Adrenal involvement in the diabetes-induced loss of growth hormone and prolactin receptors in the livers of female rats

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Summary. Streptozotocin-induced diabetes causes a decrease in growth hormone and prolactin receptors in the livers of female rats, and in the serum concentration of somatomedin-C/insulin-like growth factor-I, concomitantly with an increase in the serum testosterone levels. In this study, a possible role for adrenal androgens in the loss of receptors was examined. Rats were adrenalectomised bilaterally 3 days after the induction of diabetes with streptozotocin (100 mg/kg intravenously), and livers were removed 3 days later. Adrenalectomy had no effect on binding of ovine prolactin or bovine growth hormone to liver microsomal membranes from non-diabetic rats, but in diabetic rats it entirely abolished the 56% decrease in prolactin binding and significantly reversed the 66% decrease in growth hormone binding and the parallel fall in serum levels of somatomedin-C/insulin-like growth factor-I ($p < 0.05$). Adrenalectomy also prevented the diabetes-induced rise in serum testosterone. Daily injection of testosterone to normal and diabetic rats for 12 days significantly reduced both prolactin and growth hormone binding ($p < 0.001$), with the effect of diabetes being additive upon the testosterone effect. Implantation of testosterone-filled silastic capsules at the time of adrenalectomy (i. e. for 3 days) did not prevent the adrenalectomy-induced restoration of both growth hormone and prolactin receptors. The resulting high serum testosterone level did not reduce binding to growth hormone receptors in control rats over the 3 day period, and caused no further decrease in diabetic rats. However, binding to prolactin receptors was reduced by 47% in control animals with no further loss in diabetic animals ($p < 0.001$). Adrenalectomy prevented the loss of prolactin receptors in both testosterone-treated and diabetic rats. These results indicate that, whether or not the rise in testosterone of adrenal origin contributes to the receptor loss in diabetes, the effect also depends on another factor of adrenal origin.

Key words: Growth hormone receptor, prolactin receptor, somatomedin-C, insulin-like growth factor-I, diabetes, adrenalectomy, testosterone.

We have previously reported that both growth hormone (GH) and prolactin (PRL) receptors in liver microsomal membranes decreased in number, but not affinity, in female rats with experimental diabetes induced by streptozotocin [1, 2]. Insulin treatment restored these receptors to normal levels. The circulating level of the GH-dependent growth factor somatomedin-C (identical to insulin-like growth factor-I [3] and designated SM-C/IGF-I) also decreases in experimental diabetes [4]. It has been suggested that the loss of GH receptors might be associated with the decrease in SM-C/IGF-I, although there is some evidence that the growth factor and receptor levels fall at different rates [5]. In an earlier study, we observed that the loss of PRL receptors in diabetes was accompanied by an increase in serum testosterone, which returned to normal levels with insulin treatment [2]. Subsequently Leaming et al. [6], investigating steroid metabolism in female rats made diabetic with alloxan or streptozotocin, confirmed that diabetes increased testosterone levels. Whereas ovariectomy had

no effect on testosterone levels in diabetes, adrenalectomy prevented the rise caused by diabetes, implying that the testosterone was of adrenal origin [6].

Testosterone has been widely documented as having a suppressive effect on PRL receptors [7-11], and we have previously reported that testosterone administration decreases the number of PRL receptors in the livers of both normal and diabetic rats [2]. In contrast to prolactin receptors, little is known about the regulation of GH receptors in rat liver by sex steroids, except that livers from female animals are known to have more receptor sites that those from males when measured in either whole hepatocytes [12] or microsomal membrane preparations [1, 13].

If androgens suppress GH as well as PRL receptors in female rat liver, the increase in adrenal androgen production seen in diabetes might be responsible for the loss of both receptor types and of SM-C/IGF-I observed in earlier studies.

Table 1. Weight changes, serum glucose and insulin levels in adrenalectomised and sham-operated rats

	Sham-operated rats		Adrenalectomised rats	
	Normal ($n=7$)	Diabetic ($n=8$)	Normal ($n=7$)	Diabetic ($n=8$)
Weight change (g)	$+9.7 \pm 3.1$	-5.3 ± 8.0^c	$+8.1 \pm 5.7$	-1.0 ± 8.7^a
Serum glucose (mmol/l)	8.5 ± 3.8	34.8 ± 3.1^c	7.6 ± 2.1	$27.2 \pm 4.9^{c,d}$
Serum insulin (mU/l)	34.6 ± 9.2	16.0 ± 1.1^c	24.9 ± 5.4^b	16.1 ± 2.5^c

Values are mean $\pm$ SD
$^a p < 0.01$, $^b p < 0.005$, $^c p < 0.001$. Comparisons with sham-operated non-diabetic animals
$^d p < 0.001$. Comparisons with sham-operated diabetic animals

Table 2. Serum testosterone in adrenalectomised and sham-operated rats with and without testosterone treatment

	Serum testosterone (nmol/l)			
	Sham-operated rats		Adrenalectomised rats	
	Normal	Diabetic	Normal	Diabetic
Control rats	2.45 ± 0.96 ($n=9$)	5.30 ± 1.60^a ($n=14$)	2.49 ± 1.62 ($n=12$)	2.17 ± 0.76 ($n=14$)
Testosterone-treated rats	74 ± 19 ($n=4$)	77 ± 21 ($n=6$)	87 ± 30 ($n=6$)	106 ± 32 ($n=7$)

Values are means $\pm$ SD. Diabetic rats received streptozotocin (100 mg/kg) 6 days prior to killing
$^a p < 0.001$ Comparison with sham-operated non-diabetic animals
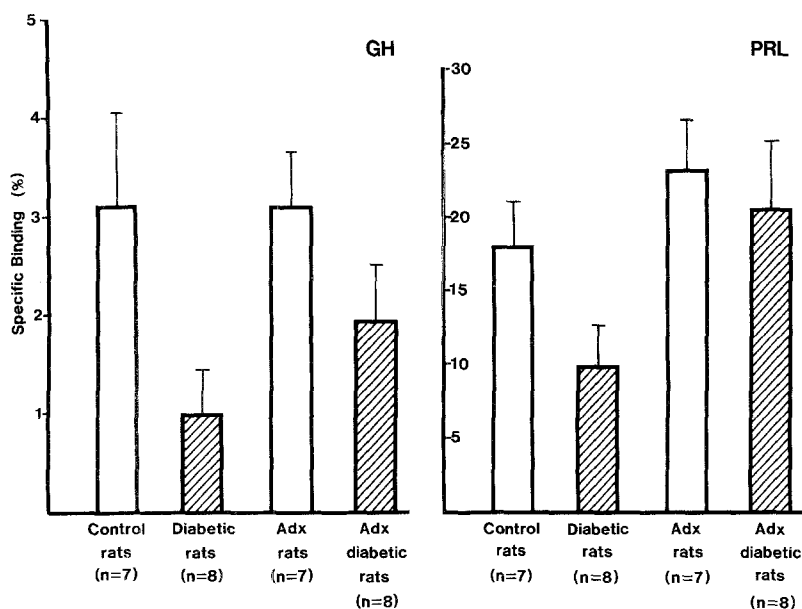
Fig. 1. Effect of adrenalectomy (Adx) on binding of bovine GH and ovine PRL tracers to liver microsomal membranes from non-diabetic (☐) and diabetic (▨) female rats. Data are expressed as mean $\pm$ SD. Significant differences refer to a comparison with the control group, with diabetic rats (GH and PRL) $p <$ 0.001, Adx rats (GH) N.S., Adx rats (PRL) $p < 0.05$, Adx diabetic rats (GH) $p < 0.01$ and Adx diabetic rats (PRL) N.S.

To investigate this hypothesis, we studied the effects of adrenalectomy and the effects of pharmacologically increasing the serum testosterone concentration on hepatic GH and PRL receptors and serum SM-C/IGF-I levels in normal and diabetic rats.

Materials and methods

Procedures

Female Wistar-Furth rats weighing 120-140 g were housed under standardised lighting conditions (light on from 07.30 to 19.30 h) and fed standard rat cubes with water ad libitum. Each experimental group contained six or seven animals. In the initial experiments designed to test the effects of androgens, rats were subcutaneously given a mixture of testosterone propionate, phenyl propionate and isocaproate (Sustanon, Organon, Sydney, Australia), 1 mg/day for 12 days, commencing 5 days before streptozotocin treatment. Diabetes was in-

duced by streptozotocin, 100 mg/kg (Calbiochem, La Jolla, California, USA), freshly dissolved in 0.1 mol/l Na citrate buffer pH 4.0 and administered by tail vein injection. Controls received buffer alone. To study the role of the adrenals, rats were adrenalectomised bilaterally 3 days after streptozotocin treatment. Non-adrenalectomised control rats were sham operated.

In a later protocol, testosterone-treated rats were implanted with testosterone-filled silastic capsules at the time of adrenalectomy or sham operation. These capsules were made from silastic tubing (No. 602-265, Dow Corning, Midland, Michigan, USA) with 1.6 mm internal diameter and 2.4 mm external diameter). Capsules were filled with testosterone (Sigma, St. Louis, Missouri, USA) to a length of 5 mm. On day 6 following streptozotocin treatment, rats were anaesthetised with Ketalar, 1 ml/kg (Parke-Davis, Sydney, Australia), blood was collected by cardiac puncture, and the livers were removed and stored at $-80 \degree C$ until used for membrane preparation.

Binding studies

Liver microsomal membranes were prepared by differential centrifugation as previously described [14]. GH and PRL receptors were mea-

Table 3. Binding to GH and PRL receptors in adrenalectomised and sham-operated rats with and without testosterone implants

	Specific GH Binding (% of Total)	Specific PRL Binding (% of Total)
Control rats		
Normal (n = 10)	3.02 ± 0.83	22.2 ± 8.0
Adrenalectomised (n = 12)	3.10 ± 0.69	25.7 ± 4.5
Diabetic (n = 14)	1.01 ± 0.42[c]	10.0 ± 2.6[c]
Adrenalectomised - Diabetic (n = 11)	1.78 ± 0.57[b,d]	22.0 ± 5.5[e]
Testosterone-treated rats		
Normal (n = 4)	2.45 ± 0.84	14.2 ± 2.5[a]
Adrenalectomised (n = 6)	3.28 ± 1.07	22.8 ± 7.1
Diabetic (n = 6)	0.73 ± 0.56[c]	9.8 ± 4.0[c]
Adrenalectomised - Diabetic (n = 7)	1.90 ± 0.76[b,d]	21.6 ± 3.1[e]

Values are expressed as mean ± SD
[a] $p < 0.01$, [b] $p < 0.05$, [c] $p < 0.001$. Comparison with normal control animals
[d] $p < 0.05$, [e] $p < 0.001$. Comparison with corresponding diabetic animals

Table 4. Effect of adrenalectomy on serum SM-C/IGF-I levels in control and diabetic rats

	SM-C/IGF-I (U/ml)
Control rats (n = 7)	0.68 ± 0.09
Adrenalectomised rats (n = 7)	0.58 ± 0.09[a]
Diabetic rats (n = 8)	0.18 ± 0.10[b]
Adrenalectomised - Diabetics (n = 8)	0.28 ± 0.10[b,c]

Values are expressed as mean ± SD
[a] $p < 0.05$, [b] $p < 0.001$. Comparison with control animals
[c] $p < 0.05$. Comparison with diabetic animals

sured on 200 µg of membrane protein, using bovine GH (bGH) and ovine PRL (oPRL) respectively as radioligands as reported earlier [1, 2]. Briefly, for either receptor type incubations were performed at 22 °C in 1.5 ml polystyrene centrifuge tubes for 16 h in a final volume of 0.3 ml of Tris-HCl (25 mmol/l), $CaCl_2$ (10 mmol/l) buffer containing 0.1% bovine albumin, pH 7.4. Tracer hormone, iodinated using chloramine T to a specific activity of approximately 100 Ci/g, was present at 10 nCi (100 pg)/tube. At the end of the incubation period, membranes were pelleted by centrifugation for 7 min at 7,000 g (Beckman Microfuge, Beckman Instruments Inc., Palo Alto, California, USA), washed in 1 ml of cold incubation buffer, and the tips of the tubes containing the pellets were cut off and counted in a gamma counter (LKB-Wallac Multigamma, Turku, Finland).

Non-specific binding measured in the presence of unlabelled hormone (0.5 µg) was typically 4% of total radioactivity for bGH tracer and 6% of total radioactivity for oPRL tracer. Although the nonspecific binding of bGH tracer was high relative to the specific binding, this figure is comparable to that seen in other published studies of bGH binding to microsomal membranes [1, 14], homogenates [5] and hepatocyte pellets [12]. The bGH for iodination (S-32-35) was donated by Dr. M.Sonenberg, Memorial Sloan-Kettering Cancer Center, New York, USA. Iodination grade oPRL (I-1) was provided by the National Hormone and Pituitary Program, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, Bethesda, Maryland, USA. Specificity studies validating the use of these ligands for GH and PRL receptor measurement have been reported previously [1, 2].

Hormone assays

Testosterone was measured using Bio-RIA (Louisville, Kentucky, USA) I^{125}-Testosterone direct (no extraction) Covalent Coat Tubes Radioimmunoassay kit. Serum oestradiol was measured after ether extraction by radioimmunoassay using 16 α-$[I^{125}]$-iodoestradiol (New England Nuclear, Boston, Massachusetts, USA) and antiserum (Endocrine Sciences, Tarzana, California, USA). Due to the large sample requirement, measurements were made on pooled specimens. Insulin was measured using the Insulin Radioimmunoassay kit from Bio-RIA. Somatomedin-C levels were determined on acid-ethanol extracted serum samples [16] by specific radioimmunoassay using antiserum Tr4 as described previously [17]. Glucose was measured using glucose oxidase and peroxidase.

Statistical analysis

Differences between control and treated animals were evaluated using Duncan's multiple range test [18] after pooling variances by analysis of variance.

Results

Weight changes and serum glucose and insulin levels in the diabetic and adrenalectomised animals are shown in Table 1. Adrenalectomy did not alter the effect of diabetes on body weight change or serum insulin levels, although it appeared to marginally ameliorate the hyperglycaemia. There was a 66% decrease in bGH tracer binding ($p < 0.001$) and a 56% decrease in oPRL binding ($p < 0.001$) in diabetic rats compared to normal rats (Fig. 1). This was accompanied by a 2.2-fold increase in serum testosterone levels (Table 2) with no change in the mean oestradiol level (control: 204 pmol/l; diabetic: 270 pmol/l). Adrenalectomy had no effect on GH binding in normal rats, but there was a slight increase ($p < 0.05$) in binding of PRL. Adrenalectomy 3 days after the induction of diabetes completely restored the binding of oPRL to control levels, whereas bGH binding was only partially restored ($p < 0.01$ compared with control rats and $p < 0.01$ compared to diabetic rats). This was accompanied by the return of serum testosterone levels to control values (Table 2). The mean serum oestradiol level was reduced by over 60% by adrenalectomy to 79 pmol/l, with diabetes causing no further change (71 pmol/l).
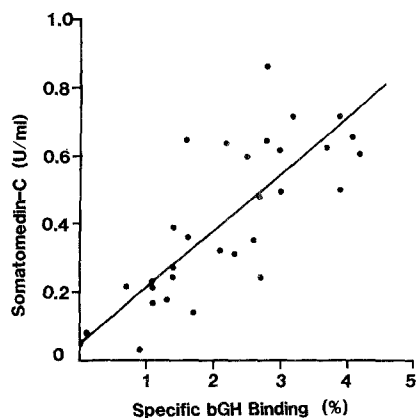
Fig. 2. Association between specific binding of tracer bovine GH to liver microsomal membranes and serum SM-C/IGF-I levels in the group of animals referred to in Fig. 1. The correlation coefficient $(r = 0.759)$ was highly significant $(p < 0.0001)$

Since the changes in serum testosterone levels were consistent with the involvement of testosterone in the modulation of GH and PRL receptors, the effect of testosterone was studied directly by giving 12 daily injections of a commercial preparation (Sustanon, 1 mg/day). Testosterone reduced specific binding to GH sites in livers from normal animals ($3.85 \pm 0.47\%$ of total, $n = 6$) by 40% $(p < 0.001)$, while diabetes caused the previously observed 67% decrease in binding $(p < 0.001)$. The effects of diabetes and testosterone appeared to be additive, as the combined effect was a further decrease in binding of 72% $(p < 0.001)$ compared to testosterone alone, and a further decrease of 49% $(p < 0.01)$ compared to diabetes alone.

As in our earlier study [2], the diabetes and testosterone effects on PRL binding interacted in a similar way. Diabetes reduced specific binding ($17.8 \pm 4.3\%$ of total, $n = 6$) by 57% $(p < 0.001)$, testosterone by 64% $(p < 0.001)$, and diabetes + testosterone by 75% $(p < 0.001)$ compared with normal animals. As seen for bGH binding, the effects of diabetes and testosterone appeared to be additive. However, due to the effect of large variance in the normal group on the pooled variance for all groups, the decrease in binding due to diabetes + testosterone, when tested using Duncan's multiple range test, was barely significant compared to diabetes alone $(p = 0.05)$ and not significant compared to testosterone alone $(p > 0.05)$. When re-tested by Student's t-test, however, the combined effect of diabetes and testosterone was indeed significantly greater than that of diabetes $(p < 0.01)$ or testosterone $(p < 0.001)$ alone. Mean $(\pm SD)$ testosterone levels in normal animals were 2.1 ± 0.6 nmol/l, increasing to 5.8 ± 0.4 nmol/l in diabetes, while in testosterone-treated animals the mean value was 33.2 ± 1.6 nmol/l for non-diabetic and 29.8 ± 2.3 nmol/l for diabetic rats.

To test the effects of simultaneous testosterone administration and adrenalectomy, testosterone filled capsules were implanted at the time of adrenalectomy.

Since maximal loss of hGH binding had previously been shown to require 12 days of testosterone treatment [7], a high testosterone concentration was used in this experiment in an attempt to produce a significant response in the 3-day experimental period. The implants resulted in serum testosterone levels between 70 and 100 nmol/l in all rats (Table 2). Despite this high level of testosterone, the binding to GH receptors in non-adrenalectomised, non-diabetic rats was decreased by only 20% in the 3 days of implantation and in diabetic rats by approximately 30% (Table 3) compared with animals not receiving testosterone. This modest effect of testosterone probably reflects the short duration of treatment [7]. Testosterone treatment did not prevent the restoration of GH receptors in diabetic rats following adrenalectomy, suggesting that the adrenal-mediated changes in diabetes were not due to increased testosterone production.

However, testosterone treatment decreased PRL binding by 47% in non-adrenalectomised, non-diabetic animals, with no further drop in diabetic animals $(p < 0.001)$ (Table 3). Adrenalectomy prevented this testosterone-induced loss of PRL binding in non-diabetic rats, as well as the diabetes-induced loss of binding. Despite the high testosterone levels in rats with implants, an increase in PRL receptors following adrenalectomy was still seen in diabetic rats.

Changes in serum SM-C/IGF-I levels due to diabetes were observed to parallel closely those in GH receptors (Table 4), with the decrease in diabetes being partly blocked by adrenalectomy. Similar results were seen in animals with testosterone implants (data not shown). The relationship between GH receptors and SM-C/IGF-I levels in individual animals is shown in Fig. 2. A highly significant association was seen $(r = 0.759, p < 0.0001)$, while between the PRL binding and SM-C/IGF-I levels there was a somewhat weaker association $(r = 0.484, p < 0.001)$.

Discussion

It has been reported that rats with chronic diabetes produced by streptozotocin show an abnormal regulation of the factors that control adrenal steroid regulation and metabolism [19]. These rats have adrenal hypertrophy with high resting levels of plasma corticosterone and an increased response to stress with changes in the feedback response [20]. More recently, Leaming et al. [6] have shown that the increase in serum testosterone that we had observed in diabetes [2] was due to increased production of androgens by the adrenals. The role of testosterone as a regulator of PRL receptors in rat liver is well documented [7-11]. In males, binding to PRL receptors is increased by castration and decreased by testosterone administration [7, 10]. In females, testosterone treatment decreases, although not to male levels, PRL binding [8, 9].

Regulation of rat liver GH receptors by testosterone has not been documented, although it has been reported that females have more receptors than males [1, 12, 13]. Our studies confirm that testosterone decreases PRL receptors, and they establish a similar role for testosterone on GH binding in the livers of female rats. Very strong negative correlations are seen between testosterone and both GH ($r=0.706$, $p<0.0001$) and PRL ($r=0.744$, $p<0.0001$) binding.

The decreased PRL binding in rat liver caused by androgen treatment has been reported to be due to the direct action of testosterone on the liver [8, 9]. However, the present study suggests that the effects of both testosterone and diabetes on liver PRL receptors and, to a lesser extent, on GH receptors are mediated by the adrenals. Whether the effects of diabetes are due to changing testosterone levels, or the regulatory pathways for testosterone and diabetes are quite separate, remains unresolved, since both effects were abolished by adrenalectomy. However, further suppression of both GH and PRL receptors by diabetes in animals which had received testosterone implants indicates that there are important additional regulatory mechanisms in the diabetic animal. Treatment of diabetic rats with anti-androgens may answer the question whether testosterone plays any part at all.

From these studies it is concluded that the regulatory effect of insulin on hepatic GH and PRL receptors is mediated, at least in part, via the adrenal glands. The partial restoration of SM-C/IGF-I levels in diabetic rats following adrenalectomy indicates an adrenal component also in the regulation of this peptide, while the failure of adrenalectomy to cause complete reversal of SM-C/IGF-I levels (or of GH receptors) suggests an additional suppressive factor, perhaps related to the relative nutritional deficiency of diabetic rats [21]. One regulatory factor known to affect both receptor types and SM-C/IGF-I is GH itself. GH secretion is decreased in both fasting [22] and diabetes [23], conditions where hepatic GH and PRL receptors, and serum SM-C/IGF-I levels [4, 21, 24], are diminished. Our results could be explained if an adrenal factor, perhaps a catecholamine, were found to be a physiological regulator of pituitary GH secretion. The demonstration that α-adrenergic blockade enhances GH release, possibly by inhibiting hypothalamic somatostatin release [25], supports this hypothesis. If this is so, the decrease in GH secretion seen in diabetes [23] would be dependent upon increased adrenal catecholamine secretion, and should be reversed by adrenalectomy.

Changes in GH secretion would directly affect both GH and PRL receptor levels and hepatic SM-C/IGF-I production. GH administration is known to induce PRL receptors in the livers of hypophysectomised rats [26], and recent studies from this laboratory have shown that both GH and PRL receptors are also induced by GH in intact animals of both sexes [27, 28]. With regard to SM-C/IGF-I, this peptide has long been recognised

as being under GH control [29]. In reports from this laboratory, a highly significant association was seen between GH secretory spikes and serum SM-C/IGF-I levels integrated over a 5-hour period [17], and in studies of SM-C/IGF-I release by rat hepatocytes in primary culture, both the GH status of the donor animal and the addition of GH in vitro have been found to modulate the rate of growth factor release [30].

Receptors for GH may also be closely involved in the regulation of SM-C/IGF-I synthesis, as indicated by the close correlation observed between hepatic GH binding and serum SM-C/IGF-I levels in hypophysectomised rats in which both GH receptors and SM-C/IGF-I were restored by GH infusion [28]. In the present study we again observed a significant relationship between GH receptors and serum SM-C/IGF-I. In particular, the partial restoration of GH receptors after adrenalectomy of diabetic rats was closely paralleled by an increase in SM-C/IGF-I. Further testing of the hypotheses resulting from these experiments will require detailed measurements of the combined effects of diabetes, adrenalectomy and testosterone treatment on GH and PRL secretory dynamics. Since both hormones are secreted in a pulsatile manner [31, 32] and are affected by stress [32, 33], measurements in random serum samples are unlikely to provide meaningful information. Therefore, resolution of these questions will require serial sampling from chronically cannulated, unrestrained rats. These studies should help to elucidate the exact role of insulin, androgens and catecholamines in modulating GH and PRL secretion and in the maintenance of hepatic receptor and serum SM-C/IGF-I levels.

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