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Inactivation and intracellular retention of the human I183N mutated melanocortin 3 receptor associated with obesity

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Abstract

Melanocortins are known to be involved in the regulation of feeding behavior. These hormones mediate their effects through G protein-coupled receptors (GPCRs) by stimulating adenylate cyclase. The melanocortin 3 receptor (MC3R) in the melanocortin receptor (MCR) family has been identified as a neural receptor subtype mainly expressed in the brain in mammals. Until now, only one heterozygous mutation (I183N) has been identified in the coding region of this receptor in two obese patients of the same family. In this study, we reported the functional characterization of the I183N mutated MC3R compared with that of the wild-type MC3R after transfection in HEK293 cells.

Our results showed that the I183N mutation totally abolished the activity of the mutated receptor to generate intracellular cAMP. Furthermore, confocal microscopy observation revealed that the mutation induced an intracellular retention of the mutated receptor. Moreover, we demonstrated for the first time by co-transfection studies that the mutated receptor could reduce the wild-type receptor activity through a dominant negative effect.

Keywords: Melanocortin-3 receptor; Obesity; Mutation; Dominant negative effect

1. Introduction

Obesity is a complex multifactorial disease caused by the interaction of multiple genetic, environmental and behavioral factors. The central melanocortin system is involved in the regulation of energy homeostasis in humans and rodents. The melanocortin receptor (MCR) family consists of five isoforms (MC1R–MC5R) identified so far [1–6] and belongs to the G protein coupled receptor (GPCR) superfamily.

The melanocortin peptides (α, β, and γ melanocyte stimulating hormones and ACTH) derived by posttranslational processing of the proopiomelanocortin (POMC) are the endogenous agonist ligands for these receptors. The MCRs exhibit distinct physiological functions and tissue-specific expression.

The respective inactivation of the mouse melanocortin 3 receptor (MC3R) and MC4R has demonstrated the critical roles of these receptors in weight regulation [7–9]. In contrast to MC4R knockout mice which exhibit increased food intake, increased somatic growth and defects in metabolism, MC3R knockout mice exhibit an exclusively metabolic syndrome. The MC3R −/− mice have increased fat mass, reduced lean mass caused by increased feed efficiency but not hyperphagia associated with reduced energy expenditure. Mice lacking both MC3R and MC4R have exacerbated obesity compared to MC4R −/− mice consistent with the non-redundant role of the two receptors in regulating body weight [9]. However, heterozygous mice for MC3R or MC4R exhibited an intermediate phenotype [7–9].

Several mutations in the MC4R leading to complete loss of functions have been associated with rare extreme obesity in humans [10,11]. Based on the structure/function similarity of the MC3R and the MC4R [3] and since a locus encoding MC3R on human chromosome 20q13 seems to contribute to body fat and insulin level in a human population[12–14], mutations in the MC3R gene may play a role in the pathogenesis of obesity. Variations of the human MC3R gene were investigated in a large cohort of extremely obese and type 2 diabetes mellitus patients and only two variants were found but none of them was associated with

Abbreviations: NDP-MSH, (Nle^4, D-Phe^7) α melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone; MCR, melanocortin receptor; EGFP, enhanced green fluorescent protein; GPCR, G protein-coupled receptor

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obesity phenotype [15–17]. Similarly, some positive associations between MC3R polymorphisms in non-coding regions and obesity-related phenotypes have also been reported [18,19].

Recently, a novel mutation (I183N) was found in heterozygosity in a 13-year-old obese girl and her father [20] but functional analysis of this MC3R mutation has not been reported. Since we have developed an in vitro expression model for the human MC3R tagged at its C terminus with the enhanced green fluorescent protein (EGFP) [21], we reported here the functional characterization of the human I183N mutated MC3R expressed in HEK 293 cells.

2. Materials and methods

2.1. Materials

Fetal calf serum (FCS), Dulbecco's Modified Eagle Medium/Ham's nutrient mixture F12 (DMEM/F12 1:1), LipofectAmine Plus reagent, and L-glutamine were purchased from Invitrogen (Cergy Pontoise, France); 3-isobutyl-1-methyl-xanthine (IBMX) was from Sigma-Aldrich (St Quentin Fallavier, France); (Nle^4, D-Phe^7)αMSH (NDP-MSH) was from Bachem (Voisins le Bretonneux, France).

2.2. Constructions of expression vectors

The full-length coding sequence of the wild-type hMC3R was amplified by polymerase chain reaction (PCR) from human genomic DNA using specific primers and subcloned into the pEGFP-N2 expression vector as described previously[21].

Mutated receptor was generated from the cloned wild-type hMC3R by site-directed mutagenesis. Primers were designed from the sequence in the second intracellular loop domain of the MC3R using recommended protocol (QuikChange site-directed mutagenesis kit, Stratagene, The Netherlands). Briefly, denatured double-stranded DNA vector was annealed to a mutagenic primer pair of forward and reverse oligonucleotides (sense 5′-CAGGTACGTCACCAACTTTTACGCGCTCCG-3′, antisense 5′-CGGAGCGCGTAAAAGTTGGTGACGTACCTG-3′) and the new strand of DNA containing the desired mutation was synthesised with *Pfu* DNA polymerase (pfu Turbo DNA polymerase, Stratagene) by PCR (conditions: 95 °C for 30 s; 94 °C for 30 s, 55 °C for 1 min, and 68 °C for 13 min repeated 14 cycles; 68 °C for 10 min). Methylated template DNA was digested with *Dpn*I enzyme for 1 h at 37 °C. Reaction mixtures were then used to transform Max DH5α competent cells (Invitrogen). Inserts of plasmid DNA of several colonies were totally sequenced confirming the presence of the mutation (I183N), and that no errors were induced by PCR. These plasmids were then used for transfection.

2.3. Transfection in HEK293 cells

The day before transfection, HEK293 cells (human embryonic kidney) were plated on six-well dishes at 2.5 10^5 cells/well in DMEM/F12 medium supplemented with FCS (7.5%) and 2 mM L-glutamine. Transfections were performed with 1 μg of plasmids in serum-free DMEM/F12 medium without antibiotics, using the LipofectAMINE PLUS reagent according to the manufacturer's protocol as previously described [22].

2.4. Northern blot analysis

The expression of the wild-type and mutated MC3R-EGFP was evaluated by Northern blot analysis after extraction of total RNA from the parental cell line and from transiently transfected HEK293 cells as previously described [23]. Hybridizations were performed using the entire coding sequence of the EGFP or the hMC3R as a probe, labeled in the presence of [α-^{32}P]-deoxy-CTP using the Rediprime DNA labeling system (Amersham Pharmacia Biotech, Orsay, France). The relative intensity of hybridization signals was quantified by image analysis and equal loading of RNA samples was confirmed by scanning the 28S negatives (SAMBA 2005, Alcatel TITN, Meylan, France).

2.5. cAMP measurement

To study the coupling of the wild-type MC3-R-EGFP, or the I183N mutated MC3R-EGFP receptor, to adenylate cyclase in the HEK293 cells transiently expressing each one of them, NDP-MSH dose-responses were performed. Forty-eight hours after transfection, cells were stimulated for 20 min with increasing concentrations of NDP-MSH in the presence of 1 mM IBMX to inhibit phosphodiesterases, then recovered and the intracellular accumulation of cAMP was measured by radioimmunological assay using ^{125}I-labeled cAMP (Beckman Coulter, Roissy, France) [24].

2.6. Fluorescence microscopy

Cells were plated on 10-cm-diameter dishes at 2.10^6 cells/dish. Transient transfections were made using the LipofectAMINE PLUS reagent as described above. Twenty-four hours after transfection, cells were observed on fluorescence confocal microscopy (Leica Microsystems SA, Rueil Malmaison, France) after stimulation with UV (488 nm).

2.7. Co-transfection studies

HEK293 cells were transiently transfected as described, with 0.1 μg of wild-type MC3R-EGFP construct in the absence or presence of different amounts of I183N mutated

MC3R-EGFP construct and pEGFP-N2 vector, such that the total amount of DNA transfected each time was identical (1 μg/well). Cells were stimulated 48 h after transfection with 0.5 nM of NDP-MSH. Intracellular cAMP production was then measured as described above.

3. Results

We have generated the I183N mutation in the human MC3R by site-directed mutagenesis from the cloned wild-type MC3R fused to EGFP on its carboxy-terminal tail previously described [21]. The wild-type MC3R and the I183N mutated receptor were transiently expressed in HEK293 cells for further functional analysis.

Northern blot analysis revealed similar levels of mRNA encoding wild-type or I183N mutated MC3R fused to EGFP in transiently transfected HEK293 cells, using an MC3R probe or an EGFP probe (Fig. 1). As expected, parental HEK293 cells exhibited no specific hybridization signal, which confirmed the absence of endogenous MC3R in this cell line.

Functional analysis of the transiently transfected HEK293 cells was realized by measuring the intracellular cAMP levels after stimulation by increasing concentrations of the agonist NDP-MSH [25]. Whereas the cells transfected with the wild-type MC3R-EGFP plasmid produced cAMP in a

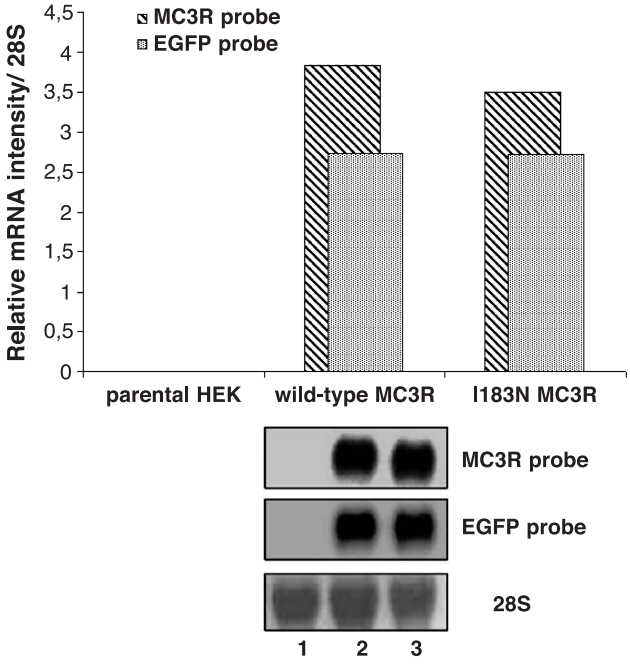

Fig. 1. Northern blot analysis. Bottom: Representative Northern blot analysis of 15-μg total RNA extracted from parental HEK293 cells (1), transiently transfected HEK293 cells with wild-type MC3R (2) or I183N mutated MC3R (3) fused to EGFP after hybridizations with the MC3R or the EGFP probes. Top: Relative intensity of hybridization signals over 28S RNA.

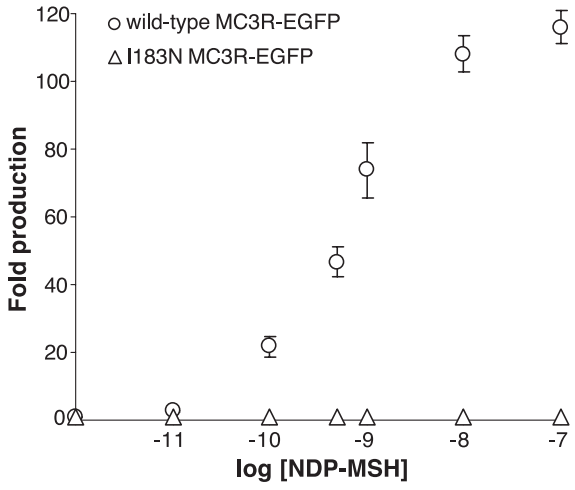

Fig. 2. cAMP accumulation assays. Intracellular cAMP production in HEK293 cells transiently expressing wild-type or I183N mutated MC3R fused to EGFP in response to increasing concentrations of NDP-MSH. Each point represents the mean ± S.E. of three independent experiments performed in triplicate. Basal cAMP production in absence of agonist stimulation was 4.6 ± 0.52 pmol/10^6 cells ($n = 3$).

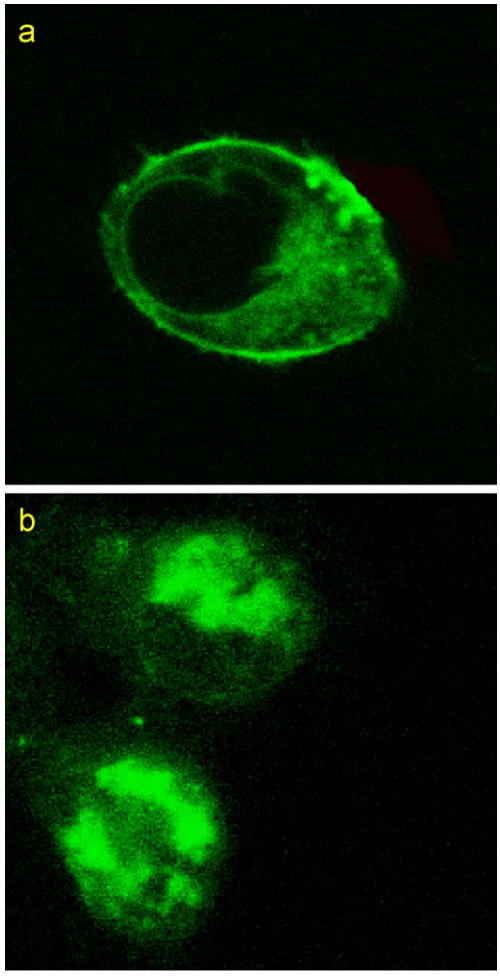

Fig. 3. Confocal microscopy observation. Fluorescent confocal microscope images of HEK293 cells transiently expressing wild-type MC3R (a) or I183N mutated MC3R (b) fused to EGFP, after stimulation with UV (488 nm).

dose-dependent manner with an EC_{50} of 0.52 ± 0.11 nM ($n = 5$), the cells transfected with the I183N mutated MC3R-EGFP plasmid did not exhibit cAMP production at any NDP-MSH concentration used (Fig. 2). These results suggest a defect in the coupling of the mutated I183N receptor to intracellular effectors. We next investigated the subcellular localization of this mutated receptor since the hMC3R-EGFP fusion protein can be easily visualized by confocal microscopy observation after stimulation with UV as previously reported [21]. As shown in Fig. 3a, in cells transfected with the wild-type MC3R-EGFP, the fluorescent signal was detected at the plasma membrane as well as in perinuclear position, confirming that the fusion protein was well expressed and addressed to the cell membrane. In contrast, in cells transfected with the I183N mutated MC3R coupled to EGFP (Fig. 3b), there was no cell surface fluorescence but a strong intracellular signal was observed, indicating that the mutated receptor accumulated intracellularly. Since the I183N mutation was found in heterozygous form and co-segregate with an obese phenotype, one hypothesis could be that the mutated receptors might exert its action through dominant-negative effects on coexpressed wild-type receptors.

To test this hypothesis, HEK293 cells were co-transfected at different ratios of the wild-type MC3R to I183N mutated receptors while maintaining a constant total DNA amount. As observed in Fig. 4, after 48 h, intracellular cAMP production in co-transfected HEK293 cells in response to 0.5 nM NDP-MSH decreased depending upon the amount of I183N mutated MC3R transfected. Since the amount of wild-type MC3R transfected was always constant, these results suggest a dominant-negative effect of the mutated receptor.

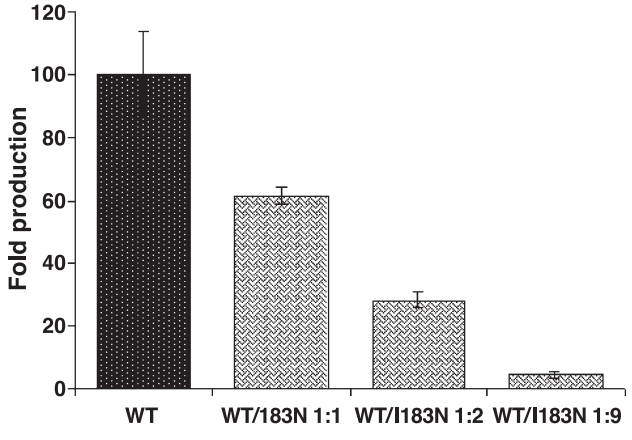

Fig. 4. Co-transfection studies. cAMP production in response to 0.5 nM NDP-MSH in HEK293 cells co-transfected at a ratio of 1:1, 1:2 or 1:9 of wild-type MC3R (WT) to I183N mutated MC3R (I183N). Data are expressed as a percentage of production of cAMP in co-transfected cells compared to cells transfected with wild-type MC3R alone. Each point represents the mean ± S.D. of one experiment performed in triplicate. The experiment was repeated three times with similar results.

4. Discussion

Different research groups reported a strong evidence for linkage between obesity phenotypes and loci in human chromosome 20q which encodes MC3R [12–14]. In the same cohort as those of Lembertas et al. [12], an insertion polymorphism in the 3′ region of MC3R was shown to be associated with the level of fat in non-obese subjects [18]. Several groups have investigated to determine the variations of the human MC3R gene in large cohort of extremely obese and type 2 diabetes individuals [15–17,19]. To date, only two variants in coding region (I81V and K6T) have been described and were not associated with diabetes or obesity. The I183N mutation was the first human MC3R mutation found in heterozygosity in a 13-year-old obese girl and her father [20]. Since this mutation was not found in 121 control individuals without obesity, it is likely that the mutated MC3R exhibits some abnormal functions.

The I183N mutation is located in the second intracellular loop of the MC3R, which is implicated in the interaction with G-protein to mediate signal transduction in other GPCR [26]. Moreover, this isoleucine is a conserved residue among all the other MCRs [27]. Therefore, it is likely that mutation of isoleucine with a nonpolar side chain to asparagine with a polar side chain could modify the function of the MC3R. To date, no mutation at the corresponding position has been described for the other MCRs, whereas several mutations have been identified in the second intracellular loop of the human MC2R and the human MC4R in familial glucocorticoid deficiency patients [22] and extremely obese patients [10,11,28,29], respectively. However, functional analysis was necessary to confirm that I183N mutation could be responsible for the metabolic syndrome observed in the carrier patients.

By transient transfection studies with the I183N mutated MC3R coupled to EGFP, we demonstrated that the mutation I183N completely abolished the activation of the mutated MC3R by the agonist NDP-MSH whereas the NDP-MSH response of the wild-type MC3R in the same experimental conditions was similar to those described previously [21]. As demonstrated by Northern blot analysis and confocal microscopy studies, these different responses to ligand were not related to different levels of transfection or expression of the fusion protein between the wild-type MC3R and the I183N mutated MC3R. In fact, the I183N mutated MC3R was intracellularly retained without expression at the cell surface, leading to the absence of agonist response. This defect seems to be a common feature of mutant MCRs. Indeed, previous studies have reported that MC4R mutations associated with obesity caused also partial or completed intracellular retention of mutant receptors [29–33].

The I183N mutation of the MC3R was found in heterozygosity in the proband and her father, although the percentage of his body fat was less elevated than for the proband [20]. So, the obese phenotype in this family could

be the result of haploinsufficiency but it could also suggest a dominant negative mechanism of the mutated MC3R over the wild-type receptor function. Such hypothesis has already been suggested for MC4R. In fact, this was strongly supported by a study which failed to detect obesity due to haploinsufficiensy of the MC4R gene in several individuals with deletion of 18q including the MC4R gene [34]. Moreover, Vaisse et al. [10] have reported that obesity-associated defects in MC4R range from loss of function to constitutive activation, indicating a variable expressivity of these mutations. In the same way, heterozygous MC3R −/+ and MC4R −/+ transgenic mice also display a variability in phenotype from intermediate phenotype to wild type or homozygous phenotype. Until recently, the hypothesis that obesity in the heterozygous carriers of the MC4R mutations resulted from haploinsufficiency was the most common [11,28,30,32]. In fact, there was no evidence that obesity-associated heterozygous MC4R mutations could exert dominant negative effect on the wild-type MC4R [30,32]. However, such dominant negative effects have been reported for several mutant receptors which are able to form homo- or heterodimers [35,36].

In conclusion, our in vitro expression studies have clearly demonstrated a loss of function of the I183N mutated MC3R, arguing that the MC3R mutation maybe the cause of the abnormal development of the fat mass in the carrier patients. These results confirm that in humans, as in mice, MC3R is mostly involved in energy storage and fat metabolism. Moreover, for the first time we have demonstrated that the mutated receptor reduced the activity of the wild-type receptor in a co-transfection assay, suggesting that probably these MCRs are able to oligomerize. These results are in favour of a dominant negative mechanism of the mutated receptor on the wild-type receptor in heterozygous carrier patients rather than haploinsufficiency which could result in variable phenotypes.

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