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Reduced ob mRNA in Hypophysectomised Rats Is Not Restored by Growth Hormone (GH), but further Suppressed by Exogenously Administered Insulin-like Growth Factor (IGF) I

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To examine whether GH and IGF-I participate in the regulation of obese (ob) mRNA expression we determined ob mRNA levels in epididydimal fat pads of hypophysectomised (hypox) rats, hypox rats treated with recombinant human (rh) GH or rhIGF-I and normal, weight-matched controls. We found that 1. ob mRNA was markedly suppressed after hypophysectomy (37 ± 25% of controls), 2. GH infusion had no effect on ob mRNA, but stimulated IGF-I mRNA in fat pads, 3. IGF-I treatment further suppressed ob mRNA (3.5% ± 0.6% of controls) and 4. serum insulin levels were decreased in all hypox groups (11.2 to 15.9% of controls). In conclusion, exogenous and GH-induced IGF-I differ in their effects on ob mRNA expression and GH is unable to restore ob mRNA towards normal at low insulin levels.

The obese (ob) gene isolated from the genetically obese ob/ob mouse by positional cloning encodes a 167 amino acid polypeptide with the structural features of a secreted protein of 146 amino acids (1). It is expressed exclusively in adipocytes (2,3), secreted into the blood stream (4) and appears to signal to the hypothalamus (5). To date two physiological roles have been attributed to the ob protein: 1. Downregulation of appetite and 2. stimulation of energy expenditure (5,6,7,8). Thus, it serves an endocrine function in body weight regulation by integrating food intake and energy consumption and storage. A positive correlation has been observed between the nutritional status and ob tissue mRNA and serum protein levels (4). ob mRNA and ob protein levels decrease in fasting rats and increase again upon refeeding (4,9,10,11,12). In adipose tissue of strepozotozin-diabetic rats, ob mRNA expression is markedly reduced, but restored by insulin treatment (10,12). Together with the fasting-refeeding experiments these in vivo data point to insulin as a major regulator of ob gene expression. However, in contrast to humans, reduced insulin levels in fasted and diabetic rats are accompanied by reduced GH levels, and refeeding of fasted rats as well as insulin treatment of diabetic rats restores the GH secretory pattern (13,14). Therefore, GH together with insulin, might contribute to the regulation of ob mRNA expression. Furthermore, adipose tissue which secretes ob protein, is also a target tissue for GH action (15). GH directly promotes lipolysis by enhancing the response of hormone-sensitive triglyceride lipase to lipolytic hormones (16). To examine whether GH plays a role in the regulation of ob mRNA expression we determined ob mRNA levels in fat pads of hypophysectomised (hypox) rats and hypox rats treated either with rhGH or with rhIGF-I. IGF-I is mainly produced in the liver under the influence of GH and mediates the effects of GH on somatic growth (17).

MATERIALS AND METHODS

Animals. Hypophysectomised male Tif RAI rats (seven weeks old) were obtained from Dr. K. Müller and M. Cortesi, Ciba-Geigy, Basel Switzerland. Rats with a weight gain of less than 2g/week were chosen for treatment.

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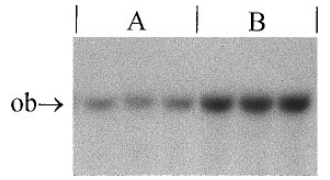

FIG. 1. ob mRNA of rats with differing weights. Northern blot of total fat pad RNA (10 μg) hybridised with a rat ob probe produced by RT-PCR. Lanes A show ob mRNA of rats with a mean body weight of 175g and lanes B with a mean body weight of 392g.

Recombinant human (rh) GH (Novo-Nordisk, Gentofte, Denmark) was dissolved in H_2O and rhIGF-I (Ciba-Geigy, Basel, Switzerland) in 0.1 M acetic acid and applied s.c. using miniosmotic pumps (model 2001, Alza Corp., Palo Alto, CA). Animals were treated for 6 days with solvent (0.1 M acetic acid), 300 μg/day of rhIGF-I or 200 mU/day of rhGH. Weight-matched normal rats served as controls. On the morning of sacrifice the animals were anesthetized with Innovar Vet (Pitman Moore, Washington Crossing, NJ) and bled by aortic puncture. After clotting (on ice) blood was centrifuged for 30 min. at 3000 $\times$ g and 4°C, and serum was stored at -20°C. Epididymal fat pads were excised, weighed, immediately frozen in liquid nitrogen and stored at -80° until RNA was extracted.

Preparation of a rat ob cDNA probe by RT-PCR. Total RNA from fat pads was reverse transcribed with oligo (dT) as primer and Superscript reverse transcriptase (GIBCO BRL, Basel, Switzerland) according to the manufacturer's instructions. The cDNA was then subjected to PCR (kit from Boehringer Mannheim, Germany) using the following oligonucleotides complementary to the mouse ob cDNA as primers: (+) strand 5′-CCTATCCAGAAAGTCCAGGA-3′, (−) strand 5′-ATGTCCTGCAGAGAGCCCTG-3′ as described (3). The 404 bp reaction product was separated by agarose gel electrophoresis and isolated by agarase digestion (Boehringer Mannheim).

Total RNA isolation and northern blotting. Total RNA was isolated from frozen fat pads by a standard CsCl centrifugation method (18). RNA was separated in a 1% agarose gel containing 2M formaldehyde, transferred to Nylon membranes (Hybond-N, Amersham, U.K.) and crosslinked by UV-radiation (19). The rat ob cDNA fragment, the rat IGF-I cDNA probe (20) and the β-actin cDNA probe (21) were labelled with [α-^{32}P]deoxy-CTP ($\sim$3000Ci/mmol, Amersham) by random primer extension using a commercial kit (Boehringer, Mannheim, Germany). Filters were prehybridised, hybridised and washed as described (20,22) and exposed to Kodak X-Omat AR films in the presence of a Cronex lightening plus enhancer screen. Optical densities of bands from autoradiograms were determined using a BIO RAD densitometer (model GS 700). ob values were corrected by normalisation with the corresponding β-actin value.

Determination of insulin, IGF-I and free fatty acids (FFA). Serum insulin was determined using a radioimmunoassay kit for rat insulin (NovoBiolabs, Gentofte, Denmark). Serum IGF-I was determined as described previously (23,24) using human rh IGF-I or rat IGF-I (gift from Dr. M. Kobayshi, Fujisawa, Japan) as a standard for determination of infused rhIGF-I or endogenous rat IGF-I, respectively. The antibody used for determination of human IGF-I does not significantly crossreact with rat IGF-I. Serum FFA levels were determined with a commercial kit (Wako Chemicals, IG Instrumenten Gesellschaft Zürich, Switzerland).

RESULTS

Figure 1 shows a representative Northern blot with fat pad RNA hybridised to the ob probe from 3 normal rats with an average body weight of 175 g (A) and of 3 rats with an average body weight of 392 g (B). The fat pad weight/100 g of the body weight amounts to a mean of 0.52 in group A and of 1.05 in group B. Ob mRNA levels were higher in the group with higher body weight and with a higher fat pad/body weight ratio. Therefore, we used weight-matched normal rats as controls for the growth-arrested hypox rats. Hypox rats and hypox rats treated with rhGH or rhIGF-I were sacrificed after 6 days of treatment and body weight, fat pad weight, serum insulin and serum IGF-I levels were determined. Circulating IGF-I is mainly produced in the liver, is regulated by GH and, together with the body weight gain, serves as an indicator of the effectiveness of hypophysectomy and of GH-treatment. Table 1 shows that hypox rats have strongly reduced serum IGF-I levels which are restored by rhGH and partly by rhIGF-I treatment. The effectiveness of the treatment with rhGH or rhIGF-I is further evident by the body weight gain of the treated hypox rats. Table 1 also shows that serum

TABLE 1
Serum IGF-I and Serum Insulin Levels, Body- and Fat Pad Weights in Hypox Rats, Hypox Rats Treated with rhIGF-I or rhGH and Weight-Matched Controls

Treatment groups	Serum IGF-I (ng/ml)	Insulin ng/ml	Weight# (g)	Fat pad (mg)
Controls	1064 ± 196†	2.32 ± 0.94	176 ± 5.6	937 ± 151
Hypox	134 ± 24†	0.37 ± 0.12	182 ± 5.4	840 ± 134
Hypox + IGF-I	395 ± 69‡	0.26 ± 0.07	201 ± 5.8	718 ± 169
Hypox + GH	1000 ± 159†	0.28 ± 0.03	207 ± 9.8	867 ± 201

† Endogenous rat IGF-I, ‡ rhIGF-I, # body weight at sacrifice. Initial body weights in the hypox, and treated hypox groups were 176 ± 4.0, 180 ± 5.9 and 176 ± 7.1 g, respectively. All values are mean values ± SD (n = 5).

insulin levels are around 10-fold lower in hypox than in normal control rats and that rhGH or rhIGF-I treatment does not significantly alter the decreased insulin levels.

Figure 2 compares relative ob mRNA levels of normal control rats, hypox rats and hypox rats treated with rhGH or rhIGF-I. All data were normalised for β-actin and represent the mean ± SD. The variations of the β-actin band intensities were minimal within a treatment group or between different treatment groups (results not shown). Therefore, β-actin was used as a standard to correct for small differences in loading of RNA. Fat pads of hypox rats have

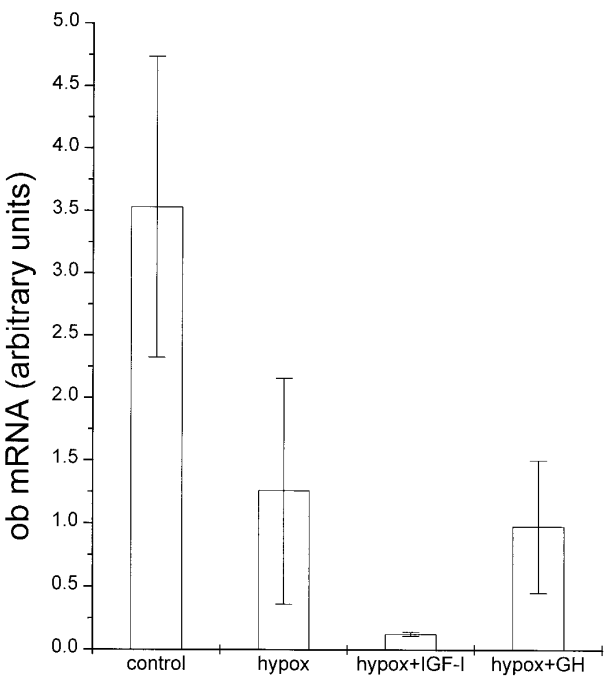

FIG. 2. ob mRNA levels of fat pads of hypox rats, hypox rats treated with rhGH or rhIGF-I and weight-matched control rats. 10 μg/lane of total RNA analysed by Northern blotting. The blots were first hybridised with the a rat ob probe and subsequently with a β-actin probe. Relative ob message levels were determined by densitometry and corrected for differences of the RNA load with actin as a standard. The bar diagram shows the mean value (arbitrary units) ± SD. Normal control rats (n = 4), hypox rats (n = 5), hypox rats treated with GH (n = 5), hypox rats treated with IGF-I (n = 3). Students t-test: controls vs hypox, $p < 0.05$; hypox vs hypox + GH, $p = 0.527$; hypox vs hypox + IGF-I, $p = 0.072$; hypox + GH vs hypox + IGF-I, $p < 0.05$.

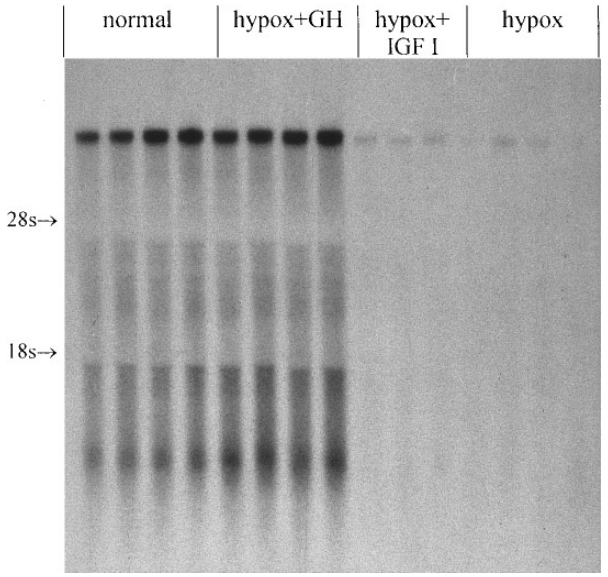

FIG. 3. IGF-I mRNA of fat pads of hypox rats, hypox rats treated with rhGH or rhIGF-I and weight-matched control rats. Northern blot of total fat pad RNA (10 μg) isolated from hypox rats, hypox rats treated with GH or IGF-I, and weight-matched control rats. The blot was hybridised with a rat IGF-I cDNA probe and exposed overnight.

significantly reduced ob mRNA levels (37 ± 25% as compared to weight-matched control rats). Treatment of hypox rats with rhGH did not significantly change ob mRNA levels (27.8 ± 14.7% of controls), whereas rhIGF-I treatment almost completely suppressed of ob mRNA levels (3.5 ± 0.6% of controls). Due to the relatively large variation of ob mRNA levels in hypox rats, the supressive effect of rhIGF-I did not quite reach the significance level of 0.05 when rhIGF-I-treated hypox rats were compared to hypox rats ($p=0.072$), although the individual values of both treatment groups do not overlap. Nevertheless, the suppressive effect of rhIGF-I in hypox rats was significant ($p<0.05$) when compared to rhGH-treated hypox rats.

Although ob mRNA expression was unresponsive to rhGH, IGF-I mRNA levels in fat pads were normalised by rhGH (Fig. 3), demonstrating that the failure of ob mRNA to increase after rhGH treatment, was not due to the unresponsiveness of the adipose tissue to rhGH.

DISCUSSION

Insulin is held to be a major positive regulator of ob-gene expression (4,9−12). In rats, changes of circulating insulin are accompanied by parallel changes of GH. Thus, streptozotocin diabetes or fasting cause low insulin and GH levels, and both are restored by insulin treatment of diabetic or refeeding of fasted rats (13,14). It was, therefore, of interest to examine whether GH alone affects ob mRNA expression. Hypox rats lack GH besides other pituitary and pituitary-dependent hormones, which are not restored by GH replacement (25). Like GH, serum insulin levels fall after hypophysectomy and, as shown in this study, are not significantly altered by rhGH or rhIGF-I treatment (Table 1). The effects of GH replacement in hypox animals thus reflect GH actions which are relatively independent of other pituitary or pituitary-dependent hormones. RhGH treatment of hypox rats partly restores arrested growth (table 1 and 25,26). This effect is mediated via the induction of endogenous IGF-I (17) and is mimicked by the administration of exogenous rhIGF-I (25,26).

Our results demonstrate 1. that fat pads of hypox rats contain significantly reduced ob mRNA levels as compared to normal weight-matched controls and 2. that rhGH and rhGH-

induced endogenous IGF-I are unable to raise or restore decreased ob mRNA levels. In contrast, fat pad IGF-I mRNA expression was restored to normal demonstrating that GH is effective in adipose tissue, the site of ob synthesis. We, therefore, conclude that GH itself is not a regulator of ob mRNA expression. Rather, low serum insulin levels in hypox and rhGH-treated hypox rats appear to be responsible for decreased ob mRNA levels consistent with the present concept that insulin is a major positive regulator of ob mRNA expression.

One of the striking findings of this study was the nearly complete suppression of ob mRNA by exogenous IGF-I. This suppression occurred at reduced serum insulin levels, which were not significantly different from those of rhGH-treated hypox animals. Thus, infused IGF-I behaves differently from GH-induced, endogenous IGF-I. Although both have similar effects on growth (see Table 1 and ref. 25,26), differences exist with respect to their actions on glucose and lipid metabolism (27). This may, at least in part, be due to a different distribution of infused and endogenous IGF-I to the various circulating IGF binding protein complexes (28,29).

The observation that rhIGF-I raises serum FFA in hypopituitary humans (27) together with the finding that FFA inhibit ob transcription in 3T3-L1 adipocytes in vitro (30), at first suggested that IGF-I might mediate the suppression of ob mRNA levels via an elevation of serum FFA. However, the latter were reduced in all hypox groups when compared to controls (561 $\pm$ 222 μmol/l) and not significantly different between hypox (196 $\pm$ 79 μmol/l) and GH- or IGF-I-infused hypox rats (138 $\pm$ 31 and 207 $\pm$ 110 μmol/l, respectively). Suppression of ob mRNA by exogenous IGF-I can, therefore, not be due to increased circulating FFA. It is also unlikely that exogenous IGF-I directly suppresses ob mRNA in adipocytes, because adipocytes lack functional type 1 IGF receptors (31) and because IGF-I crossreacts poorly with the insulin receptor (32). The latter has a low affinity for IGF-I, and an insulinomimetic effect of IGF-I would result in a stimulation rather than a suppression of ob mRNA expression. The mechanism responsible for the supressive effect of exogenous IGF-I is, therefore, presently unexplained.

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REFERENCES

1. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. M. (1994) *Nature* **372,** 425–423.
2. Masuzaki, H., Ogawa, Y., Isse, N., Satoh, N., Okazaki, T., Shigemoto, M., Mori, K., Tamura, N., Hosoda, K., Yoshimasa, Y., Jingami, H., Kawada, T., and Nakao, K. (1995) *Diabetes* **44,** 855–858.
3. Ogawa, Y., Masuzaki, H., Isse, N., Okazaki, T., Mori, K., Shigemoto, M., Satoh, N., Tamura, N., Hoseda, K., Yoshimasa, Y., Jingami, H., Kawada, T., and Nakao, K. (1995) *J. Clin. Invest.* **96,** 1647–1652.
4. Frederich, R. C., Löllmann, B., Hamann, A., Napolitano-Rosen, A., and Kahn, B. B., Lowell, B., and Flier, J. S. (1995) *J. Clin. Invest.* **96,** 1658–1663.
5. Campfield, L. A., Smith, F. J., Guisez, Y., Devos, R., and Burn, P. (1995) *Science* **269,** 546–549.
6. Weigle, D. S., Bukowski, T. R., Foster, D. C., Holderman, S., Kramer, J. M., Lasser, G., Lofton-Day, C. E., Prunkard, D. E., Raymond, C., and Kuijper, J. L. (1995) *J. Clin. Invest.* **96,** 2065–2070.
7. Rentsch, J., Levens, N., and Chiesi, M. (1995) *Biochem. Biophys. Res. Comm.* **214,** 131–136. J
8. Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995) *Science* **269,** 540–543.
9. Saladin, R., DeVos, P., Guerre-Millo, M., Leturque, A., Girard, J., Staels, B., and Auwerx, J. (1995) *Nature* **377,** 527–529.
10. Becker, D. J., Ongemba, L. N., Brichard, V., Henquin, J. C., and Brichard, S. M. (1995) *FEBS-Lett.* **371,** 324–328.
11. Trayhurn, P., Thomas, M. E., Duncan, J. S., and Rayner, D. V. (1995) *FEBS-Lett.* **368,** 488–490.
12. MacDougald, O. A., Hwang, C.-S., Fan, H., and Lane, M. D. (1995) *Proc. Natl. Acad. Sci.* **92,** 9034–9037.
13. Tannenbaum, G. S., Rorstad, O., and Brazeau, P. (1979) *Endocrinol.* **104,** 1733–1738.
14. Carlsson, L. M. S., Clark, R. G., Skottner, A., and Robinson, I. C. A. F. (1989) *Endocrinol.* **122,** 661–670.

15. Goodman, H. M. (1968) *Ann. NY Acad. Sci.* **148,** 419–440.
16. Dietz, J., and Schwartz, (1991) *J. Metab. Clin. Exp.* **40,** 800–806.
17. Chochinov, R. H., and Daughaday, W. H. (1976) *Diabetes.* **25,** 994–1007.
18. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) in: Molecular Cloning p. 196–205, Cold Spring Harbour, NY.
19. Church, G. M., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci.* **81,** 1991–1995.
20. Böni-Schnetzler, M., Schmid, Ch., Meier, P. J., and Froesch, E. R. (1991) *Am. J. Physiol.* **260,** E846–E851.
21. Cleveland, D. W., Lopata, M. A., Mach Doanald, R. J., Cowan, N. J., Rutter, W. J., and Kirschner, M. W. (1980) *Cell* **20,** 95–105.
22. Peter, M. A., Winterhalter, K. H., Böni-Schnetzler, M., Froesch, E. R., and Zapf, J. (1993) *Endocrinol.* **133,** 2624–2631.
23. Zapf, J., Walter, H., and Froesch, E. R. J. (1981) *Clin. Invest.* **68,** 1321–1330.
24. Zapf, J., Hauri, C., Futo, E., Hussain, M., Rutishauser, J., Maack, Ch., and Froesch, E. R. (1995) *J. Clin. Invest.* **95,** 179–186.
25. Hunziker, E. B., Wagner, J., and Zapf, J. (1994) *J. Clin. Invest.* **93,** 1078–1086.
26. Guler, H.-P., Zapf, J., Scheiwiller, E., and Froesch, E. R. (1980) *Proc. Natl. Acad. Sci.* **85,** 4889–4893.
27. Hussain, M. A., Schmitz, O., Mengel, A., Glatz, Y., Christiansen, J. S., Zapf, J., and Froesch, E. R. (1994) *J. Clin. Invest.* **94,** 1126–1133.
28. Zapf, J. (1995) *Eur. J. Endocrinol.* **312,** 645–654.
29. Zapf, J., Hauri, Ch., Waldvogel, M., Futo, Häsler, H., Binz, K., Guler, H. P., Schmid, C., and Froesch, E. R. (1989) *Proc. Natl. Acad. Sci.* **86,** 3813–3817.
30. Rentsch, J., and Chiesi, M. (1996) *FEBS Lett.* **379,** 55–59.
31. Caro, J. F., Poulos, O., Ittoop, W. J., Pories, E. G., Flickinger, E. G., and Sinha, M. K. (1988) *J. Clin. Invest.* **81,** 976–783.
32. Zapf, J., Schoenle, E., and Froesch, E. R. (1978) *Eur. J. Biochem.* **87,** 285–296.