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Upregulation of GH receptor and GH binding protein during pregnancy in the GH deficient rat

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During pregnancy there are dramatic changes in the endocrine and metabolic status of the mother: growth hormone (GH) is an important regulator of growth and development. A proportion of GH is bound by specific GH binding proteins (GHBP) that closely resemble the GH receptor (GHR). In the rodent both GHBP and the GHR are considered to be GH dependent, and consequently during pregnancy the increase in serum GH is associated with an increase in GHBP. To examine whether an increase in maternal GH is obligatory for elevation of maternal GHBP or GHR during pregnancy, we used a unique GH-deficient (GHD) strain of rats, to avoid the methodological complications of hypophysectomy and assessed serum GH, GHBP and hepatic GHR binding during the course of pregnancy. In GH normal rats, serum GH concentrations increased twofold and GHBP levels increased threefold; there was no change in hepatic GHR binding. In GHD rats, serum GH concentrations were low and did not increase during pregnancy. Nonetheless, levels of both serum GHBP and hepatic GHR binding increased to that measured in normal rats. Thus, an increase in maternal GH concentration is not required for the gestational upregulation of maternal GHBP or hepatic GHR binding, suggesting that other hormones may be essential in modulating the GH axis during pregnancy.

Keywords: GH deficiency; GH binding protein; pregnancy

Introduction

Requirements for energy and nutrients increase during gestation. Early pregnancy is an anabolic phase, a period of fat and protein storage while the fetus is small, whereas late pregnancy may be considered a catabolic phase, where the increasing demands of rapidly growing fetuses cause redistribution of maternal nutrients (Freinkel, 1988; Metcalfe *et al.*, 1988). Growth hormone (GH), a single polypeptide of 22 kDa, is a key endocrine regulator of growth and nutrient partitioning. During human pregnancy, GH levels increase in the plasma due to placental expression of a GH variant (Frankenne *et al.*, 1988; Erikson, 1989). Similarly, in the pregnant rat, plasma concentrations of GH increase (Kishi *et al.*, 1991), while in the hypophysectomised pregnant rat GH levels do not (Carlsson *et al.*, 1990), suggesting that during pregnancy in the rat, maternal GH is pituitary derived. In the blood, GH is found associated with a GH binding protein (GHBP), a glycoprotein that increases the half life of GH and that inhibits the interaction of GH with its receptor (reviewed Rogol, 1990, 1991; Baumann, 1991). During pregnancy, circulating levels of the GHBP increase. Studies using hypophysectomized mice suggested a role for GH in the pregnancy associated increase in GHBP (Sanchez-Jimenez *et al.*, 1990). More recent studies have shown that although the elevation in GHBP begins on day 9, maternal GH levels do not increase significantly until day 13 of pregnancy in the mouse (Cramer *et al.* 1992a). This suggests that the increase in GHBP could be initiated by other factors. On the basis of correlative studies, placental lactogens have been suggested as initiators of the pregnancy associated increase in GHBP. (Cramer *et al.*, 1992b). Similarly, mouse hepatic GH receptor levels also increase during pregnancy (Cramer *et al.*, 1992a), although the mechanism of regulation and the specific factors responsible remain to be elucidated. Using a unique GH-deficient strain of Sprague-Dawley rats, (avoiding the methodological complications of hypophysectomy), and normal rats, we have examined whether an increase in maternal GH is an obligatory requirement for elevation of maternal GHBP or GH receptor binding during pregnancy. To achieve this we have measured serum GH and GHBP concentrations, as well as hepatic GH receptor binding through gestation.

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Results

To assess the circulating serum GH concentrations, serum was subjected to a specific radioimmunoassay (Figure 1). In GH normal rats the mean GH levels were 18.9 ± 4.3 ng/ml (mean $\pm$ SEM) compared with 5.8 ± 0.3 ng/ml in GH deficient (Figure 1, upper). During the course of pregnancy, serum GH concentrations are known to increase (Kishi *et al.*, 1991). Similarly, in normal rats, serum GH concentrations had increased twofold by day 21 of pregnancy, whereas, in the GHD rats the serum levels remain low and unchanged (Figure 1, lower).

GH binding proteins (GHBP) are the serum carriers of GH and are thought to modulate the activity of GH (Lim *et al.*, 1990). To determine if GHBP levels are influenced by changes in GH or other factors, GHBP was assessed using a specific RIA (Figure 2). There was no significant difference between non pregnant normal (521 ± 50 ng/ml, mean $\pm$ SEM) or GH deficient (SDR) rats (571 ± 168 ng/ml) (upper panel). Interestingly, the concentration of GHBP in the female SDR rats used in this study (571 ± 168 ng/ml) is not significantly different from the concentration reported for the GH deficient Lewis dwarf rat (542 ± 32 ng/ml) (Barnard *et al.*, 1994). The striking finding of the present work was that during the course of pregnancy in both GH normal and GH deficient rats, concentrations of GHBP significantly increased by day 14 of gestation and remained elevated on day 21. This increase was evident as assessed by ANOVA, multiple comparison tests, or when taking into consideration the variable of time using least squared regression analysis. This increase in GHBP was clearly not dependent upon changes in serum GH levels.

To determine if the serum GHBP or serum GH levels were correlated with the concentration of hepatic GH receptor levels, we assessed specific [^{125}I]-GH binding to hepatic microsomal membranes (Figure 3). As there was wide variation between animal size and thus liver size, the % specific binding is expressed per mg of membrane protein. As observed with the serum GH levels, hepatic GH receptor levels were

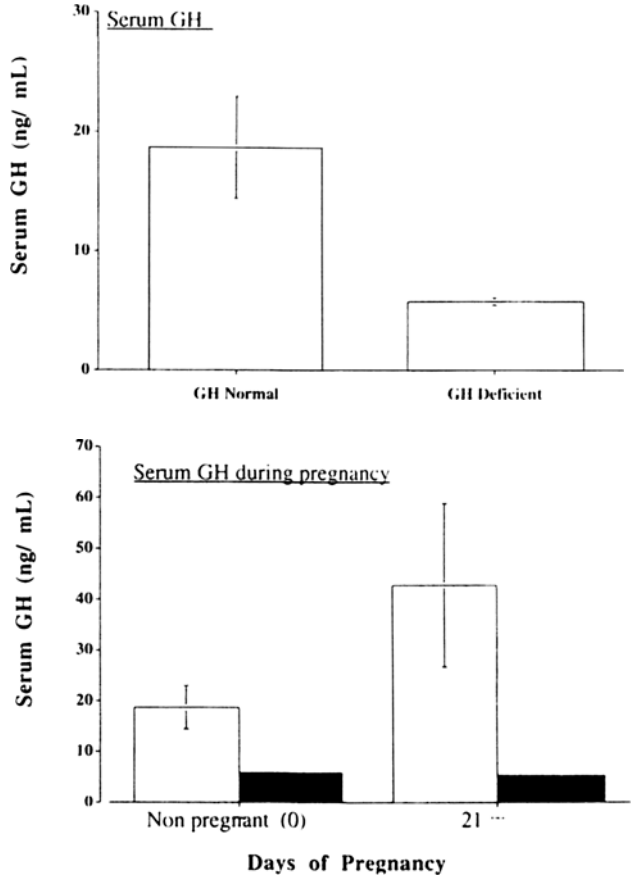

Figure 1 Serum GH. Serum GH was measured using the GH assay and standards as provided by the NIDDK. The UPPER panel shows serum from non pregnant GH normal ($n = 10$) and GH deficient rats ($n = 9$). The LOWER panel compared serum from pregnant animals ($n = r$) with non pregnancy animals. The open bars represent GH normal rats, while the solid bars represent GH deficient rats. Mean ± SEM is shown.

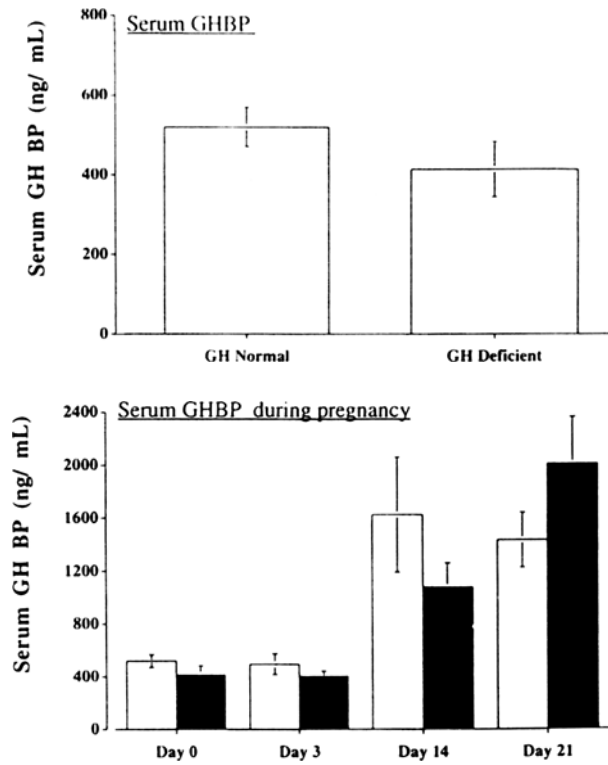

Figure 2 Serum growth hormone binding protein. Serum GHBP is assessed by a specific RIA. GHBP levels, measured in the serum of non pregnant rats (GH normal ($n = 10$) and GH deficient rats ($n = 9$) are shown in the Upper panel. The Lower panel compared serum from pregnant rats across gestation ($n = 5-9$) to non pregnancy animals. The open bars represent GH normal rats, while the solid bars represent GH deficient rats. Mean ± SEM is shown.

reduced in the non pregnant GH deficient rat (upper). During the course of pregnancy in the normal rat there was no significant change in the % specific binding, whereas in the GH-deficient rat, the % specific binding of bGH to the hepatic receptors progressively increased to equal that of normal rats (lower). This was significant by day 14 and the % binding remained elevated at day 21. This increase in GH receptor binding was significant as assessed by ANOVA, multiple comparison tests, or when taking into consideration the variable of time using least squared regression analysis. Again these changes in GH receptor occur despite the unaltered serum GH concentrations.

Discussion

We have examined the interaction of GH, GHBP and hepatic GH receptor binding during pregnancy by comparing normal rats with rats from an isolated GH-deficient strain. Four previous animal models of decreased pituitary GH production include the Ames (Robert & Gowen, 1961), Snell (1929), Little (Eicher & Beamer, 1980) and Lewis (Charlton *et al.*, 1988) strains. However, the Snell and Ames mice also have prolactin and thyroid stimulating hormone deficiencies; the Little mice are associated with an abnormality of growth hormone releasing factor; and the Lewis rat shows 20–25% of the normal levels of GH mRNA in the pituitary (Charlton *et al.*, 1988). In contrast, the spontaneous dwarf rats used in this study have an isolated GH deficiency. The expression of pituitary GH mRNA is only 3–6% of that of control animals (Okuma & Kawashima, 1980; Nogami *et al.*, 1989; Takeuchi *et al.*, 1990). We measured low levels of GH in the GHD rats that approached the assay sensitivity levels. Nonetheless, these levels were higher than those previously published (Okuma & Kawashima, 1980). This may be due to the fact that Kim and coworkers (1992) measured pituitary GH levels not circulating levels, or that the molecular form of GH in the serum is modified either by glycosylation, proteolysis or exists as a variant which may alter the biological activity of GH but not the immunoreactivity (Hughes & Friesen, 1985; Sinha & Jacobsen, 1987). Moreover, it is also important to note that the GH in our GHD rat model has a frame shift mutation in the region coding for helix 3 and is missing helix 4 (Takeuchi *et al.*, 1990) which would make the GH molecule biologically inactive as it could not dimerize the GH receptor. Thus, the GH in the serum of GHD rats may be measurable but not functionally active, such that measurable concentrations do not reflect physiological significance.

During late pregnancy in the human, GH is secreted from the placenta which results in significantly elevated serum concentrations of GH, while pituitary expression and GH pulsatility is almost completely suppressed (Frankenne *et al.*, 1988; Erikson, 1989). In the rat, the increase in serum GH concentrations are solely derived from the pituitary (Carlsson *et al.*, 1990). In the normal rats we measured increased serum GH levels between day 0 and 21 of pregnancy and found that in our GHD rat model that serum GH concentrations did not change during the course of pregnancy. These findings add weight to the finding that the rat placenta does not express GH and that the pituitary is the sole source of circulating concentrations in the pregnant rat.

GH exists in the circulation bound to a GHBP which is biochemically and immunologically related to the GH recep-

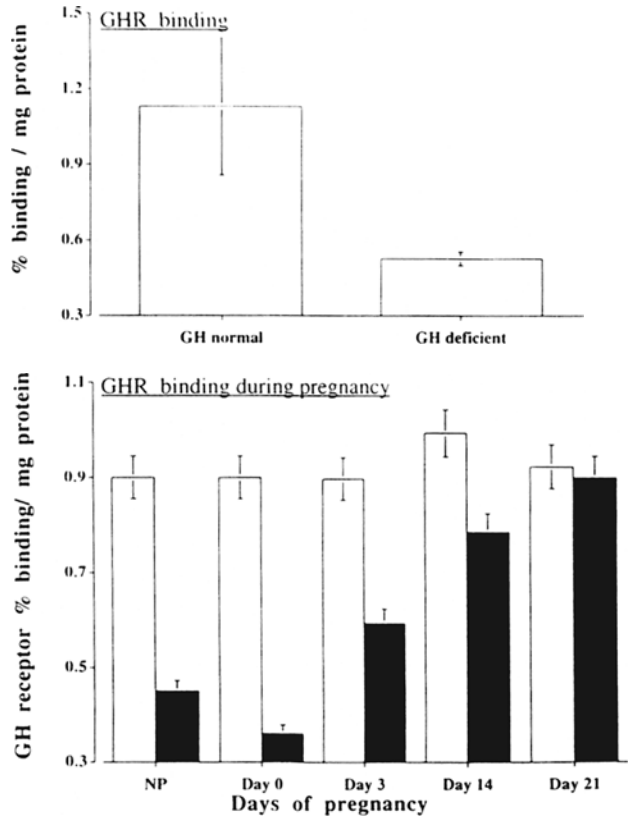

Figure 3 Hepatic growth hormone receptor binding. Hepatic microsomal membranes were prepared from the livers of GH Normal and GH deficient rats. Due to the variation in liver size, the results have been corrected for mg protein/liver. The upper panel shows the % binding/mg protein for $n = 9/10$ non pregnant animals, while the lower panel shows the change in receptor binding during the course of pregnancy ($n = 3-4$ animals per time). The open bars represent GH normal rats, while the solid bars represent GH deficient rats. Mean ± SEM is shown.

tor (Leung *et al.*, 1987; Barnard & Waters, 1986). It has been documented that rat and mouse GHBP levels are elevated during the course of pregnancy, both at the peptide and mRNA level (Tiong & Herrington, 1991; Cramer *et al.*, 1992a), and in the normal rat or drawf rat, GH replacement can change the serum GHBP and GH receptor levels. However, the modality of GH administration is essential to the changes that are measured: twice daily injection will up regulate the GH receptor without effecting the GHBP, whilst continuous infusion of GH will upregulate both GHBP and the GH receptor levels (Carmignac *et al.*, 1992; Barnard *et al.*, 1994). Clearly, in our GHD rat model the elevation in both GHBP and GH receptor binding are not likely to occur through GH as serum concentrations do not increase during the course of pregnancy.

The gestational increase in GHBP was initially suggested to be dependent upon GH (Sanchez-Jimenez *et al.*, 1990) on the basis of replacement of GH in hypophysectomised animals. However the findings of Cramer *et al.* (1992a,b) implied that the regulation of GHBP during pregnancy is more complex. The elevation in GHBP begins on day 9, maternal GH levels do not increase significantly until day 13 of pregnancy in the mouse (Cramer *et al.*, 1992a). Thus, these studies provide more evidence that GH is not a key effector in the changes in serum GHBP levels, and that other effectors are involved. We can discount the role of estrogen as Gabrielsson *et al.*, (1995) have recently shown that induction of GHBP and GH receptor by estrogen is GH dependent, and that the estrogen-mediated induction is reduced in both dwarf and hypophysectomised rats. Thus, the possibility of estrogen mediating the increase in GHBP and GH receptor in our GHD rat seems unlikely. Studies by Cramer *et al.* (1992a,b,c) have shown that hypophysectomy reduces GHBP concentration only on day 14 of mouse gestation and that other factors (mPLI and MPLII) correlated better with GHBP on day 9. Thus, although the rodent placental lactogens (rPL) do not bind appreciably to the GH receptor, PLs dramatically increase from mid-gestation (Kishi *et al.*, 1991) and may be involved in the upregulation of GHBP, possibly through their own receptors. Alternatively, GHBP may be upregulated due to the increasing body mass of the pregnant rat during the course of gestation, thus the increase in GHBP does not discriminate between the GH deficient or normal rat. This is supported by studies (Barnard *et al.*, 1994; Leung *et al.*, 1995) that have shown a relationship between body weight and serum GHBP levels, and that independent of the assay used, that the dynamics of GH and GHBP interaction may contribute to differences in growth rate and body weight. Thus, in the presence of low, unaltered serum concentrations of GH, GHBP levels increase strikingly during the course of pregnancy in GHD rats in parallel with normal rats.

Interestingly, the levels of GHBP and GH receptors are not co-ordinately expressed during pregnancy (Cramer *et al.*, 1992a). In view of the observation that the GHBP levels increased in both normal and GHD rats during pregnancy, we assessed the hepatic GH binding. In normal rats we saw no significant change during pregnancy which is consistent with other reports (Tiong & Herrington, 1991) and in the GHD rats, GH receptor binding was significantly lower than in the GH normal rats. This contrasts with findings in hypophysectomised rats where hepatic binding of GH has been reported to be elevated (Hochberg *et al.*, 1990). This discrepancy may be due to the fact that our animal model has very low levels of serum GH and is otherwise pituitary replete, allowing other regulatory factors to interact with the GH axis. During the course of pregnancy GH receptor binding increased in the GHD rat to similar levels of GH normal rats. Our data suggest that the upregulation of hepatic GH receptors during pregnancy is independent of pituitary GH and may be regulated by placental factors. Increased concentrations of hepatic GH receptors during pregnancy may be required to facilitate the partitioning of nutrients in the maternal compartment to support the increasing demand for placental and fetal growth. Cramer *et al.* (1992b) have shown that hepatic GH receptor and GHBP increase with increasing litter size; however, GHD rats have small litters of 4–7 pups in contrast to the GH normal litter of 10–14 pups (Gargosky *et al.*, 1992). Since litter size in rodents is partially determined by the presence of adequate plasma concentrations of IGF-I during the pre-ovulatory phase (Kroonsberg *et al.*, 1989) and GHD rats have been shown to have very low plasma concentrations throughout life and pregnancy (Gargosky *et al.*, 1992), we would anticipate that the low litter size observed in these animals is unrelated to pregnancy associated changes in plasma GHBP and hepatic receptor concentrations. Clearly, litter size can not explain the upregulation of the GHBP as litter size is small but GHBP levels increase. Clearly further investigation is required to assess GH receptor mRNA expression levels, tissue source and the effect of other regulators and pregnancy-associated factors in this model.

In conclusion, an increase in maternal GH concentration is not essential for the gestational increase in maternal GHBP concentrations or GH receptors during pregnancy in the rat.

Materials and methods

Animals

The strain of Spontaneous Dwarf Rat (SDR) was generously provided by Roussel Morishita Pharmaceutical Co., Ltd.,

Shiga, Japan. These rats carry an autosomal recessive mutation in the GH gene, represented by dr. Their expression of pituitary GH mRNA is only 3–6% that of control animals, due to an aberrant splice site in the mRNA. Furthermore, this truncated mRNA is not translated into the mature protein due to premature termination of translation, such that little GH peptide can be measured in the anterior pituitary gland of SDR (Okuma & Kawashima, 1980; Nogami *et al.*, 1989; Takeuchi *et al.*, 1990). Unlike other GH-deficient dwarf rat strains available, these rats are unique, since they lack only functional GH and are otherwise pituitary-sufficient.

The phenotypes of the SDR animals are distinctive. Adult dr/dr males or females are significantly smaller than litter mates (40% of control). For Dr/dr or Dr/Dr animals, neither somatic growth nor pituitary GH levels differed (Kim *et al.*, 1992). Thus, the GH-normal rats, Dr/dr and Dr/Dr, were indistinguishable and subsequently used interchangeably, while the dr/dr rats were classified as GH-deficient.

Animal handling

Rats were housed in the Department of Laboratory and Animal Medicine facilities at Oregon Health Sciences University. Temperature was constant at 25°C, with a cycle of 12 h light: 12 h darkness and standard food and water were available *ad libitum*.

Rats were mated as follows: (1) female GH-deficient (dr/dr) were crossed with GH-deficient (dr/dr) males; (2) female GH-normal (Dr/dr, Dr/Dr) were mated with GH-normal (Dr/dr, Dr/Dr) males. Detection of one or more vaginal plug(s) was considered day 1 of pregnancy. Animals were exsanguinated and cervically dislocated. Blood was clotted at room temperature for 30 min and sera were harvested after centrifugation at 10 000 g for 10 min. Sera were stored at −20°C. Livers were weighed and frozen immediately in liquid nitrogen for future receptor analysis.

Serum GH measurement

Serum GH levels were measured by radioimmunoassay (RIA) as previously described using materials obtained from the NIDDK (Kaeshoh *et al.*, 1989). Samples were run in duplicate in a single assay. The intra-assay co-efficient of variation was $<10\%$. The minimal detectable concentration of GH was <2 ng/ml. Samples were measured against a GH-RP2 standard.

Serum GH binding proteins

Total serum GH BP was measured using a radioimmunoassay described in detail previously (Barnard *et al.*, 1994). This assay uses a synthetic peptide (corresponding to the hydrophilic C-terminus of GHBP as standard (Cramer *et al.*, 1992c) and a monoclonal antibody reactive with this peptide (MAb 4.3, Sadeghi *et al.*, 1990) as the primary antibody.

GH binding to hepatic receptors

Hepatic GH receptor (GHR) assays were performed as described previously (Breier *et al.*, 1988) and validated for rat liver (Singh *et al.*, 1992). In brief, microsomal membranes were prepared as follows: liver tissue was thawed at 4°C, cut into small pieces (~1 g) and washed in cold (4°C) 0.3 M sucrose. The tissue was then weighed and homogenised (1:3 wt/vol) in 0.3 M sucrose containing aprotinin (10^6 kallikrein inhibitor units [KIU]/l; Trasylol, Bayer Pharmaceuticals, Botany, Australia). The homogenate was centrifuged at 1500 g for 20 min at 4°C and the resulting supernatant was centrifuged sequentially at 15 000 g for 20 min and 100 000 g for 90 min at 4°C. The 100 000 g pellet was incubated with 4 M $MgCl_2$ (1:2 wt/vol ratio of initial liver weight) for 20 min at 4°C to remove exogenously bound ligand (Breier *et al.*, 1988). The preparation was then centrifuged at 48 500 g for 60 min at 4°C, and the resulting pellet was suspended in 0.025 M HEPES buffer, pH 7.6, and centrifuged again at 48 500 g for 20 min at 4°C. Aliquots of the final pellets were resuspended in cold 0.25 M HEPES buffer pH 7.6 containing aprotinin (10^6 KIU/l) at a ratio of 1 mL buffer/g wet wt initial liver tissue and frozen in aliquots at −20°C until further analysis. Binding analysis was performed in triplicate using 50 uL of membrane preparation (= 50 mg initial wet liver weight) per tube. The membrane preparations were incubated with approximately 3000 c.p.m. of iodinated bovine GH (bGH) at a final incubation volume of 0.5 mL for 20 h. Non-specific binding was determined by addition of excess of unlabeled bGH (1000 ng/tube). Incubation was terminated by adding 3 mL of ice-cold 0.025 M TRIS, 0.01 M $CaCl_2$ buffer, pH 7.6. Bound and free hormone were separated by centrifugation at 3000 g for 45 min at 4°C. Equilibrium was reached under these conditions and specific binding was completely reversible by the addition of excess of unlabeled bGH. The intra- and inter-assay coefficients of variation were 6.7% and 9.8%, respectively. Protein concentrations of the membrane preparations for individual animals were determined by a modified Lowry method (Singh *et al.*, 1992), and the specific binding has been corrected for protein concentration.

Statistics

All data were analysed by a one way analysis of variance as the ANOVA which assumes that all data comes from a population with equal SDs. Subsequently a Bonferroni Multiple Comparison test was performed, as well as a least squared regression analysis for Figures 2 (GHBP) and Figure 3 (GHR binding) to assess the effect of time. Analysis the data as a natural log enabled the homoscedacity test to be passed, stabilizing the variances and providing a better r squared. Analysis was done using InStat, a statistical software package provided by GraphPad Software (San Diego, CA, USA).

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