Body weight, white adipose tissue weight, food intake, and water intake

Measurements of BW, food, and WI determinations were performed every morning between 0700 and 0800 h. FI and WI were measured by subtracting the remaining amounts of food or water from the fixed amount provided to rat. Food spillage was carefully

calculated and accounted for in the measurements. At the end of experiment, epididymal white adipose tissue (WAT) was quickly dissected and immediately weighted. Blood was also sampled.

Brain preparation

Rats were anesthetized with 1.5 ml mixture containing 20 mg/ml ketamine and 2.5 mg/ml xylazine. Without delay, they were intracardially perfused with 20 ml ice-cold isotonic saline followed by 120 ml paraformaldehyde (4%) solution. The brains were removed at the end of perfusion and kept in paraformaldehyde for an additional period of 7 days. They were then transferred to a solution containing paraformaldehyde (4%) and sucrose (10%) before being cut 12 h later using a sliding microtome (HistoSlide 2000, Reichert-Jung, Heidelberg, Germany). Brain sections were taken from the olfactory bulb to the brain stem. Thirty micrometer thick sections of brain were collected and stored at -30°C in a cold cryoprotecting solution containing sodium phosphate buffer (50 mM), ethylene glycol (30%), and glycerol (20%).

In situ hybridization histochemistry

In situ hybridization histochemistry was used to localize CRF, CRF₁R, and CRF₂R mRNAs on tissue sections taken from the entire brain. The protocol used was largely

Figure 1 The optical densities (ODs) of the hybridization signals of CRF mRNA in the parvocellular and magnocellular parts of the PVN and in the supraoptic nucleus (SON). The main and interaction effects of insulin status (I; non-diabetic or diabetic) and corticosterone status (C; sham-operated or adrenalectomized) were determined using 2×2 ANOVA, followed by *a posteriori* Fisher's posterior list of significant difference. *Significantly different from non-diabetic rats within the same adrenal status; †Significantly different from sham-operated rats within the same insulin status, $P < 0.05$.

adapted from the technique described by Simmons *et al.* (1989). Briefly, one out of every five brain sections were mounted onto poly-L-lysine-coated slides and allowed to desiccate overnight under vacuum. The sections were then successively fixed for 20 min in paraformaldehyde (4%), digested for 30 min at 37 °C with proteinase K (10 µg/ml in 100 mM Tris-HCl containing 50 mM EDTA, pH 8.0), acetylated with acetic anhydride (0.25% in 0.1 M triethanolamine, pH 8.0), and dehydrated through graded concentrations (50, 70, 95, and 100%) of alcohol. After air drying for at least 2 h,

90 µl hybridization mixture, which contains an antisense ³⁵S-labeled cRNA probe (10⁷ c.p.m./ml), were spotted on each side. The slides were sealed under a coverslip and incubated overnight at 60 °C in the slide warmer. The next day, the coverslips were removed and the slides rinsed four times with 4× SCC (0.6 M NaCl, 60 mM trisodium citrate buffer, pH 7.0), digested for 30 min at 37 °C with RNase-A (20 µg/ml in 10 mM Tris-500 mM NaCl containing 1 mM EDTA), washed in descending concentrations of SSC (2×, 10 min; 1×, 5 min; 0.5×, 5 min; 0.1×, 30 min at 60 °C) and

Figure 2 Dark field photomicrographs of coronal brain sections taken from the hypothalamic paraventricular nucleus, illustrating CRF mRNA. The brain sections (30 µm thick) were obtained from sham-operated (top panels) or adrenalectomized (ADX; bottom panels) non-diabetic (left panels) or diabetic (right panels) rats. PVNp and PVNm are the parvocellular and magnocellular parts of the PVN respectively; 3v, third ventricle. The scale bar corresponds to 200 µm.

dehydrated through graded concentrations of alcohol. After a 2-h period of vacuum drying, the slides were exposed on an X-ray film (Kodak) for periods varying between 24 and 72 h, depending upon the nature of the probes used. Once removed from the autoradiography cassettes, the slides were defatted in xylene and dipped in NTB2 nuclear emulsion (Eastman Kodak). Again depending on the probe used, the slides

were exposed from 7 to 21 days, before being developed in D19 developer (Kodak) for 3·5 min at 14–15 °C and fixed in rapid fixer (Kodak) for 5 min. Finally, tissues were rinsed in running distilled water for 1–2 h, counterstained with thionin (0·25%), dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with DPX.

Figure 3 Dark field photomicrographs depicting CRF mRNA hybridization signal on the coronal brain sections (30 µm thick) taken at the level of the SON in rats with intact adrenal glands (top panels) or after adrenalectomy (ADX; bottom panels), non-diabetic (left panels), or diabetic (right panels). Ox, optic chiasm. The scale bar corresponds to 200 µm.

Non-diabetic

Diabetic

Combination of immunocytochemistry with *in situ* hybridization

Immunocytochemical detection of immunoreactivity for oxytocin (OT-ir) and arginine vasopressin (AVP-ir) was combined with detection of CRF mRNA in the PVN or SON to determine whether OT or vasopressin was colocalized with CRF in diabetic rats. Brain sections were first processed for immunocytochemical detection of OT-ir and AVP-ir using a conventional avidin–biotin–immunoperoxidase method. Briefly, brain slices were washed in sterile 50 mM potassium PBS (KPBS) that was treated with diethylpyrocarbonate. They were then incubated for 24 h at 4 °C with an OT or AVP antibody (rabbit polyclonal IgG; Star Technologies Inc., Minneapolis, MN, USA). The OT and AVP antibodies were used at a 1:1000 and 1:10 000 dilution respectively, in KPBS with heparin (0.25%), Triton X-100 (0.4%), and BSA (2%). Following incubation at 4 °C with the first antibody, the brain slices were rinsed in sterile KPBS and incubated with a mixture of KPBS, Triton X-100, heparin, and biotinylated goat antirabbit IgG (1:1500 dilution; Vector Labs, Burlingame, CA, USA) for 60 min. Sections were then rinsed with KPBS and incubated at room temperature for 60 min with an avidin–biotin–peroxidase complex (Vectastain ABC Elite Kit; Vector Labs). After several rinses in sterile Tris–HCl (50 mM, pH 7.6), the brain slices were allowed to react in a mixture containing sterile Tris, the chromagen 3,3'-diaminobenzidine tetrahydrochloride (0.05%), and 1% hydrogen peroxide. Thereafter, tissues were rinsed in KPBS, mounted onto poly-L-sine-coated slides, desiccated overnight under vacuum, fixed in paraformaldehyde (4%) for 30 min, and digested for 30 min at 37 °C with proteinase K (10 µg/ml in 100 mM Tris–HCl (pH 8.0), and 50 mM EDTA). Prehybridization, hybridization, and posthybridization steps were performed as described above except for the dehydration step, which was shortened to avoid decolorization of OT-ir or AVP-ir cell. After vacuum drying for 2 h, sections were exposed on X-ray film, defatted in xylene, and dipped in the NTB2 nuclear emulsion. Slides were exposed for 7 days, developed in D19 developer for 3.5 min at 14 °C, and fixed in rapid fixer for 5 min. Thereafter, tissues were rinsed in running distilled water for 1–2 h, rapidly dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with DPX.

Antisense ³⁵S-labeled cRNA probes

The CRF cRNA probe was generated from the 1.2 kb EcoRI fragment of a rat CRF cDNA (Dr Mayo K, Northwestern University, Evanston, IL, USA) subcloned into a pGEM4 vector (Stratagene, La Jolla, CA, USA), and linearized with HindIII and EcoRI (Pharmacia Biotech Canada, Inc.) for antisense and sense probes respectively. The CRF₁R cRNA probe was generated from a 1.3 kb PstI–PstI fragment of the rat prCRF PP1.3-BS cDNA (Dr Vale W, Peptide Biology Laboratory, The Salk Institute) subcloned into a pBluescript II-SK vector (Stratagene) and linearized with BamHI and HindIII to generate antisense and sense probes respectively. The rat CRF₂R cRNA probe was prepared from a 275 bp fragment of the 5'-region cDNA of the CRF₂ receptor (Dr Lovenberg T W, Neurocrine Biosciences, Inc.) subcloned into a pBluescript SK+ vector (Stratagene) and linearized with EcoRI and BamHI for antisense and sense probes respectively. The radioactive antisense riboprobes were synthesized by incubation of 250 ng linearized plasmids in 6 mM MgCl₂, 40 mM Tris (pH 7.5), 10 mM NaCl, 10 mM dithiothreitol, 0.2 mM ATP/GTP/CTP, and α-³⁵S UTP, 40 U RNase inhibitor (Promega), and 20 U SP6, T7 or T3 RNA polymerase for CRF, CRF₁R, or CRF₂R antisense probes for 60 min at 37 °C. The DNA templates were treated with 100 µl DNase solution (1 µl DNase, 5 µl of 5 mg/ml tRNA, 94 µl of 10 mM Tris/10 mM MgCl₂). The preparation of the riboprobes was accomplished through phenol–chloroform extraction and ammonium acetate precipitation. The specificity of each probe was confirmed by the absence of positive signal in sections hybridized with sense probe.

Quantitative analyses of the hybridization signals

The hybridization signals revealed on NTB2 dipped nuclear emulsion slides were analyzed and quantified under a light microscope (Olympus, BX50) equipped with a black and white video camera (Sony, XC-77) coupled to a Macintosh computer (Power PC 7100/66) using Image software (version 1.55 non-FPU, Wayne Rasband, NIH, Bethesda, MD, USA). The optical density (OD) of the hybridization signal was measured under dark field illumination. Brain sections were matched for rostrocaudal levels as closely as possible using the atlas of the rat brain (Paxinos & Watson 1997). The OD for each specific region was corrected

Figure 4 Bright field photomicrographs demonstrating oxytocin immunoreactivity (OT-ir; brown staining) and CRF mRNA hybridization signal (silver grains) in the paraventricular hypothalamic nucleus (PVN) and SON in non-diabetic (four top panels) and diabetic (four bottom panels) rats with intact adrenal glands. The right panels are the high-magnification photomicrographs depicting neurons outlined by rectangles on the left panels. White arrowheads indicate some neurons displaying OT-ir only; black arrowheads indicate representative neurons coexpressing OT protein and CRF mRNA. Note the absence of CRF hybridization signal in the OT neurons in the magnocellular PVN and in the SON in non-diabetic rats. PVNp and PVNm are the parvocellular and magnocellular part of the PVN respectively; 3v, third ventricle; ox, optic chiasm. The scale bars correspond to 20 µm for right panels and 100 µm for left panels.

for the average background signal, which was determined by sampling unlabeled area outside the areas of interest. When no hybridization signal was visible under dark field illumination, the brain structures of interest were outlined under bright field illumination and then subjected to densitometric analysis under dark field illumination. Saturation of the hybridization signal was avoided by the creation of the calibrate density profile plot for the strongest hybridization signals sampled for each region in every series. The system luminosity was then corrected so that for this strongest hybridization signals the reading OD did not exceed the half of the pixel value maximum, and this luminosity was conserved throughout the analysis of entire series. In the PVN (parvocellular and magnocellular parts), ODs were measured for each animal on two to three brain sections, taken between 1.6 and 2.0 mm caudal to bregma (Paxinos & Watson 1997). In the SON and VMH, ODs were measured for each animal on four brain sections taken between 1.3–1.8 mm for SON and 2.8–3.3 mm for VMH caudal to bregma (Paxinos & Watson 1997). Measurements were done on both left and right hemispheres. The values of the hemispheres and then those of the sections were averaged (four to eight measurements) to calculate individual scores. These scores were used to determine the mean $\pm$ S.E.M. for each group.

Plasma determinations

The intracardial blood samples were taken in anesthetized rats immediately before the intracardial perfusion with saline. Plasma glucose was determined (glucose oxidase method) using a glucose analyzer (Beckman, Palo Alto, CA, USA). Insulin levels were determined by RIA (sensitivity, 0.035 nmol/l; interassay coefficient of variation, 9.2%) using reagent kits from Incstar (Stillwater, MN, USA). Plasma corticosterone was determined by a competitive protein-binding assay (sensitivity, 0.058 nmol/l; interassay coefficient of variation, 9.0%) using plasma from a dexamethasone-treated female Rhesus monkey as the source of transcortin (Murphy 1967).

Statistical analysis

Results are expressed as the mean $\pm$ S.E.M. for each group. A 2 $\times$ 2 ANOVA was used to examine the main and interaction effects of insulin status (I: non-diabetic, diabetic) and corticosterone status (C: sham-operated, ADX) on the various dependent variables measured in this study. A *posteriori* comparisons between groups were realized using the Fisher's protected least squares difference test. Each experimental group included five to seven animals.

Figure 5 The optical densities (ODs) of the hybridization signals of the CRF₁R mRNA in the parvocellular and magnocellular parts of the paraventricular hypothalamic nucleus (PVN) and in the SON. The main and interaction effects of insulin status (I; non-diabetic or diabetic) and corticosterone status (C; sham-operated or adrenalectomized) were determined using 2 $\times$ 2 ANOVA, followed by a *posteriori* Fisher's posterior list of significant difference. *Significantly different from non-diabetic rats within the same adrenal status; †Significantly different from sham-operated rats within the same insulin status, $P < 0.05$.

Results

Body weight, weight of the white adipose tissue, food intake, and water intake

The values in Table 1 show the different effects of diabetes or ADX on the BW, BW gain, weight of epididymal WAT, and relative WAT/BW, FI, and WI in sham-operated and adrenalectomized non-diabetic or diabetic rats. The BW, BW gain, WAT weight, and WAT/BW were markedly lower in diabetic rats than in non-diabetic animals. The absolute value of diurnal FI appeared to be equivalent in STZ-treated

and non-diabetic rats within the same adrenal status. However, when absolute FI measurements were corrected for BW differences, they revealed that in sham-operated groups, the diabetic rats consumed higher amount of food than did non-diabetic rats. Adrenalectomized rats consumed less food compared to the animals with intact adrenals. The WAT weight and WAT:BW ratio decreased in response to diabetes and ADX. STZ treatment significantly increased WI. This effect of STZ on WI was more important in sham-operated rats (sixfold increase) compared to ADX animals (twofold increase).

Figure 6 Dark field photomicrographs of coronal brain sections taken from the PVN, depicting CRF₁R mRNA. The brain sections (30 μ m thick) were obtained from sham-operated (top panels) or adrenalectomized (ADX; bottom panels) non-diabetic (left panels) or diabetic (right panels) rats. PVNp and PVNm are the parvocellular and magnocellular parts of the PVN respectively; 3v, third ventricle. The scale bar corresponds to 200 μ m.

Plasma glucose, insulin, and corticosterone levels

Plasma glucose levels were significantly higher in diabetic rats compared to non-diabetic animals, whereas ADX slightly decreased plasma glucose (Table 1). The STZ-treated rats had very low plasma insulin levels. There was a non-significant tendency for a reduction in plasma insulin after ADX. STZ resulted in a threefold increase in corticosterone levels in rats with intact adrenal glands.

CRF mRNA

CRF expression in the parvocellular part of the PVN was lowered and enhanced after respectively STZ and ADX (Figs 1 and 2). ANOVA revealed a significant effect of both corticosterone ($P=0.001$) and insulin ($P=0.004$) status on the expression of the CRF mRNA in the parvocellular PVN. In the magnocellular PVN (Figs 1 and 2) as well as in the SON (Figs 1 and 3), CRF expression was low in non-diabetic rats, but markedly

Figure 7 Dark field photomicrographs illustrating CRF₁R hybridization signal on the coronal brain sections (30 μ m thick) taken at the level of the SON in rats with intact adrenal glands (top panels) or after ADX (bottom panels), non-diabetic (left panels) or diabetic (right panels). ox, optic chiasm. The scale bar corresponds to 200 μ m.

increased in diabetic animals. ADX strongly enhanced CRF transcription in the parvocellular PVN of both non-diabetic and diabetic animals (Figs 1 and 2), however, it significantly decreased CRF transcript levels in the magnocellular PVN and SON in diabetic rats. Combination of immunohistochemistry for OT and AVP with *in situ* hybridization for CRF mRNA revealed that CRF expression in magnocellular neurons in diabetic rats was confined to oxytocinergic neurons (Fig. 4). Following STZ treatment, the majority of OT-ir cells in the PVN and SON coexpressed CRF mRNA. There was no colocalization of AVP-ir and CRF mRNA in either the PVN or the SON of diabetic and non-diabetic rats (data not shown).

CRF₁R mRNA and CRF₂R mRNA

Diabetes induced the expression of the CRF₁R in both the PVN and the SON. The CRF₁R transcript was barely detectable in the PVN and SON of control non-diabetic rats. After STZ treatment, CRF₁R mRNA expression was strongly induced in the magnocellular PVN (Figs 5 and 6) and in the SON (Figs 5 and 7). Also, levels of the CRF₁R transcript moderately but significantly rose in the parvocellular part of the PVN (Figs 5 and 6). ADX partially blunted STZ-induced expression of CRF₁R mRNA in the PVN and SON.

The expression of the CRF₂R in the VMH was significantly lower in diabetic animals (Fig. 8). This reduction was not dependent on the corticosterone status and was seen in both, sham-operated and ADX diabetic rats (Fig. 9).

Discussion

The results of the present study clearly demonstrate an induction of CRF and CRF₁R in the magnocellular part of the PVN and in the SON of STZ-treated rats. In contrast, the parvocellular part of the PVN of diabetic rats expressed less CRF mRNA. ADX significantly increased CRF expression in the parvocellular PVN independent of the insulin status and blunted CRF and CRF₁R induction in the magnocellular PVN and SON of STZ-treated rats.

A significant decrease in CRF mRNA expression after STZ treatment in the parvocellular PVN is in agreement with previous reports (Sipols *et al.* 1995, Schwartz *et al.* 1997), which provide evidence for a decrease in the parvocellular CRF expression in diabetic rats despite increases in plasma ACTH levels and in adrenal gland weight. The reduction in CRF mRNA expression in the parvocellular PVN in uncontrolled diabetes most probably depends on the levels of plasma corticosterone (Sipols *et al.* 1995, Schwartz *et al.* 1997). Corticosterone levels increased by three times following STZ

treatment and were accompanied with a decrease in parvocellular CRF expression. In contrast, ADX led to a strong activation of the CRF mRNA synthesis in the parvocellular neurons. Corticosterone replacement has been reported to dose-dependent reverse the ADX-stimulating effects on the CRF PVN gene expression in diabetic rats (Schwartz *et al.* 1997).

The mechanisms responsible for the activation of the magnocellular system in animals with insulin deficiency has yet to be fully described, but seems to be associated with the hyperosmotic challenge resulting from the hyperglycemia that occur in uncontrolled diabetes mellitus (Musabayane *et al.* 1995). It is noteworthy that the CRF expression in the magnocellular neurons in diabetic rats was likely not attributable to normal saline drinking as sham animals, which were also provided with this saline, did not demonstrate any magnocellular expression of CRF. Furthermore, it is well known that normal saline does not alter plasma osmolality or hypothalamic CRF content (Imaki *et al.* 1992). In diabetic rats, the drinking of saline has been reported not to change plasma insulin levels, blood pressure, glomerular filtration rate, and kidney weight

Figure 8 The optical densities (ODs) of the hybridization signals of the CRF₂R mRNA in the ventromedial hypothalamic nucleus (VMH). The main and interaction effects of insulin status (I; non-diabetic or diabetic) and corticosterone status (C; sham-operated or adrenalectomized) were determined using 2×2 ANOVA, followed by *a posteriori* Fisher's posterior list of significant difference. *Significantly different from non-diabetic rats within the same adrenal status.

(Dai *et al.* 1994, Vallon *et al.* 1997). It is notable that the expression of CRF in the magnocellular neurons of diabetic rats was partially reversed by ADX.

As revealed through double labeling, CRF mRNA expression in the magnocellular PVN and SON was confined specifically to OT but not to AVP neurons. The mechanism for such selectivity in CRF expression within a particular subgroup of magnocellular neurons in diabetic rats is not clear. Uncontrolled diabetes apparently triggers the synthesis of both OT and AVP (Luo *et al.* 2002). The selectivity for CRF expression in OT-ir but not AVP-ir neurons has been reported in osmotic stress (Dohanics *et al.* 1990, Imaki *et al.* 2001a), whereas insulin-induced hypoglycemia promotes CRF and AVP colocalization (Paulmyer-Lacroix *et al.* 1994). The impact of an enhanced CRF expression in OT neurons is as yet undetermined. Magnocellular CRF may directly affect the adenohypophysis by means of local secretion from the magnocellular axons in passage through the external zone of the median eminence (Holmes *et al.* 1986). In addition, magnocellular CRF via the neurohypophysis may reach the circulation and directly stimulate the adrenal glands using the extrapituitary mechanisms (Fehm *et al.* 1988). It is not clear as to whether CRF can activate the release of OT, which could either activate or block the HPA-axis

activity (Petersson *et al.* 1999, Windle *et al.* 2004, Laguna-Abreu *et al.* 2005) depending on various factors. At the level of anterior pituitary, OT has been reported to act as an ACTH secretagog, with significant effects seen at low concentrations (Schlosser *et al.* 1994). There is also evidence that endogenous brain OT enhances the long lasting, but not the acute HPA-axis response (Nakashima *et al.* 2002). In STZ rats, OT may be involved in the maintenance of the HPA-axis hyperactivity. Diabetic rats exhibit an enhanced basal HPA-axis activity but a blunted response to an acute CRF, ACTH, or dexamethasone test (Chan *et al.* 2002). Basal HPA axis may also be affected by magnocellular CRF that directly stimulates the AVP secretion through a paracrine mechanism at the level of neurohemal zone of the neurohypophysis (Alzein *et al.* 1984).

In order to better understand the central CRF pathways sensitive to insulin and corticosterone deficiencies, we examined the expression of CRF receptors in diabetic and adrenalectomized rats. Diabetic rats demonstrated a dramatic increase in CRF₁R mRNA expression in the SON and magnocellular PVN, and a modest enhancement in the parvocellular PVN. The CRF₁R is broadly expressed throughout the brain, but it is barely detected in the hypothalamic parvocellular and magnocellular neurons under basal conditions

Figure 9 Film autoradiograms of coronal brain sections demonstrating CRF₂R transcript at the level of the VMH. The brain sections (30 μ m thick) were obtained from sham-operated (top panels) or adrenalectomized (ADX; bottom panels) non-diabetic (left panels) or diabetic (right panels) rats. The scale bar corresponds to 1 mm.

(Perrin *et al.* 1993). However, the expression of the CRF₁R in the parvocellular PVN has been reported to be triggered by different stressful conditions (Rivest *et al.* 1995, Richard *et al.* 1996, Timofeeva & Richard 1997). The mechanism underlying CRF₁R expression in the magnocellular neurons of diabetic rats is not yet clear, but it may involve CRF, which is also induced in the magnocellular system of these animals. Indeed, CRF may modulate its own biosynthesis as well as that of its type-1 receptor through an ultra-short positive feedback loop (Mansi *et al.* 1996, Imaki *et al.* 2001b).

The present study demonstrates the significant decrease in the expression of the CRF₂R in the VMH of STZ-treated rats. The VMH is a brain region seemingly involved in the modulation of insulin secretion as ablation of the VMH led to increase in the parasympathetic tone to the pancreas and insulin oversecretion (Penicaud *et al.* 1996). It is, however, not clear whether a decrease in CRF₂R at the levels of the VMH in diabetic rats is related to the control exerted by the VMH on insulin secretion. There is evidence that the CRF₂R in the VMH mediates the anorectic effects of CRF (Richard *et al.* 2000, 2002). We have found a decrease in the CRF₂R expression in food-deprived rats and in genetically obese Zucker rats (Richard *et al.* 1996, Timofeeva & Richard 1997). Fasting rats, genetically obese rats, and diabetic animals are all hyperphagic and have impaired or low insulin signaling. In the brain, insulin inhibits feeding (Strack *et al.* 1995) and potentiates the anorectic effects of CRF (Richardson *et al.* 2002). The present results suggest that at least a part of insulin central effects may be mediated through its regulation of CRF₂R VMH expression.

In conclusion, the present study provides clear evidence that the systemic deficit in insulin and corticosterone results in opposite effects on the central expression of CRF and the CRF₁R. Uncontrolled diabetes led to a decrease in CRF expression in the parvocellular PVN, but to the induction of CRF and CRF₁R in the magnocellular system. In contrast, ADX increased parvocellular CRF, but significantly blunted STZ-induced activation of the magnocellular neurons. The levels of the CRF₂R mRNA in the VMH were significantly lower in diabetic rats without any noticeable effects of ADX. The present results suggest that insulin and corticosterone deficiency have the opposite effects on the hypophysiotropic CRF and CRF₁R, whereas the expression of CRF₂R is mostly related to the insulin, but not to the corticosterone, status. Alterations in the brain CRF system due to insulin deficiency may contribute to the hyperphagia, hyperdipsia, and the hyperactivity of the HPA axis in diabetes.

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