SHORT COMMUNICATION

Full Title: Agouti related protein (AgRP) is upregulated in Cushing’s syndrome

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Abstract

Alpha melanocyte stimulating hormone (α-MSH) is an agonist at the melanocortin 3 (MC3-R) and melanocortin 4 (MC4-R) receptors. α-MSH stimulates corticosterone release from rat and human adrenal cells. Patients with Cushing’s syndrome have elevated levels of serum α-MSH. Agouti related protein (AgRP) is an endogenous antagonist at the MC3-R and MC4-R and is expressed in the rat adrenal cortex. AgRP antagonises α-MSH-induced corticosterone release from rat and bovine adrenal cells. This suggests that AgRP may have an inhibitory paracrine role in the adrenal gland. We measured adrenal AgRP mRNA expression and circulating AgRP in 2 patients with Cushing’s syndrome and controls. Adrenal AgRP mRNA expression and plasma AgRP were higher in the patients with Cushing’s syndrome compared to controls. Plasma AgRP in the patients with Cushing’s syndrome following bilateral adrenalectomy and hydrocortisone replacement were similar to the levels seen in controls. Our results suggest that AgRP may have a novel inhibitory paracrine role in the human adrenal gland.
Introduction

Adrenocorticotropic hormone (ACTH) and alpha melanocyte stimulating hormone (α-MSH) are produced by post-translational cleavage of the precursor pro-opiomelanocortin (POMC). ACTH potently stimulates glucocorticoid synthesis and secretion from adrenal glands by stimulating the melanocortin 2 receptor (MC2-R). Alpha MSH also stimulates glucocorticoid release from rat and human adrenal cells at near physiological concentrations (Vinson et al., 1981; Vinson et al., 1983; Henville et al., 1989). Alpha MSH is a potent agonist of the melanocortin 1 receptor (MC1-R), MC3-R and MC4-R (Schioth et al., 1996). The presence of MC3-5R’s in the rat adrenal gland (Dhillo et al., 2003; Liakos et al., 2000) have recently been reported. Agouti related protein (AgRP) is an endogenous antagonist at the MC3-R and MC4-R and does not bind to the MC2-R (Ollmann et al., 1997). AgRP is primarily expressed in the arcuate nucleus of the hypothalamus and is a potent orexigen (reviewed in (Barsh and Schwartz, 2002)). In humans and rats the adrenal glands are the site of highest AgRP mRNA expression after the hypothalamus (Ollmann et al., 1997) and AgRP is expressed in the adrenal cortex (Bicknell et al., 2000). AgRP antagonises α-MSH-induced glucocorticoid production in rat (Dhillo et al., 2003) and bovine adrenal cells (Doghman et al., 2004) suggesting that α-MSH may mediate its effects via the MC3-R or MC4-R. Adrenal AgRP mRNA is increased in rats treated with dexamethasone (Dhillo et al., 2003). This data suggests that AgRP may have an inhibitory paracrine role in the rat and bovine adrenal gland. The role of AgRP in the human adrenal gland is not known. To investigate the role of AgRP in the human adrenal gland we measured adrenal AgRP mRNA and circulating AgRP in
patients with Cushing’s syndrome and controls and determined the presence of the MC3-R and MC4-R in human adrenal tissue.
Materials and Methods

Patient 1

This patient had biochemical Cushing’s syndrome secondary to ectopic ACTH secretion from an unknown source. She was 27 years old and presented with increasing weight gain (BMI 28.1) and generalised weakness. Twenty four hour urine for cortisol excretion was grossly elevated (31,000; normal 0-270 nmol / 24 hours). Her 9am cortisol following a standard low dose dexamethasone suppression test (LDDST) did not suppress appropriately (time = 48 hours (t=48) cortisol 1291 nmol/l; normal suppression to <50 nmol/l) confirming Cushing’s syndrome. Following a high dose dexamethasone suppression test (HDDST) her baseline cortisol of 1472 nmol/l fell to 649 nmol/l suggesting that a pituitary source of her Cushing’s syndrome was unlikely. An MRI scan of her pituitary, CT scan of her chest, abdomen and pelvis, octreotide scan and PET scan were normal. Bilateral inferior petrosal sinus sampling with CRH administration (IPSS) demonstrated no central to peripheral ACTH gradient confirming an ectopic ACTH source causing her Cushing’s syndrome. Since the primary source of ectopic ACTH was not found she was treated with laparoscopic bilateral adrenalectomy and hydrocortisone replacement. This patient consented to a blood sample being taken prior to and 7 days after her operation. She also consented to her adrenal tissue being used for measurement of AgRP mRNA.
**Patient 2**

This patient had Cushing’s disease as part of MEN-1. She was diagnosed with Cushing’s disease and underwent two transphenoidal attempts at pituitary surgery at another hospital. Despite this the patient remained Cushingoid and was referred to our hospital. She was 23 years old with a BMI of 30.0 and biochemically Cushingoid (following LDDST her t=48 cortisol was 275 nmol/l; normal suppression to <50nmol/l). Following a HDDST her baseline cortisol of 649 nmol/l fell to 84 nmol/l in keeping with a pituitary source of her Cushing’s syndrome. Furthermore, IPSS demonstrated a significant central to peripheral ACTH gradient confirming a pituitary source of her Cushing’s syndrome. MRI pituitary revealed only minimal residual pituitary tissue. As such she was treated with bilateral adrenalectomy as a definitive treatment for her Cushing’s disease and given hydrocortisone replacement. This patient consented to a blood sample being taken prior to and 7 days after her operation. She also consented to her adrenal tissue being used for measurement of AgRP mRNA.

All samples were obtained following written informed consent and approval by the Hammersmith, Queen Charlotte’s & Chelsea Hospitals Research Ethics Committee (Registration number: 2002/6410).

**(i) Measurement of adrenal AgRP mRNA**

Adrenal gland tissue from the 2 patients with Cushing’s syndrome described above was used. Total RNA from the tissue was isolated using Tri-reagent (Helena Biosciences, Sunderland, UK) according to the manufacturers protocol (Dhilllo et al., 2003). Human adrenal gland total RNA used as a control was obtained commercially from Ambion
(Huntingdon, UK) and BD Biosciences Clontech (Ontario, Canada). The adrenal RNA sample from Ambion was from a 62 year old Hispanic woman who had died suddenly of an intracerebral haemorrhage. The adrenal RNA sample from BD Biosciences Clontech was a pooled sample taken from 61 subjects that were all healthy during life but had died suddenly (for example in a road traffic accident). Northern blot analysis was used to quantify the amount of AgRP mRNA expression in each sample.

*Northern blot analysis:*

Northern blot analysis of RNA from adrenal glands was performed as previously described (Taheri et al., 2001). Briefly, 50μg of total RNA was size separated on a denaturing MOPS [3-(n-morpholino) propane-sulphonic acid]/formaldehyde gel (1% agarose) and transferred to a Hybond-N membrane (Amersham Biosciences, Buckinghamshire, UK). The RNA was fixed by baking at 80°C for 2 h before probing with a riboprobe corresponding to nucleotides 508-688 of the human AgRP cDNA sequence (accession number U89485) containing the coding region for AgRP (Shutter et al., 1997). The riboprobe was synthesised using [α-32P]-CTP using T7 RNA polymerase (Promega Corporation, Southampton, UK). Hybridization was carried out overnight at 55°C in a mixture of 2.5mM ethylenediaminetetra-acetic acid (EDTA) (pH8), 0.5% dried milk, 0.25M sodium phosphate buffer (pH 7.2), 5% sodium dodecyl sulphate (SDS) and 25μM aurin tricarboxylic acid. Non-specific hybridisation was removed by increasingly stringent washes the final one being in 0.1x saline sodium citrate (SSC)/ 0.1% (w/v) SDS at 70°C for thirty minutes. RNA was quantified using phosphorimager analysis and
ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Blots were reprobed with oligo(dT)$_{12-18}$ to enable differences in RNA loading to be corrected.

(ii) Measurement of plasma hormones

Measurement of plasma AgRP

Plasma AgRP was measured in the 2 patients with Cushing’s syndrome described and 11 normal female volunteers. Volunteers were matched for BMI to patients with Cushing’s syndrome since plasma levels of AgRP are increased in obese subjects (Katsuki et al., 2001) (BMI (mean ± S.E.M) in patients with Cushing’s syndrome 29.0 ± 0.9, volunteers 27.1 ± 5.4). Venous blood was sampled from all subjects between 9am and 12pm following breakfast since fasting is known to increase AgRP levels (Shen et al., 2002). AgRP in plasma samples was measured using a commercially available RIA kit (AgRP(83-132)-NH2 (Human) RIA kit; Phoenix Pharmaceuticals, Inc., Belmont, CA, USA) as previously described ((Katsuki et al., 2001; Shen et al., 2002). Briefly, 100μl of standard (synthetic human AgRP(83-132) fragment) or plasma were added to tubes coated with primary antibody (rabbit antiAgRP serum) and incubated at 4°C for 18 h. One hundred microlitres of $^{125$I labelled peptide was then added to each assay tube and incubated at 4°C for an additional 18 h. Following this 100μl goat anti-rabbit IgG serum and normal rabbit serum were added to each tube and incubated for 90 min at room temperature. After centrifugation at 3000 rpm for 20 min at 4°C, the supernatant radioactivity was counted. The values of plasma AgRP were then extrapolated from a curve drawn using standard concentrations of AgRP. This assay showed no significant cross-reactivity with, or interference by leptin, orexin A, orexin B, neuropeptide Y, α-
MSH, melanin-concentrating hormone, and calcitonin gene related peptide. The intra- and interassay coefficients of variation were 6.0% and 8.9%, respectively.

Measurement of plasma alpha-MSH

Plasma alpha-MSH was measured in the 2 patients with Cushing’s syndrome described prior to and following bilateral adrenalectomy using a radioimmunoassay as previously described (Bewick et al., 2005). The alpha-MSH antibody was obtained from Chemicon International (Temecula, CA; catalog no. AB5087) and was used at a final dilution of 1:320,000. This antibody has been previously characterized (Elias et al., 1998). Briefly, the antiserum is specific for alpha-MSH with no cross-reactivity with melanin-concentrating hormone and is dependent on the amidated C-terminal region for recognition. Cross-reactivities of the antiserum with related peptides are desacetyl- alpha-MSH, 82–100%; alpha-MSH-free acid, 0.018%; ßMSH (monkey), 0.0018%; αMSH (11–13), γ1MSH, and γ2MSH (each undetectable at 1 µM); ACTH (1–10), 0.018%; ACTH (1–24), 0.02%; ACTH (1–39) (human), 0.022%; ß-lipotropin (human), 0.022%; and ß-endorphin (human) and γ-endorphin (each undetectable at 1 µM). The sensitivity of the assay was 1 fmol/ml. The specific activity of iodinated αMSH as measured by the self-displacement assay was 55 Bq/fmol. The intra- and interassay variation was 7 and 8%, respectively.

(iii) To determine the presence of the MC3-R and MC4-R in human adrenal tissue

To determine the presence of the MC3-R and MC4-R in adrenal gland mRNA from a control subject (using the sample from Ambion) and a patient with Cushing’s syndrome
RT-PCR was performed using nested primers as previously described (Dhillo et al., 2003). Briefly, 20μg of total RNA was reverse transcribed using avian myoblastoma virus reverse transcriptase (RT) (Promega, Southampton, UK) in a reaction using oligo(dT)_{12-18}. Half of the RT reaction was subjected to PCR using primers obtained from the published sequence of the human MC3-R and MC4-R (Accession numbers NM019888 and NM005912, respectively). The primers were synthesised by Oswell DNA services (Southampton, UK). The primers used in the reaction for the MC3-R were 5’ tgtctttcctgtgagcagca and 3’ ccatgtgctggataaactgg (corresponding to positions 36 to 55 and 441 to 460 of the GenBank nucleotide sequence) and for the MC4-R 5’acttctctgcacctctggaa and 3’ tcaacatagacccgtcgaac (corresponding to positions 31 to 50 and 511 to 530 of the GenBank nucleotide sequence). The conditions used for the PCR were 95°C for 45 s, 58°C for 30 s, and 72°C for 60 s. Following the first round of PCR 1/20 of the reaction was subjected to a second round of PCR using internal primers for the MC3-R (5’ tcagccaacactgcctaatg and 3’ ggctgcagagaaagaagtac, corresponding to positions 141 to 160 and 330 to 349 of the GenBank nucleotide sequence) and MC4-R (5’ tcctgaggttttgtgactc and 3’ cagatcaccgagtcaatgac, corresponding to positions 141 to 160 and 370 to 389 of the GenBank nucleotide sequence). Using these primers an amplified fragment of 208 bp for the MC3-R and 248 bp for the MC4-R would be expected. Controls were conducted at the same time and treated in an identical way but no RT added to the reverse transcription reaction. All of the PCR products were analysed on 1% agarose gels.
Results

(i) Measurement of adrenal AgRP mRNA in patients with Cushing’s syndrome and controls

Adrenal AgRP mRNA levels were higher in the samples from the 2 patients with Cushing’s syndrome compared to controls. Mean adrenal AgRP mRNA in the Cushing’s group was $0.10 \pm 0.004$ arbitrary units compared to $0.03 \pm 0.01$ arbitrary units in controls (Figure 1).

(ii) Measurement of plasma hormones in patients with Cushing’s syndrome

Plasma AgRP was higher in the 2 patients with Cushing’s syndrome (prior to their operation) compared to mean plasma AgRP in controls (patient 1 plasma AgRP pre-operatively 246 pg/ml; patient 2 plasma AgRP pre-operatively 370 pg/ml vs. normal controls (mean $\pm$ SEM) 209 $\pm$ 17 pg/ml, n=11). There was a fall in plasma AgRP in both patients with Cushing’s syndrome following normalization of their raised cortisol levels by bilateral adrenalectomy and physiological hydrocortisone replacement therapy (patient 1 plasma AgRP post-operatively 152 pg/ml; patient 2 plasma AgRP post-operatively 184 pg/ml).

Plasma alpha-MSH was higher in the 2 patients with Cushing’s syndrome prior to bilateral adrenalectomy compared to levels following the operation (plasma alpha-MSH: patient 1 pre-operatively 2.5 pmol/L; post-operatively 0.1 pmol/L. Patient 2 pre-operatively 3.1 pmol/L; post-operatively 2.2 pmol/L).
(iii) To determine the presence of the MC3-R and MC4-R in human adrenal tissue from a control subject and a patient with Cushing’s syndrome

RT-PCR demonstrated the presence of both the MC3-R and MC4-R in human adrenal gland mRNA from a control subject (using the sample from Ambion) and from a patient with Cushing’s syndrome (patient 1). To check for possible artefacts generated by amplification of remnants of genomic DNA, control RT-PCR were performed and treated in an identical way but with no RT added to the RT-reaction mixture. No MC3-R and MC4-R PCR products were detected in these reactions (Figure 2). The MC3-R and MC4-R products were sequenced (Advanced Biotechnology Centre, Imperial College, London, UK) and demonstrated 100% sequence homology with the MC3-R and MC4-R coding sequences in the National Centre for Biotechnology (NCBI) Blast database.
Discussion

AgRP has previously been reported to be present in the human adrenal gland (Ollmann et al., 1997; Bicknell et al., 2000). However, the role of adrenal AgRP in humans is not known. We investigated whether human adrenal AgRP mRNA levels are altered in Cushing’s syndrome. Our results demonstrate that there was an approximate three-fold increase in mean adrenal AgRP mRNA expression in the 2 Cushing’s patients studied compared to controls. These results are in keeping with data from rats in which adrenal AgRP mRNA is upregulated by dexamethasone treatment (Dhillo et al., 2003). AgRP antagonises α-MSH-induced glucocorticoid secretion in rat and bovine adrenal cells (Dhillo et al., 2003; Doghman et al., 2004). It is possible that the upregulation of adrenal AgRP in Cushing’s syndrome antagonises α-MSH-induced cortisol release. Patients with Cushing’s disease and ectopic ACTH secretion may have adrenal hyperplasia. Whilst it is possible that this could be a confounding factor, this is unlikely since we controlled for this by using the same amount of RNA from patients with Cushing’s syndrome and controls for Northern blot analysis to quantify the relative amount of adrenal AgRP expression.

Alpha MSH has been shown to stimulate glucocorticoid release from rat and human adrenal cells at near physiological concentrations and it has been suggested that it may have a physiological role in the control of adrenocortical function (Vinson et al., 1981; Vinson et al., 1983; Henville et al., 1989). Plasma α-MSH-IR was increased in our patients with Cushings’ syndrome in accord with the findings of Coates et al. (Coates et al., 1989). Plasma AgRP levels in our
study were also higher in the two patients with Cushing’s syndrome (pre-operatively) compared to controls. In both patients with Cushing’s syndrome, plasma AgRP decreased following cure of their Cushing’s syndrome by bilateral adrenalectomy and hydrocortisone replacement to levels similar to that of the control group. The cellular source of circulating AgRP is not known. AgRP expression has been detected in a range of human tissues including the brain, adrenal glands, testis, lung and kidney (Shutter et al., 1997). AgRP-like protein activity is secreted by adrenal fasciculata-reticularis cells (Doghman et al., 2004) and therefore adrenal glands may contribute to circulating levels of AgRP. The elevated serum AgRP levels in patients with Cushing’s syndrome fell following bilateral adrenalectomy to levels similar to those observed in the control group but not to undetectable levels. This suggests that the adrenal glands are not the major source of circulating AgRP in normal subjects, but in states of cortisol excess the adrenal glands may be the source of elevated serum AgRP.

Previous studies demonstrate expression of MC3-R and MC4-R mRNA in H295R cells (a human adrenal tumour cell line) but only MC4-R in human adrenal tissue (Doghman et al., 2004). Our results demonstrate that MC3-R and MC4-R mRNA are both expressed in the human adrenal gland from a control subject and a patient with Cushing’s syndrome. We used RT-PCR using nested primers which increases the sensitivity of this technique, and this may have allowed the detection of MC3-R mRNA in human adrenal mRNA in our study. The presence of the
MC3-R and MC4-R in human adrenal tissue provide a mechanism to explain the effects of α-MSH and AgRP on glucocorticoid release from human adrenal cells.

In conclusion, previous reports demonstrate that AgRP antagonises α-MSH-induced glucocorticoid secretion. Our results suggest that AgRP is upregulated in Cushing’s syndrome suggesting a possible novel inhibitory paracrine role in the human adrenal gland, which may be mediated via the MC3-R or MC4-R.
Acknowledgements

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Figure Legends

Figure 1: Adrenal AgRP mRNA levels (arbitrary units) measured by Northern blot analysis in control subjects (●) and patients with Cushing’s syndrome (▼).

Figure 2: MC3-R and MC4-R RT-PCR products from human adrenal glands from a control subject and a patient with Cushing’s syndrome.

For lanes 1-4 adrenal gland mRNA from a patient with Cushing’s syndrome was used. Lanes 1: adrenal gland mRNA – RT + MC4-R primer; 2: adrenal gland mRNA + RT + MC4-R primer; 3: adrenal gland mRNA – RT + MC3-R primer; 4: adrenal gland mRNA + RT + MC3-R primer.

For lanes 5-8 adrenal gland mRNA from the control subject was used. Lanes 5: adrenal gland mRNA – RT + MC4-R primer; 6: adrenal gland mRNA + RT + MC4-R primer; 7: adrenal gland mRNA – RT + MC3-R primer; 8: adrenal gland mRNA + RT + MC3-R primer. Lane 9: 1kb DNA ladder.

(+ or – indicates the presence or absence of reverse transcriptase enzyme (RT) in the reaction). MC-R = melanocortin receptor. The size of the expected RT-PCR product for the MC3-R was 208 base pairs (bp) and for the MC4-R was 248 bp.
References


Bewick GA, Dhillo WS, Darch SJ, Murphy KG, Gardiner JV, Jethwa PH, Kong WM, Ghatike MA, Bloom SR. Hypothalamic cocaine- and amphetamine-regulated transcript (CART) and agouti related protein (AgRP) neurons co-express the NOP1 receptor and nociceptin alters CART and AgRP. Endocrinology 2005; 146: 3526-3534.


Figure 1
Figure 2