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Hypothalamic agouti-related protein immunoreactivity in food-restricted, obese, and insulin-treated animals: evidence for glia cell localization

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Abstract

Agouti-related protein (AGRP) has been implicated in the regulation of metabolic balance. Overexpression of this peptide leads to obesity. Its activity is mediated via the melanocortin-4 (MC-4) receptor where it acts as an MC-4 receptor antagonist. In this study, we characterized the AGRP brain distribution and cellular localization in control, food-restricted, obese, and insulin-treated rats using immunohistochemistry. AGRP immunostaining was found selectively in regions of the arcuate and ventromedial hypothalamic nuclei. These regions were stained less intensely in food-restricted rats than in controls. AGRP-positive cells in the hypothalamus of obese animals were three times more numerous than in control rats. Also, insulin treatment acted to decrease AGRP immunostaining. Analysis of AGRP cellular localization demonstrated its presence in the cytoplasm of numerous small (7–12 μm) cell bodies of putative protoplasmic astrocytes as well as in nerve fibers. Glia fibrillary acidic protein (GFAP) immunostaining of sections adjacent to those stained for AGRP revealed astrocytes with morphology similar to AGRP-positive cells. A few AGRP-positive nerve cell bodies were also found in the arcuate nucleus of obese rats. We conclude that AGRP hypothalamic content is decreased by fasting and intracerebroventricular (ICV) insulin treatment and increased in obesity. In addition to its presence in nerve fibers, AGRP localization in astroglia-like cells suggests a possible role for these elements in its synthesis or its sequestration from the neuronal compartment.

Keywords: Agouti; Melanocortin receptor; Obese; Insulin; Hypothalamus; Rat (Wistar)

Introduction

Agouti-related protein (AGRP) is an endogenous melanocortin-4 receptor antagonist (Bagnol et al., 1999; Beck, 2000). Its overproduction has been associated with increased feeding leading to obesity (Beck, 2000; Kim et al., 2000; Moussa and Claycombe, 1999). Our laboratory and others have investigated the role of the obesity hormone leptin and the proopiomelanocortin (POMC) products alpha melanocyte-stimulating hormone (αMSH) and beta-endorphin in the regulation of metabolic balance and their regulation of the sympathetic nervous system (Dunbar and Lu, 1999; Dunbar et al., 1997; McMinn et al., 2000). We have demonstrated that leptin can act to increase sympathetic nervous activity, and this response is mediated by increasing hypothalamic POMC products, especially αMSH (Dunbar and Lu, 1999; Dunbar et al., 1997). It has also been demonstrated that pretreatment with AGRP antagonizes both leptin and αMSH activation of sympathetic nervous activity and increased blood pressure (Dunbar and Lu, 2000a).

It has been demonstrated that fasting is associated with an increase in AGRP gene expression especially in the hypothalamus (Hahn et al., 1998; Mizuno et al., 1999; Wilson et al., 1999a). On the other hand, obesity has been reported to be associated with lower hypothalamic AGRP while diabetes has also been associated with an increase in

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AGRP expression (Hahn et al., 1998; Wilson et al., 1999a,b). However, other studies have demonstrated that extraction of AGRP from hypothalamic tissue homogenates from obese animals has an increase in AGRP content while food-restricted rats have a decrease (Harrold et al., 1999). Insulin has been demonstrated to be a major regulator of CNS peptides regulating feeding and metabolic balance at the level of proopiomelanocortin (POMC) (Broberger et al., 1998; Dunbar and Lu, 2000b; Havel et al., 2000). However, a more direct role of insulin in the regulation of AGRP has not been established. We previously conducted studies on the mechanistic interactions of leptin and the hypothalamic POMC products (Dunbar and Lu, 1999). Since leptin via αMSH exerts at least a portion of their action via melanocortin-4 receptors and AGRP is a MC-4 receptor antagonist, we decided to investigate the effect of food restriction, obesity, and intracerebroventricular (ICV) insulin on hypothalamic AGRP.

Materials and methods

All animal studies were conducted following institutional guidelines and the protocols were approved by the Institutional Animal Investigation Committee. Male Wistar rats (Harlan, Indianapolis, IN) weighing between 275 and 300 g were housed two to a cage with a 12-h light–dark cycle and an ambient temperature of 23°C. The control animals were fed laboratory rodent chow and had free access to water. A second group of animals was fed a high fat diet (40% fat by weight) for 8 weeks to produce hypertrophic obesity. A third group of animals was maintained the same as obese rats but was food restricted 72 h before sacrifice. A fourth group of control animals was anesthetized using ketamine (80 mg/kg) and xylazine (5.0 mg/kg), and the lateral ventricle cannulated using standard coordinates (Paxinos and Watson, 1998) with stainless steel guide cannulas (Plastic Products, Roanoke, VA) and allowed to recover for 4–5 days. Following the recovery period, the rats were treated twice daily with insulin for 3 days. Each animal was injected with 5.0 mU of insulin dissolved in 5 μl of artificial cerebrospinal fluid (ACF). All animals were weighed weekly, and a blood sample was collected before being euthanized for plasma glucose and insulin determination. Plasma glucose and insulin were measured as previously described (Lu et al., 2001).

Immunohistochemistry: AGRP

Each rat (controls, obese, food restricted, and insulin treated) was anesthetized with chloral hydrate (400 mg/kg body weight, IP). The rats were perfused transcardially with 50 ml of 0.1 M PBS (pH 7.5) to flush the blood from the vascular system, followed by 200 ml of 4% paraformaldehyde in 0.1 M sodium phosphate-buffered solution (pH 7.5). One hour following completion of perfusion, the brain was carefully removed and stored in the perfusion fixative at 4°C for a minimum of 24 h. The brain was sectioned coronally using a vibratome, and the 50-μm slices were stored at 4°C until immunohistochemistry procedures were performed. A profile of ten 50-μm sections separated by 500-μm intervals through the hypothalamus from controls, obese, food-restricted, and insulin-treated rat brains was processed at the same time to ensure uniformity of immunostaining. Brain sections were washed three times in 0.1 M PBS (pH 7.5), treated with 3% hydrogen peroxide to quench endogenous peroxidases, washed three times again with PBS, and then treated with 0.1% Triton X-100 for 15 min. The sections were incubated in blocking solution (Vector Laboratories, Inc., Burlingame, CA) for 30 min and then incubated with a rabbit anti-AGRP serum (1:500, Phoenix Pharmaceutical, Inc., Mountain View, CA) at 4°C overnight. The following morning the sections were treated with a secondary antibody consisting of diluted biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories) at room temperature for 1 h. Sections were then immersed in a avidin–biotin complex (ABC, Vector Elite Kit; amplification solutions made according to Vector's instructions in the kit) for 30 min and stained for 10 min with 3,3 diaminobenzidine and H_2O_2 solution. Each slice was placed on a slide containing glycerol/PBS solution and cover-slipped. Control sections, omitting the primary antibody, were included in every experiment.

GFAP

Sections (50 μm) adjacent to those stained for AGRP were treated in a similar manner but immunostained with a glial fibrillary acidic protein (GFAP) rabbit polyclonal antibody (1:100, Sigma, St. Louis, MO) at 4°C overnight. Secondary antibody and chromogen reactions were carried out as above. Comparisons of arcuate nucleus astrocytes with AGRP cells were made from photomicrographs taken with the X100 oil immersion objective at a total magnification of ×1300.

AGRP cell counts

In representative, coronally sectioned series of control, obese, and starved hypothalamus stained for AGRP, all immunopositive cells in both sides of the hypothalamus were located and counted with a Leitz drawing tube attachment at a magnification of ×250. Each series consisted of ten 50-μm consecutive sections separated from each other by 50-μm intervals and extended approximately from 2 to 3.5 mm posterior to Bregma (Paxinos and Watson, 1998). The thickness of each section and the intervals used precluded counting the same cell more than once. Using similar hypothalamic levels in the three paradigms allowed comparisons of total cells at each of the selected levels of the arcuate nucleus.