Disturbances of growth hormone-insulin-like growth factor axis and response to growth hormone in acidosis

KARINA J ANDZISZAK,1 CARLOS SUAREZ,1 ETHAN WASSERMAN,1 ROSS CLARK,2 BONNIE BAKER,3 FRANCES LIU,3 RAYMOND HINTZ,3 PAUL SAENGER,3 AND LUC P. BRION1

1Department of Pediatrics, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, New York 10461; 2Genentech, South San Francisco 94080; and 3Department of Pediatrics, Stanford University, Stanford, California 94304

Jandziszak, Karina, Carlos Suarez, Ethan Wasser- man, Ross Clark, Bonnie Baker, Frances Liu, Raymond Hintz, Paul Saenger, and Luc P. Brion. Disturbances of growth hormone-insulin-like growth factor axis and response to growth hormone in acidosis. Am. J. Physiol. 275 (Regul. Integrative Comp. Physiol. 44): R120–R128, 1998.—Severe chronic metabolic acidosis (CMA) in rats is associated with poor food intake and downregulation of growth hormone (GH), insulin-like growth factors (IGFs), and liver receptors; the administration of recombinant GH (rGH) fails to improve the growth failure. In mice with carbonic anhydrase II deficiency (CAD), a model of moderate CMA with food intake close to normal, we studied serum levels of GH, IGFs, and IGF-binding proteins, and the growth response to rGH. CAD was associated with low serum levels of GH in males. Randomized administration of rGH from −5 to −12 wk to CAD mice improved food efficiency and increased serum IGF-I levels, final length, and weight compared with placebo without affecting blood pH. Although administration of rGH also increased linear growth in healthy animals, the effect was less than that in CAD mice and was only observed when started before 6 wk of life. Thus growth failure in CAD mice is associated with a decrease in GH secretion in males but not in females. Long-term administration of rGH increases linear growth in CAD mice despite persistent CMA.

somatotropin; insulin-like growth factors I and II; insulin-like growth factor-binding proteins; carbonic anhydrase II deficiency; renal tubular acidosis; growth hormone, recombi- nant

GROWTH FAILURE IN CHILDREN with chronic metabolic acidosis (CMA) may result from negative nitrogen balance; alterations of collagen metabolism; bone demineralization and negative mineral balance despite normal levels of 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3); and low serum levels of insulin-like growth factor (IGF)-I, thyroxine, or triiodothyronine (2, 12, 13, 16, 24, 30, 31, 37). In patients with chronic renal failure, CMA contributes to growth failure by increasing protein degradation (36). Children with renal tubular acidosis (RTA) have blunted secretion of growth hormone (GH), low serum IGF-I levels, and a low response of GH to arginine while acidotic but a normal response after correction of acidosis (7, 32). Growth failure in most children with CMA responds to chronic administration of alkali (15, 16, 22, 30, 35). However, alkali administration has variable success on growth in patients in whom RTA is due to congenital carbonic anhydrase II deficiency (CAD) (40). Furthermore, alkalinization could aggravate osteopetrosis.

Models of Severe CMA With Food Deprivation

In rats severe (pH 7.1–7.2) CMA induced by high-dose ammonium chloride (NH4Cl) or uremia is associated with growth failure, a 29–37% reduction in food intake, poor food efficiency, high protein catabolism, low 25-hydroxyvitamin D3 [25-(OH)D3] 1α-hydroxylase activity, low pulsatile GH secretion, low serum IGF-I levels, and low expression of hepatic IGF-I and GH-receptor mRNAs (10, 11, 18, 26–28). Most of these changes are similar to those observed in pair-fed, nonacidotic animals. Growth in these rats improves by increasing caloric intake or correcting the acidosis but not by administering supramaximal doses of recombinant GH (rGH, 350 U/kg of body wt daily) for a short duration (2 wk) without correcting the acidosis (18, 23).

Models of CMA Without Malnutrition

In adult rats subjected to surgical stress, the administration of rGH or IGF-I reduces systemic acid production, thereby correcting the acidosis and improving weight gain (17). CMA in rabbits loaded with 15 mM·kg−1·day−1 of NH4Cl results in several adaptation mechanisms, including upregulation of urinary acidification and of several acid base-related mechanisms, e.g., renal and liver carbonic anhydrase (6). In adult humans loaded with NH4Cl, rGH administration reduces endogenous organic acid production and further increases renal NH3 excretion, thereby partially correcting the CMA (39). In mice, the CAD mutation causes RTA (characterized by CMA with failure to acidify the urine but without renal failure), upregulation of carbonic anhydrase, and growth failure but, unlike in humans with that disease, is not associated with osteopetrosis (4, 5, 25).

The present study was designed to analyze the role of the GH-IGF axis in growth retardation associated with moderate CMA. For this purpose, we used CAD mice, which have a blood pH of 7.25 ± 0.04 (n = 5) compared with 7.37 ± 0.01 (n = 6) in controls (P < 0.05), and a serum bicarbonate concentration of 17.6 ± 1.2 mM (n = 6) compared with 21.1 ± 0.5 mM (n = 10) in controls (P < 0.05) (4).

Our first hypothesis was that without primary bone disease, renal failure, or limitation of food intake, growth retardation in mild to moderate chronic CMA results in part from hormonal mechanisms. The second hypothesis was that the administration of rGH improves final length and weight in animals with a moderate CMA and normal food intake.
METHODS

Design

Baseline measurements. We compared food intake and hormonal levels in 3- to 5-mo-old homozygous CAD mice with those of B6AF1 controls. To assess the effect of CAD on growth rate and adult size, we measured length and weight in 2- to 18-wk-old B6AF1 mice studied under baseline conditions. We calculated the means ± SD of weight and length for each one-half-week interval; we then used nonlinear regression analysis to construct smoothed growth curves for weight and for length. For each CAD mouse, we calculated weight SD score and length SD score using the formula

\[
\text{SD score} = \frac{\text{observed value} - \text{mean value in age- and gender-matched controls}}{\text{SD in age- and gender-matched controls}}
\]

We defined the end of the rapid linear growth phase as the age at which average length was within ±5% of adult length both in controls and in CAD mice.

Randomized administration of rGH. We randomly assigned two groups of mice to receive either rGH or placebo: 1) mice in the CAD colony between the time of sampling for diagnosis of CAD (~5 wk of life) and 12 wk of life and 2) genetically normal B6AF1 mice, which were matched for age with the CAD mice. Animals were removed from the study if they were later found to be heterozygous for carbonic anhydrase II (CA II), developed renal failure, became moribund and received euthanasia, or died before the end of the study period. We used an open, gender-stratified, random allocation, with a weighting factor of 4 to 1 to limit any imbalance among the four groups: 1) controls on placebo, 2) controls on rGH, 3) CAD on placebo, and 4) CAD on rGH. Because the rate of growth during rGH administration may not accurately predict final adult size (1), we designed the study to last until the end of the rapid linear growth phase.

Our main hypothesis was that rGH administration would result in a significant increase in final (i.e., at 12 wk of life) length compared with placebo-treated animals. To take into account individual differences in age (both at entry and at the end of the study) and in initial size, we normalized length and weight using the SD score. The secondary hypothesis was that CMA would not reduce the effect of rGH on growth.

We analyzed whether rGH had other effects on these animals. First, rGH may cause excessive weight gain due to water retention. Second, upregulation of IGF-I by rGH administration may increase renal blood flow and glomerular filtration rate, and thus compromise long-term renal function (21). Third, IGF-I increases tubular reabsorption of phosphate and 1α-hydroxylation of 25-(OH)D3 (9, 38). Finally, rGH has been reported to stimulate urinary acidification, specifically NH4 secretion, and to partially correct CMA induced by NH4Cl in adult humans (39); such correction of acidosis could then mediate an improvement in growth. Thus we obtained serial weight measurements and, at the end of the study, serum levels of IGF-I and IGF-binding proteins (IGF-BP), blood acid-base status, and plasma concentrations of creatinine, calcium, and phosphate.

Procedures

CAD. CAD mice used in this study had been originally obtained by mating heterozygotes (Car-2+/+) from Dr. R. P. Erickson (Ann Arbor, MI) with B6AF1 hybrids (8). CAD mice were bred by mating one homozygous male with one heterozygous female and one homozygous female; this method, selected because of the low fertility and high mortality of homozygous females, necessitated that every animal be tested for CA II expression. Animals were diagnosed as heterozygous (healthy) or homozygous (CAD) by Western blot analysis on a drop of tail blood using as primary antibody a rabbit polyclonal antiserum to rat CA II provided by W. Cammer (8) and as secondary antibody a goat antiserum to rabbit IgG that was conjugated to peroxidase. Bands were then visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL) (4).

We weaned heterozygous animals after obtaining the results of the Western blot analysis (i.e., at an average of 5 wk of life) and CAD mice at 5–6 wk. Attempts at earlier weaning of CAD mice led to death of all CAD mice from starvation. We excluded from this study animals with abnormal kidney anatomy as determined at the time of autopsy. We compared these CAD mice to age- and gender-matched control B6AF1j mice (purchased from Jackson Laboratories, Bar Harbor, ME), which were weaned at 3 wk of life. All animals were kept at the Animal Institute of the Kennedy Building, where lights are turned off from 8 PM to 8 AM.

Measurement of growth. Animals were weighed with a precision of 0.02 g. Their nose-to-anus length was measured with a precision of 0.1 cm. For this purpose the animals were placed flat on a ruler placed on the bench top and did not receive anesthesia.

Blood sampling. Preliminary samples were obtained in 3- to 6-mo-old animals at 2 PM for measurement of GH. To analyze the secretory pattern of GH (14), we later obtained serial blood samples on alternate hours around the clock over a 1-wk period. In a larger group of animals, we obtained serial measurements of GH every other hour during a 12-h period, 2 days in a row. Blood was collected from the tail without anesthesia, and bleeding was stopped by applying silver nitrate.

The total amount of blood taken was <6 ml/kg body wt.

Blood samples for acid-base measurements and for serum levels of IGFs and IGF-BPs were obtained when the mice were killed, immediately after pentobarbital anesthesia. All blood samples but those for blood gas measurements were centrifuged, and plasma was kept in the freezer at −20°C until analysis.

Laboratory methods for measuring hormone levels. Serum levels of GH were measured by murine-specific ELISA (29). Because the sensitivity of the assay was initially 0.1 to 1.0 ng/ml, preliminary data obtained at 2 PM were left censored; the sensitivity was subsequently improved to 0.05 ng/ml for serial measurements of GH concentration.

Plasma concentrations of IGF-I and IGF-II were measured by RIA (14, 20). Plasma levels of IGF-BPs were measured by Western ligand blot analysis (20); the density of each band was corrected for background and normalized using a standard band in each gel. Three discrete bands were observed, migrating, respectively, at ~22 kDa (IGF-BP4), ~32–29 kDa (presumably IGF-BP2), and ~40 kDa (IGF-BP3).

Administration of rGH and placebo. Animals in the rGH group received rGH (10 U·kg⁻¹·day⁻¹ 5 days a week and 20 U/kg 1 day a week) subcutaneously. This dose was selected according to the study of Mehls et al. (33), which had shown that a dose of 5–10 U·kg⁻¹·day⁻¹ reached maximal effects in uremic rats. A 6 U/ml solution of Genotropin (Lot 95L12FY, a gift from Pharmacia Adria-SP, Albuquerque, NM) was prepared every other week and kept refrigerated. A 1:10 dilution in isotonic saline was prepared fresh daily, kept on ice, and protected from the light. Animals in the placebo group received the same volume of isotonic saline.

Approval. These protocols have been approved by the Animal Institute of the Albert Einstein College of Medicine and by the Institutional Animal Care and Use Committee of

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RESULTS

asserted at release 6.0, SPSS, Chicago, IL). Statistical significance was assessed at P < 0.05 using two-tailed tests.

We compared frequencies among various groups using likelihood ratio χ² analysis; segmentation modeling was done using the default settings, which include Bonferroni adjustment of the P value. We defined peak GH level as the 95th percentile of all uncensored serum levels obtained in control B6AF1 mice (of either gender) under baseline conditions.

For continuous variables with normal distribution, we used Student's t-tests or ANOVA followed by Tukey tests if variances were equal, and Dunnett's T3 tests in the other cases as appropriate. For variables with distribution skewed to the right (e.g., IGF-BPs), we used logarithmic transformation before statistical analysis. Values are expressed as means ± SE.

We tested the hypothesis that baseline levels of IGFs or IGF-BPs in adult mice would differ between males and females and between CAD mice and B6AF1 animals. For this purpose, we used two-way ANOVA to analyze IGF levels and IGF-BP densitometry, with gender and CA status (i.e., B6AF1 vs. CAD) as factors, and assessed the effects of each factor and their interactions on levels of IGFs and IGF-BPs.

We also tested the hypothesis that rGH administration would affect levels of IGF-I, IGF-BPs, or both. For this purpose, we used three-way ANOVA, with gender, treatment, and CA status as factors for randomized animals, and assessed the effects of each factor and their interactions on levels of IGFs and IGF-BPs.

For continuous variables with left censoring (i.e., measurements of serum GH concentration at 2 PM), we used the Mann-Whitney test; the value of P was the upper limit of the 99% confidence interval using Monte Carlo sampling method. Values are shown as median (percentiles and range).

To assess the effect of rGH on growth, we first compared the change in SD score (ΔSD score) for length or for weight from the time of entry until the end of the study observed in the placebo group with that in the rGH group, using unpaired Student's t-test. We needed 17 animals per group to detect a rGH-related effect size of 1, using a two-tailed Student's t-test with an α of 0.05 and a power of 80%. We expected to need at least twice as many animals from the CAD colony, because of the presence of heterozygous siblings at the time of randomization (to be eliminated after obtaining diagnosis of CA status) and because of the high mortality rate of CAD mice. One might expect rGH to be less effective when initiated late in life, so that we further divided the rGH group in two (entry up to 6 wk of age vs. after 6 wk of age); we compared the three resulting groups by one-way ANOVA, followed by Tukey test for paired comparisons.

Second, we used analysis of covariance (ANCOVA) with as dependent variable length SD score or weight SD score at the time of entry into the study, and as factors gender, genetic status (B6AF1 vs. CAD) and treatment.

RESULTS

Baseline Measurements in Adult Animals

Food intake in adult CAD mice was 82.8% of that in controls (0.19 ± 0.01 g of food/g wt daily, n = 9, vs. 0.23 ± 0.01 g/g daily, n = 6, P = 0.064).

Measurements of serum GH concentration at 2 PM showed higher values (P < 0.004) in B6AF1 mice than in CAD mice (median for 74 controls: 1 ng/ml, with 10th and 90th percentiles of 0 and 28.0, respectively, and a range of 0–89.8; median for 38 CAD mice: 0.3 ng/ml, with 10th and 90th percentiles of 0 and 4.6, respectively, and a range of 0–18.8).

Serial measurements in male B6AF1 controls (n = 6) showed that the 12-h average GH concentration was similar during the day and at night (Fig. 1). Therefore, further serial data were all obtained during the day. The average GH concentration in control males was twice that in control females and that in CAD mice of either gender (P < 0.05; Table 1). The value of peak serum GH level, i.e., the 95th percentile of all (n = 328) uncensored GH levels in adult B6AF1 mice was 5 ng/ml. The frequency of peak GH levels in control males was more than twice that in the other three groups studied (χ² = 14.76 using segmentation modeling, adjusted P < 0.001; Fig. 1).

Under baseline conditions, levels of IGF and of IGF-BPs were independent of gender and of CA status (Table 2). A typical gel is shown in Fig. 2.

Effect of Moderate Acidosis on Growth Rate

We obtained 286 measurements of length in control males, 196 in control females, 142 in CAD males, and 108 in CAD females under baseline conditions. We obtained 346 measurements of weight in control males, 248 in control females, 178 in CAD males, and 114 in CAD females. The best fit of the growth curve for weight and length was obtained using the formula

weight = e^(a + b(age))

where a and b are constants that are specific for each group (Fig. 3). Both in controls and in CAD mice, rapid growth ended at ~84 days of age (12 wk) for length and after 120 days (17 wk) for weight. Length increased <5% (0.5 cm) after 12 wk of life, whereas weight increased by 15–30% (~2–5 g) between 12 and 18 wk. Almost all measurements of length (Fig. 3) and weight in CAD mice were >2 SD below the mean for B6AF1; this difference persisted into adulthood (data not shown).

Effect of Daily Administration of rGH on Growth

A total number of 82 CAD and 56 B6AF1 mice were entered into the study. Among CAD mice, 45 were eliminated because of heterozygosity (n = 36), sickness (n = 2 on placebo and 4 on rGH), renal failure (hydronephrosis, n = 1 on rGH), or loss (n = 2). Among B6AF1 mice, seven animals were eliminated because of sickness (n = 2 on saline and 3 on rGH) or loss (n = 2, Table 3). The final analysis included 37 CAD animals (80% of all randomized CAD mice) and 49 B6AF1 (87% of all randomized B6AF1 mice). Although the age when mice were killed was significantly greater in B6AF1 than in CAD animals, this small difference is physiologically insignificant.
In CAD mice, rGH resulted in a significant increase in ΔSD score, even in animals entered into the study after 6 wk of life (Fig. 4). In CAD mice on placebo, ANCOVA showed that final SD score depended on initial SD score \( (P = 0.033) \) and on gender (adjusted final SD score in females was 4.41 lower than that in males, 95% confidence interval (CI) 1.54–7.28, \( P < 0.005 \)). The administration of rGH increased final length SD score (adjusted for gender and for initial SD score at entry into the study) by 5.61 (95% CI 2.98–8.24, \( P < 0.001 \)) and final weight SD score by 2.97 (95% CI 1.87–4.06, \( P < 0.005 \)). The effect of rGH on final length and weight SD scores was independent of gender (data not shown).

Food intake at 11 wk was similar in rGH- and placebo-treated animals, whereas food efficiency in the rGH-treated group was three times as high as in the placebo group (Table 4). Blood pH and bicarbonate concentration at 12 wk of life in rGH-treated CAD mice were similar to CAD mice in the placebo group (Table 4) and to those reported in another study for CAD mice under baseline conditions (4). In addition, rGH increased plasma concentration of phosphate (Pi) from 7.2 ± 0.3 mg/dl, \( n = 8 \), to 8.7 ± 0.3 mg/dl, \( n = 9 \), \( P < 0.02 \), without affecting those of creatinine and calcium.

In contrast, in B6AF1 mice, rGH administration increased ΔSD score only when initiated by 6 wk of life (Fig. 4). ANCOVA showed that the administration of rGH increased final length SD score (adjusted for gender and for initial SD score at entry into the study) by 2.81 (95% CI 1.67–3.95, \( P < 0.001 \)) and final weight SD score by 2.36 (95% CI 1.55–3.17, \( P < 0.001 \)). At 11 wk of life, food intake and food efficiency were similar in rGH-treated and in saline-treated animals (data not shown).

The effect of rGH on ΔSD score was not less in CAD mice than in B6AF1 mice; in fact, it was significantly greater (Fig. 4). Linear growth during the last week of

### Table 1. GH levels in adult control and CAD mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>B6AF1</th>
<th>CAD</th>
<th>B6AF1</th>
<th>CAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH level, ng/ml</td>
<td>1.83 ± 0.23</td>
<td>0.94 ± 0.15</td>
<td>0.99 ± 0.16</td>
<td>0.95 ± 0.18</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 8 \) each group. ANOVA followed by Dunnett's T3.

### Table 2. Serum levels of IGFs and IGF-BPs in adult mice studied under baseline conditions

<table>
<thead>
<tr>
<th>Variable</th>
<th>B6AF1</th>
<th>CAD</th>
<th>B6AF1</th>
<th>CAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I, ng/ml</td>
<td>247 ± 27</td>
<td>208 ± 27</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>IGF-II, ng/ml</td>
<td>12.4 ± 1.2</td>
<td>17.4 ± 1.4</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>log IGF-BP2</td>
<td>−0.27 ± 0.08</td>
<td>−0.54 ± 0.21</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>log IGF-BP3</td>
<td>0.06 ± 0.15</td>
<td>−0.30 ± 0.16</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>log IGF-BP4</td>
<td>−0.87 ± 0.06</td>
<td>−0.86 ± 0.15</td>
<td>14</td>
<td>10</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 10 \) each group. ANOVA followed by Dunnett's T3.

### References

1. R123HORMONAL MECHANISMS OF GROWTH FAILURE IN ACIDOSIS by 10.220.32.246 on April 19, 2017 http://ajpregu.physiology.org/ Downloaded from

Fig. 1. Serial measurements of growth hormone (GH) levels in adult mice under baseline conditions. Plasma GH levels were obtained every other hour over a 2-day period in 16 male B6AF1 mice (A), 16 female B6AF1 mice (B), 8 male carbonic anhydrase II-deficient (CAD) mice (C), and 8 female CAD mice (D). Serial data from same animal appear as identical symbols. Frequency of peak GH levels (>5 ng/ml) between 8 AM and 7 PM in control mice (−2%) was more than twice that in the other animals studied (−2%). Bonferroni-adjusted \( P < 0.001 \), likelihood ratio \( \chi^2 \) analysis.
rGH administration was similar in B6AF1 mice and CAD mice: B6AF1 mice grew by 0.11 \pm 0.02 cm/wk (1.1 \pm 0.2%) and CAD mice by 0.13 \pm 0.03 cm/wk (1.4 \pm 0.3%). Neither CAD status nor administration of rGH or isotonic saline affected body proportions; indeed, the plot of weight vs. length was similar in the rGH-treated group, in the placebo group, and in animals studied under baseline conditions (Fig. 5).

ANOVA showed a significant interaction effect between treatment and CA status on IGF-I levels; these

Table 3. Randomized trial of GH: population studied

<table>
<thead>
<tr>
<th></th>
<th>B6AF1 Controls</th>
<th>CAD Colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. animals randomized</td>
<td>56</td>
<td>82</td>
</tr>
<tr>
<td>No. losses</td>
<td>7</td>
<td>45</td>
</tr>
<tr>
<td>No. in final analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rGH (males/females)</td>
<td>24 (12/12)</td>
<td>18 (10/8)</td>
</tr>
<tr>
<td>Placebo (males/females)</td>
<td>25 (12/13)</td>
<td>19 (10/9)</td>
</tr>
<tr>
<td>Age at entry, wk</td>
<td>5.5 \pm 0.2</td>
<td>4.9 \pm 0.3</td>
</tr>
<tr>
<td>Age when killed, wk</td>
<td>12.7 \pm 0.1</td>
<td>12.3 \pm 0.1*</td>
</tr>
</tbody>
</table>

Values are means \pm SE; rGH, recombinant growth hormone. *P < 0.05 vs. B6AF1 controls (2-tailed unpaired Student's t-test).

rGH administration was similar in B6AF1 mice and CAD mice: B6AF1 mice grew by 0.11 \pm 0.02 cm/wk (1.1 \pm 0.2%) and CAD mice by 0.13 \pm 0.03 cm/wk (1.4 \pm 0.3%). Neither CAD status nor administration of rGH or isotonic saline affected body proportions; indeed, the plot of weight vs. length was similar in the rGH-treated group, in the placebo group, and in animals studied under baseline conditions (Fig. 5).

ANOVA showed a significant interaction effect between treatment and CA status on IGF-I levels; these

Fig. 2. Western ligand blot for insulin-like growth factor-binding protein (IGF-BP) in adult mice studied under baseline conditions. A: B6AF1 male mice. B: B6AF1 female mice. C: CAD male mice. D: CAD female mice. Blood was collected when the mice were killed, and plasma was kept frozen until analysis. Electrophoresis was done at 125 V for 2 h on a 12% Tris-glycine-SDS-PAGE, using 0.5 µl of plasma/lane. Transfer was done at 75 A for 60 min, using nitrocellulose membranes with 0.45-µm pores. Membranes were dried, washed, and incubated overnight at 4°C with 1 \times 10^6 counts/min ^{125}\text{I}-IGF. Membranes were then washed, dried, and exposed to Reflection NEF Autoradiography Film Du Pont (Boston, MA) at \(22\)°C for 2 days. We observed 3 discrete bands migrating, respectively, at \(22\) kDa (IGF-BP4), \(32–29\) kDa (presumably IGF-BP2), and \(40\) kDa (IGF-BP3). Densitometric analysis took into account interassay variability in background and in contrast (using the molecular weight markers, not shown). The figure shows a large variability in densitometry of the bands, in both B6AF1 and CAD mice.

Fig. 3. Linear growth rate in B6AF1 and in CAD male (A) and female (B) mice under baseline conditions. Lines represent smoothed curves (means \pm 2SD) for B6AF1 mice. Each dot represents single measurement in CAD mouse. CAD was associated with slow rate of growth and smaller size at 12 wk of life.

Fig. 4. Effect of recombinant GH (rGH) on length (A) and weight (B). Each bar represents change in SD score (ΔSD score), i.e., average difference between final (i.e., at end of study) and initial (i.e., at time of randomization) SD score; line represents SE. In CAD mice administration of rGH increased ΔSD score compared with placebo, even in animals entered into study after 6 wk of life. In contrast, in B6AF1 mice, administration of rGH significantly affected ΔSD score compared with placebo only in animals entered into study by 6 wk of life. Among animals treated with rGH, values of ΔSD score observed in CAD mice were higher than those in B6AF1 mice. *Significantly different from placebo (P < 0.05).
Table 4. Randomized trial of GH: physiological variables in CAD mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo</th>
<th>rGH</th>
<th>n</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pH</td>
<td>7.26 ± 0.03</td>
<td>8</td>
<td>7.30 ± 0.10</td>
<td>5</td>
</tr>
<tr>
<td>Blood bicarbonate concentration, mM</td>
<td>16.8 ± 1.6</td>
<td>8</td>
<td>18.2 ± 2.1</td>
<td>5</td>
</tr>
<tr>
<td>Food intake, g food·g body wt⁻¹·wk⁻¹</td>
<td>1.3 ± 0.1</td>
<td>6</td>
<td>1.4 ± 0.4</td>
<td>6</td>
</tr>
<tr>
<td>Food efficiency, g wt gain/100 g of food</td>
<td>1.7 ± 0.8</td>
<td>6</td>
<td>5.7 ± 2.7</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. animals. Food intake and food efficiency were measured at 11 wk of life. Blood pH and bicarbonate concentration were obtained when mice were killed at 12 wk of life. *Two-tailed unpaired Student’s t-test.

DISCUSSION

Baseline serial measurements of serum GH are in agreement with data in human and in rat, which show higher and more frequent peaks in males than in females (14, 19, 34). Data in the rat have shown that growth is primarily stimulated by GH pulses; this could account in part for growth differences between genders in that species (19). The hormonal changes observed in CAD mice differ from those observed in uremic patients, in whom a decreased IGF-I-to-IGF-BP3 ratio often results from an increase in IGF-BP3 concentration (3). Our results in this model of moderate CMA in the mouse also differ from those in rats with severe CMA, in which food intake is severely decreased and GH, IGF, and liver receptors are downregulated.

In the present study we showed that moderate CMA in CAD mice did not decrease the effect of rGH administration on linear growth and weight gain. Because of the study design (inclusion of heterozygous animals in the randomization) and of the characteristics of the major outcome variable (i.e., length at 12 wk of life), final analysis could only be done on animals completing the study, possibly leading to bias. Nevertheless, 80% of randomized CAD mice and 87% of B6AF1 mice completed the study. Furthermore, there was no significant difference in the number of dropouts between animals randomized to rGH and those randomized to placebo.

The effect of rGH on growth in acidotic mice was mediated at least in part by an increase in serum IGF-I levels and in food efficiency and was not associated with any changes in food intake, in blood pH, or in serum concentrations of IGF-BPs. The slight increase in phosphatemia in rGH-treated CAD mice in the absence of changes in plasma creatinine or calcium concentration might result from an increase in tubular reabsorption of Pi and in serum levels of 1,25-(OH)₂D₃ (9, 38).

In contrast with the present data, previous studies in rats with uremic or NH₄Cl-induced acidosis did not show any effect of rGH on growth (23). This discrepancy may have resulted from several differences in design. First, the type of acid-base disturbance was not identical: although blood pH was similar in both studies, bicarbonate concentration was 17 mM in CAD mice compared with 12 mM in acidic rats, consistent with a compensated metabolic acidosis. Second, food intake per gram of body weight in CAD mice was >80% as much as controls, compared with 60% as much as controls in acidic rats. Third, the dose of rGH used in the study in acidic rats was ~35 times as high as the dose use in the present study, i.e., 10 U·kg⁻¹·day⁻¹; we had selected the minimum dose that according to Mehls et al. (33) yielded the best increase in linear growth in uremic rats. This dose in rats is still higher than that recommended in humans (0.3–1.0·kg⁻¹·wk⁻¹). Fourth, the rates of growth (expressed in g/wk or cm/wk) in the various groups of rats were not adjusted for age or for size at initiation of the study. In contrast, our multivariate analysis allowed us to adjust growth rate for differences in age and in size at the time of entry into the study. In addition, our design allowed us to correct for possible overestimation of the effect of rGH due to early malnutrition (41). Finally, the duration of rGH administration in rats in previous studies was 2 wk, compared with an average duration of 7 wk (range 4–9 wk) in the present study. This allowed us to use as main outcome variable the length at the end of the rapid linear growth phase, rather than the rate of growth, which was used in previous studies in rats. Studies in humans have shown that the rate of growth may not
accurately predict final size (1). Our data show that the rate of growth in mice under baseline conditions decreases progressively with age and that their response to rGH depends on the age of initiation, the duration of the treatment, or both.

We considered the possibility that the difference in response to rGH observed in CAD and genetically normal mice > 6 wk old at the time of entry into the study could have resulted in part from a delay in puberty associated with CAD. Indeed, in our colony, pregnancies were observed as early as 10 wk of age in genetically normal B6AF1 mice, but only after 14 wk in CAD mice. Nevertheless, by 12 wk of life, the rate of linear growth had already slowed down considerably in animals under baseline conditions (see Fig. 3), as well as in those receiving rGH, as shown by the slow rate of growth during the last week of treatment (see RESULTS).

We have previously shown that CAD mice present with distal RTA. The latter was shown by the association of CMA with normal plasma creatinine concentration, a urine pH > 7.5 vs. < 7.5 in controls, negative values for titratable acid excretion and for net acid excretion, and limitation of the increase in NH₄⁺ excretion in response to acidosis (4); an additional proximal component could be shown in some animals. Whereas rGH has been reported to stimulate urinary acidification, specifically NH₄⁺ secretion, and partially to correct CMA induced by NH₄Cl in adult humans (39), we did not observe any improvement of the acid-base status in CAD mice injected with rGH, presumably because of their distal RTA. This observation allowed us to show that the effect of rGH on growth in CAD mice was observed in the absence of correction of the acidosis.

In summary, growth failure in CAD mice is associated with chronic acidosis and adequate nutrition and may result in part from a decrease in pulsatile GH secretion in males. In CAD mice, chronic acidosis does not impair the growth response to prolonged administration of rGH; the effect of rGH on growth appears to be mediated by an increase in serum IGF-I levels and by an increase in food efficiency.

### Perspectives

Growth failure in CAD mice is associated with low serum levels of GH in adult males but normal levels of IGF-I, IGF-II, and IGF-BPs in adult animals of both genders. These data do not exclude the possibility of changes in serum levels of IGFs or IGF-BPs during the growth period.

Analysis of our data and of previous literature suggests that the presence of moderate chronic acidosis with normal food intake, in contrast with severe acidosis with food deprivation, does not prevent the effect of rGH on growth. Nevertheless, we do not advocate the use of rGH in all acidotic patients with growth retardation, because the rate of growth in many of these patients improves in response to correction of the acidosis and subsequent increase in caloric intake. Randomized studies are needed to assess whether acidosis affects the response to rGH administration in patients with renal failure and renal tubular dysfunction and in patients with end-stage renal disease.

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Address for reprint requests: L. P. Brion, Albert Einstein College of Medicine and Montefiore Medical Center, Weiler Hospital, Rm. 725, 1825 Eastchester Rd, Bronx, NY 10461.

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### Table 5. Serum levels of IGFs and IGF-BPs at 12 wk of life in randomized mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Saline</th>
<th>rGH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B6AF1</td>
<td>CAD</td>
</tr>
<tr>
<td>IGF-1, ng/ml</td>
<td>330 ± 56</td>
<td>11</td>
</tr>
<tr>
<td>IGF-2, ng/ml</td>
<td>14.6 ± 5.8</td>
<td>11</td>
</tr>
<tr>
<td>IGF-BP2</td>
<td>0.13 ± 0.06</td>
<td>11</td>
</tr>
<tr>
<td>IGF-BP3</td>
<td>0.50 ± 0.44</td>
<td>11</td>
</tr>
<tr>
<td>IGF-BP4</td>
<td>-0.54 ± 0.51</td>
<td>11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1, ng/ml</td>
<td>314 ± 58</td>
<td>10</td>
</tr>
<tr>
<td>IGF-2, ng/ml</td>
<td>15.3 ± 3.4</td>
<td>10</td>
</tr>
<tr>
<td>IGF-BP2</td>
<td>-0.06 ± 0.82</td>
<td>10</td>
</tr>
<tr>
<td>IGF-BP3</td>
<td>0.24 ± 0.80</td>
<td>10</td>
</tr>
<tr>
<td>IGF-BP4</td>
<td>-0.57 ± 0.61</td>
<td>10</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. animals. Statistical analysis, done by 3-way ANOVA, showed significant (P < 0.05) effect of gender on serum levels of IGF-1 and IGF-2; a significant effect of CA status on IGF-1, IGF-BP2, IGF-BP3, and IGF-BP4, a significant interaction between treatment and CA status on serum levels of IGF-1, and a significant interaction between treatment, CA status and gender on serum levels of IGF-2.
REFERENCES


2. Bailлер, P. E., M. A. Mcnulran, H. N. Hulter, S. E. Ander-


9. Cammer, W., and H. Zhang. Comparison of immunocyto-


23. Kleinkecht, C., S. Maniar, X. Zhou, V. Motel, D. Deau-


29. McDowell, R. S., K. A. Elbias, M. S. Stanley, D. J. Burdick, J. P. Burnier, K. S. Chan, W. J. Fairbrother, R. G. Ham-


31. McSherry, E. J., M. Weberman, S. Kaplan, and M. Grum-


