Chronic food-restriction alters the expression of somatostatin and growth hormone-releasing hormone in the ovariectomised ewe

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Abstract

Changes in the secretion of GH induced by long-term alterations in nutritional status are thought to result from alterations in somatostatin (SRIF) and growth hormone-releasing hormone (GHRH) at the level of the hypothalamus. To date however, the effect of nutrition on the gene expression of SRIF and GHRH in a species where GH secretion is increased by food restriction, as is the case for the sheep and human, remains unknown. We determined the effect of under-nutrition on the expression of SRIF and GHRH in the hypothalamus of sheep. Ovariectomised ewes were randomly divided into two groups and either fed an *ad lib* diet ($n=6$) or a restricted diet of 500 g lucerne chaff per day (food-restricted; $n=5$)

for 7 months. *In situ* hybridisation was used to study hypothalamic gene expression for GHRH, SRIF and galanin (GAL). The food-restricted animals had elevated plasma concentrations of GH; this was associated with an increase in GHRH mRNA levels in the arcuate nucleus (ARC) and reduced SRIF in the rostral periventricular nucleus and ventromedial hypothalamic nucleus. The level of gene expression of GAL in the ARC and SRIF in the caudal periventricular nucleus was similar in *ad lib* and food-restricted animals. In conclusion, the effect of chronic food-restriction on the secretion of GH reflects increased GHRH and reduced SRIF synthesis in the hypothalamus.

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Introduction

The secretion of GH is highly susceptible to changes in nutritional status in all species studied to date. Under-nutrition increases the secretion of GH in man (Stoving *et al.* 1999) and sheep (Foster *et al.* 1989, Thomas *et al.* 1990). Conversely, plasma GH levels are reduced in food-restricted or fasted rodents (Tannenbaum *et al.* 1979). In the obese state, both basal and stimulated secretion of GH is reduced in man (Williams *et al.* 1984) and rodents (Renier *et al.* 1990).

The episodic release of GH from the somatotroph is predominantly controlled by somatostatin (SRIF) and growth hormone-releasing hormone (GHRH), which are secreted from the hypothalamus to exert inhibitory and stimulatory effects respectively. To what extent both SRIF and GHRH mediate the altered secretion of GH due to nutritional status remains unknown; no studies have been performed in species where GH is increased by under-nutrition. Some progress, however, has been made in the fasted rodent in which GHRH mRNA and peptide levels are down-regulated (Bruno *et al.* 1990, Brogan *et al.* 1997) by acute under-nutrition. Despite this, there is only a small effect (Brogan *et al.* 1997), if any (Bruno *et al.* 1990), on

the level of SRIF mRNA and peptide. On the other hand, food-restricted sheep have reduced SRIF concentrations in the hypophyseal portal circulation whereas GHRH levels in this model appear to be maintained (Thomas *et al.* 1991).

It is important to note that previous mRNA studies have been performed on whole rodent hypothalamic tissue. SRIF-containing neurons are abundant throughout the hypothalamus in rats (Bennett-Clarke *et al.* 1980), sheep (Willoughby *et al.* 1995) and man (Langevin & Emson 1982) and previous studies have not accounted for the specific regional variations in expression that one might expect. Indeed, neuronal tracing studies in rodents have shown that the SRIF neurons from the rostral periventricular nucleus (PeVN) are solely responsible for the projections to the median eminence (Kawano & Daikoku 1988).

The current study aimed to determine the effects of chronic food-restriction on the expression of GHRH in the ARC and SRIF in the rostral and caudal periventricular nucleus (rPeVN, cPeVN) and the ventromedial hypothalamic nucleus (VMH). The effect of nutrition on the mRNA levels for galanin (GAL) was also measured, since this peptide stimulates GH secretion (Ottlecz *et al.* 1986, Bauer *et al.* 1986, Spencer *et al.* 1994).

Materials and Methods

Ethics

This work was approved in advance by the Animal Experimentation Ethics Committees of Monash University and Victorian Institute of Animal Science.

Animals

Ovariectomised Corriedale ewes were randomly divided into two groups and fed either an *ad lib* diet of lucerne chaff and 1 kg lupin grain per week or a restricted diet of 500 g of chaff per day as previously described (Henry *et al.* 2001). At the time of experimentation the food-restricted animals ($n=5$) had significantly ($P<0.001$) lower body weights compared with the *ad lib* fed animals ($n=6$) (30.8 ± 1.3 kg vs 53.4 ± 2.2 kg) and elevated plasma levels of GH ($P<0.001$) (11.6 ± 0.6 ng/ml vs 5.5 ± 0.9 ng/ml), as previously reported (Henry *et al.* 2001). Serial blood samples were taken every 10 min for 8 h for the measurement of GH. Although the animals were previously treated with leptin (Henry *et al.* 2001), GH determinants were performed prior to this and the animals were killed 1 month later (to ensure that there was no confounding effect of the leptin infusion). Throughout this period the animals were maintained on either the restricted or *ad lib* diet.

Tissue collection preparation and *in situ* hybridisation

The animals were killed by humane means and the brains perfusion fixed with a series of paraformaldehyde solutions (Henry *et al.* 2000). Frozen sections were cut at 20 μ m using a cryostat and then stored at -20°C in 2% PFA/cryoprotectant solution. At least two sections were anatomically matched, pair-mounted onto Super Frost Plus slides (Menzel-Glaser, Braunschweig, Germany) and dried overnight at room temperature. *In situ* hybridisation was performed using ^{35}S -dUTP-labeled (Amersham Pharmacia Biotech, Sydney, Australia) riboprobes following the method of Simmons *et al.* (1989). The cDNA and plasmid inserts used were a 220 bp human galanin insert in BSKII (Evans *et al.* 1992), a 160 bp rat GHRH insert in pGemT-easy and a 420 bp rat SRIF insert in pGem-4 (Fuller & Verity 1989). The rat GHRH cRNA probe was PCR cloned from hypothalamic tissue using the primers 5' hGHRH-105 (AGA TGC CAT CTT CAC CAR CAG CT) and 3' hGHRH-250 (ATT TGC TTT TGY TCT GCC CAC AY) (Gibco BRL, New York, USA). Amplification, purification and linearisation of plasmid DNA were performed using standard techniques (Sambrook *et al.* 1989). All cRNA probes were synthesised using a Gemini system II kit (Promega Corp., Annandale, New South Wales, Australia). Hybridisation signal was detected using a Molecular Dynamics Storm Phosphor-Imager (Amersham Pharmacia Biotech, UK Ltd,

Buckinghamshire, England). Sections hybridised for SRIF were exposed for 1 day, whereas those for GHRH and GAL were exposed for 3 days. Slides were then dipped in photographic emulsion (Ilford Australia, Mount Waverly, Victoria, Australia) and exposed at 4°C for 2, 14 and 21 days for SRIF, GAL and GHRH respectively. The slides were then developed using Ilford Phenisol X-ray developer, stop-bath and Hypam fixer, then counterstained with 1% cresyl violet, dehydrated and coverslipped using DPX.

Data and statistical analysis

For each sheep one section representing each region was selected and 20 cells were randomly chosen (unless the total number of cells was less than 20) for silver grain analysis. A cell was deemed labeled if the number of silver grains exceeded the adjacent background reading by 7 times. This was performed at 400x magnification using a microcomputer imaging device (MCID) M1 system from the Imaging Research, Inc. (Brock University, St Catharines, Ontario, Canada). The total number of labeled cells was counted at 20x magnification. Direct comparisons of silver grains/cell were made between *ad lib* and food-restricted animals using a single factor ANOVA after checking for homogeneity of variance; SRIF silver grains/cell for the VMH were subjected to log transformation. Data for the number of labeled cells were analysed by the Mann Whitney U test for unpaired samples. Correlation analysis was performed to determine the association between plasma levels of GH and gene expression.

FIGURE 1 Representative darkfield micrographs (2x magnification) of GHRH-labelled cells in the ARC (A,B) and SRIF-labelled cells in the rPeVN (C,D). Panels A and C correspond to *ad lib* and panels B and D the food-restricted groups. 3 V: third ventricle.

FIGURE 2 The effect of nutritional status on the gene expression of GHRH and SRIF in *ad lib* (n=6; closed bars) and food-restricted (n=5; open bars) sheep. All data are presented as mean ($\pm$ S.E.M.). * $P < 0.05$, *** $P < 0.0001$

Results

Examples of hybridisation signals for GHRH and SRIF are shown in Fig. 1. The number of GHRH-labeled cells in the ARC was higher ($P < 0.01$) in the food-restricted animals but the number of GHRH silver grains/cell was similar between the two groups (Fig. 2). The number of SRIF-labeled cells in all three regions, the rPeVN and cPeVN and the VMH, was similar in the food-restricted and *ad lib* fed animals (Fig. 2). The number of silver grains/cell for SRIF in the rPeVN and the VMH was lower ($P < 0.01$) in the food-restricted group, with no effect of nutritional status on the number of silver grains/cell for SRIF in the cPeVN (Fig. 2). Expression of GAL mRNA was similar in the two groups (data not shown).

Mean plasma GH concentrations were negatively correlated ($P < 0.01$) to the number of silver grains per SRIF cell in the rPeVN and the VMH ($r = -0.77$, $P < 0.001$; data not shown) but not the cPeVN (Fig. 3). There was no significant correlation between GH levels and the number of SRIF-labeled cells for any given region. Neither was the number of GHRH-labeled cells nor the number of silver grains per cell for GHRH correlated with mean plasma GH levels (Fig. 3).

Discussion

We show an effect of nutrition on the expression of SRIF mRNA, which is specific to particular hypothalamic nuclei; there was a reduction in the rPeVN and the VMH, but not the cPeVN in food-restricted sheep. Furthermore, the changes in the number of silver grains/cell for SRIF in the rPeVN and the VMH were negatively correlated with alterations in plasma GH levels. The expression of GHRH mRNA was increased in the ARC in food-restricted animals, but these changes were not correlated to the secretion of GH. Whilst gene expression and the pulsatile

release of these neurohormones may not be correlated in the strictest sense, our results are consistent with an upregulation of the GH axis at the hypothalamic level in the food-restricted animals. Previous studies in rodents have shown that SRIF cell bodies in the rPeVN project to the median eminence (Kawano & Daikoku 1988) and are most likely to be involved in the regulation of GH secretion; the current results are consistent with this notion. The expression of SRIF was reduced in the rPeVN of the food-restricted animals concordant with increased GH secretion in this group. In contrast, SRIF projections from the VMH to the median eminence have not been identified (Kawano & Daikoku 1988) and whether decreased SRIF mRNA levels in this area relate to increased

FIGURE 3 Correlation studies in *ad lib* (Closed squares) and food-restricted (open squares) animals. The SRIF silver grains per cell in the rPeVN were negatively correlated ($P < 0.01$) with the mean plasma concentrations of GH.

GH levels in food-restricted animals remains unknown. SRIF is expressed throughout the central nervous system, is implicated in various bodily functions, and is thought to act as a central neurotransmitter (Olpe *et al.* 1980).

Central administration of SRIF has been shown to regulate food intake in rodents but results remain controversial, with SRIF reported to decrease (Lin *et al.* 1987, Ho *et al.* 1989), to increase (Danguir 1988), or to induce a biphasic effect (Aponte *et al.* 1984) on feeding behaviour. The effects of SRIF on feeding appear to be dose-dependent, with low doses stimulating and high doses inhibiting food intake (Feifel & Vaccarino 1990). The VMH is integral to the regulation of feeding (Luiten *et al.* 1987) and decreased expression of SRIF in this region may relate to altered appetite drive in food-restricted animals. It is possible to invoke a mechanism, whereby decreased SRIF mRNA levels in the VMH, and low SRIF levels, may be translated into increased hunger-drive in the food-restricted group. These animals are indeed hungry as when fed an *ad lib* diet of lucerne hay their voluntary food intake doubles (Henry *et al.* 2001).

A role for SRIF in the VMH in mediating the secretion of GH can not be dismissed however. In the current study the expression of SRIF in the VMH was negatively correlated to the mean plasma GH concentrations. Although there are no direct projections from the VMH to the median eminence (Kawano & Daikoku 1988), SRIF may exert an effect on the secretion of GH by modulating GHRH release. Radiolabeling studies have identified SRIF binding sites in the ventrolateral ARC and approximately 35% of the GHRH cells in the ARC contain SRIF-receptors (McCarthy *et al.* 1992). It is possible that decreased SRIF expression in the VMH could lead to a lowered inhibitory input to GHRH cells of the ARC and a consequent increase in GH secretion. In addition to the alterations in SRIF, the expression of GHRH mRNA was increased in the ARC of the food-restricted animals, consistent with the higher plasma GH levels in this group. Despite this, however, expression of GHRH mRNA was not correlated to the mean plasma concentrations of GH suggesting that SRIF may be the primary controller in mediating the increased secretion of GH in food-restricted animals. In accordance with this, previous studies from our laboratory have shown that the hypophyseal portal concentrations of SRIF are lower in under-fed ewes but the concentrations of GHRH are unchanged (Thomas *et al.* 1991). It remains possible that small changes in the secretion of GHRH may occur, but levels in portal blood are very low and close to the limit of detection (Thomas *et al.* 1991).

Cells containing GHRH have been shown to project to multiple sites within the central nervous system (Merchenthaler *et al.* 1984) apart from the median eminence, suggesting that GHRH is involved in mediating functions other than the secretion of GH. Like SRIF, GHRH has been implicated in the control of feeding

behaviour, having been shown to enhance food intake in rats (Vaccarino *et al.* 1985) and sheep (Ruckebusch & Malbert 1986). In addition to the regulation of GH secretion, increased GHRH mRNA levels may precede an up-regulation in hunger-drive in food-restricted sheep.

We investigated expression of GAL due to the well-documented stimulatory effects of this peptide on the secretion of GH (Ottlecz *et al.* 1986, Spencer *et al.* 1994). The expression of GAL in the ARC was similar in the *ad lib* and food-restricted animals. Evidence suggests that the effects of GAL on the secretion of GH involve SRIF-ergic inhibition (Loche *et al.* 1990, Tanoh *et al.* 1993) and GAL cells in the ARC project to the SRIF neurons in the PeVN (Liposits *et al.* 1993). It is likely, therefore, that any effect on the GAL system may manifest as changes in the levels of SRIF in the PeVN and our results for the rPeVN are consistent with this.

In conclusion, the effects of chronic under-nutrition to elevate the secretion of GH may result from increased GHRH and decreased SRIF synthesis. Furthermore, the effects of nutrition on the expression of SRIF are specific to particular regions of hypothalamus and it is likely that SRIF in the rPeVN is most relevant to the control of the secretion of GH. In addition to effects on the secretion of GH, both GHRH and SRIF could possibly be involved in the regulation of feeding behaviour; decreased gene expression for SRIF in the VMH may be linked to such a function.

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