A POMC variant implicates β-melanocyte-stimulating hormone in the control of human energy balance

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Summary

The melanocortin-4 receptor (MC4R) plays a critical role in the control of energy balance. Of its two pro-opiomelanocortin (POMC)-derived ligands, α- and β-MSH, the majority of attention has focused on α-MSH, partly reflecting the absence of β-MSH in rodents. We screened the POMC gene in 538 patients with severe, early-onset obesity and identified five unrelated probands who were heterozygous for a rare missense variant in the region encoding β-MSH, Tyr221Cys. This frequency was significantly increased (p < 0.001) compared to the general UK Caucasian population and the variant cosegregated with obesity/overweight in affected family members. Compared to wild-type β-MSH, the variant peptide was impaired in its ability to bind to and activate signaling from the MC4R. Obese children carrying the Tyr221Cys variant were hyperphagic and showed increased linear growth, both of which are features of MC4R deficiency. These studies support a role for β-MSH in the control of human energy homeostasis.

Introduction

The pro-opiomelanocortin (POMC) gene is expressed in neurons originating in the arcuate nucleus of the hypothalamus and in the nucleus tractus solitarius (NTS) of the caudal medulla, as well as in the corticotrophs of the anterior pituitary and in the skin and lymphoid system (Hadley and Haskell-Luevano, 1999). POMC undergoes extensive and tissue-specific posttranslational processing by proprotein convertases (PCs) to yield a range of biologically active peptides (Bertagna, 1994; Pritchard et al., 2002; Seidah and Chretien, 1999). Pituitary corticotrophs express prohormone convertase 1 (PC1), but not PC2, resulting in the production of N-terminal peptide, joining peptide, ACTH, and β-lipotropin. In contrast, the expression of PC2 within the hypothalamus leads to the production of α-, β-, and γ-MSH (the melanocortins) but not ACTH (Bertagna, 1994; Pritchard et al., 2002). The melanocortins mediate their effects through a family of five related G protein-coupled receptors, two of which, MC3R and MC4R, are highly expressed within the central nervous system (Gantz and Fong, 2003). Genetic defects impairing the synthesis and processing of POMC and in the receptors for its constituent melanocortin peptides have clearly established that the melanocortin system plays a critical role in energy homeostasis in rodents and humans (Cone, 2005).

Of the two centrally expressed melanocortin receptors, the MC4R is the one most closely linked to energy homeostasis. Thus, humans and mice lacking the MC4R are markedly obese and hyperphagic as well as showing increased linear growth and severe hyperinsulinaemia from a young age (Farooqi et al., 2003; Huszar et al., 1997). Mice lacking the MC3R have subtle abnormalities of body composition, which occur later in life (Butler et al., 2000; Chen et al., 2000), and no MC3R mutations convincingly linked to monogenic human obesity have been described.

Mutations affecting POMC have also helped to illuminate the role of the melanocortin system in energy balance (Coll et al., 2004). Mice (Challis et al., 2004) and humans (Krude et al., 1998) genetically lacking all POMC-derived peptides are severely obese, but their phenotype is complicated by the severe glucocorticoid deficiency that results from the concomitant absence of ACTH. Importantly, heterozygous null *POMC* mutations in mice (Challis et al., 2004) and humans (Krude et al., 2003) predispose to obesity but do not necessarily result in a severe phenotype. In contrast to the clear hierarchy of melanocortin receptors in the control of energy balance, there is still uncertainty regarding the relative importance of particular POMC-derived melanocortin ligands in these processes.

In vitro studies have established that γ-MSH preferentially binds to MC3R with a 50-fold higher affinity than to MC4R, which

it binds to poorly (Abbott et al., 2000). In comparison, α- and β-MSH both bind to the MC4R with high affinity and with similar IC_{50} values (Abbott et al., 2000). Indeed, some studies suggest that β-MSH has higher affinity for MC4R in stably expressing cell lines (Schioth et al., 1996) and in rat hypothalamic homogenates (Harrold et al., 2003). In rats, icv administration of β-MSH induced early growth response factor-1 expression in hypothalamic nuclei (Millington et al., 2001). Several in vivo studies have demonstrated that rodents given a single intracerebroventricular (icv) injection of α-MSH display a dose-dependent reduction in food intake (Poggioli et al., 1986). In direct comparisons of icv administration of melanocortin peptides, β-MSH has been shown to reduce food intake at equimolar doses (Abbott et al., 2000). Despite this, attention has been principally focused on α-MSH as the probable endogenous ligand in rodents (Mountjoy and Wong, 1997). This is largely because rodents (the most common experimental species) lack the proximal di-basic site that is necessary for the proteolytic cleavage event that produces β-MSH in humans (mouse accession number P01193, rat accession number AAA41903). Thus, it is possible that an important role for β-MSH in the control of energy balance has been overlooked.

In order to determine whether missense/nonsense mutations within the melanocortin peptides might predispose to obesity, we screened the coding regions of the POMC gene for mutations in 538 UK Caucasian subjects with severe early-onset obesity in the absence of obvious glucocorticoid deficiency (mean BMI SDS 3.8 ± 0.9, age of onset < 5 years) using a combination of direct sequencing and denaturing high-performance liquid chromatography (Transgenomic WAVE DNA fragment analysis). We also screened 300 UK Caucasian non-obese adult controls.

Results

Identification of missense mutations in *POMC*
We identified a number of sequence variants in *POMC* in severely obese children (Table 1). As well as a number of synonymous SNPs and a common 9 bp insertion that has been reported previously (Challis et al., 2002; Del Giudice et al., 2001; Echwald et al., 1999), we identified seven rare nonsynonymous SNPs. Three of these missense mutations directly affect regions of the POMC gene that encode melanocortin peptides (Figure 1A). R236G was identified in three patients but also two controls. We have previously shown that this mutation disrupts a di-basic cleavage site between β-MSH and β-endorphin, resulting in a β-MSH/ β-endorphin fusion protein that binds to MC4R but has reduced ability to activate the receptor (Challis et al., 2002). Its presence in both obese probands and controls reflects previous studies that show that this is not a highly penetrant cause of inherited obesity but may increase the risk of obesity in carriers (Challis et al., 2002; Echwald et al., 1999). Novel heterozygous missense mutations were found in β-MSH (Tyr221Cys, five subjects) and α-MSH (His143Gln, one subject). Other mutations were rare and/or occurred in regions of the POMC pro-peptide of uncertain function. Our further studies concentrated on the α- and β-MSH variants.

Tyr221Cys in β-MSH is more common in obese subjects than in controls
A novel heterozygous missense mutation (Tyr221Cys) in β-MSH was found in five unrelated UK Caucasian probands (Table 1).

Table 1. Identification of variants in *POMC*

POMC variants	Obese children (n = 538)	Controls (n = 300)
Nonsynonymous SNPs & insertion		
Leu37Phe	1	0
9 bp insertion (c.297-298insAGCAGCGGC)	51 (9.48%)	28 (9.33%)
Pro132Ala	1	0
His143Gln	1	1 (0.33%)
Ala195Thr	1	0
Glu214Gly	8 (1.49%)	2 (0.67%)
Tyr221Cys	5 (0.93%)	1 (0.33%)
Arg236Gly	3 (0.56%)	2 (0.67%)
Synonymous SNPs		
Cys6Cys	5	0
Ser94Ser	4	0
Ala195Ala	4 (0.74%)	1 (0.33%)
Leu116Leu	8 (1.49%)	7 (2.33%)

Synonymous and nonsynonymous variants in severely obese children and controls.

This variant was also found in 1/300 non-obese UK Caucasian adults. To establish the frequency of this allele in an unselected UK Caucasian population, we genotyped a random subset of 5000 subjects recruited to the EPIC-Norfolk study (Khaw et al., 2001). 4,852 subjects were successfully genotyped, and we identified 3 heterozygotes, all of whom were non-obese. Thus, Tyr221Cys is found at significantly higher frequency in a population of UK Caucasians subjects with early-onset obesity than in unselected UK Caucasian controls (5/538 versus 4/5152, Chi-Square 22.4, p < 0.001). The Tyr221Cys variant was not found in 1132 obese French Caucasian subjects (597 morbidly obese adults [BMI 47.5 ± 7.53] and 535 obese children [BMI sds = 4.37 ± 1.31\], nor in 723 lean French Caucasian subjects (BMI 23.29 ± 2.74). These observations suggest that Tyr221Cys is likely to be a rare variant in Caucasian populations.

Does the Tyr221Cys mutation in β-MSH cosegregate with obesity in families?
To examine whether this variant was linked to obesity/overweight in families, first-degree relatives were contacted, and those who agreed to be studied were examined and genotyped (Figure 1B). Thirteen relatives were heterozygotes for Tyr221Cys, 11 of which were obese, two overweight, and none lean. Five relatives were wild-type, three of which were obese and two lean. Thus, in total, in these families, 0/20 Tyr221Cys carriers and 2/5 wild-type subjects were lean.

Tyr221Cys β-MSH mutation alters three-dimensional structure of β-MSH, binding, and signaling through MC4R
Tyr221 is a highly conserved residue, being present in the β-MSH sequence of species from teleost fish through to mammals (accession numbers: Zebrafish NP_852103, Chicken BAA34366, Mouse P01193, Rat AAA41903, Human P01189). A tyrosine is also present in the equivalent position in all melanocortins from all mammalian species. To determine whether Tyr221 is necessary for the normal three-dimensional structure of β-MSH, we undertook NMR studies of wild-type and a mutant peptide analog replacing Tyr221 with Serine (to avoid intermolecular disulfide formation common with Cys-containing
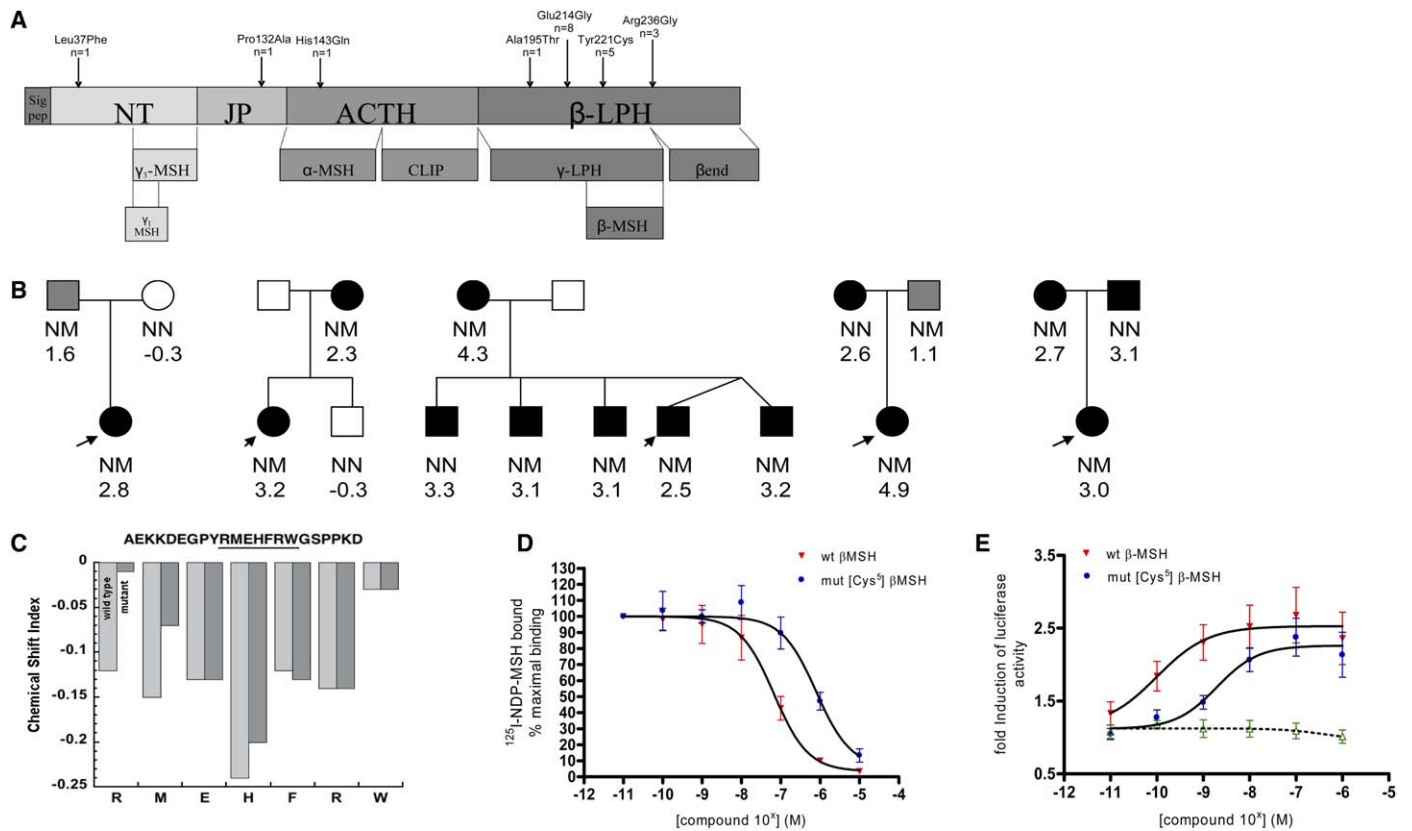
Figure 1. Identification and characterization of Tyr221Cys β-MSH mutation

A) Structure of *POMC* and location of rare missense mutations.

B) Cosegregation of Tyr221Cys β-MSH mutation with obesity (black symbols) and overweight (gray symbols) in families. Genotype (N = wild-type allele, M = mutant allele) and BMI sds is denoted. In adults, overweight defined as BMI 25–30 kg/m², obesity as BMI > 30 kg/m². In children, overweight defined as >91st and obesity as >99th percentile for age-adjusted BMI. Arrows indicate the proband of each family.

C) Comparison of the Chemical Shift Index (CSI = experimental chemical shift—consensus random coil chemical shift) values between wild-type and mutant β-MSH. Throughout the segment Arg-Met-Glu-His-Phe-Arg-Trp, the CSIs for both peptides were indicating the presence of turns (values ranging from −0.01 to −0.24). For the receptor binding motif, His-Phe-Arg-Trp, both wild-type and mutant peptides gave approximately identical values. However, for the Arg-Met residues following the mutation, but preceding the receptor binding motif, the wild-type sequence showed a significantly greater tendency to form turns relative to the mutant.

D) [Cys⁵] β-MSH binds to MC4R with lower affinity than β-MSH. Whole HEK293 cells stably expressing wild-type MC4R were exposed to tracer amounts of [^{125}I]NDP-MSH, and the ability of increasing concentrations of β-MSH or [Cys⁵] β-MSH to inhibit radio-ligand binding was measured as described previously. Data are expressed as a percentage of maximum counts of [^{125}I]NDP-MSH binding to MC4R. Each point represents the mean (±SEM) of four independent experiments in triplicate.

E) [Cys⁵] β-MSH has a markedly reduced ability to stimulate production of cAMP. Graphs indicate responses of wild-type, mutant, and control constructs to a logarithmic increase in β-MSH concentration. cAMP/Luciferase reporter assays were performed as described previously (Yeo et al., 2003). Open symbols denote mock transfected controls. Each point represents the mean (±SEM) of eight independent experiments performed in quadruplicate.

peptides). Chemical Shift Index (CSI) values were used to probe average structural properties (Mielke and Krishnan, 2004; Wishart et al., 1992). Compared to wild-type β-MSH, the mutation resulted in a significantly reduced helical turn propensity for two of the three residues between the mutation site and the His-Phe-Arg-Trp receptor binding motif (see Figure 1C and Supplemental Data available with this article online). When compared to wild-type β-MSH, a synthetic peptide incorporating the Tyr221Cys mutation showed reduced binding to MC4R stably expressed in HEK293 cells (Figure 1D) (IC_{50} of mutant β-MSH = 797.1 ± 261.0 nM compared to 67.2 ± 43.0 nM of wt β-MSH, p = 0.006). Consistent with these findings, the addition of wild-type β-MSH led to a dose-dependent increase in cAMP accumulation in HEK293 cells transiently expressing MC4R, while Tyr221Cys generated significantly less cAMP (Figure 1E) (EC50 mutant β-MSH = 1.95 nM compared to 0.1 nM of wt β-MSH, p = 0.04).

Clinical phenotype of subjects with the Tyr221Cys mutation in β-MSH

To establish whether obese children carrying the β-MSH mutation were hyperphagic, we admitted six children heterozygous for Tyr221Cys β-MSH (five probands and one relative) to the Addenbrooke's Hospital Clinical Research Facility. The six children underwent an ad libitum test breakfast. Ad libitum caloric intake, expressed per kg lean mass, was markedly increased compared to normal weight control children (Figure 2A) and similar to that seen in subjects heterozygous for *MC4R* mutations (Farooqi et al., 2003). Basal metabolic rate of affected subjects was not significantly different from that predicted using age- and gender-specific equations (data not shown). In order to determine whether obese children carrying the β-MSH mutation had other phenotypic similarities to children with known defects in MC4R signalling, we assessed body composition using standard procedures (Farooqi et al., 2003). The children with the
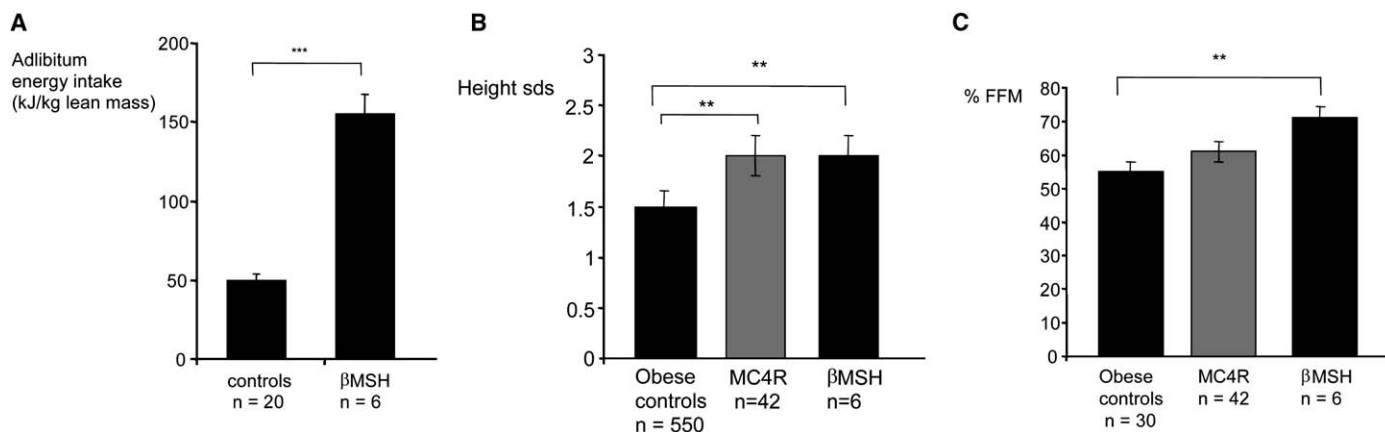
Figure 2. Phenotypes of subjects with mutations in POMC

A) Food intake at an 18MJ ad libitum test meal compared to normal weight controls. Food intake is expressed per kg lean body mass measured by dual energy X-ray absorptiometry (DXA) to allow comparison between subjects of different ages (Farooqi et al., 2003) ***p < 0.001.

B) Height SDS in children with the Tyr221Cys mutation in β-MSH compared to heterozygotes for MC4R mutations and controls of comparable age and degree of obesity. **p < 0.01

C) Fat-free mass (FFM) measured by DXA in children with the Tyr221Cys mutation in β-MSH compared to heterozygotes for MC4R mutations and controls of comparable age and degree of obesity (unpublished data). **p < 0.01

β-MSH mutation more closely resembled MC4R heterozygotes than equivalently obese children (with normal *MC4R* and *POMC* sequence) in terms of accelerated linear growth and increased fat-free mass (Figures 2B and 2C).

Characterization of a mutation in α-MSH

As described above, one UK Caucasian proband was found to have a missense mutation (His143Gln) in α-MSH (Figure 1A). However, this was also present in one of the 300 UK Caucasian controls. The grandfather of the proband carried the mutation and was obese, but the transmitting parent was lean (Figure 3A). The mutation replaces a highly conserved residue within the classical His-Phe-Arg-Trp receptor binding motif of the melano-

cortins and would be expected to impair function. Consistent with this, a synthetic mutant peptide has marked reduced affinity for the MC4 receptor (Figure 3B) (IC_{50} of mutant α-MSH = 1624 ± 624 nM compared to 15.2 ± 14.0 nM of wt α-MSH, p < 0.0001). The His143Gln mutant α-MSH also had reduced ability to stimulate cAMP generation from MC4R (Figure 3C) with a reduced maximal response, although the EC50 was not significantly different from wild-type (EC50 mutant α-MSH = 3.8 nM compared to 2.2 nM of wt α-MSH, p = ns). Thus, while heterozygous disruption of α-MSH function may play a contributory role in the obesity of the proband, the presence of this mutation in a lean transmitting family member implies that it is insufficient, in itself, to fully explain the obese phenotype.
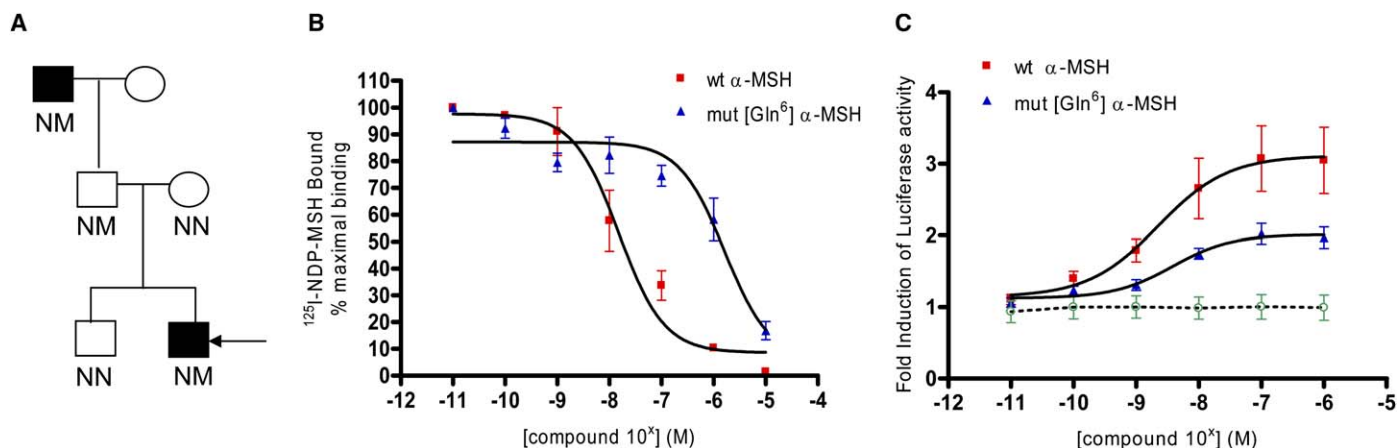

Figure 3. His143Gln α-MSH mutation

A) Cosegregation of His143Gln α-MSH mutation with obesity (black symbols). N = wild-type allele, M = Mutant allele.

B) [Gln⁶] α-MSH binds to MC4R with lower affinity than α-MSH (methods as in Figure 1D). Data is expressed as a percentage of maximum counts of [^{125}I]NDP-MSH binding to MC4R. Each point represents the mean (±SEM) of four independent experiments in triplicate.

C) [Gln⁶] α-MSH has a markedly reduced ability to stimulate production of cAMP (methods as in Figure 1E). Open symbols denote mock-transfected controls. Each point represents the mean (±SEM) of eight independent experiments performed in quadruplicate.

Discussion

Tyr221Cys mutation in β-MSH is associated with human early-onset obesity

These studies provide compelling evidence that β-MSH is likely to be a physiologically relevant, endogenous ligand for the MC4R in humans. Firstly, the Tyr221Cys variant is enriched in a population of UK Caucasian subjects with early-onset obesity compared to its background prevalence in the UK population. The overrepresentation of this mutation in obese subjects is supported by independent studies in a German population (Biebermann et al., personal communication). The fact that we did not find this variant at all in a large French population suggests that this variant may have arisen relatively recently in European ancestry.

Further support for the importance of β-MSH in energy homeostasis is provided by our previous observation of an association of a cleavage site mutation affecting β-MSH with obesity although, in contrast with Tyr221Cys, that mutation resulted in the production of a peptide with the potential to interfere with signaling at the MC4R in a dominant-negative manner (Challis et al., 2002). It is unlikely that Tyr221Cys would result in a dominant-negative interference with wild-type β-MSH so, in this case, one would have to hypothesize that this mutation simply reduces the amount of β-MSH tone at the MC4R and that this in itself is sufficient to predispose to obesity. The plausibility of such a model is supported by the increased prevalence of obesity and overweight in carriers of POMC null mutations (Krude et al., 2003), a finding which we ourselves have recently replicated in a further large pedigree (unpublished data).

It is important to note that the Tyr221Cys mutation is unlikely to be a highly penetrant cause of monogenic obesity, but that apparent enrichment in obese populations is likely to occur because it is an allele that increases the risk of obesity. Our reasons for stating this include (1) the fact that human and murine heterozygotes for null mutations in POMC show a propensity to increased adiposity but are not all frankly obese (Krude et al., 2003) (and unpublished data) and (2) the fact that some carriers of the Tyr221Cys β-MSH mutation are not obese.

Both α-MSH and β-MSH influence melanocortinergic tone in humans

It is noteworthy that the vast majority of the literature concerning the POMC system and energy homeostasis focuses on the possible role of α- rather than β-MSH as the endogenous ligand (Coll et al., 2004; Cone, 2005). This is understandable, as in rodents the N-terminal cleavage site necessary for the generation of β-MSH is absent. In contrast, β-MSH has been established to be present in the human hypothalamus (Bertagna et al., 1986). Interestingly, we found a missense mutation in α-MSH in a single proband, which had a major deleterious effect on its function. However, this variant was found in one lean family member and one lean unrelated control. While it is likely that this variant is contributing to the obesity of the proband, it is notable that our human genetic studies provide more compelling evidence for a specific role for β-MSH than α-MSH in the control of human energy balance. Finally, the similarities in clinical phenotype between obese children carrying the Tyr221Cys β-MSH mutation and those with MC4R mutations (Farooqi et al., 2003) suggest that both mutations are affecting shared physiological pathways. In conclusion, β-MSH is likely to be an important and physiologically relevant endogenous ligand for the human MC4R. These findings illustrate how human genetics can provide insights into the important physiological roles of particular species-restricted peptides that are not obtainable through the study of rodent models.

Experimental procedures

Human genetics and cohorts

Five hundred and thirty-eight UK Caucasian subjects with severe early-onset obesity were screened using a combination of direct nucleotide sequencing and denaturing high-performance liquid chromatography (Transgenomic WAVE DNA fragment analysis) as previously described (Challis et al., 2002). Subjects were recruited as part of the Genetics of Obesity Study (GOOS) cohort (O'Rahilly et al., 2003), and mutations in leptin, leptin receptor, and MC4R had been excluded by direct nucleotide sequencing. The mean BMI SDS of this group was 3.8 ± 0.9, mean age of onset < 5 years. BMI sds was calculated using age- and gender-specific UK population data (Cole et al., 1995).

For comparison of allele frequency, 300 randomly selected non-obese adult controls from the MRC Ely Study (Wareham et al., 2000) were screened using the same techniques.

Genotyping of the Tyr221Cys variant was performed in 5000 subjects from a UK Caucasian population-based cohort, the EPIC-Norfolk study (Khaw et al., 2001). These subjects were screened using a custom-made assay based on TaqMan chemistry (Applied Biosystems, Warrington, United Kingdom).

Genotyping of the Tyr221Cys variant was also performed in two groups of French obese subjects: 597 morbidly obese adults (BMI 47.5 ±7.53), 535 obese children (BMI sds = 4.37 ± 1.31) and also in 723 lean controls (BMI 23.29 ± 2.74). These subjects were genotyped using a FRET (Fluorescence Resonance Energy Transfer) based assay using the LightTyper technology (Roche, Meylan, France and Mannheim, Germany) as previously reported (Meyre et al., 2005). Probes for LightTyper were synthesized by TIB Molbiol Syntheselabor Germany.

NMR studies

Two-dimensional ^{1}H NMR (2D-NOESY and 2D-TOCSY) performed on peptides corresponding to the β-MSH segment of POMC was used to assign chemical shifts in both wild-type and a mutant with a Tyr221Ser mutation. Ser was used instead of Cys since, at NMR concentrations, free -SH groups tend to form disulfides. For flexible peptides, a negative CSI indicates a turn or helical structure and a positive CSI indicates the presence of extended, β sheet conformers (Mielke and Krishnan, 2004; Wishart et al., 1992). See Supplemental Data for additional experimental procedures.

Competitive binding studies

Whole HEK293 cells stably expressing wild-type MC4R were exposed to tracer amounts of [^{125}I]NDP-MSH, and the ability of increasing concentrations of β-MSH or [Cys^5] β-MSH to inhibit radioligand binding was measured as described previously (Yeo et al., 2003). All wild-type and mutant peptides were obtained from Bachem.

Receptor activation studies

HEK293 cells were transiently transfected with wt MC4R and cotransfected with a cAMP-dependent luciferase reporter construct. cAMP/Luciferase reporter assays were performed as described previously (Yeo et al., 2003).

Physiological studies

Measurements of food intake and body composition were undertaken after appropriate informed consent and using protocols previously described (Farooqi et al., 2003).

Supplemental data

Supplemental data include text and one table and can be found with the article online at http://www.cellmetabolism.org/cgi/content/full/3/2/135/DC1/.

SHORT ARTICLE

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