Endocrine, *vol. 16, no. 2, 139–143, November 2001* 0969–711X/01/16:139–143/$11.25

Growth Hormone Therapy During Neonatal Hypoxia in Rats

Body Composition, Bone Mineral Density, and Insulin-like Growth Factor-1 Expression

Hershel Raff,[1,3] Eric D. Bruder,[1] Barbara Jankowski,[1] Martin K. Oaks,[2] and Ricki J. Colman[4]

[1]Endocrine and [2]Transplant Research Laboratories, St. Luke's Medical Center, Milwaukee, WI; [3]Department of Medicine, Medical College of Wisconsin, Milwaukee, WI; and [4]Wisconsin Regional Primate Research Center, University of Wisconsin, Madison, WI

Hypoxia from birth results in a decrease in body weight gain, body size, and bone mineral density (BMD). The purpose of the present study was to determine whether short-term administration of growth hormone (GH) (rat GH; 100 µg/d) could attenuate some of these effects of neonatal hypoxia. Rat pups (with their lactating dams) were exposed to hypoxia (vs normoxic control) from birth. Hypoxia was continued until 14 d of age, with rat GH (vs vehicle control) administered daily. Hypoxia significantly inhibited body weight gain; GH therapy did not reverse this effect. GH therapy did reverse the inhibitory effect of hypoxia on tail length but not on body length. Hypoxia decreased BMD analyzed by dual X-ray absorptiometry (DXA); this effect was not reversed by GH therapy. Both GH therapy and hypoxia decreased the percentage of body fat analyzed by DXA, the effects of which were additive when combined. There were minimal effects of hypoxia and GH therapy on plasma insulin-like growth factor-1 (IGF-1), IGF-binding protein-3, and hepatic IGF-1 mRNA expression. We conclude that some of the effects of hypoxia on body habitus are reversed by GH therapy, but that short-term GH therapy did not prevent a loss of BMD. GH therapy for more than 14 days may be necessary to appreciate fully its potential in the treatment of the sequelae of neonatal hypoxia.

Key Words: Lean body mass; somatotropin; growth rate; insulin; insulin-like growth factor; hypoxia.

Introduction

Chronic hypoxia results in significant weight loss in humans and a significant decrease in weight gain in growing rats *(1–4)*. This effect occurs from the neonatal through the adult rat. It is probably owing to the profound anorexia that accompanies hypoxia, although changes in metabolism may be contributory *(1,4–9)*.

We previously demonstrated that neonatal hypoxia from birth resulted in a significant decrease in body weight, body fat, and bone mineral density (BMD) in rat pups *(8)*. This could have been the result of an alteration in lipid and/or glucose metabolism *(2,6–10)*; a decrease in pup food intake; or a decrease in maternal food intake; since maternal food restriction or malnutrition can decrease body weight in the neonatal rat *(11–13)*.

A recent study suggested that growth hormone (GH) therapy could reverse a portion of the hypoxia-induced growth retardation in the newborn rat *(11)*. We hypothesized, then, that GH therapy could alter the effect of hypoxia on body composition and weight gain in neonatal rats. Since GH therapy may be useful in human infants during recovery from hypoxia, we were interested in the effectiveness of GH given during vs after hypoxic exposure.

Therefore, the purpose of the present study was to analyze the effects of GH therapy on changes in body weight, BMD, body composition, organ weights, insulin-like growth factor-1 (IGF-1) expression, and IGF-binding proteins (IGFBPs) in rats exposed to hypoxia from birth to 14 d of age.

Results

Table 1 shows body habitus and organ weights (normalized to body wt) of 14-d-old rats exposed to normoxia or hypoxia and treated with vehicle or rat GH from birth to 14 d of age. Exposure to hypoxia resulted in a significant decrease in body length (nose to tail base [anus]). Daily GH treatment resulted in significant increases in both tail length and the ratio of tail length to body length in normoxic and hypoxic pups. Therefore, administration of GH to hypoxic pups restored tail length to values similar to those of normoxic controls. When normalized to body weight, liver weight was unaffected by hypoxia or GH and, if anything, was increased when hypoxia and GH were combined. The only significant effect on kidney weight after normalization to body weight was a small increase with combined hypoxia and GH. Hypoxia resulted in a large increase in heart weight (normalized to body weight) in rats; this effect was not altered by administration of GH. Both hypoxia and

Received September 27, 2001; Revised October 31, 2001; Accepted November 7, 2001.

Author to whom all correspondence and reprint requests should be addressed: Dr. Hershel Raff, Endocrinology and Diabetes, St. Luke's Physician's Office Building Suite 245, 2801 W KK River Parkway, Milwaukee, WI 53215. E-mail: hraff@mcw.edu

Table 1
Body Habitus and Organ Weights of 14-d-Old Rats Exposed to Normoxia (N) or Hypoxia (H) from Birth to 14 d of Age and Treated with Vehicle (V) or Rat GH Subcutaneously (100 µg/d) from Birth to 14 d of Age[a]

	N-T (cm)	TL (cm)	TL/N-T	Liver/body wt (×100)	Kidney/body wt (×100)	Heart/body wt (×100)	Body wt (g)
N-V	9.8 ± 0.1	5.2 ± 0.1	0.53 ± 0.01	3.2 ± 0.1	1.1 ± 0.1	0.5 ± 0.1	36.2 ± 1.2
N-GH	9.7 ± 0.1	5.6 ± 0.1[c]	0.58 ± 0.02[c]	3.0 ± 0.1	1.1 ± 0.1	0.6 ± 0.1	32.5 ± 1.0[c]
H-V	8.5 ± 0.1[b]	4.5 ± 0.1[b]	0.54 ± 0.01	3.0 ± 0.1	1.2 ± 0.1	1.3 ± 0.1[b]	21.5 ± 0.8[b]
H-GH	8.1 ± 0.1[b,c]	4.9 ± 0.2[b,c]	0.62 ± 0.01[b,c]	3.4 ± 0.1[b,c]	1.4 ± 0.1[b,c]	1.4 ± 0.1[b]	17.7 ± 0.8[b,c]

[a]N-T (length from nose to base of tail); TL (tail length); (body wt). Each mean ± SE is $n = 5$.
[b]N vs H different ($p < 0.05$).
[c]V vs GH different ($p < 0.05$).

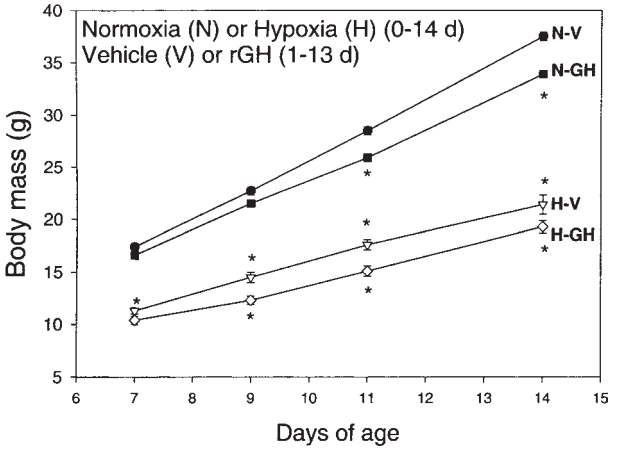

Fig. 1. Body mass measured from 7 to 14 d of age in rats exposed to normoxia (N) or hypoxia (H) from birth to 14 d of age and vehicle (V) or rat GH (rGH) injections (100 µg subcutaneously) daily from 1 to 13 d of age ($n = 8$ to 9). *Significantly different from next higher mean at same time point.

GH led to a decrease in body weight, the effect of which was additive when combined.

Figure 1 shows the body mass of rat pups between 7 and 14 d of age. Exposure to hypoxia (H-V) and GH therapy (N-GH) independently resulted in lower body weight. When combined (H-GH), the inhibitory effects of hypoxia and GH on weight gain were additive.

Figure 2 shows body weight, BMD, percentage of fat, and percentage of lean body mass in a subset of rat pups exposed to normoxia or hypoxia from birth to 14 d of age, and treated daily with vehicle or rat GH on d 1–13. Hypoxia led to a decrease in body weight and, when combined with GH therapy, further decreased body weight. GH therapy did not result in a significant change in BMD in normoxic rats. Fourteen days of hypoxia resulted in a decrease in BMD. Administration of GH resulted in a further decrease in BMD when given to hypoxic rat pups. GH therapy resulted in a decrease in percentage of fat in both normoxic and hypoxic rat pups. There was a tendency for GH therapy to increase percentage of lean in normoxic rat pups. Hypoxia resulted in a decrease in percentage of lean; this effect was not altered by GH therapy.

There was no effect of hypoxia and/or daily rat GH therapy (1–13 d of age) on plasma IGF-1 or IGFBPs (data not shown). Note that GH was not injected on the morning of sacrifice (14 d of age).

Figure 3 shows hepatic IGF-1 mRNA expression in 14-d-old rats exposed to normoxia or hypoxia from birth to 14 d of age and given vehicle or GH injections daily (d 1–13). Although there was a tendency for GH to increase IGF-1 mRNA in normoxic rat pups, this was not statistically significant. Hypoxia for 14 d decreased hepatic IGF-1 mRNA levels. This effect of hypoxia was no longer apparent after GH treatment.

There was no effect of hypoxia and/or GH therapy on plasma cholesterol (data not shown). Hypoxia from 0 to 14 d of age resulted in significant hypertriglyceridemia (322 ± 8 mg/dL) as compared with normoxic control (84 ± 6 mg/dL). GH therapy had no effect on this hypertriglyceridemia. Neither glucose nor insulin was significantly altered after 14 d of hypoxia, without or with GH therapy.

Discussion

Our study focused on the effect of hypoxia and/or GH treatment on body weight gain, body habitus, organ weights, body composition, plasma IGF-1 and IGFBPs, and hepatic IGF-1 mRNA expression. The main findings were as follows: First, hypoxia significantly inhibited body weight gain; GH therapy did not reverse this effect. Second, GH reversed the inhibitory effect of hypoxia on tail length but did not reverse the inhibitory effect on body length. Third, GH had no effect on the increase in heart weight that occurs during 14 d of hypoxia from birth. Fourth, hypoxia for 14 d from birth significantly decreased BMD. Fifth, GH and hypoxia from birth each decreased percentage of body fat; the effect of the combination of GH and hypoxia on decreased percentage of body fat was additive. Finally, GH had minimal effects on plasma IGF-1 and hepatic IGF-1 mRNA expression.

The inhibitory effect of hypoxia on growth and weight gain in rats has been extensively studied *(1,3,4,10,11)*. The mechanism has not been established definitively, but these

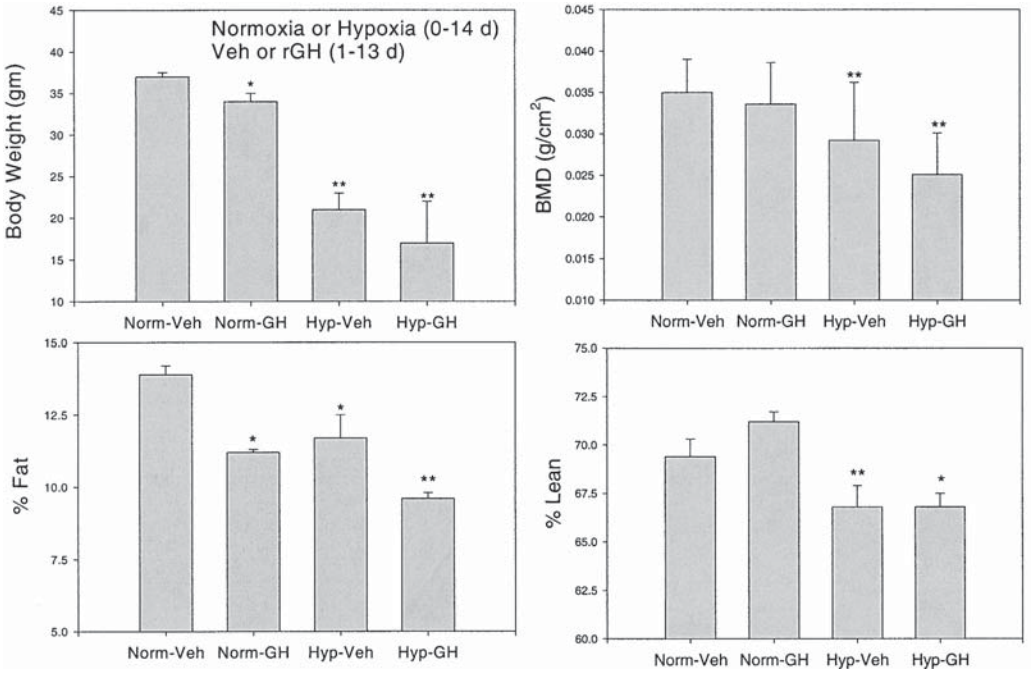

Fig. 2. Body weight, BMD, and percentage of fat and percentage of lean measured by DXA in 14-d-old rats exposed to normoxia (Norm) or hypoxia (Hyp) from birth to 14 d of age, and vehicle (Veh) or rat GH (rGH) injections (100 µg subcutaneously) daily. *Different from Norm-Veh (control); **different from next higher value and Norm-Veh (control). *n* = 5/mean.

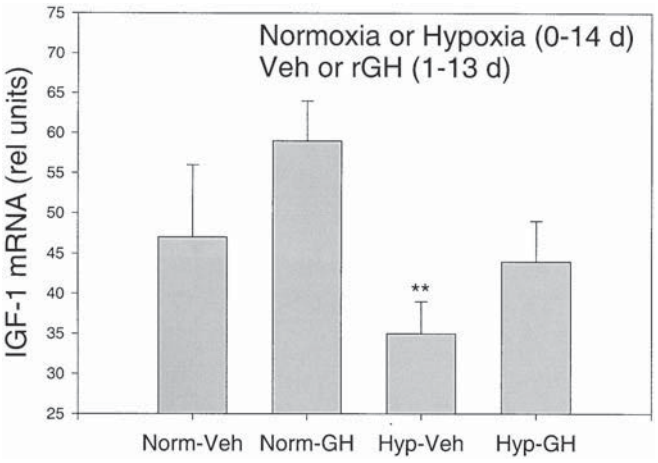

Fig. 3. Summary of reverse transcriptase polymerase chain reaction (RT-PCR) of hepatic IGF-1A mRNA in 14-d-old rats exposed to normoxia (Norm) or hypoxia (Hyp) from birth to 14 d of age, and vehicle (Veh) or rat GH (rGH) injections (100 µg subcutaneously) daily. **Different from next higher value and Norm-Veh (control). *n* = 4/mean.

decreases have been attributed to a decrease in food intake (anorexia), and a decrease in the release of GH and IGF-1 *(2,3,4,10,11,14–16)*. A previous study using slightly different timing, but essentially the same dose and source of GH, suggested that GH might reverse some of the effects of hypoxia on attenuating weight gain *(11)*; however, we found no such effect. Some of the effect on the hypoxic pups may have been mediated by a decrease in maternal food intake *(11)*. The design of our environmental chambers does not allow analysis of maternal or pup food intake, but we presume both were decreased.

Rat GH given for 14 d clearly had biologic effects since tail length was increased, as has been shown by others *(11, 17)*. Interestingly, administration of GH appeared to reverse the effects of 14 d of hypoxia on tail length. GH therapy has been proposed to treat short stature owing to intrauterine growth retardation *(18,19)*, to enhance recovery from neonatal malnutrition *(12,13)*, and to improve brain recovery from neonatal hypothyroidism *(20)*. It is also likely that GH is less effective in the very young rat pup, and that GH therapy in older rats might be more effective *(21)*.

Hypoxia for 14 d from birth had a dramatic effect on heart weight, a previously described phenomenon occurring secondarily to pulmonary hypertension and increased right heart afterload *(3)*. GH therapy had no effect on the increase in heart weight during hypoxia.

Body composition analysis by dual X-ray absorptiometry (DXA) (PIXI) scan revealed several novel findings. Hypoxia for 14 d from birth led to a small but significant decrease in BMD. This confirms our previous study with only 7 d of hypoxia *(8)*. GH therapy by itself had no significant effect on BMD but caused an additional decrease in BMD in hypoxic rats. Although long-term GH therapy increases BMD *(22)*, a decrease in BMD has been demonstrated early in GH therapy in growing rats probably owing to an early increase in resorption not yet coupled to accretion *(23)*. Although

we do not yet know why hypoxia decreases BMD in rat pups, it is likely owing to a combination of factors including decreased milk intake, alterations in local factors within bone, and possibly alterations in the hormonal controllers of bone turnover *(1,3,9,22)*. Regardless of the mechanism, the GH-induced augmentation of the decrease in BMD during hypoxia is an important finding if short-term GH therapy is ever considered for children recovering from hypoxia.

Daily administration of GH resulted in a decrease in the percentage of fat and a tendency to increase percentage of lean, again consistent with previous studies and the well-known effects of GH *(17,23,24)*. When combined with hypoxia, GH led to a further decrease in percentage of fat. This can be explained at least partially by the well-known anorectic effect of hypoxia and the lipolytic effect of GH. Again, a decrease in body fat may not be desirable in hypoxic neonates considering their energy requirements. Furthermore, GH seemed to minimize the decrease in percentage of lean that occurred during hypoxia, although this effect was quite small. The DXA method for analysis of body composition in rats treated with GH has recently been revalidated against direct chemical analysis, so we are convinced that our data are accurate *(25)*.

We previously failed to find an effect of 7 d of hypoxia on plasma GH or IGF-1 levels. The current study also did not find an effect of hypoxia for 14 d on plasma IGF-1. The lack of an effect of 14 d of GH administration on plasma IGF-1 and hepatic IGF-1 mRNA during the first 14 d of life is interesting. Most previous studies carried GH injections from birth into older ages of rat pups *(11–13,17,20)*. Even then, only a small increase in plasma IGF-1, and no change in IGFBPs, was observed *(18)*. It seems likely, then, that younger rat pups do not respond to GH as well as rats after weaning *(21)*. However, shorter-term GH injections into neonatal rats have been shown to exert significant effects *(24)*. Note that neonatal pigs do express hepatic GH receptors *(26)*. The most recent review of this topic suggested that it is local IGF-1 stimulated by GH, rather than circulating IGF-1 from the liver, that is critical for growth *(21)*. The dose of GH was the same as previously used *(10)*, and considerably higher than others *(12,13,17)*. A much lower dose than we used doubled plasma GH in 45-d-old rats *(20)*. About 10 times the total dose (that we used) given to adult rats, increased GH to >1000 μg/L *(23)*, whereas the same total dose given to adult rats increased GH to >50 μg/L *(25)*. Therefore, the dose used in the present study increased plasma GH in the pharmacologic range in the 7-d-old rats we studied. GH administration for 14 d did have the expected effects on tail length and percentage of fat, so the duration and dose of the GH regimen was clearly effective. The source of GH used in our study was the same as in previous studies, so it is unlikely that its failure to promote large effects was owing to a potency problem and/or generation of antibodies in such young rats. Longer-duration GH therapy will be necessary to evaluate these issues further.

In conclusion, GH therapy can reverse some of the changes in body habitus that occur during neonatal hypoxia (e.g., tail length), but it actually accentuates other effects of hypoxia (e.g., decrease in BMD and percentage of body fat). Much more work is necessary to determine whether GH therapy of longer duration during the recovery period can be useful in ameliorating some of the detrimental effects of hypoxia on growth and development in the neonate.

Materials and Methods

Animal Treatment and Exposure to Hypoxia

Timed, pregnant Sprague-Dawley rats (Harlan Sprague-Dawley, $n = 8$) were obtained at 14 d of gestation and maintained on a standard sodium diet and water ad libitum in a controlled environment (lights on 6:00 AM to 6:00 PM). Parturition usually occurred on the afternoon of gestational d 21, during which rats were kept under observation. As soon as a litter was completely delivered, the pups were counted and weighed. The litters were matched by weight and time of birth, culled to ~nine/litter, and randomly assigned to treatment groups. Each dam and her pups were moved to an environment chamber and exposed to normobaric normoxia (21% O_2) or hypoxia (12% O_2) as described in detail previously *(27–29)*. We have previously shown that this exposure leads to arterial P_{O_2} levels in adults of about 50–55 torr with sustained hypocapnia and alkalosis *(27,29)*.

Rat pups, with their lactating dams, were maintained for 14 d (0–14 d of age) in a normoxic or hypoxic environment *(30)*. Chambers were opened daily at ~9:00 AM starting at 1 d of age, at which time rat pups were injected with vehicle (0.01 *M* sodium bicarbonate) or rat GH (100 μg subcutaneously obtained through National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, and Dr. A. L. Parlow). Cages were cleaned on days 4, 8, and 12. Pups were weighed at birth, and at 7, 9, 11, and 14 d of age.

At 8:00 AM at 14 d of age, rat pups were quickly weighed, decapitated, and trunk blood from three to four pups was pooled for plasma measurements. Pups were not injected with vehicle or GH on the morning of sacrifice. Livers were quickly removed, weighed, and frozen for IGF-1 mRNA measurements. Kidney (both) and heart weights were also obtained. Some of the rat pups were euthanized by an overdose of halothane anesthesia, weighed, analyzed for body (nose to anus) and tail (anus to tail tip) length, and then quickly frozen for future body composition analysis.

Plasma Assays

Plasma cholesterol, triglycerides, and glucose were measured spectrophotometrically using reagents purchased from Sigma (St. Louis, MO). Plasma IGF-1 was measured after extraction by radioimmunoassay (RIA) using reagents purchased from Diagnostic Systems (Webster, TX) as described previously *(8)*. Plasma insulin was measured by RIA using

reagents purchased from Linco (St. Charles, MO) as described previously *(8)*.

Plasma IGFBP profiles were characterized as described previously *(12)*. Thirty microliters of a 1:10 dilution of plasma were prepared for electrophoresis by the addition of Laemmli sample buffer and were applied to a 4% stacking gel and separated through a 12% gel under nonreducing conditions overnight at 50 V, 4ºC. Following electrophoresis, proteins were electrotransferred onto 0.45-µm nitrocellulose (Micron Separations, Westborough, MA). Nitrocellulose membranes were sequentially blocked and incubated overnight at 4ºC with [^{125}I] IGF-1 in Tris-buffered saline. Membranes were washed, air-dried, and IGFBP visualized by exposure to Kodak X-Omat AR film (Rochester, NY) with an intensifying screen for 5 d at −70ºC. Because IGFBP-3 bands were light, membranes were reexposed for 13 d. Autorads were digitized and scanned using an Alphaimager System (Alpha Innotech, San Leandro, CA).

Body Composition

Total body composition was analyzed by DXA scans, utilizing PIXImus (GE/Lunar, Madison, WI). Scans were acquired and analyzed with GE/Lunar PIXI software (version 1.45). The animals were placed supine on the scanner bed with arms and legs extended outward from the body. The analysis was performed as previously described *(8)* except that the scan excluded the head to minimize positioning error. This analysis provided an assessment of BMD, and lean and fat tissue masses.

IGF-1 mRNA by RT-PCR

Extraction of mRNA and RT-PCR were performed as previously described *(31)* except that primers were annealed at 62ºC. The primers used in these studies were previously described and extensively characterized *(15)*. Our analysis focused on the IGF-1A isoform, which is the most abundant form expressed in the liver. Gels were digitized and scanned using an Alphaimager System (Alpha Innotech). Data were normalized to β-actin mRNA expression, thus providing semiquantitative analysis of IGF-1 expression.

Statistical Analyses

Data were analyzed by two-way analysis of variance and Duncan multiple range test. $p < 0.05$ was considered significant. Data are presented as the mean ± SEM.

Acknowledgments

We thank Sharon Donovan at the University of Illinois for performing the IGFBP analyses. This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-54685.

References

1. Elia, R., Elhoyhen, A. B., Bugallo, G., Rio, M. E., and Bozzini, C. E. (1985). *Acta Physiol. Pharmacol. Ther. Latinoam.* **35,** 311–318.
2. Gloster, J., Hasleton, P. S., Harris, P., and Heath, D. (1974). *Environ. Physiol. Biochem.* **4,** 251–4258.
3. Moromisato, D. Y., Moromisato, M. Y., Zanconato, S., Roberts, C. T., Brasel, J. A., and Cooper, D. M. (1996). *Crit. Care Med.* **24,** 919–924.
4. Singh, S. B., Sharma, A., Sharma, K. N., and Selvamurthy, W. (1996). *J. Appl. Physiol.* **80,** 1133–1137.
5. Cheng, N., Cai, W., Jiang, M., and Wu, S. (1997). *Pediatr. Res.* **41,** 852–856.
6. Fushiki, T., Kano, T., Ito, K., Hirofuji, C., Inoue, K., Moritani, T., and Sugimoto, E. (1992). *Can. J. Physiol. Pharmacol.* **70,** 1522–1524.
7. Raff, H., Bruder, E. D., and Jankowski, B. M. (1999). *Endocrine* **11,** 37–39.
8. Raff, H., Bruder, E. D., Jankowski, B. M., and Colman, R. J. (2001). *Horm. Metab. Res.* **33,** 151–155.
9. Raff, H., Bruder, E. D., Jankowski, B. M., and Goodfriend, T. L. (2000). *Am. J. Physiol. Regul. Integrat. Comp. Physiol.* **278,** R663–R668.
10. Mlekusch, W., Paletta, B., Truppe, W., Paschke, E., and Grimus, R. (1981). *Horm. Metab. Res.* **13,** 612–614.
11. Moromisato, D. Y., Moromisato, M. Y., Brasel, J. A., and Cooper, D. M. (1999). *Crit. Care Med.* **27,** 2234–2238.
12. Zhao, X. and Donovan, S. M. (1995). *J. Nutr.* **125,** 2773–2786.
13. Zhao, X., Unterman, T. G., and Donovan, S. M. (1995). *J. Nutr.* **125,** 1316–1327.
14. Chen, X.-Q. and Du, J.-Z. (2000). *Neurosci. Lett.* **284,** 151–154.
15. Zhang, J., Whitehead, R. E., and Underwood, L. E. (1997). *Endocrinology* **138,** 3112–3118.
16. Zhang, Y.-S. and Du, J.-Z. (2000). *Neurosci. Lett.* **279,** 137–140.
17. Rynikova, A., Koppel, J., Kuchar, S., Mozes, S., Noskovic, P., and Boda, K. (1988). *Exp. Clin. Endocrinol.* **91,** 105–108.
18. Muaku, S. M., Thissen, J. P., Gerard, G., Ketelslegers, J.-M., and Maiter, D. (1997). *Pediatr. Res.* **42,** 370–377.
19. Stanhope, R. (2000). *Clin. Endocrinol.* **53,** 665, 666.
20. Savard, P., Blanchard, L. M., Merand, Y., and Dupont, A. (1984). *Dev. Brain Res.* **15,** 239–245.
21. LeRoith, D., Bondy, C., Yakar, S., Liu, J. L., and Butler, A. (2001). *Endocr. Rev.* **22,** 53–74.
22. Ohlsson, C., Bengtsson, B.-A., Isaksson, O. G. P., Andreassen, T. T., and Slooteweg, M. C. (1998). *Endocr. Rev.* **19,** 55–79.
23. Rosen, H. N., Chen, V., Cittadini, A., Greenspan, S. L., Douglas, P. S., Moses, A. C., and Beamer, W. G. (1995). *J. Bone Miner. Res.* **10,** 1352–1358.
24. Alvarez, C., Escriva, F., and Pascual-Leone, A. M. (1992). *Horm. Res.* **37,** 39–44.
25. Johansen, P. B., Flyvbjerg, A., Wilken, M., and Malmlof, K. (2000). *Growth Horm. IGF Res.* **10,** 342–348.
26. Lewis, A. J., Wester, T. J., Burrin, D. G., and Dauncey, M. J. (2000). *Am. J. Physiol. Regul. Integrat. Comp. Physiol.* **278,** R838–R844.
27. Raff, H. and Chadwick, C. J. (1986). *Clin. Exp. Pharmacol. Physiol.* **13,** 827–830.
28. Raff, H., Jankowski, B. M., Bruder, E. D., Engeland, W. C., and Oaks, M. K. (1999). *Endocrinology* **140,** 3147–3153.
29. Raff, H., Sandri, R. B., and Segerson, T. P. (1986). *Am. J. Physiol. Regul. Integrat. Comp. Physiol.* **250,** R240–R244.
30. Thomas, T. and Marshall, J. M. (1995). *J. Physiol.* **487,** 513–525.
31. Oaks, M. K., Hallett, K. M., Penwell, R. T., Stauber, E. C., Warren, S. J., and Tector, A. J. (2000). *Cell. Immunol.* **201,** 144–153.