Acute food deprivation reduces expression of diazepam-binding inhibitor, the precursor of the anorexigenic octadecaneuropeptide ODN, in mouse glial cells

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Abstract

In the central nervous system of mammals, the gene encoding diazepam-binding inhibitor (DBI) is exclusively expressed in glial cells. Previous studies have shown that central administration of a DBI processing product, the octadecaneuropeptide ODN, causes a marked inhibition of food consumption in rodents. Paradoxically, however, the effect of food restriction on *DBI* gene expression has never been investigated. Here, we show that in mice, acute fasting dramatically reduces *DBI* mRNA levels in the hypothalamus and the ependyma bordering the third and lateral ventricles. I.p. injection of insulin, but not of leptin, selectively stimulated DBI expression in the lateral ventricle area. These data support the notion that glial cells, through the production of endozepines, may relay peripheral signals to neurons involved in the central regulation of energy homeostasis.

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Introduction

Hypothalamic neuropeptide signaling systems play an important role in the control of food intake and energy expenditure in mammals. These systems notably include two interconnected populations of neurons located in the arcuate nucleus (ARC), one producing the orexigenic neuropeptide Y (NPY) and the other one producing the anorexigenic peptide α -melanocyte-stimulating hormone (α -MSH), a processing product of proopiomelanocortin (POMC; Schwartz *et al.* 2000).

It is now recognized that astroglial cells can release various biologically active molecules, called gliotransmitters, that participate in the regulation of neuronal activity (Volterra & Meldolesi 2005). In particular, astrocytes express the polypeptide diazepam-binding inhibitor (DBI) which generates through proteolytic cleavage several regulatory peptides including the anorexigenic octadecaneuropeptide (ODN; De Mateos-Verchere *et al.* 2001, Do Rego *et al.* 2007, Matsuda *et al.* 2007). DBI is highly expressed in astroglial cells located in the hypothalamic regions which play a major role in the control of food intake, i.e. the ARC, the dorso- and ventromedial nuclei, and the lateral area of the hypothalamus (Malagon *et al.* 1993). I.c.v. administration of ODN in rats provokes a reduction of

NPY mRNA and an increase of POMC mRNA levels (Compère *et al.* 2003), suggesting that the anorexigenic action of ODN is mediated through inhibition of NPY neurons and/or activation of POMC neurons.

Acute food deprivation causes a reduction in circulating leptin and insulin levels (Bi *et al.* 2003). During re-feeding, insulin and leptin act as feedback signals stimulating the activity of hypothalamic POMC neurons and inhibiting the activity of NPY neurons (Schwartz *et al.* 2000).

Although there is clear evidence that the endozepine ODN is a potent anorexigenic peptide (De Mateos-Verchere *et al.* 2001, Do Rego *et al.* 2007, Matsuda *et al.* 2007), the possible involvement of satiety signals in the control of *DBI* gene expression has never been reported. Therefore, in the present study, we have examined the effect of food deprivation as well as of insulin and leptin administration on *DBI* mRNA levels in astroglial cells.

Materials and methods

The effects of fasting, insulin and leptin administration on *DBI* mRNA levels were investigated on 12–14-week-old male C57BL/6 mice (Charles River Laboratories,

St Constant, Quebec, Canada). All animal procedures were approved by the Laval University Animal Welfare Committee.

Treatments

To study the effect of fasting, 12 mice were divided into two groups. In the control group, mice ($n=6$) had free access to standard rodent chow and drinking tap water, and in the second group, mice ($n=6$) had free access to drinking tap water, but were food deprived for 24 h.

To examine the effects of insulin and leptin, 36 mice were food deprived for 24 h. Then, animals were divided into three groups. In the first group, mice ($n=12$) received an i.p. injection of murine leptin (100 µg/0.1 ml; Sigma). In the second group, mice ($n=12$) received an i.p. injection of bovine insulin (0.1 UI/0.1 ml; Sigma). In the third group, mice ($n=12$) received an i.p. injection of vehicle (5 mM sodium citrate, pH 4.0; 0.1 ml). The effect of treatments on *DBI* mRNA was analyzed 1 and 4 h after leptin or insulin administration.

Animals were deeply anesthetized with ketamine–xylazine, and were then perfused transcardially with 4% paraformaldehyde in 0.2 M phosphate buffer. The brains were removed and post-fixed in the same fixative overnight at 4 °C, and were then placed in 0.1 M phosphate buffer containing 15% sucrose overnight at 4 °C. Thereafter, the tissues were frozen on dry ice in a support medium (OCT, Bayer Corp.), and were conserved at –80 °C until *in situ* hybridization and immunohistochemistry experiments.

In situ hybridization

Frontal brain sections (10 µm) were mounted on Superfrost/PLUS microscope slides (Fisher Scientific, Montreal, Quebec, Canada), and were stored at –80 °C until use. *In situ* hybridization was performed as described previously (Compère *et al.* 2003). The vector used for the production of the cRNA probe was constructed by insertion of a 266 bp fragment of mouse *DBI* cDNA (GenBank no. NM_007830, nucleotides 215–461) into pCR-BluntII-TOPO (Invitrogen). After hybridization, the sections were dehydrated and coated with liquid photographic emulsion (Kodak NTB-2), and were exposed for 12 days. Semi-quantitative analysis of the hybridization signal was carried out on nuclear emulsion-dipped slides over the reactive cells in the hypothalamus using a Zeiss Optical System coupled to a Macintosh computer (Power PC 7500/100) and the Image Software (version 1.60 non-FPU, W Rasband, NIH, Bethesda, MD, USA). The optical density of the hybridization signal

measured in each specific region was corrected for the average background signal. Quantitative data are presented as mean ($\pm$ S.E.M.) calculated from the measurements obtained from 12 to 18 sections per mouse ($n=6$) in the same hypothalamic region. Comparison of the mRNA levels between experimental groups was performed by an ANOVA (Statview, Abacus Concepts, Berkeley, CA, USA), and differences were considered significant at $P<0.05$. Incubation of brain sections with the sense *DBI* probe did not produce any detectable labeling.

Figure 1 Dark- and bright-field photomicrographs showing the expression of *DBI* mRNA in the hypothalamus and the lateral ventricle of normally fed and food-deprived mice. Frontal sections through the hypothalamus (A and C) and the lateral ventricle (B and D) of *ad libitum* fed (A and B) and acute food-deprived mice (C and D) were hybridized with the *DBI* antisense riboprobe. The effects of food deprivation on *DBI* mRNA were quantified by measuring the integrated optical density (OD) of the signal in each area (E; $n=6$). (F and G) Brain sections were hybridized with the *DBI* antisense riboprobe (F), and adjacent sections were labeled (brown staining) with antibodies against GFAP (G). The arrows point to identified cells expressing *DBI* mRNA positively stained with GFAP. V, third ventricle; Va, area bordering the third ventricle; ARC, arcuate nucleus; ME, median eminence; LV, lateral ventricle; LVA, area bordering the lateral ventricle; and bv, blood vessel. Scale bars = 250 µm (A–D) and 10 µm (F and G). ** $P<0.01$, *** $P<0.001$ versus controls. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-09-0176>.

Figure 2 Bright-field photomicrographs showing the presence of insulin and leptin receptors in *DBI*-expressing cells in the arcuate nucleus of the mouse hypothalamus. Brain sections were labeled (brown staining) with antibodies against insulin receptors (A) or leptin receptors (B), and adjacent sections were hybridized with the *DBI* antisense riboprobe (C and D). The arrows point to identified cells expressing *DBI* mRNA positively stained with insulin (A and C) or leptin (B and D) receptors. V, third ventricle. Scale bars = 10 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-09-0176>.

Immunohistochemistry

Sections adjacent to those used for *in situ* hybridization were first hydrated, and were then incubated overnight at 4 °C with a monoclonal antibody raised against mouse leptin receptor (Ob-R; 1:500; sc-8391, Santa Cruz Biotechnology, Santa Cruz, CA, USA), purified polyclonal antibodies raised against the β -subunit of the insulin receptor (1:500; sc-711, Santa Cruz Biotechnology), or polyclonal antibodies raised against glial fibrillary acidic protein (GFAP; 1:1000; Dako, Glostrup, Denmark). Western blot analysis of mouse tissue/cell extracts indicated that the Ob-R antibody detects two bands corresponding to the long and short forms of Ob-R (sc-8391; Santa Cruz Biotechnology; Magni *et al.* 1999), and insulin receptor antibodies detect two bands corresponding to the β -subunit of the insulin receptor and insulin receptor precursor (sc-711; Santa Cruz Biotechnology). Sections were then washed in PBS (pH 7.6), incubated at room temperature for 10 min with the ultra streptavidin–HRP reagent (Signet, Dedham, MA, USA), and stained with 3,3'-diaminobenzidine. Brain sections incubated with phosphate buffer or non-immune serum, instead of with the primary antibodies, did not exhibit any detectable labeling.

Results and discussion

As previously shown in rats (Tonon *et al.* 1990), in the mouse brain, the expression of the *DBI* gene is restricted to astroglial cells notably ependymocytes bordering the third and lateral ventricles, and tanycytes of the median eminence (Fig. 1A and B), where *DBI* mRNA colocalized with the astroglial cell marker GFAP (Fig. 1F and G). The present work demonstrates, for the first time, that food deprivation strongly reduces the level of *DBI* mRNA in the outer area of the ARC, in the median eminence, and in the area bordering the lateral ventricle (Fig. 1A–E). It has been previously

Figure 3 Effects of i.p. injections of insulin or leptin on *DBI* mRNA levels in food-deprived mice. The *in situ* hybridization signal was quantified by measuring the integrated optical density (OD) of the signal in each area, i.e. arcuate nucleus (A and B), median eminence (C and D), and area bordering the lateral ventricle (E and F). The effect of treatment was analyzed 1 h (A, C, and E) and 4 h (B, D, and F) after insulin or leptin administration. * $P < 0.05$ versus controls ($n = 6$).

demonstrated that food deprivation inhibits central anorexigenic pathways, and notably reduces the expression of POMC and thus the production of the anorexigenic neuropeptide α -MSH in the hypothalamus (Swart *et al.* 2002, Bi *et al.* 2003). The present observation that fasting profoundly inhibits DBI expression in the hypothalamus provides additional support for a physiological role of the anorexigenic DBI-derived peptide ODN in the central control of energy homeostasis.

The two hormones insulin and leptin are known to convey information about the status of body energy stores to the brain (Niswender & Schwartz 2003, for review), and the hypothalamic POMC or NPY neurons express insulin and leptin receptors (Hakansson *et al.* 1996, Baskin *et al.* 1999, Pardini *et al.* 2006). However, the precise localization of these two receptors in hypothalamic astroglial cells has not been fully described yet. Cheunsuang & Morris (2005) have shown the occurrence of the leptin receptor protein in astrocytes in the ARC. Although insulin receptors are expressed by cultured rat astrocytes (Schechter & Yanovitch 1999), the presence of this receptor in glial cells *in situ* has never been reported. Insulin binding sites have only been described in freshly isolated human brain tumors of glial origin (Grunberger *et al.* 1986). Here, we have shown by an immunohistochemical approach that insulin receptors are present in numerous cells in the area bordering the third ventricle (Fig. 2A). We also found that leptin receptors are predominantly located in the ependyma area (Fig. 2B). Consecutive sections hybridized with the DBI probe revealed that in the mouse brain, most of the cells expressing DBI mRNA were also stained with antibodies against insulin or leptin receptors (Fig. 2C and D).

Since acute food deprivation provokes a reduction in plasma insulin and leptin concentrations (Bi *et al.* 2003), these results led us to investigate the effects of i.p. administration of these two hormones on DBI mRNA expression in food-deprived mice. Leptin and insulin had no effect on hypothalamic DBI mRNA levels (Fig. 3A–D). In contrast, insulin, but not leptin, significantly increased DBI mRNA expression in the area bordering the lateral ventricle (Fig. 3E and F). These data suggest that the inhibitory effect of food deprivation on DBI mRNA level in the lateral ventricle area is mediated, at least in part, by a decrease of plasma insulin concentration. There is now clear evidence that the lateral septum, in particular the area bordering the lateral ventricle, is involved in the control of feeding behavior (Pankey *et al.* 2008, Scopinho *et al.* 2008). In the lateral septum, dendrites of NPY-expressing neurons penetrate the ependyma to reach the liquor space (Kovacs *et al.* 2007). Since ODN reduces the level of NPY mRNA in the ARC (Compère *et al.* 2003, 2005), DBI-derived peptides might also exert an inhibitory

effect on NPY neurons in the septum. Thus, these data suggest that extrahypothalamic DBI could be involved in the regulation of energy homeostasis.

In conclusion, we demonstrate for the first time that acute food deprivation markedly reduces DBI mRNA levels in the mouse hypothalamus, and that insulin, but not leptin, stimulates DBI expression in astroglial cells bordering the lateral ventricle. Altogether, these data suggest that DBI-expressing astroglial cells relay hormonal signals from the periphery to the central nervous system.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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