Chronic upper airway resistive loading induces growth retardation via the GH/IGF-I axis in prepubescent rats

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Chronic upper airway resistive loading induces growth retardation via the GH/IGF-I axis in prepubescent rats. J Appl Physiol 102: 913–918, 2007. First published November 30, 2006; doi:10.1152/japplphysiol.00838.2006.—The effect of upper airway loading on longitudinal bone growth and various components of the growth hormone (GH)/insulin-like growth factor I (IGF-I) axis has not been fully elucidated. In the present study, the effect of chronic resistive airway loading (CAL) in a prepubescent rat model on linear bone growth and weight gain was investigated. We hypothesize that CAL induced in prepubescent rats will lead to impaired longitudinal growth due to impairment in circulating and liver GH/IGF-I parameters. The tracheae of 22-day-old rats were obstructed by tracheal banding to increase inspiratory esophageal pressure. The GH/IGF-I markers were analyzed using ELISA, RT-PCR, and Western immunoblot analysis 14 days after surgery. Animals exhibited impaired longitudinal growth as demonstrated by reduction of tibia and tail length gains by 40% (P < 0.0001) and body weight gain by 24% (P < 0.0001). No differences were seen in total body energy balance, i.e., oxygen consumption, daily food intake, or arterial blood gases. Circulating GH, IGF-I, and IGF binding protein-3 (IGFBP-3) levels were reduced by 40% (P = 0.037), 30% (P < 0.006), and 27% (P = 0.02), respectively, in the CAL group. Liver IGF-I mRNA level decreased by 20% (P < 0.0002), whereas GH receptor mRNA and protein expression were unchanged. We conclude that impaired longitudinal growth in prepubescent CAL rats is related to a decrease in GH, IGF-I, and IGFBP-3 levels.

Animal Preparation

Our laboratory has previously used a surgical technique to induce CAL (12, 13, 27, 35) in 22-day-old prepubertal Sprague-Dawley rats. The study was approved by the Ben-Gurion University of the Negev Animal Use and Care Committee and complied with the American Physiological Society guidelines. For all surgical procedures, animals were anesthetized with tribromoethanol (200 mg/kg ip). Sham surgery performed on the control group consisted of tracheal dissection without placement of the tracheal band. In the experimental group, increased tracheal resistance was imposed by a surgical technique for placement of a circumferential tracheal band. Supplemental oxygen was supplied via a cannula placed in the upper airway to prevent hypoxemia during surgery. A midline ventral cervical incision was made, and the trachea was exposed and dissected so as not to damage adjacent structures. A circumferential plastic band 0.5 cm long was placed around the trachea. A suture was looped around the band and tightened, thus constricting the trachea so as to increase inspiratory esophageal pressure swings two- to threefold. The suture was then tied and the wound bathed with penicillin G solution; an intramuscular injection of penicillin G was given in the hindlimb. The skin incision was sutured, and the animals were returned to their cages for recovery and given food and water ad libitum, under standard laboratory conditions (12:12-h light-dark cycle, lights on at 0600).

Respiratory Parameters and Food Intake

Inspiratory swings in pleural pressure and respiratory rate were measured before and immediately after CAL surgery, while animals were still anesthetized (tribromoethanol, 200 mg/kg ip). (35) Fifteen breaths were used to analyze inspiratory swings of pleural pressure and respiratory rate before and after surgery. Pleural pressure, as approximated by esophageal pressure, was measured by means of a saline-filled catheter placed in the lower one-third of the esophagus and connected to a pressure transducer. Our laboratory has previously demonstrated that tracheal resistance is approximately three times greater in the obstructed group than in the sham-operated control animals (12).

Total Body Energy Balance

Food intake and total body oxygen consumption (V\(\text{O}_{2}\)) were assessed (12). Food intake (energy supply) was assessed on days 12 and

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13 post surgery. Food intake was determined by first cleaning the cages and changing the bedding. Animals were housed individually in cages and given 30 g/day of standard rodent chow (Harlan, Jerusalem, Israel). The water bottle for each cage was kept full. Food placed into the feeder at the beginning and any left over at the end of each 24-h period were weighed. Any visible food in the cage was scavenged and included in the measurements. \( V_{O2} \) consumption (energy demand) was measured using an open system. Animals were placed in a 350-ml Lucite container through which airﬂow of 350 ml/min was directed. This ﬂow is more than 10-fold greater than the normal ventilation of 22- to 36-day-old rats. Measurements on day 14 were obtained following acclimatization sessions of 30 min between 1000 and 1300 for 3 days. The outﬂow was directed through a water vapor datering chamber and vented through a T-shaped connector to an oxygen analyzer (Maxtec Oxygen Analyzer, Salt Lake City, UT). The rats were allowed to acclimate to their surroundings for 30 min before \( V_{O2} \) measurements were taken. Two Lucite chambers were used in parallel, one for the control and the other for CAL animals. All measurements were taken with the animals awake and quietly resting in the chambers, as assessed by observation; laboratory temperature was maintained at 24.5°C (SD 0.5) during all measurements. Food and water were not available during measurement.

Longitudinal Growth

Somatic growth was measured before and at day 14 of observation by measuring body weight and tibia and tail lengths, using a digital caliper as previously described by our group. (31, 35).

At death 14 days postsurgery (between 1000 and 1200), animals were decapitated, and serum was separated and frozen at \(-20°C\) for later measurements of GH, IGF-I, IGFBP-3, and lactate. (6) The serum was also used to determine glucose, total protein, albumin, and cholesterol levels. In a subset of five CAL and five control animals, arterial blood gases (\(pH, PCO2, PO2,\) and HCO\(_3^{-}\)) were determined 14 days after surgery. In this subset group, we also measured inspiratory swings of pleural pressure and respiratory rate before and immediately after surgery, while animals were still anesthetized. The livers were carefully removed, immediately frozen in liquid nitrogen, and used for mRNA and protein level determinations.

Immunoassays

Serum GH determination. Serum GH was measured by ELISA kit (Diagnostic Systems Laboratories, Webster, TX). The sensitivity was 0.13 ng/ml.

Serum IGF-I determination. Plasma concentration of rat IGF-I was measured by enzyme immunoassay (Diagnostic Systems Laboratories). The sensitivity was 30 ng/ml; the intra-assay coefficient of variation for sera with IGF-I levels of 171, 446, and 1,352 ng/ml were 7.8, 9.1, and 5.3%, respectively.

Serum IGFBP-3 determination. IGFBP-3, which binds \(95\)% of the IGF-I in serum, was measured by ELISA kit (Diagnostic Systems Laboratories). The sensitivity was 0.04 ng/ml; the intra-assay coefficient of variation for sera with IGFBP-3 levels of 4.6, 27.4, and 74.4 ng/ml were 9.6, 9.5, and 7.3%, respectively.

mRNA Studies and Western Immunoblot Analysis

Total RNA was prepared from frozen tissues by the TriReagent method (Molecular Research Center, Cincinnati, OH). Liver IGF-I, GHR, and IGF binding protein-1 (IGFBP-1) mRNA were determined by reverse transcription-polymerase chain reaction (RT-PCR) method as previously described (18). PCR products were quantitated densitometrically using Fluorchem software (Alpha-Innotech, San Leandro, CA). To correct for differences in loading, we corrected densitometric values of IGF-I, GHR, and IGFBP-1 cDNAs with corresponding values of GAPDH cDNA, and the GHR/GAPDH, IGF-I/GAPDH, and IGFBP-1/GAPDH ratios were calculated.

Protein analysis was determined by Western immunoblot method as previously described (18). Protein expression was quantitated densitometrically using Fluorchem software.

Data Analysis

Data were compiled and tested for normal distribution (Kolmogorov-Smirnov test) and expressed as means (SD). Signiﬁcance between these variables was analyzed by \(t\)-test for independent groups. Differences in body weight over 14 days of observation were evaluated by two-way ANOVA followed by a post hoc analysis (Newman-Keuls) to determine the source of signiﬁcance. Null hypotheses were rejected at the 5% level.

RESULTS

During the surgical procedure, the mortality rate of the CAL group was 10%, and an additional 10% mortality was observed 2–5 days after surgery. A total of 50 animals were included in this study; the ﬁnal numbers of animals were 22 and 21 for the CAL and sham control groups, respectively.

As expected, immediately following CAL, inspiratory swings in esophageal pressure increased and respiratory rate decreased signiﬁcantly (Table 1). Thus the measured changes in inspiratory swings in esophageal pressure and respiratory rate indicate that resistive loading had been produced. CAL animals all demonstrated audible wheezing, especially after activity, but no signs of gasping were observed. Arterial blood gases obtained 14 days after surgery in CAL and control animals were similar and within normal physiological range (Table 2). Hemoglobin and lactate levels were unchanged between the groups [11.3 g/dl (SD 1.0) vs. 11.1 g/dl (SD 0.5) \((P = 0.5)\) and 2.9 mM (SD 0.37) vs. 3.3 mM (SD 0.78) \((P = 0.31)\) in the control and CAL groups, respectively].

Measurements of \(V_{O2}\) were taken when all animals were awake and quietly resting. Fourteen days postsurgery, \(V_{O2}\) was 38.9 ml-kg\(^{-1}\)-min\(^{-1}\) (SD 5.4) and 37.6 ml-kg\(^{-1}\)-min\(^{-1}\) (SD 7.2) in the control and CAL animals, respectively \((P = 0.8)\). Daily food intake expressed as grams of food per kilogram of body weight is presented in Fig. 1B. Fourteen days postsurgery, food intake was 130.6 g food-kg\(^{-1}\)-day\(^{-1}\) (SD 18.3) and 138.8 g food-kg\(^{-1}\)-day\(^{-1}\) (SD 10.7) in the control and CAL animals, respectively \((P = 0.7)\). The \(V_{O2}\)-to-food intake ratio (Fig. 1C) was 0.28 ml-min\(^{-1}\)-g food\(^{-1}\) (SD 0.05) and 0.29 ml-min\(^{-1}\)-g food\(^{-1}\) (SD 0.07) in the control and CAL animals, respectively \((P = 0.8)\). No statistical significance were found in serum biochemistry between CAL and control groups, i.e., glucose, total protein, albumin, and cholesterol levels [179.5 mg/ml (SD 33.3) vs. 165.4 mg/ml (SD 17.6), 4.7 g/dl (SD 0.3) vs. 4.5 g/dl (SD 0.4), 2.8 g/dl (SD 0.1)

<table>
<thead>
<tr>
<th>Table 1. Respiratory effort</th>
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<tr>
<td>Group</td>
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<tr>
<td>Sham controls ((n = 12))</td>
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<tr>
<td>Airway loading ((n = 11))</td>
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<tr>
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<td>Airway loading ((n = 11))</td>
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Values are means (SD); \(n\), no. of rats. \(\Delta\)PES, inspiratory swings in esophageal pressure.
vs. 2.7 g/dl (SD 0.2), 99.5 mg/dl (SD 12.1) vs. 105.5 g/dl (SD 15.0), respectively.

Both CAL and control groups had similar body weight before surgery [55 g (SD 8) and 56 g (SD 5), respectively; P = 0.99]. Fourteen days postsurgery, CAL and control group body weights were statistically different [105 g (SD 23) and 138 g (SD 18), respectively; P < 0.0001]. Differences between the growth curves of CAL and sham controls are significant (P < 0.0001) at all time intervals after surgery (Fig. 2; 2-way ANOVA). Before surgery, the tail lengths of the CAL and sham control animals were not statistically different [90.7 mm (SD 9.0) and 88.5 mm (SD 10.2), respectively; P = 0.52], and tibia lengths were 30.7 mm (SD 1.6) and 30.9 mm (SD 1.9), respectively (P = 0.92). Fourteen days postsurgery, both tibia and tail length gains were 40% less (P < 0.0001) in CAL animals compared with controls (Fig. 3).

Serum GH levels (Fig. 4A) showed a 40% difference between CAL and sham control animals [125.2 ng/ml (SD 30.0) vs. 207.2 ng/ml (SD 18.4), respectively; P = 0.037]. Serum IGF-I (Fig. 4B) levels showed a 30% difference between CAL animals and sham controls [979 ng/dl (SD 98) in controls vs. 1,406 ng/ml (SD 89) in CAL; P = 0.006]. IGFBP-3 levels showed a 27% difference between CAL animals and sham controls [157 ng/ml (SD 9) in controls vs. 200 ng/ml (SD 14) in CAL; P = 0.025]. Liver IGF-I mRNA levels were decreased by 20% in CAL animals (Fig. 4, C and D) compared with the sham controls (P < 0.0002). CAL does not impair the synthe-

Table 2. Arterial blood-gas tensions 14 days postsurgery

<table>
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<th>Sham Controls (n = 5)</th>
<th>Airway Loading (n = 5)</th>
<th>P Value</th>
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</thead>
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<tr>
<td>Arterial PO2, Torr</td>
<td>90.7 (14.3)</td>
<td>87.9 (15.2)</td>
<td>0.79</td>
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<tr>
<td>Arterial PCO2, Torr</td>
<td>38.8 (3.3)</td>
<td>42.8 (3.1)</td>
<td>0.1</td>
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<tr>
<td>Arterial pH, units</td>
<td>7.41 (0.01)</td>
<td>7.39 (0.05)</td>
<td>0.58</td>
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<tr>
<td>Arterial HCO3, meq/l</td>
<td>25.2 (2.8)</td>
<td>26.1 (1.6)</td>
<td>0.54</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>11.3 (1.0)</td>
<td>11.1 (0.5)</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Values are means (SD); n, no. of rats. Data were collected on day 14 postsurgery. HCO3, calculated arterial bicarbonate.

Fig. 1. Total body energy balance. A: total body oxygen consumption (V̇O2). B: daily food intake (standard rat chow). C: ratio of energy expenditure (V̇O2) to food intake. No significant changes were noted between control and chronic resistive airway loading (CAL) groups in daily food intake, V̇O2, or ratio of V̇O2 to food intake. Data were obtained from 36-day-old rats, 14 days postsurgery. Bars are means (SD).

Fig. 3. Somatic growth in sham control (n = 16) and CAL (n = 17) rats. Tibia (A) and tail (B) length gains 14 days postsurgery are shown relative to presurgery values. Bars are means (SD). *P < 0.0001, unpaired t-test.

Fig. 2. Growth curves of sham control (n = 16) and CAL (n = 17) rats. Surgery was performed on 22-day-old rats. The growth curve of the obstructed rats (CAL) was significantly less than that of the controls over the duration of the observation period. Error bars represent SD. *P < 0.0001, by 2-way analysis of variance.
sis of liver GHR (Fig. 5); liver GHR mRNA analyzed by RT-PCR and protein level analyzed by Western blot analysis were found to be unchanged between the groups. The CAL animals compared with controls showed no difference in liver IGFBP-1 mRNA levels [3.2 units (SD 0.4) and 3.4 units (SD 0.1), respectively; \( P < 0.54 \)]. A similar pattern was observed for liver IGFBP-1 protein content [0.8 unit (SD 0.15) and 0.9 unit (SD 0.05) in CAL animals vs. controls, respectively; \( P = 0.71 \)].

**DISCUSSION**

In the present study, upper airway obstruction caused a significant decrease in weight gain and bone and tail longitudinal growth in prepubertal CAL rats. Upper airway obstruction was associated with a significant decrease in serum GH, IGF-I, and IGFBP-3 levels and in liver IGF-I gene expression. In addition, no change was found in liver GHR and IGFBP-1 levels.

**The Experimental Model**

The CAL rat model was previously used to investigate adaptive changes in respiratory system function. (12, 13, 24, 26, 27, 34, 35). Similar to adult CAL rats (12, 13, 27), our animals did not become hypoxic. Immediately following surgery, inspiratory swings in esophageal pressure increased and respiratory rate decreased, similar to the adult CAL rat model. Thus resistive loading had been produced (12, 13, 27, 34, 35). Previously, it was demonstrated that CAL led to decreased respiratory rate and plethysmography inspiratory effort (index for tidal volume) accompanied with elevation of arterial Pco2 (12, 13). The unchanged arterial Pco2 and reduced respiratory rate in our study suggest that the trachea was less severely obstructed, and animals were able to maintain their ventilation by increasing the tidal volume. We recognize that our CAL model is not a model of sleep-disordered breathing or chronic obstructive pulmonary disease. In addition, we also recognize that the respiratory changes following CAL are not sleep related. The increased swings in esophageal pressure in the absence of gas-exchange abnormalities seen in our animals may be relevant for a variety of respiratory disorders in children, including tracheomalacia, tracheal stenosis, and upper airway resistance syndrome (i.e., prolonged large swings in esophageal pressure in the absence of frank apnea, hypopnea, or gas exchange abnormalities (1, 14)). Finally, the fact that \( \text{V}\text{O}_2\) during quiet wakefulness did not change should be interpreted with caution. \( \text{V}\text{O}_2\) measurement during quiet wakefulness may not accurately reflect total body energy demand. It is conceivable that rats with CAL have decreased total sleep time or impaired sleep architecture. An increase in wakefulness and superficial sleep during the 24-h period may increase energy expenditure and lead to a negative energy balance. Further studies are needed to explore the effects of CAL on \( \text{V}\text{O}_2\) during periods of sleep and activity.

Somatic growth was assessed in this study by measuring tibia and tail length gains, which better reflect linear growth (31). In adult rats, it was not surprising that no significant effect on body, tibia, or tail length gain was noted, because at this age somatic growth is complete and animals at this stage of development are only gaining body weight (35).

Hypoxia can serve as a potential trigger of reduced dietary intake (8, 15, 30) and decreased circulating IGF-I and IGFBP-3 (5, 16, 21, 22). Total body energy balance (\( \text{V}\text{O}_2/\text{daily food intake} \)), hemoglobin and lactate levels, and arterial oxygenation were similar to those of the control group, within normal physiological range (12, 27). However, when severe upper airway loading was induced, adult rats exhibited a considerable decrease in body weight immediately after surgery that was
related to reduction of food and water intake and hypoxemia (23). Hypoxia plays an important role in mediating IGFBP-1 levels, leading to a considerable increase (29). We found no evidence for increased liver IGFBP-1 gene expression or protein levels in our CAL animals. Thus changes in IGF-I and IGFBP-3 levels are unlikely to be related to hypoxemia.

GH is secreted in a series of pulses throughout 24 h; within the usual nighttime sleep and daytime wake routine, a major pulse occurs shortly after sleep onset in temporal association with slow-wave sleep (10, 17, 28). The classic endocrine effect of pituitary-secreted GH is the induction of IGF-I and IGFBP-3 synthesis. IGFBP-3 is the predominant IGF binding protein in circulation and plays an important role in the prolongation of the plasma half-life of IGF-I. IGF-I and IGFBP-3 levels are both highly correlated with the 24-h mean GH levels (3, 7). It is accepted (11) that global reduction of GH level will result in reduction of both serum and organ IGF-I and IGFBP-3 content. In our study, GH, IGF-I, and IGFBP-3 circulating levels were all significantly reduced in CAL animals. Thus our data suggest that the growth retardation observed in our CAL model is compatible with global reduction of GH, resulting in reduction of IGF-I and IGFBP-3 expression. Because the liver is a main organ for IGF-I synthesis, our data suggest that impairment of GH secretion from the anterior pituitary somatotrophic cells and impairment of the liver GH/IGF-I axis are involved in longitudinal growth failure in our animals. Recently, it was demonstrated that GH receptor antagonist pegvisomant induced general GH deficiency in wild-type mice, leading to reduced IGF-I and IGFBP-3, and severe growth deficit (19). Nevertheless, our data should be interpreted with caution. The liver IGF-I-deficient mice and acid-labile-subunit mouse models clearly exhibited normal growth, despite having >65% reduction in serum IGF level. This may suggest that the involvement of liver IGF-I in this longitudinal growth failure is controversial (36). Growth may also be mediated by the autocrine/paracrine actions of IGF-I, as well as by some nonhepatic sources of circulating IGF-I.

What could lead to a global reduction of GH secretion in our CAL model? The plasma concentration of many hormones displays sleep-related variations, suggesting that sleep influences hormone secretion. Slow-wave sleep function is critical for the neuroendocrine consequences of anabolism, GH secretion, and maintenance of growth (28). We assume that alterations in sleep function, mainly reduction of slow wave sleep function, as a result of CAL, lead to suppression of global GH level. Indeed, partially sleep-deprived rats lose body weight, despite increased food intake, due to elimination of GH pulses during slow-wave sleep and suppression of global daily GH level. (4, 10) Although sleep-disordered breathing (including upper airway resistance syndrome) in children occurs primarily during rapid-eye movement, (33) the reduction of serum IGF-I (2, 37) was postulated to be related to shortening of slow wave sleep duration (2, 20). Significant increases in serum IGF-I levels and body weight were noted following adenotonsillectomy (2). Further studies are needed to localize the effects of CAL on longitudinal bone growth and on global GH/IGF-I parameters during the sleep-wake cycle.

Finally, growth may be indirectly affected following increased expression of oxygen-derived free radicals and cytokines as a result of respiratory muscles’ strenuous contraction (26, 32, 38). Muscle strenuous contraction induces protein loss and cachexia (25). However, we found no evidence to support respiratory muscle overloading (as discussed above), which could lead to increased expression of muscle cytokines. No change in serum pro-inflammatory factors, such as IL-6 and TNF-α in adult CAL rats, were found (35). Further studies are needed to determine whether CAL induces oxidative stress and activation of pro-inflammatory processes in respiratory muscles.

GRANTS
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REFERENCES


