ACTIVATION OF FOS IN HYPOCRETIN NEURONS OF THE RAT BY INSULIN-INDUCED HYPOGLYCEMIA

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Objective. The aim of the present study was to investigate the extent of activation of hypocretin (HCRT) synthesizing neurons after a single intraperitoneal administration of insulin using Fos-HCRT dual immunohistochemistry. In addition, there was also an attempt to depict the spatial organization of activated HCRT perikarya within the whole portion of the medial and lateral hypothalamic (LHA) areas.

Methods. The animals (rats) were fixed 90 min after i.p. administration of insulin (2.5 IU/kg). The brains were removed, and sectioned through the hypothalamus into 40 μm thick alternate coronal sections. Fos-HCRT perikarya were double immunostained with avidin-biotin-peroxidase (ABC) technique using Nickel-DAB and single DAB as the two chromogens. For the mapping of Fos-HCRT double-labeled perikarya a light microscopy was employed. Counting of Fos-labeled HCRT perikarya was performed manually and blindly with no insight into the treatment of the animals.

Results. The data demonstrate that in the early phase of the acute hypoglycemia, the number of the dually labeled Fos-HCRTir perikarya in the entire LHA was only moderately increased from 9.54 to 15.64 % in spite of the fact that within the same period the plasma glucose levels were declined by more than 70 %. Moreover, within the LHA, the distribution of activated double-labeled Fos-HCRTir perikarya did not show any special spatial organization.

Conclusion. The present data indicate that a large fall in plasma glucose in early phase of acute hypoglycemia does not represent an appropriate stimulus for massive activation of HCRT neurons in the LHA of rats.

Key words: Fos - Hypocretin (orexin) - Dual immunohistochemistry - Lateral hypothalamus - Glucose - Insulin induced hypoglycemia - Rat

Hypocretins (orexins) have been described as a new family of neuropeptides derived from the same precursor (DE LECEA et al. 1998; SAKURAI et al. 1998) and localized exclusively in neuronal cell bodies dispersed along the lateral hypothalamus (LHA) including the perifornical area and adjacent zona incerta (PEYRON et al. 1998). The presence of HCRT containing terminals and HCRT receptors in different brain structures (TREVIDI et al. 1998; CUTLER et al. 1999; DATE et al. 1999) indicates for multiple functions. Actually, current studies have affirmed that hypocretins may play a role in many neuronal, autonomic, and neuroendocrine processes with implication in the sleep-wake regulation (WILLIE et al. 2001), cardiovascular function (CIRIELLO et al. 2003) and sympathetic outflow (ANTUNES et al. 2001). In addition, the presence of HCRT in the LHA, generally designated as a feeding center, indicates also their involvement in the regulation of appetite (GRIFFOND et al. 1999; LIU et al. 2001; CAI et al. 2001), energy homeostasis (WILLIAMS et al. 2000, 2001), and glucose metabolism (CAI et al. 1999).

Hypoglycemia has repeatedly been shown to induce Fos-positive signal, as a general indicator of cell activity, in many brain areas. In the LHA, hypoglycemia mainly activates glucose-sensitive neurons which accounts for ~ 25 % of the LHA neurons (OOMURA et al. 1974; BAHJAOUI-BOUHADDI et al. 1994). Recent evidence suggests that HCRT containing neurons also respond

to hypoglycemia. In rodents, intracerebroventricular administration of HCRT peptides (orexins A,B) increases food intake (Cai et al. 1999; Haynes et al. 1999) and conversely, plasma glucose fall (Moriguchi et al. 1999) or food restriction increases the HCRT expression in the LHA (Sutcliffe and de Lecea 2000). As there are differences between orexin-A and orexin-B responsiveness to hypoglycemia (Cai et al. 2001) they may play specific roles in behavioral and neuroendocrine responses to hypoglycemia. However, prepro-orexin mRNA responses (Cai et al. 1999) indicate that HCRT cells are more sensitive to a longlasting rather than short-lasting food absence or insulin-induced hypoglycemia. The aim of the present study was to investigate the extent of activation of hypocretin (HCRT) synthesizing neurons after a single intraperitoneal administration of insulin using Fos-HCRT dual immunohistochemistry. In addition, there was also an attempt to depict the spatial organization of activated HCRT perikarya within the different portions of the LHA.

Materials and Methods

Animals. Adult male Wistar rats weighing 260-280 g were used in the experiments. The animals were purchased from Charles River Wiga (Silzfeld, Germany) and housed four per cage in a room with controlled light (14 h/day), humidity (70 %) and temperature (23 °C). They received regular rat chow (dry pellets) and tap water *ad libitum*. Principles of laboratory animal care and the experimental procedures used have been approved by Animal Care Committee of the IEE SAS Bratislava, Slovak Republic. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Hypoglycemia. After 7 days of adaptation, the animals were withheld of food for 11 hrs (from 10.00 PM of the day before the experiment to 9.00 AM of the next day). They then received either an acute injection of insulin (2.5 IU/kg i.p. in 0.1 ml/100 g b.w.) or saline (controls) as described previously (Jezova et al. 1987). Ninety min after insulin injection the animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Before starting the transcardial perfusion 1 ml of blood was taken from the heart and collected in vials containing 5 ml of EDTA. Then the rats were perfused with 50 ml of ice-cold isotonic saline containing 450 ml of heparin (5000 IU/l) followed by 150 ml of 0.1 M phosphate buffer (PB, pH 7.4) containing 4 % paraformaldehyde. The brains were removed from the skull and post-fixed overnight in fresh fixative. Thereafter, the brains were washed overnight in PBS at 4 °C, infiltrated with 30 % sucrose-KPBS and sectioned into 40 mm thick serial coronal sections.

Immunohistochemistry. After rinsing in 0.05M KPBS (pH 7.4) for 3 x 15 min, and in 5 % swine serum-KPBS containing 0.3 % Triton X-100 and 1 % bovine serum albumin (BSA) for 20 min, the sections were incubated in a specific polyclonal Fos antiserum (Mikkelsen et al. 1998) diluted 1:4,000. Thereafter the sections were rinsed for 3 x 10 min in KPBS containing 0.1 % Triton X-100 and 0.3 % BSA. Then the sections were incubated in biotinylated swine-anti rabbit IgG (code E353, Dakopatts, Copenhagen, 1:600) for 60 min, rinsed in KPBS, and incubated in a streptavidin-biotin-peroxidase complex (ELITE kit; Vector, Burlingame, 1:500) for 60 min. After a thorough rinse in the wash-buffer for 10 min, KPBS for 10 min and 50 mM Tris/HCl (pH 7.6) for 10 min, the sections were incubated in a 1 ml imidazole-acetate buffer containing diaminobenzidine (DAB, 125 mg/l Tris/HCl buffer), 1 M $NiSO_4$ and 0.3 % H_2O_2 for 20 min.

In case of dual staining the sections were washed for 5 x 10 min in KPBS and incubated in the second antiserum against HCRT-1 (code no. 99006) diluted 1:4,000 in KPBS containing 0.3 % Triton X-100 and 1 % bovine serum albumin and reacted with DAB alone. Both the antisera, Fos and HCRT-1, were generated in our conditions and have been characterized elsewhere (Mikkelsen et al. 1998, 2001).

Analysis and statistical evaluation. The total number (counts from every 6th section/animal) of Fos immunoreactive cells was counted manually. The data from one animal were taken as the average of total number of immunoreactive cells in a standardized region in the LHA. Three sections containing a rostral, middle and caudal part of the LHA, where labeled neurons are present, were analyzed. For detection of dual-labeled cells an observer blind to the treatment counted manually the number of neurons with and without Fos-positive nuclei. The percentage of dual-labeled cells from one animal was averaged for the two measurements. The results are presented as means ± S.E.M. of values obtained from 6 animals per group. Statistical differences among groups were determined by one way analysis of variance (ANOVA) followed by *post hoc* Fischer PLSD tests (Fos-HCRT calculations) or by Student's *t*-test (plasma glucose analysis). Statistical significance was defined as $p< 0.05$.

Results

Blood samples taken from the hypoglycemic rats 90 min after insulin administration revealed significant fall (by more than 70 %) in the circulating levels of glucose in comparison with saline injected controls (Table 1). In both groups of animals the HCRTir neurons were dispersed along the lateral hypothalalmus, i.e. they were observable from the caudal level of the paraventricular hypothalamic nucleus up to the caudal extent of the dorsomedial hypothalamic nucleus. Many neurons of the LHA elicited Fos induction after insulin treatment.

Table 1
Plasma glucose levels in individual animals measured 90 min after a single i.p. administration of insulin. In each insulin-injected animal the decline of plasma glucose level exceed 70 % of saline injected controls.

	Plasma glucose levels (mmol/l)	
	Saline controls	**Insulin injected**
	7.71	2.07
	7.26	1.81
	8.42	2.89
	6.67	1.80
	10.02	1.33
	7.90	1.03
mean	**8.0 ± 1.03**	**1.8 ± 0.77 ***

* $p < 0.001$ (Student's *t*-test)

Fos-HCRT response in the entire LHA. In saline injected control rats, in total (sum of every 6th section/animal), 4338±457 HCRTir perikarya were detected at all three, i.e. rostral, middle and caudal, levels of the lateral hypothalamic area, from which 414±173 Fos-HCRTir cells were recognized as double-labeled. This amount of double-labeled cells represented approximately 9.54 % of the total number (4338) of HCRTir plus Fos-HCRTir double-labeled perikarya.

In insulin-treated animals, in the same anatomical extent, in total, 3336±678 HCRTir perikarya were distinguished, from which 522±148 Fos-HCRTir cells were recognized as double-labeled. This amount of double-labeled cells represented approximately 15.65 % of the total number (3336) of HCRTir plus Fos-HCRTir double-labeled perikarya.

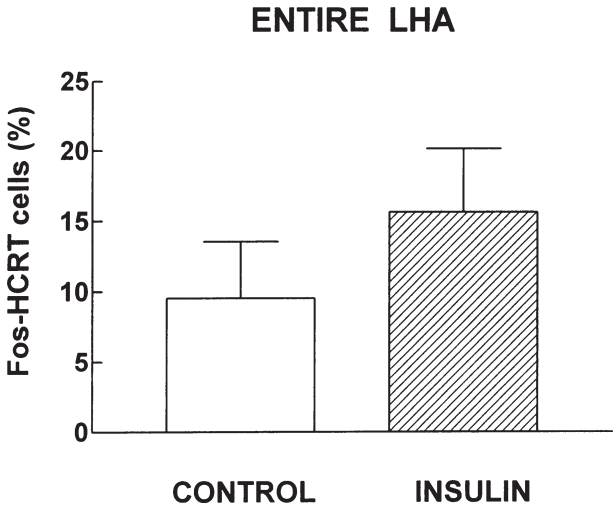

Fig. 1 Number of activated, i.e. Fos-positive, HCRT cells in the whole LHA 90 min after insulin administration. Insulin-induced hypoglycemia induced only a moderate, statistically insignificant, increase in the number of Fos-positive HCRT cells.

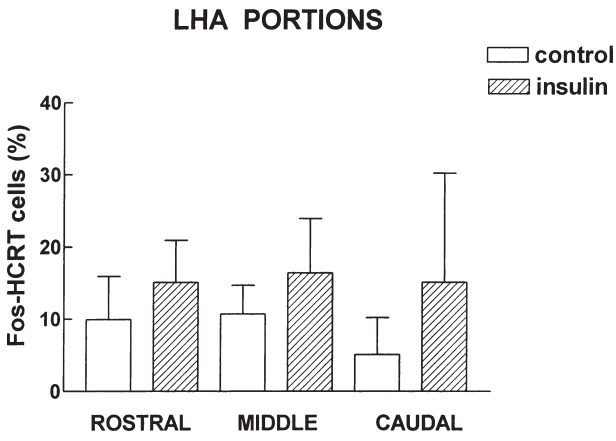

Fig. 2 Comparison of the number of Fos activated HCRT-immunopositive cells at three different, i.e. frontal, middle, and caudal, levels of the LHA. None of the combinations exhibited statistically significant increase in the number of Fos-positive HCRT cells.

Statistical comparison of the number of Fos activated HCRT cells between controls and hypoglycemic animals did not reach statistical significance (Fig. 1).

Fos-HCRT response within the LHA portions. In control rats the incidence of HCRTir cells was at the rostral level 846±84, middle level 2634±288 and caudal level 852±43, from which 84±72.0, 282±104.4 and 43±43.0 were double-labeled, respectively.

In hypoglycemic animals the incidence of HCRTir cells was at the rostral level 1800±270, middle level 1098±515 and caudal level 438 ± 437, from which 270±104.4, 180±82.8, and 66±64.8 were double-labeled, respectively.

In spite of the indications for the possible quantitative regional differences in the occurrence of HCRTir cells, the detailed comparison between the incidence of Fos-HCRTir cells in the individual portions of the LHA failed to reveal significancy (Fig. 2).

The distribution of activated Fos-HCRTir double-labeled perikarya did not show any special and anatomically definable spatial organization within the three portions of the LHA. Generally, they were quite evenly scattered among the single stained HCRTir cells at each level of the LHA.

Discussion

The data of the present study demonstrate that acute insulin treatment elicited Fos induction in many neurons of the LHA. However, the double stainings with anti-Fos and anti-HCRT sera revealed that early phase of hypoglycemia was insufficient to induce HCRT activation, neither for entire nor individual portions of the LHA, despite the profound decrease in circulating glucose levels (by more than 70 %).

The LHA is involved in the control of energy homeostasis since it contains distinct neuronal populations that express specific appetite-stimulating neurotransmitters including hypocretins. Although, hyperphagic effect of HCRT peptides is less distinct in comparison with some other hypothalamic peptides, notably NPY peptide (Edwards et al. 1999), it is still reasonable to believe that increased release of the appetite-stimulating HCRT could contribute to hypoglycemia-induced hyperphagia. However, in the lateral hypothalamus, besides HCRT melanin-concentrating hormone and PRL-like peptide containing neurons have been shown to express Fos during acute hypoglycemia (Moriguchi et al. 1999). In addition, while only a few Fos-positive signals were observed in melanin-concentrating hormone producing cells, more than 80 % PRL-like immunoreactive peptide containing neurons exhibited Fos signal during acute hypoglycemia indicating differences in the sensitivity to glucose decline among the individual phenotypes. HCRT-containing neurons have also been shown to be activated by hypoglycemia; however, Fos-signal has been reported only in around 33 % of HCRT neurons after injection of a single dose of insulin (Moriguchi et al. 1999). These data indicate that the HCRT cells do not represent the first line of defense in response to the fall of peripheral glucose levels. In addition, the HCRT neurons response to increased hunger requires specific circumstances and significantly increased prepro-orexin mRNA levels have been shown after a prolonged fasting (48 h), and 6 h acute fasting (Cai et al. 1999). Moreover, the HCRT-producing neurons are only a part of an extensive neuronal network including the hypothalamic paraventricular (PVN) and arcuate nuclei involved in the regulation of energy homeostasis. Thus, for instance, one of the most sensitive cells in response to insulin-induced hypoglycemia located outside of the LHA are the corticotrophin releasing hormone (CRH) synthesizing neurons of the hypothalamic paraventricular nucleus since Fos mRNA levels in the PVN CRH area have been shown to be significantly elevated already at 30 min, peaked at 60 min, and returned to baseline at 120 min after a single injection of insulin. However, regardless the many informations collected, the hierarchy of the known central systems involved in the feeding regulation has not been established yet (Williams et al. 2000, 2001).

Based on data of others (Cai et al. 1999) and present study it can be speculated that the response of HCRT cells to insulin induced-hypoglycemia might not need to be a prompt process depending only on the initial profound fall of circulating levels of glucose. Since the HCRT activation is not feasible in fed animals it can not be excluded that the duration of fasting period before or during the hypoglycemia could represent an important factor influencing the response of HCRT neurons to insulin-induced hypoglycemia. Thus in contrast to 90 min time-point used in our study with more than 70% of glucose decline, Cai and co-workers (2001) have shown a more profound Fos expression in the HCRT neurons of the LHA as late as 5 h after insulin-induced hypoglycemia with around only 50 % glucose fall in the peripheral circulation. Prominent response in Fos immunoreactivity has also been already reported 180 min after acute insuline administration (Niimi et al. 1995). On the other hand, Pacak and Palkovits (2001) reported only a limited number of Fos expression in other areas of the brain 90 min after insulin administration. Resuts of our study as well as the literature data indicate that at least three events, i.e. 1. the dosage of insulin applied, 2. the time-point studied, and 3. the sensitivity of the area selected, might markedly influence the final effect of in-

sulin-induced hypoglycemia on central structures including the population of HCRT neurons of the LHA. We, therefore, conclude that the HCRT neurons are not the primary site of action, but rather linked to events later and secondary to the acute metabolic change.

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