A possible role for melanocortin peptides in longitudinal growth

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Abstract

Clinical and in vitro data suggest a link between the elevation of the melanocortin peptide, ACTH, and longitudinal growth. Overproduction of ACTH in familial glucocorticoid deficiency (FGD) is associated with increased growth and ACTH increases the differentiation of chondrocytes along the endochondral pathway in vitro. Using the leptin-deficient obese (ob/ob) mouse along with lean control littermates (n=9–10), we investigated the effects of adrenalectomy (ADX)-induced elevated ACTH with and without peripheral administration of the MC3-R-specific agonist, γ2-melanocyte stimulating hormone (γ2-MSH), on longitudinal growth. Naso-anal and tibial growth were measured together with growth plate parameters; both total and zonal heights together with the proliferative index. Data were analyzed using two-way ANOVA with post hoc comparisons made using the Bonferroni correction. ADX significantly increased naso-anal length in lean mice and ADX plus γ2-MSH administration significantly increased naso-anal length above ADX alone in ob/ob mice. γ2-MSH administration to ADX lean and ob/ob mice significantly increased tibial length. In ob/ob mice, these changes occurred in the context of reduced food intake. Analysis of total and zonal growth plate heights suggest an increase in hypertrophic differentiation and an overall increase in growth plate turnover in ADX lean and ob/ob mice. These in vivo data show that ADX enhances linear growth and the results of γ2-MSH treatment suggest that the melanocortin system plays a role in linear growth.


Introduction

Longitudinal growth is accomplished through the process of endochondral ossification, which occurs in the cartilaginous growth plate located at both ends of the vertebrae and long bones. It follows a sequential pattern of biological events, beginning with chondrocyte maturation and ending in the development of mature hypertrophic chondrocytes. The growth plate is comprised of three distinct groups of chondrocytes: round periarticular chondrocytes, proliferating columnar chondrocytes, and hypertrophic chondrocytes. In developing growth plates, the periarticular chondrocytes near the articular surface proliferate, differentiate into flat proliferating columnar chondrocytes, eventually stop dividing, and differentiate into hypertrophic cells (Kobayashi et al. 2002). We have hypothesized that ACTH positively influences this process of differentiation along the endochondral pathway.

A direct correlation between elevated ACTH production and increased longitudinal growth is observed with the clinical syndrome, familial glucocorticoid deficiency (FGD). FGD is an ACTH-insensitivity disorder characterized by an overproduction of ACTH, enhanced linear growth, and advanced bone age (Clark & Weber 1998, Elias et al. 2000). In FGD, systemic levels of ACTH are extremely elevated due to a mutation in the ACTH-receptor/melanocortin-2 receptor (MC2-R). When the FGD patients are treated with glucocorticoid, their ACTH levels are reduced and their growth patterns normalize somewhat (Elias et al. 2000).

In vitro, ACTH directly affects chondrocyte development; it increases the differentiation of resting chondrocytes and mesenchymal chondroprogenitors (Evans et al. 2004). ACTH also evokes transient elevations in intracellular free calcium [Ca2+]i, and increases basal [Ca2+]i (Evans et al. 2005) and differentiation of chondrocytes along the endochondral pathway is associated with an incremental increase in basal [Ca2+]i (Zuscik et al. 2002). The MC3-R/MC4-R antagonist, SHU9119, attenuates ACTH evoked increases in [Ca2+]i (Evans et al. 2004) and the MC3-R is highly expressed by chondrocytes and chondrocyte progenitors (Clark & Weber 1998). To investigate the effects of elevated ACTH in vivo, both leptin-deficient obese mice (ob/ob) and their lean littermates were adrenalectomized (ADX) or adrenalectomized and given the MC3-R-specific agonist, γ2-melanocyte stimulating hormone (γ2-MSH) (50 µg/day, s.c.), for 21 days. The MC3-R/MC4-R antagonist, SHU9119, attenuates ACTH evoked increases in [Ca2+]i, and γ2-MSH administration to ADX lean and ob/ob mice significantly increased tibial length. In ob/ob mice, these changes occurred in the context of reduced food intake. Analysis of total and zonal growth plate heights suggest an increase in hypertrophic differentiation and an overall increase in growth plate turnover in ADX lean and ob/ob mice. These in vivo data show that ADX enhances linear growth and the results of γ2-MSH treatment suggest that the melanocortin system plays a role in linear growth.
produces transient elevations in [Ca^{2+}], through an undefined MC-R (Lanouche et al. 2001). Therefore, γ2-MSH activation of the MC3-R or another MC-R on growth plate chondrocytes could translate to increased hypertrophy and lead to increases in linear growth. After treatment, nasal-anal and tibial length were measured along with growth plate parameters, i.e. total and zonal heights and the proliferative index.

The ob/ob mouse model of obesity was used in these studies because these mice have been described as 5–10% shorter than their lean littermates (Cone 2000). It was thought that this would allow room for significant changes in growth with ADX and ADX plus γ2-MSH treatment. Additionally, with the use of this model, we were able to determine if ADX and ADX + γ2-MSH treatment enhance linear growth parameters in the absence of hypothalamic and peripheral leptin signaling.

Materials and Methods

Animals

Forty obese (ob/ob) and 40 lean (+/?) female 5-week-old mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). The exact genotype of the lean littermate control mice was not determined, therefore this group was likely a mixture of the +/+ and +/- leptin genotypes.

Lean and ob/ob animals were divided into four groups of ten animals each. Five-week-old basal control (basal), age-matched sham-operated control (Sham), adrenalectomized (ADX), and adrenalectomized with 50 μg/day γ2-MSH administration (ADX+γ2-MSH). Lean controls and ob/ob mice were adrenalectomized by the vendor. Sham operation was also performed by the vendor. ADX served to remove endogenous corticosterone and significantly elevate ACTH levels. ADX groups were maintained on drinking water supplemented with 0.9% NaCl and received an s.c. 50 μg corticosterone pellet (Innovative Research of America, Sarasota, FL, USA) designed to deliver 2.4 μg corticosterone per day over 21 days implanted to maintain basal metabolism. The γ2-MSH peptide (acetyl-(lys8,Nleθ)-γ2-MSH amide, BACHEM, King of Prussia, PA, USA) was used in these experiments because it is a specific MC3-R agonist and the MC3-R has been detected in cultured chondrocytes. The ADX + γ2-MSH treatment groups received approximately 50 μg/day acetyl-(lys8,Nleθ)-γ2-MSH amide over the 21-day experimental period delivered through an s.c. pellet (Innovative Research of America) containing a total of 1,050 mg peptide. The pellets were implanted at the time of surgery. The γ2-MSH dose used in this study can be calculated as 12.5 pmol/ml blood volume per min in lean control mice and 8.42 pmol/ml blood volume per min in ob/ob mice, or 1.07 nmol/kg per min and 0.672 nmol/kg per min respectively. These values are ~500 and 337 times greater respectively than known circulating levels of immunoreactive γ-MSH in mice (Ni et al. 2003).

ACTH, corticosterone, and leptin measurements

Blood was collected in EDTA containing tubes via intra-cardiac puncture and stored at −70 °C until assay. To confirm completeness of adrenalectomy, ACTH and corticosterone were measured via RIA using kits from MP Biomedicals, Inc (Irvine, CA, USA). ACTH levels in plasma from ADX and ADX + γ2-MSH groups were significantly elevated and thus were outside the range of standards. Therefore, these samples were run a second time at a 10X dilution along with samples from the SHAM groups. Since the volume of plasma collected from these mice was limited, data were available for only seven animals per ADX and ADX + γ2-MSH groups. To confirm animal genotype, leptin was measured via RIA with a kit from Linco Research (St Charles, MO, USA).

Histomorphometry

After being thoroughly cleaned of soft tissues, distal portions of each right femur were cut using an Isomet saw (Buehler, Lake Bluff, IL, USA) and stained at 4 °C using Villanueva Osteochrome Bone Stain (Polysciences, W arrington, PA, USA) for 5 days. The bones were then destained and dehydrated in sequential changes (70, 95, and 100%) of ethanol solutions and xylene and then cold embedded in methylmethacrylate (Erben 1997). Distal femora were frontal cut longitudinally to 8 μm with a microtome (Leica RM2155, Germany). Sections were placed on gelatin-coated slides and cover slipped using Eukitt (Calibrated Instruments, Hawthorne, NY, USA). Growth plate width was measured using the ‘Osteomeasure’ (Osteometrics) digitizing morphometry system and the standard nomenclature (Parfitt et al. 1987).
Immunohistochemistry

Eight micrometers methylmethacrylate embedded sections were de-plastified using 2-methoxyethyl acetate overnight followed by 5-min incubation in acetone. Sections were re-hydrated through decreasing graded concentrations of EtOH ending with distilled water. De-calcification was carried out in 10% EDTA in PBS for 2 h followed by washing in 1XPBS. Sections were exposed to proteinase K for 15 min at room temperature prior to a 3 h blocking with 2.5% horse serum. Avidin D (Vector Laboratories) was included in the blocking serum according to the manufacturer's instructions. After washing thrice with 0.5% Tween in PBS (PBST), sections were incubated overnight in 1:100 dilution of mouse monoclonal anti-BrdU antibody (Sigma) in blocking serum. Biotin (Vector Laboratories, Burlingame, CA, USA) was included with the primary antibody solution according to the manufacturer's instructions. The appropriate normal IgG at the same concentration as the primary antibody was used as a negative control. Secondary antibody from the RTU Universal Vectastain kit (Vector Laboratories) was diluted in the ratio of 1:1 in blocking serum and incubated for 30 min followed by 30-min incubation with ABC reagent from the same kit. Three 10-min washes with PBST followed all steps including and subsequent to blocking. Dab was used as the chromogen substrate. Nuclear-associated BrdU labeling was analyzed in four random growth plate fields of one femoral section from each animal. As an additional negative control, sections from mice not injected with BrdU were processed according to the described protocol. No staining was observed in these sections. Data were expressed as number of cells positive for BrdU divided by the total number of cells×100 = % BrdU positive cells (proliferative index).

Statistical analysis

Data were analyzed using one-way or two-way ANOVA as indicated. With two-way ANOVA, treatment and genotype were used as the main factors. Post hoc test P values were adjusted using the Bonferroni correction and a nominal significance level of 0.05 was used. When analyzing growth plate parameters, one significant outlier was identified using Grubbs' test (Iglewicz & Hoaglin 1993). The outlier was detected in the SHAM-operated lean control group. Data from this animal were removed from all analyses.

Results

Obese (ob/ob) mice demonstrate reduced longitudinal growth compared with lean littermate controls

Body weight and longitudinal growth parameters were measured in both ob/ob and lean mice at 5 weeks of age (basal; Table 1). As expected, ob/ob mice showed significantly greater body weight than their lean littermates. Conversely, ob/ob mice demonstrate significantly decreased naso-anal length and tibial length compared with lean controls. These data are consistent with previous reports on somatic and longitudinal growth in ob/ob mice (Cone 2000). Examination of total growth plate height and height of individual growth zones indicate that total growth plate height is reduced in ob/ob animals due to a significant reduction in hypertrophic zone height (Table 1, Fig. 1). This reduction appears to be due to significantly less hypertrophic cells in number but not in size in growth plate of the ob/ob mice (Table 1).

Adrenalectomy (ADX) alone and ADX plus the administration of \( \gamma \)-MSH reduces body weight and food intake in obese (ob/ob) mice

Pro-opio melano cortin (POMC) peptides have known anorexigenic effects (Millington et al. 2001). This may, in turn, impact linear growth, as calorie, restriction is known to limit linear growth (Brochman et al. 2003). Therefore, body weight and food intake were monitored in sham-operated and adrenalectomized (ADX) groups upon arrival in our animal care facility and until the end of the experimental period of 3 weeks. No significant changes in body weight were observed among the lean controls with the exception that the ADX group had a slightly lower body weight upon arrival in our animal care facility (Fig. 2A). This was likely due to the combined stresses of surgery and shipping as body weight in this group was comparable with sham-operated controls after

Table 1 Somatic and linear growth parameters in 5-week-old (basal) lean and obese (ob/ob) mice. Data are presented as means ± s.d. with n = 10 animals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lean</th>
<th>Obese</th>
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<tr>
<td>Body weight (g)</td>
<td>17.42 ± 0.62</td>
<td>31.13 ± 2.4*</td>
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<tr>
<td>Naso-anal length (mm)</td>
<td>85.62 ± 1.29</td>
<td>82.07 ± 0.80*</td>
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<td>Tibial length (mm)</td>
<td>16.34 ± 0.283</td>
<td>15.32 ± 0.175*</td>
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<tr>
<td>Total growth plate height (µm)</td>
<td>165.72 ± 16.61</td>
<td>148.04 ± 11.62*</td>
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<tr>
<td>Stem zone height (µm)</td>
<td>24.76 ± 6.19</td>
<td>21.63 ± 4.68</td>
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<td>Proliferating zone height (µm)</td>
<td>71.01 ± 10.60</td>
<td>73.43 ± 9.36</td>
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<td>Hypertrophic zone height (µm)</td>
<td>69.19 ± 10.72</td>
<td>53.21 ± 7.59*</td>
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<td>Hypertrophic cells (no. per field)</td>
<td>55.14 ± 8.39</td>
<td>42.08 ± 7.58*</td>
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For growth plate measurements, two sections were measured per animal. For hypertrophic cell number, four random fields were analyzed per section. Statistical significance between groups was determined using Student's unpaired t-test, *P<0.001.
3 days. On the other hand, significant differences in body weight were observed among ob/ob groups (Fig. 2B). ADX significantly reduced body weight among ob/ob mice and ADX + γ2-MSH supplementation decreased body weight significantly below ADX alone.

As with body weight measurements, neither ADX nor ADX + γ2-MSH affected food intake among lean controls (Fig. 3). Conversely, ADX significantly reduced food intake in obese animals. The administration of γ2-MSH to ADX ob/ob mice also reduced food intake to a level significantly below ADX alone. In fact, food intake among ADX + γ2-MSH-treated ob/ob mice was not significantly different from food intake of lean control mice (Fig. 3).

**ADX and ADX + γ2-MSH increase longitudinal growth in both lean and obese (ob/ob) mice**

As an index of longitudinal growth, the changes in naso-anal length and tibial length from basal measurements were analyzed (Fig. 4). ADX increased Δtibial length in both ob/ob and lean animals compared with sham-operated controls, yet among lean mice this effect was not quite significant (Fig. 4). However, the administration of γ2-MSH to ADX lean mice significantly increased tibial length compared with SHAM-operated controls. ΔNaso-anal length in the ADX and ADX + γ2-MSH lean groups were also significantly greater than the SHAM-operated controls. Administration of γ2-MSH to the ADX lean animals did not significantly increase Δtibial length or Δnaso-anal length above ADX alone. In the ob/ob groups, however, γ2-MSH administration to the ADX animals significantly increased Δnaso-anal length above ADX alone. Two-way ANOVA with treatment and genotype as the main factors show no interaction effect on either Δnaso-anal length or Δfinal tibial length. However, both treatment ($F_{(2,51)} = 13.92, P < 0.001$) and genotype ($F_{(1,51)} = 56.17, P = 0.0001$) significantly affected Δnaso-anal length. Treatment ($F_{(2,51)} = 16.89, P < 0.0001$) and genotype ($F_{(1,51)} = 27.34, P < 0.0001$) were also significant factors in Δtibial length.

**Figure 1** Growth plate height is significantly reduced in obese ob/ob mice versus lean control mice. Representative sections of femoral growth plate from ob/ob and lean mice. Note that the reduction in ob/ob growth plate height is primarily due to a significantly decreased hypertrophic zone height. S, stem cell zone; P, proliferating cell zone; H, hypertrophic cell zone.

**Figure 2** Adrenalectomy (ADX) and ADX + γ2-MSH administration significantly reduce body weight in obese (ob/ob) mice. Body weight measurements in lean (A) and ob/ob (B) mice after arrival in our animal care facility to the end of the experiment. SHAM, sham-operated control; ADX, adrenalectomized; ADX + γ2-MSH, adrenalectomized and administered γ2-MSH. Data presented as means ± S.E.M. Statistical significance determined after one-way ANOVA using the Bonferroni correction, $n = 9–10$. *Statistically significant from Sham ($P < 0.05$), †Statistically significant from ADX ($P < 0.05$).

**Figure 3** Total and individual zone measurements of the growth plate reveal differential responses to treatment among ob/ob and lean control mice

To gain insight into how melanocortin peptides may increase linear growth, the effects of ADX and ADX + γ2-MSH on the zones of the growth plate were examined (Table 2, Fig. 5).
height and proliferating and hypertrophic zone heights were observed between γ2-MSH-treated ADX ob/ob mice and ADX ob/ob mice. These significant increases were not observed among ADX + γ2-MSH-treated compared with ADX lean mice. Interestingly, no significant interaction effect was found for stem cell zone height (F(2,108) = 1.01, P = 0.3672), and treatment (F(2,108) = 26.38, P < 0.0001) but not genotype had a significant main effect on the height of this zone. Therefore, the stem cell zone of the tibial growth plate experienced a similar increase in height in lean and ob/ob mice.

**ADX and ADX + γ2-MSH increase the proliferative index of the growth plate in both lean and obese (ob/ob) mice**

In vivo ACTH increases the proliferation of chondrocyte precursors and increases their differentiation toward the chondrogenic phenotype (Evans et al. 2004). To confirm an in vivo effect of elevated melanocortin on proliferation of growth plate cells, both treated and untreated lean and obese mice were injected with 50 mg/kg BrdU 24 h prior to euthanasia and DNA-incorporated BrdU was detected using immunohistochemistry. In the stem cell and proliferating cell zones, the percentage of BrdU positive cells (proliferative index) was significantly increased by ADX in lean mice (Fig. 6A and B). Treatment with γ2-MSH significantly increased the proliferative index above ADX alone in the proliferating zone (Fig. 6B) but not in the stem cell zone of lean animals (Fig. 6A). In obese mice, ADX showed a trend toward increasing the proliferative index of the stem and proliferating cell zones but this was not significant. However, the proliferative index of both stem and proliferating zones of γ2-MSH-treated ADX ob/ob mice was significantly increased above SHAM (Fig. 6A and B).

**Plasma measurements confirmed leptin deficiency and completeness of ADX**

Plasma was collected from each animal at the time of euthanasia and circulating levels of leptin, ACTH, and corticosterone were measured via RIA. Leptin levels of ob/ob animals were at undetectable levels (data not shown) confirming the ob/ob genotype. Corticosterone and ACTH levels in the ADX groups reflect the absence of adrenal tissue confirming the melanocortin deficiency. The ADX + γ2-MSH group had significantly lower ACTH levels (Fig. 7A) but no difference in corticosterone levels was observed (Fig. 7B). Plasma ACTH levels were elevated compared with published basal values (Maor et al. 2002), which indicate that sample collection occurred post stress induction. Interestingly, a significant interaction effect of treatment and genotype on plasma ACTH level was found (F(2,40) = 7.86, P = 0.0013). This effect can be attributed to a significantly lower ACTH level found in the lean ADX + γ2-MSH group. No interaction effect between treatment X genotype was found with plasma corticosterone and treatment was responsible for the main effect on this variable (F(2,51) = 65.56, P < 0.0001) with no effect of genotype (F(1,51) = 0.74, P = 0.3928).
Buffett & Wyman (1954) were the first to report adrenalectomy-induced increases in the width of the rat epiphyseal growth plate. Since then others have documented increases in longitudinal growth after ADX and have attributed these changes to the removal of glucocorticoid (Li et al. 1996a, b).

Here, we introduce the possibility that melanocortin signaling, i.e. ACTH, at the level of the growth plate contributes to ADX-induced increases in linear growth. In this study, ADX increased both the proliferative index and the hypertrophic zone height of the femoral growth plate demonstrating that the removal of glucocorticoid along with elevated ACTH exposure increases the overall turnover of the growth plate leading to an overall increase in longitudinal growth. In vitro, ACTH increases the proliferation of chondrocyte precursors and increases their differentiation toward the chondrogenic phenotype (Evans et al. 2004) suggesting that ACTH in vivo acts on the tibial stem cell zone in a similar manner. Consistent with this is the fact that stem cell zone height is increased in ADX ob/ob and lean mice, and in lean mice the proliferative index of both the stem

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<th>Table 2</th>
<th>Growth plate measurements in adrenalectomized (ADX) and ADX + ( \gamma_2 )-MSH-treated lean and obese (ob/ob) mice. Data presented in ( \mu \text{m} ) and as means ± s.d., two sections were analyzed per group with and ( n=9–10 ) animals.</th>
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<tr>
<td></td>
<td>Lean</td>
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<td>SHAM</td>
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<td></td>
<td>SHAM</td>
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<tr>
<td>Total growth plate height</td>
<td>96.2 ± 14.1</td>
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<td>99.8 ± 11.8</td>
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<tr>
<td>Stem zone height</td>
<td>12.9 ± 4.6</td>
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<td>13.6 ± 4.2</td>
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<tr>
<td>Proliferating zone height</td>
<td>58.8 ± 8.3</td>
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<td></td>
<td>69.1 ± 7.5(^c)</td>
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<tr>
<td>Hypertrophic zone height</td>
<td>24.7 ± 6.9</td>
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<td>17.0 ± 4.4(^c)</td>
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Results of two-way ANOVA are presented in Results. Post hoc analysis with the Bonferroni correction was used to determine differences between individual groups. \(^a\)Significantly different from Sham of same genotype (\( P<0.05 \)); \(^b\)significantly different from ADX of same genotype (\( P<0.05 \)); \(^c\)significantly different from lean counterpart (\( P<0.05 \)).

Discussion

Buffett & Wyman (1954) were the first to report adrenalectomy-induced increases in the width of the rat epiphyseal growth plate. Since then others have documented increases in longitudinal growth after ADX and have attributed these changes to the removal of glucocorticoid (Li et al. 1996a, b). Here, we introduce the possibility that melanocortin signaling, i.e. ACTH, at the level of the growth plate contributes to ADX-induced increases in linear growth. In this study, ADX increased both the proliferative index and the hypertrophic zone height of the femoral growth plate demonstrating that the removal of glucocorticoid along with elevated ACTH exposure increases the overall turnover of the growth plate leading to an overall increase in longitudinal growth. In vitro, ACTH increases the proliferation of chondrocyte precursors and increases their differentiation toward the chondrogenic phenotype (Evans et al. 2004) suggesting that ACTH in vivo acts on the tibial stem cell zone in a similar manner. Consistent with this is the fact that stem cell zone height is increased in ADX ob/ob and lean mice, and in lean mice the proliferative index of both the stem

Figure 5  Growth plate height is significantly increased in adrenalectomized (ADX) lean and obese (ob/ob) mice. The administration of \( \gamma_2 \)-MSH significantly elevates growth plate height over ADX alone in ob/ob mice. Representative sections of femoral growth plate from ADX and ADX + \( \gamma_2 \)-MSH administered to lean and ob/ob mice after 3 weeks of treatment. Note increases in hypertrophic zone height and stem cell zone height in both the treatment groups compared with sham controls. S, stem cell zone; P, proliferating cell zone; H, hypertrophic cell zone.
and the proliferating growth plate zones are significantly increased by ADX. In vivo findings also show that elevated exposure to ACTH increases hypertrophic differentiation among chondrocytes likely through an increase in intra-
cellular calcium (Evans et al. 2004, 2005). These data are consistent with in vivo findings where the hypertrophic zone of both the ADX ob/ob and the lean mice are significantly greater than the sham-operated controls pointing toward a 
direct action of ACTH at the growth plate. Further studies are necessary to definitively determine if ACTH does indeed contribute to ADX-induced increases in growth plate height.

However, several factors suggest that melanocortin stimulation at the level of the growth plate can lead to changes in longitudinal growth. First, \( \gamma_2 \)-MSH administration to ADX obese mice increased naso-anal length above ADX alone; secondly, \( \gamma_2 \)-MSH administration to both lean and obese mice enhanced tibial growth above ADX alone, and lastly, \( \gamma_2 \)-MSH administration increased growth plate proliferative indices in ADX lean and ob/ob mice. All the five melanocortin receptors are expressed in the mouse growth plate (Zhong et al. 2005); however because \( \gamma_2 \)-MSH is an MC3-R-specific agonist, our data implicate the MC3-R as a receptor involved in the regulation of endochondral growth. This is supported by both in vivo and in vitro findings. MC3-R-deficient mice experience decreased linear growth. Chen et al. (2000) have reported that the average femoral length in female MC3-R-deficient mice was significantly shorter than wild-type littermates suggesting growth is stunted in the absence of MC3-R. Additionally, the MC3-R is highly expressed by both chondrocytes and chondrocyte precursors (Evans et al. 2004). Moreover, the MC3-R-/MC4-R-specific inhibitor, SHU9119, attenuates ACTH-evoked transient elevations in intracellular calcium in resting chondrocytes and the RCJ3.1C5.18 chondrogenic cell line (Evans et al. 2005).

However, studies of the cardiovascular system and of brain indicate the possible existence of an undefined \( \gamma \)-MSH-responsive MC-R. \( \gamma_2 \)-MSH, when centrally administered, evokes cardiovascular responses even in the presence of the SHU9119 antagonist (Li et al. 1996a,b). SHU9119 is also an antagonist of the other two melanocortin receptors expressed in brain. This suggests that \( \gamma_2 \)-MSH can act through a melanocortin receptor other than the five receptors that have been described. In vitro data support the existence of another yet to be described \( \gamma \)-MSH-responsive melanocortin receptor. The pituitary cell line, GH3, responds to \( \gamma \)-MSH with transient elevations in calcium and a high dose of SHU9119 does not block this response (Languouche et al. 2001). These data introduce the possibility that this undefined MC-R is expressed in the growth plate and that \( \gamma_2 \)-MSH exerts its effects through it.

Regardless of the receptor through which \( \gamma_2 \)-MSH exerts its effects, it likely augments adrenalectomy effects on growth through a mechanism involving an elevation in intracellular calcium. All the MC-Rs, including the putative new \( \gamma \)-MSH-responsive MC-R, are known to evoke transient elevations in intracellular calcium or calcium influx upon activation (Languouche et al. 2001, Mountjoy et al. 2001).
Al–Majed et al. 2004). In the case of chondrocytes, ACTH stimulation of the MC3-R also leads to increases in basal intracellular calcium (Evans et al. 2005). Chondrocyte hypertrophic differentiation is accompanied by an incremental increase in basal intracellular calcium and increased hypertrophy due to the activation of MC-R among growth plates cells would translate to increases in linear growth. The possibility that γ2-MSH augments the response to adrenalectomy through increasing hypertrophic differentiation is supported by the fact that hypertrophic zone height is increased in γ2-MSH-treated ADX ob/ob mice when compared with ADX ob/ob mice.

The changes in linear growth parameters of ADX and ADX + γ2-MSH-treated ob/ob mice occurred in the context of significantly decreased food intake and body weight. The effect of ADX on food intake and body weight gain in ob/ob mice has been previously documented (Makimura et al. 2000); however, the significant reduction in these parameters with ADX and concomitant γ2-MSH treatment has not been reported. It is not clear why ADX and γ2-MSH treatment did not also produce body weight changes in lean mice. Our results however, are consistent with previous findings where ADX of lean mice did not reduce body weight or food intake (Saito & Bray 1984).

As mentioned, the ob/ob mouse model has been reported to be 5–10% shorter than its lean littermates (Langouche et al. 2001) and our data confirm this finding (Table 1). A role for leptin in longitudinal growth has recently been defined (Gat-Yablonski et al. 2004). Therefore, the possibility exists that the absence of leptin is responsible for the growth deficiency of the ob/ob mouse. In fact, researchers have shown that administration of peripheral leptin ameliorates decreased tibial length in ob/ob mice (Stephan et al. 2000). On the other hand, glucocorticoid levels are elevated in ob/ob mice (Sainsbury et al. 2002) and adrenal steroids are known to inhibit endochondral growth (Chrysis et al. 2003, Smink et al. 2003). These data create the possibility that linear growth in ob/ob mice is inhibited through mechanisms related to elevated glucocorticoid. Our present data support this latter possibility. Removal of glucocorticoid by ADX enhanced tibial growth in ob/ob mice independently of leptin. Previous findings, where endogenous leptin treatment improved tibial growth in ob/ob mice may have been due to the fact that leptin treatment normalizes circulating levels of glucocorticoid (Halaas et al. 1995, Pelleymounter et al. 1995).

Contrary to what was expected, at the end of the study period the SHAM-operated lean mice and the SHAM ob/ob mice had similar naso-anal lengths. Stress among lean controls could have contributed to this discrepancy. This is supported by the lack of weight gain by this group during the last 10 days of the study. Another possibility is that hyperphagia among the ob/ob mice led to a more rapid increase in naso-anal length between 5 and 8 weeks. The precise role of the melanocortin system is yet to be determined but it is tempting to speculate that it is involved in relaying signals of nutritional status at the level of the growth plate. Expression of the precursor to the melanocortin peptides, POMC (Solomon 1999), in the hypothalamus is highly regulated by food intake, i.e. expression increases with increased food intake and decreases in response to caloric restriction (Ziotopoulou et al. 2000, Bi et al. 2003). An increase in POMC expression in the ob/ob mice as a result of increased food intake could lead to increased circulating melanocortin peptide and signal an increased growth rate at the level of the growth plate. This along with increased stress or poor health among the controls could explain the discrepancy.

Greater changes in naso-anal length were also observed among γ2-MSH–treated ADX ob/ob mice and greater changes in tibial length were observed in ADX and γ2-MSH–treated ADX ob/ob mice compared with lean controls. In vitro data have shown that ACTH–evoked [Ca2+]i transients and increases in basal calcium are greater among populations of chondrocytes that have been primed with the glucocorticoid dexamethasone (Evans et al. 2005). As stated above, ob/ob mice have increased circulating levels of glucocorticoid (Sainsbury et al. 2002). Therefore, the possibility exists that because growth plate chondrocytes of ob/ob mice have been exposed to increased levels of glucocorticoid prior to exposure to elevated ACTH they are more responsive to its effects. More data are necessary to confirm this hypothesis.

Both ACTH and corticosterone levels in sham–operated lean mice were elevated compared with published basal values (Millington et al. 2001), which indicates that sample collection occurred post stress induction. The restraint required for injection of the ketamine/xylazine anesthesia combined with the stress of the injection itself is the likely cause of the elevated ACTH and corticosterone values. ADX will induce hypersecretion of ACTH and stress to ADX animals will increase ACTH levels further (Mukherjee et al. 2004). In this study, ADX mice experienced significant increases in ACTH; however, in the γ2-MSH–treated lean control mice, these values were significantly less than ADX alone. This may be explained by melanocortin effects on the hypothalamic–pituitary–adrenal axis (HPA). Central γ-MSH administration can inhibit IL–1β activation of the HPA (Cragnolini et al. 2004). In addition, the MC3-R, the hypothalamic target of γ2-MSH, may act as an autoinhibitory receptor on POMC neurons (Cone et al. 2001). Therefore, it is possible that γ2-MSH administration reduced the stress response to restraint. Although γ2-MSH was administered peripherally, it likely reached its target receptor among POMC hypothalamic neurons. POMC neurons are located in the arcuate nucleus of the hypothalamus, which functions as a circumventricular organ, existing functionally outside the blood–brain barrier (Cone et al. 2001). This is supported by the fact that γ2-MSH treatment reduced food intake in ob/ob mice. Central administration of γ2-MSH is known to reduce food intake in fasted rats (Millington et al. 2001). The reduction in plasma ACTH level in response to stress by γ2-MSH was not observed among ob/ob mice likely reflecting a functional dependence on leptin signaling.

While plasma ACTH levels among sham–operated ob/ob mice reflect a response to stress, corticosterone levels in these mice are not significantly elevated above published basal values for these mice (Sainsbury et al. 2002). In ob/ob mice, a smaller
fractional increase in restraint–induced cortisol increase is observed (Rowland & Dunn 1995) along with an increased clearance of 3H-corticosterone from the plasma (Naeser 1975). These two factors may have contributed to the reduced cortisol response to stress observed in the ob/ob mice of this study. These findings are similar to those in obese humans where elevated plasma ACTH is associated with a paradoxically low plasma cortisol in response to hydrocortisone injection (Jesop et al. 2001) and where an increased metabolic clearance of cortisol is observed (Dunklemann et al. 1964).

In summary, clinical data suggest a link between the melanocortin system and linear growth. This and other studies performed in rodents show that ADX, which removes glucocorticoid and elevates ACTH, results in increased longitudinal growth. Our in vivo data taken together with the present in vivo data support a role for the melanocortin, ACTH, in ADX-induced changes in growth plate height (Evans et al. 2004, 2005). Additionally, γ2-MSH treatment of ADX ob/ob and lean mice resulted in increases in tibial length above ADX alone, suggesting that the melanocortin system plays a role in endochondral ossification. However, the precise role of the melanocortin system in longitudinal growth is yet to be determined.

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