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Evidence for direct actions of melanocortin peptides on bone metabolism

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Abstract

Expression of melanocortin-4 receptor (MC4R) mRNA in developing rat limb buds, teeth, and skull bone first indicated a possible role for MC4R in bone metabolism. We therefore investigated whether MC4R mRNA was expressed in the rat osteosarcoma UMR106.06 cell line and in primary rat osteoblast cells. Reverse transcriptase-polymerase chain reaction (RT-PCR), Northern blot analysis, and ribonuclease protection assay (RPA) were used to demonstrate MC4R mRNA expression in UMR106.06 and primary osteoblast cells. MC4R mRNA was found to be localized to the periosteum of mouse bone using in situ hybridization. We also used RT-PCR and rat specific MC2R and MC5R oligonucleotides to amplify the correct size DNA fragments for these melanocortin receptors from rat primary osteoblasts. In conclusion, melanocortin receptor expression in mouse periosteum and rat osteoblasts suggests a direct role for POMC derived peptides in bone development and bone metabolism.

Keywords: POMC; α-MSH; Melanocortin receptor; Agouti protein

1. Introduction

Genetic studies in mouse models and human diseases suggest roles for pro-opiomelanocortin (POMC) derived peptides and melanocortin-4 receptor in bone formation. The yellow agouti obese mouse [2,11], the MC4R knockout mouse [9], the POMC knockout mouse [28], humans with defective MC4R alleles [30], or humans with defective MC2R alleles [8] all exhibit increased body size. However, the precise role of POMC peptides in bone formation is not understood.

The pro-opiomelanocortin gene is expressed at physiologically significant levels in numerous mammalian tissues including anterior and intermediate pituitary, brain, skin, heart, testis, and the immune system and is proteolytically cleaved into a range of smaller peptides in a tissue-specific manner. The melanocortin peptides derived from POMC, melanocortin receptors (MCRs), and the MCR antagonists, agouti protein and agouti gene related peptide (AGRP), represent one of the most complex systems in human endocrinology. The melanocortin system is critical for physiological processes such as pigmentation, energy homeostasis, adrenal function, immune function, and exocrine gland function [20].

POMC-derived peptides are downstream effectors of the leptin signal generated in peripheral fat reserves and acting on the hypothalamus. Signals acting through the neural MC3R, and the more widely distributed neural MC4R are

Abbreviations: POMC, pro-opiomelanocortin; MCR, melanocortin receptors; α-MSH, α-melanocyte stimulating hormone; AGRP, agouti gene related peptide; FGF, fibroblast growth factor

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critical for leptin regulating energy homeostasis. Other central effects of leptin include the regulation of neuroendocrine and immune systems, hematopoiesis, brain and bone development [1]. The absence of leptin signaling in ob/ob and db/db mice revealed enhanced bone formation [7]. Peripheral administration of leptin to these mice was reported to dramatically increase cortical bone formation compared with control animals [24]. On the other hand, Ducy et al. [7] reported that leptin administered into the brain is a potent inhibitor of bone formation and this is believed to be acting through the central nervous system and mediated by the sympathetic nervous system [25]. It has been hypothesized that leptin exerts dual effects on bone metabolism; early in life leptin could stimulate bone growth and size and later may decrease bone remodeling in the mature skeleton when trabecular bone turnover is high. The central negative effects may counterbalance the peripheral positive effects of leptin on bone metabolism.

It is unknown whether central MC4R plays a role in leptin's inhibition of bone formation. However, in addition to MC4R mRNA expression in discrete nuclei throughout all regions of the brain [15], spinal cord and autonomic nervous system [16], and cardiorespiratory system, it is also expressed in integumental systems [19], including developing limb buds, skull bone, and teeth of fetal rats [19]. We have previously shown that α-MSH, but not desacetyl-α-MSH, significantly stimulated rat osteoblast cell proliferation [6,18]. These results and the reported expression of MC4R mRNA in developing bone cells led us to investigate in this study whether rat osteoblast cells express melanocortin receptors and thus provide evidence for a direct role for POMC peptides on bone formation.

2. Experimental

2.1. Cells

Rat osteosarcoma UMR106.06 cells were grown in Dulbecco's modified Eagle's Medium (DMEM) (GIBCO BRL, Rockville, MD) supplemented with 10% fetal calf serum (FCS) (Invitrogen, Auckland, New Zealand) and 50 U/ml penicillin plus 50 μg/ml streptomycin. Cells were maintained at 37 °C in 5% CO_2 and passaged every week.

Primary rat osteoblast cells were isolated from 20 days fetal rat calvariae. The use of animals for these studies was approved by the Auckland Animal Ethics Committee. Calvariae were excised and the frontal and parietal bones, free of suture and periosteal tissue were collected and sequentially digested using collagenase as previously described [5]. Primary rat osteoblast cells were grown in DMEM supplemented with 10% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. After 48 h, the medium was changed to MEM. Confluence was reached within 5–6 days, at which time the cells were subcultured into 10 cm culture plates for RNA preparation.

2.2. Preparation of mRNA

Total RNA was extracted from adult rat brain, skin, UMR106.06, or primary rat osteoblast cells using the guanidine thiocyanate method [3]. Poly $(A)^+$ mRNA was purified from the total RNA using the PolyATract mRNA Isolation System (Promega, Madison, WI).

2.3. Northern blot analysis

Primary rat osteoblast poly $(A)^+$ (5 μg) and rat brain poly $(A)^+$ were size separated alongside lambda *Eco*RI–*Hin*dIII markers by electrophoresis on a 2.2 M formaldehyde, 1.2% agarose gel, transferred to a Magnacharge Nylon membrane (MSI, Westborough, MA), and hybridized with a rat specific MC4R gene DNA fragment spanning transmembrane domains III–VII [15]. Hybridization conditions were 50% formamide, 1 mM NaCl, 50 mM Tris–HCl (pH 7.5), sodium pyrophosphate (0.1%), SDS (0.2%), salmon sperm DNA (100 μg/ml), 10× Denhardts and 10% dextran sulphate at 42 °C for 18 h. A digital image of MC4R transcripts was obtained after 10 days exposure to a phosphoscreen by using the Storm imaging system scanner (Molecular Dynamics).

2.4. PCR amplification of reverse transcribed mRNA (RT-PCR)

Poly $(A)^+$ mRNA was DNase treated twice using 10 U RQ1 RNase-free DNase (Promega Corp., Madison, WI) per mg poly $(A)^+$ mRNA for 30 min at 37 °C each time. First strand cDNA was synthesized using 1 μg poly $(A)^+$ mRNA, 200 U SuperScript II $RNaseH^-$ reverse transcriptase (GIBCO BRL, Rockville, MD) and oligo $(dT)_{12–18}$ (Pharmacia Biotech AB, Uppsala, Sweden) at 42 °C for 1 h in a final volume of 20 μl. To test for DNA contamination of the RNA, a reaction was carried out with 1 μg poly $(A)^+$ mRNA and all the reagents but no reverse transcriptase (control reaction). The cDNA and control reaction (2 μl) were used as templates for PCR with rat melanocortin receptor specific oligonucleotides described in Table 1. The PCR conditions were 94 °C for 3 min, 40 cycles of 94 °C for 40 s, annealing for 40 s, and 72 °C for 1 min, followed by 72 °C for 10 min. The amplified cDNA products were separated on a 1.2% agarose gel alongside a *Eco*RI–*Hin*dIII-digested lambda DNA ladder and stained with ethidium bromide.

2.5. Ribonuclease protection assay (RPA)

The cDNA templates used to synthesize the antisense rMC4R and rMC1R riboprobes were generated from 562 and 270 bp, respectively, nucleotide DNA fragment spanning transmembrane I–VII and III–VI domains subcloned into pBKS (Stratagene, USA). These recombinant DNA templates were linearized with *Eco*RI and *Sal*I and then transcribed with [α-^{32}P]UTP (Amersham Life Science, Buckinghamshire, UK) using T 7 RNA polymerase to generate