Comparison of Hypoglycaemic Responses to Human Growth Hormone and the Synthetic 4–15 Fragment Between 16–18 Day-Old and 45–50 Day-Old Rats

F. M. Ng and J. Bornstein

Department of Biochemistry, Monash University, Clayton, Victoria, Australia

Summary. The hypoglycaemic response to human growth hormone and its synthetic 4–15 fragment were studied in 16–18 day-old and 45–50 day-old normal male rats using three different bioassay system: (1) intravenous insulin tolerance tests, (2) insulin binding to specific receptor of hepatic plasma membranes, and (3) activation of insulin-dependent glycogen synthase in muscle. The mean level of the endogenous growth hormone in the 16–18 day-old group was 11 ± 4 ng/ml and that of the 45–50 day-old group was 164 ± 35 ng/ml. A hypoglycaemic action of human growth hormone was observed in the baby animals but not in the adults. The synthetic 4–15 fragment was found, at a similar dose to that of the intact molecule, to enhance hypoglycaemia in both groups of

rats. In exact parallel, growth hormone increased hepatic insulinbinding and activated muscle glycogen synthase only in baby rats but the synthetic 4–15 fragment affected both baby and adult animals in the same way. The molecular mechanism for the diminution of the responses to the intact molecule of growth hormone in normal adult rats remains to be identified. However, the present studies provide the first evidence on the hypoglycaemic response to growth hormone in vivo in normal rats without any previous chemical or surgical treatment.

Key words: Rats, human growth hormone, hypoglycaemia, insulin receptors, glycogen metabolism.

Numerous studies have demonstrated that the levels of endogenous growth hormone (GH) influence insulin action in vitro and in vivo [1–4]. Current evidence, however, suggests that the entire molecule of GH is not always required to elicit the biological activities of the hormone [5, 6]. Studies with various synthetic fragments of human GH showed that amino-terminal fragments containing the sequence H_2N -Arg-Leu-Phe-Asp-Asn-Ala-COOH (human GH 8–13) potentiate the action of insulin by increasing the binding of insulin to specific receptors at target tissues and modulating the activities of insulin-dependent enzymes such as glycogen synthase and phosphorylase [7–9]. In the present study we have compared two groups of normal rats with different levels of endogenous GH in response to the hypoglycaemic action of human GH and a synthetic fragment of the molecule, H_2N -Ile-Pro-Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala-Met-Leu-COOH (human GH 4–15).

Materials and Methods

Materials

Human GH was a gift from Institut Choay, Paris, France. Human GH 4–15 was synthesized and purified as described previously [10]. Twice

recrystallized beef insulin was generously supplied by the Commonwealth Serum Laboratories, Melbourne, Australia. Sodium [^{125}I]-iodide was obtained from the Radiochemical Centre, Amersham, UK. Insulin was iodinated to a specific activity of 80–100 $\mu Ci/\mu g$ according to the method of Freychet et al. [11].

Animals

Male albino Wistar rats (aged 45–50 days) with a body weight ranging from 100–120 g were used as the adult animals. They were housed under control conditions at a constant temperature (25 °C), a normal 12-h light cycle and fed according to a synchronized schedule as described previously [12]. The pre-weaning baby rats were housed with their mothers for 16–18 days after birth, and removed shortly before use. At this age they weighed 25–30 g. The male baby rats in each litter were equally divided into control and test groups for experiments.

Studies in Vivo

The acute effects of human GH and synthetic peptide human GH 4–15 on basal blood glucose and plasma insulin levels were determined on 16–18 day-old baby rats and 45–50 day-old, fasted overnight, adult rats. The test substances (0.10–0.15 $\mu mol/kg$ body weight) were given intravenously, and blood samples (25–100 μl) were collected from the tail vein of pentobarbital-anaesthetized animals 30 min after IV injection. Blood glucose was estimated immediately on collection by the glucose oxidase method using a Yellow Springs Model 23 AM glucose analyser. Plasma insulin was assayed by the double-antibody radioimmunoassay [13]. Plasma GH was determined by ra-

Table 1. Effect of human growth hormone and the 4–15 fragment on basal blood glucose levels and activities of muscle glycogen synthase in 45–50 day-old (adult) and 16–18 day-old (baby) normal male rats

Rats	Blood glucose (mmol/l)–			Activity ratio $\left(\frac{I}{I+D}\right)$ of glycogen synthase		
	Control	Human GH	Human GH 4–15	Control	Human GH	Human GH 4–15
45–50 day-old	5.96 ± 0.22 (16)	6.05 ± 0.41 (8) NS	5.30 ± 0.20 (8) $p < 0.025$	0.308 ± 0.018 (6)	0.313 ± 0.015 (6) NS	0.401 ± 0.022 (6) $p < 0.0025$
16–18 day-old	6.18 ± 0.18 (14)	5.70 ± 0.20 (8) $p < 0.05$	5.45 ± 0.26 (6) $p < 0.0125$	0.265 ± 0.015 (6)	0.308 ± 0.015 (6) $p < 0.0125$	0.342 ± 0.028 (6) $p < 0.01$

Saline or test substances at a dose as indicated in the text were given IV 30 min before taking blood samples for glucose estimation. The activities of glycogen synthase from control and treated rats are expressed as the fraction of enzyme present in the active form (I). Results expressed as mean ± SEM; number of animals in each group is given in parentheses. Significance of comparison with control was determined by Student's t-test for unpaired values. NS = not significant

dioimmunoassay, using materials supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, USA.

Intravenous insulin tolerance tests were performed on control and treated rats of both age groups. As previously described [14], all IV insulin tolerance tests were carried out at 09.45 h, 45 min after induction of anaesthesia by the IP injection of pentobarbital at a dose of either 60 mg (for adult rats) or 40 mg (for baby rats)/kg body weight. The basal blood glucose levels of all animals used for IV insulin tolerance tests were within the range 5.2–5.8 mmol/l.

Preparation of Hepatic Plasma Membrane

Overnight fasted 16–18 day-old baby rats or 45–50 day-old adult rats were divided into three groups. One test group was injected IV with human GH (0.15 µmol/kg); the other test group with synthetic human GH (0.10 µmol/kg) and the control group with an equivalent volume of physiological saline (0.9% NaCl). Thirty minutes after injection, blood samples were collected for glucose and insulin analyses and immediately after, the animals were killed by decapitation. Hepatic plasma membranes were prepared following the method of Ray [15] and stored at –70 °C. Membrane protein was measured by the method of Lowry et al. [16].

Insulin Binding Studies

Studies of binding of insulin to hepatic plasma membranes of control and pretreated rats of both age groups were conducted at 25 °C in 50 mmol/l Tris-HCl buffer (pH 7.5) containing 0.5% bovine serum albumin according to the technique of Freychet et al. [17]. Aliquots of plasma membrane (200 µg/ml of membrane protein) were incubated with [¹²⁵I]-insulin (1 ng/ml) in the presence or absence of unlabelled hormone for 30 min. Immediately after incubation, the membrane-bound hormone was recovered by centrifugation of triplicate samples for 2 min with a Beckman Microfuge B and radioactivity estimated using a Philips automatic gamma analyser (model PW 4520).

Assays of Muscle Glycogen Synthase

Both control and test rats of the two different age groups were anaesthetized and pretreated as those for insulin binding studies. From the animals a portion of hind leg muscle was removed [18], tissue homogenates prepared according to the method of Danforth et al [19] and the enzyme activity assayed as described by Thomas et al [20].

Statistical Analysis

Results are presented as mean ± SEM. Differences in means were analyzed by the Student's t-test. All p values of < 0.05 were accepted as statistically significant.

Results

Basal Levels of Blood Glucose, Plasma Insulin and Growth Hormone

The 40–50 day-old and the 16–18 day-old groups of normal rats had similar basal levels of blood glucose (6.2 ± 0.4 versus 5.8 ± 0.3 mmol/l) and plasma insulin (40.1 ± 9.4 versus 36.8 ± 6.0 mU/l), but the levels of plasma GH in the adult rats were markedly higher than those of the baby rats (164 ± 35 versus 11 ± 4 ng/ml).

Effects of Human GH and Human GH 4–15 on Blood Glucose and Muscle Glycogen Synthase Activity

Single IV injection of rats with either intact human GH (0.15 µmol/kg) or synthetic human GH 4–15 (0.10 µmol/kg) produced no significant change in the levels of plasma insulin in both groups of rats (results not shown). In baby rats similar acute treatment with the same dose of GH lowered the levels of blood glucose for 45 min reaching a minimum 30 min after injection, but in adult rats it had no observable effect in the same period of time. Synthetic human GH 4–15 induced a significant decrease of blood glucose from 6.18 ± 0.18 to 5.45 ± 0.26 mmol/l ($p < 0.0125$) in baby rats and from 5.96 ± 0.22 to 5.30 ± 0.20 mmol/l ($p < 0.025$) in adult rats (Table 1). The data also showed that synthetic human GH 4–15 increased the proportion of the active form of muscle glycogen synthase in both groups of normal rats. Acute treatment with intact human GH in baby rats similarly activated the glycogen-metabolizing enzyme, but in adult rats no alteration of the enzyme activity was observed (Table 1).

Effects on Insulin Sensitivity

The hypoglycaemic effect of human GH and human GH 4–15 was demonstrated with IV insulin tolerance tests on the two groups of rats (Fig. 1). Both the intact hormone and the synthetic peptide significantly poten-

Fig. 1. Effect of human growth hormone and the synthetic 4–15 fragment in 45–50 day-old (adult) and 16–18 day-old (baby) rats during IV insulin tolerance tests. Data are expressed as mean $\pm$ SEM of 12 animals from each group. Human growth hormone (right-hand panel, $\square$) and the synthetic 4–15 fragment (left-hand panel, Δ) were administered IV 5 min before the tests. From 15–60 min after the commencement of the IV insulin tolerance tests, the blood glucose in the baby rat pretreated with human growth hormone or the synthetic 4–15 fragment and the adult rats pre-treated with the synthetic 4–15 fragment were significantly lower ($p < 0.05$) than that of the respective control groups ($\circ$). No significant change was detected at any time between the adult rats pre-treated with human growth hormone and the corresponding control rats ($\circ$)

tiated the action of exogenously administered insulin in 16–18 day-old baby rats. The synthetic human GH 4–15 showed similar activity but GH was devoid of such effect in 45–50 day-old adult rats.

Insulin Binding

Figure 2 depicts the effect of human GH and human GH 4–15 in modulating the binding of [125 I]-insulin to hepatic plasma membrane receptors *in vivo* in the two different age groups of normal rats. In exact parallel with the results of IV insulin tolerance tests, marked increases were observed in the binding of insulin by hepatic plasma membranes prepared from all pretreated rats with the exception of GH-treated 45–50 day-old rats. The actual experimental data are presented as bound to free ratio (B/F) of [125 I]-insulin as a function of bound hormone (B) as described by Scatchard [21]. The plots suggested that the differences in binding were due to an increased number of active receptors.

Fig. 2a and b. Insulin binding to hepatic plasma membranes from control ($\circ$), human growth hormone-treated ($\square$) and the synthetic 4–15 fragment-treated (Δ) rats of two different age groups. **a** The percentages of [125 I]-insulin bound to various membrane preparations in the presence of increasing concentrations of unlabelled insulin. Data are corrected for non-specific binding and represent the mean $\pm$ SEM of eight rats from each group. **b** Scatchard analysis of data derived from the same series of experiments as in **a**

Discussion

A hypoglycaemic response to GH occurs consistently in hypophysectomized animals, and attempts to demonstrate this physiological response in normal animals have been proved disappointing. However, it is currently suggested that the response may be related to the elimination of endogenous GH [2, 22–25]. As hypophysectomy induces drastic alterations in the regulatory mechanism of the endocrine systems, it is difficult to ascertain that the induced hypoglycaemic response of the hypophysectomized animals is not due to physiological factors other than GH. Studies in animals without any prior chemical or surgical intervention would certainly provide a better understanding of the actions of GH under normal physiological conditions. Experiments in the present study were designed specifically to maintain these normal physiological conditions, thus utilizing only normal animals. For many years it has been recognized that the levels of endogenous GH in rats vary with

age and are at the lowest from days 5–20 after birth [26, 27]. Normal baby rats of 16–18 days of age were, therefore, chosen for comparison with adult rats of 45–50 days of age in the present studies of the hypoglycaemic actions of human GH and its synthetic fragment human GH 4–15. The mean level of endogenous GH in the baby rats was significantly lower than that of the adult animals. The hypoglycaemic responses to exogenous intact GH are also quite different in the two different age groups of rats.

Acute GH administration had no significant effect on blood glucose levels in adult rats; this is consistent with previous evidence obtained from investigations in rats [28] and in man [4, 29]. The absence of a correlation between endogenous GH and blood glucose in normal animals also cast doubt on the role of the intact GH molecule in glucose homeostasis. In the studies of lipolytic activity of highly purified GH, Frigeri observed that the biological activity is not due to the intact peptide but rather a low molecular weight fragment or a cleaved form of GH [30]. This observation *in vitro* is supported by the present finding *in vivo* that the synthetic fragment of human GH 4–15, but not intact GH, induced hypoglycaemia in normal adult rats. The data reported here not only substantiate the concept that the amino-terminal region of the human GH molecule might be responsible for the hypoglycaemic action of the hormone [31], but also suggest the further possibility that the absence of hypoglycaemic response to human GH in adult rats is associated with the impairment of the cellular mechanism to initiate the proteolytic activation of GH and subsequently to release the active hypoglycaemic fragments from the molecule for the regulation of glucose metabolism. The only evidence in this regard to date is the isolation of a urinary peptide fraction which had the same biological actions as the synthetic fragment [14]. Unfortunately, it is still impossible to identify the urinary material definitively, and elucidation must wait for its purification and the sequencing of its structure.

The results with the 16–18 day-old rats show that the baby animals responded to the hypoglycaemic action of both human GH and the synthetic human GH 4–15 fragment equally well. The exogenous GH at pharmacological doses clearly enhanced insulin-sensitivity in insulin tolerance tests, increased binding of insulin to the specific receptors, and activated the insulin-dependent intracellular glycogen synthase. At similar doses the synthetic human GH 4–15 appears to be equally, if not more, potent as intact human GH in all parameters tested. While our experiments unequivocally show the difference in the hypoglycaemic response to GH between baby and adult rats, the molecular mechanisms underlining this occurrence remain essentially unknown. Our observations are consistent with the idea of Goodman, that the insensitivity to the hypoglycaemic effect of growth hormone in normal animals is produced by growth hormone itself [22]. Our experiments cannot rule

out the influence of other physiological factors such as changes in hormonal profiles other than GH and insulin in the two different age groups of animals. However, these studies provide the first evidence *in vivo* on the hypoglycaemic response to GH in normal rats without any prior chemical or surgical treatment. The present findings suggest that an impaired mechanism(s) in the proteolytic release of the hypoglycaemic fragments from the intact GH or the proteolytic modification of the molecule may contribute to the absence of response in adult animals.

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Dr. Frank M. Ng
Department of Biochemistry
Monash University
Clayton, 3168
Victoria, Australia