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Hepatic estrogen and androgen receptors and binding proteins in streptozotocin-diabetic male Wistar rats

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Summary. We have previously shown that there are decreases in the sex differences seen in certain hepatic drug and steroid metabolising enzymes in rats with early (4 day) streptozotocin-induced diabetes [31]. We postulated that hepatic sex hormone receptors or binding proteins might be involved in modulation of the sex differences noted in metabolism. In the present study, we measured the binding kinetics of the hepatic cytosolic estrogen receptor and androgen receptor, along with the high capacity-low affinity estrogen binding protein. At 4 or 10 days post-streptozotocin (60 mg/kg intravenously), there was no change in the maximum binding capacity of the estrogen receptor, nor in the hormone affinity of any of the three proteins. However, the binding capacity of the androgen receptor and estrogen binding protein in the diabetic animals was decreased to less than half of control levels. This effect could not be reversed by hormone replacement with any of the following regimens: protamine zinc insulin, 10 U/kg subcutaneously once a day; Toronto insulin, 15 U/kg subcutaneously twice a day; testosterone enanthate, 1 mg/kg s.c. once a day; triiodothyronine, 30 μg/kg s.c. daily; ovine growth hormone: 0.02 U/h s.c., 30 μg s.c. 7 times daily, 30 μg i.v. 4 times daily; or various combinations of these hormones. Stress, such as 4 intravenous injections of saline per day, was noted to decrease the binding capacity of the estrogen binding protein. Therefore, we measured the basal serum corticosterone levels, which were not significantly different from control values in untreated or insulin-treated diabetic rats. In addition, the hepatic cytosolic glucocorticoid receptor capacity was not significantly changed in the diabetic animals. This provides evidence that at 4 days post-streptozotocin, the diabetic state is not so stressful as to result in major alterations in these two parameters.

In summary, because insulin is known to restore the sex differences in hepatic drug and steroid metabolism to control levels but does not restore the capacity of the cytosolic androgen receptor or estrogen-binding protein, we conclude that they are not of primary importance in regulation of the metabolic enzymes.

Key words: Streptozotocin, diabetes, liver, estrogen receptor, androgen receptor, high capacity-low affinity estrogen binding protein.

The rate and route of catabolism of steroid hormones and many drugs are controlled by sex hormones and growth hormone in rat liver [1-4]. The effect of testosterone is well known and will not be discussed here. The interaction with growth hormone appears to involve androgenic stimulation of somatostatin release which, along with growth hormone releasing factor, modulates the release of growth hormone. This produces the sex differences seen in growth hormone patterns, i.e. the peak and trough ultradiene pattern of the male and the continuous presence of growth hormone in the plasma of the female [5-8]. These different patterns of growth hormone release result in differences seen in the metabolism of some steroids and drugs [9, 10].

The male rat liver contains several sex steroid binding proteins, the estrogen receptor [11-14], the high capacity-low affinity estrogen binding protein (HCLA) [15-19], and the androgen receptor [20-22]. There is a sex difference only in the levels of the HCLA and androgen receptor [15, 18, 19, 21, 22]. Manipulations which abolish the hepatic sex differences in steroid and drug metabolism in adult rats, for example neonatal castration, hypophysectomy and gonadectomy, also abolish the sex differences seen in the levels of the androgen receptor or HCLA [18, 19, 23-27]. These observations led us to postulate that hepatic sex steroid binding proteins may be involved in modulation and control of the sex differences seen in metabolism.

It is of interest that in the diabetic state, there are also alterations in hepatic steroid and drug metabolism [27-31]. Many substrates which are normally metabolised faster in males than females, are decreased in catabolic rate by about 50% in the diabetic male rat

liver [31]. In addition, diabetic male rats have decreased serum levels of testosterone and triiodothyronine, and a feminised pattern of growth hormone secretion [31–34]. Therefore, we formulated the hypothesis that some of the differences in hepatic steroid and drug catabolism known to occur in the diabetic state are mediated by changes in the capacity or affinity of the sex steroid binding proteins. To test this hypothesis, we examined streptozotocin-treated rats to determine the effects of diabetes on the hepatic cytosolic sex steroid binding proteins at a time point when alterations in metabolism are known to occur [31].

Materials and methods

Chemicals and reagents

Biofluor scintillation cocktail; [6,7-^{3}H(N)]-estradiol, 40–60 Ci/mmol; [6.7-^{3}H(N)]-dexamethasone 35–50 Ci/mmol; 17-α [methyl-^{3}H] methyltrienolone (R1881), 86–87 Ci/mmol; and R1881 were obtained from New England Nuclear (Boston, Mass, USA). [Monoethyl^{3}H] diethylstibesterol (DES) was purchased from Amersham (Oakville, Ont, Canada), dextran T-70 was obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Ammonium sulfate, ultrapure, was purchased from ICN Biomedical (Montreal, Quebec, Canada), and Alzet osmotic-minipumps, Model 2001 were obtained from Alza Corporation (Palo Alto, Calif, USA). Ovine growth hormone (1.5 IU/mg) was a gift of the National Institute of Diabetes, Digestive, and Kidney Diseases (Baltimore, Md, USA). Protamine zinc and Toronto insulin were products of Connaught Laboratories (Toronto, Ont, Canada), and Tes-Tape of Eli Lilly and Co. (Scarborough, Ont, Canada). Most other chemicals and hormones were obtained from Sigma Chemical Company (St. Louis, Mo, USA).

Care and treatment of animals

Male Wistar rats weighing 300–350 g, were obtained from Canadian Breeding Farms (Montreal, Quebec, Canada). Animals were housed in a separate animal room on Lobund bedding (Paxton Processing Ltd, Paxton, Ill, USA) under controlled light (0600 hours on, 2000 hours off) and temperature (22 °C). They were allowed free access to food (Purina Laboratory Chow, Ralston Purina of Canada Ltd, Woodstock, Ont, Canada) and tap water ad libitum. Rats were allowed a minimum of 3–4 days to equilibrate after shipment before starting treatment.

Streptozotocin was administered 4 or 10 days prior to animal use via a tail vein injection of 60 mg/kg in citrate buffer under light ether anaesthetic. Control animals were injected with vehicle only under similar conditions. Hormone replacement experiments were done using 4-day post-streptozotocin injected diabetic rats. All rats injected with streptozotocin had a glycosuria measurement of 0.5% or greater as determined by Tes-Tape, except those treated with insulin. The following hormones were injected subcutaneously once a day: protamine zinc insulin (10 U/kg), testosterone enanthate (1 mg/kg in corn oil), triiodothyronine (T_3; 30 μg/kg in normal saline at pH 8.5). Toronto insulin was administered subcutaneously twice a day (15 U/kg). Ovine growth hormone (oGH) was dissolved in normal saline (pH = 8.5). It was either administered in a dose of 30 μg/s.c. injection seven times per day at 0615, 1015, 1415, 1815, 2100, 2340, and 0240 hours or by continuous infusion of ovine growth hormone 0.02 U/h, via Alzet Model 2001 minipumps implanted subcutaneously in the rostral half of ether-anaesthetized rats. For i.v. injection oGH was administered via the tail vein in conscious animals 4 times per day (0900, 1300, 1700, 2100 hours) in a dose of 30 μg/injection.

Assays

a) Sex hormone receptors. Cytosol was prepared according to the method of Sunahara et al. [22] with the following modifications. The 105,000 *g* centrifugation for the estrogen receptor and HCLA was for 30 min. An equal volume of saturated ammonium sulfate solution in buffer 1 was added dropwise over 30 min to the androgen receptor and estrogen receptor cytosolic fraction which was stirred on ice. This was centrifuged at 12,000 *g* for 30 min. The resulting pellet was resuspended with buffer 1 to the original volume of cytosol (see "d" below). The cytosols, protein concentration 1–3 mg/ml, were assayed for the androgen receptor (ligand-^{3}[H]-methyltrienolone, 0.05–5 nmol/l) and HCLA (ligand-^{3}[H]-estradiol, 10–200 nmol/l) according to the method of Sunahara et al. [22]. The addition of 100-fold molar excess of triamcinolone acetonide was used to mask any glucocorticoid receptor interference with the androgen receptor assay. Higher concentrations of triamcinolone did not result in any change in capacity, and therefore were not necessary: binding capacity fmol/mg ± SEM at 100-fold excess = 20.76 ± 1.49, 500-fold = 16.65 ± 6.91, 1000-fold = 19.08, n = 5–43. The estrogen receptor was assayed in a similar manner with [^{3}H]-DES as the ligand, final concentration 0.1–2.0 nmol/l, and a 100-fold molar excess nonlabelled DES as the competitor. The incubation was for 1.5 h, and 0.5% dextran-coated charcoal (0.5% charcoal, 0.05% dextran) was used to treat the mixture at the end of the incubation. Measurement of radioactivity and analysis of data were carried out as described by Sunahara et al. [22] using Scatchard analysis [31], resulting in values for the affinity (K_d) and binding capacity (B_{max}). Protein concentrations were determined by the method of Bradford [36]. In all cases, control experiments were carried out to ensure that equilibrium conditions were attained, and that saturation was achieved. In addition, control samples were assayed in parallel each day that experimental groups were assayed in order to check that vehicle treatment did not affect the results.

b) Serum corticosterone. Each animal was decapitated at 0800 hours in a quiet room, separate from all other animals. Serum was frozen and assayed for total corticosterone by the method of Kaneko et al. [37].

c) Glucocorticoid receptor. The hepatic glucocorticoid receptor was measured according to the methods of Beato and Feigelson [38] and Rosner and Polimeni [39]. Cytosol was prepared using buffer 2.

d) Buffers. Buffer 1: 0.05 mol/l tris-base; 1.5 mmol/l ethylenediamine tetraacetic acid disodium; 0.5 mmol/l dithiothreitol; 10% glycerol; 20 mmol/l sodium molybdate (for androgen receptor). Buffer 2: 10 mmol/l tris-base; 2.5 mmol/l ethylenediamine tetraacetic acid disodium; 5 mmol/l dithiothreitol; 10% glycerol; 20 mmol/l sodium molybdate (for glucocorticoid receptor).

Statistical analysis

Data were analysed using ANOVA and the Newman-Kuells multiple range test. Differences were considered significant from control at $p < 0.05$. All samples were assayed in duplicate or triplicate.

Results

The effect of streptozotocin-induced diabetes on hepatic cytosolic estrogen receptor, androgen receptor, and HCLA

At 4 days post-induction of diabetes by streptozotocin there was no effect on the binding capacity or K_d of the estrogen receptor (Table 1). A similar result was obtained after 10 days (data not shown). However, the

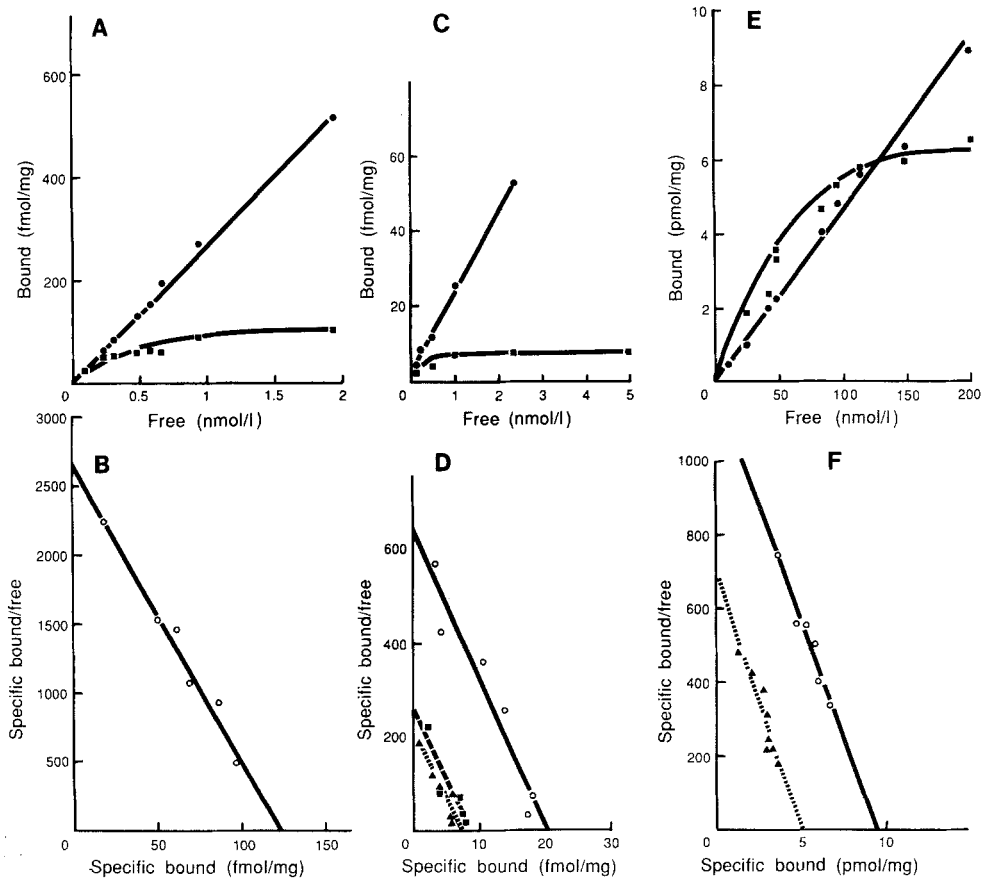

Fig. 1 A-F. Examples of saturation curves and Scatchard plots of rat hepatic cytosolic estrogen (**A, B**) and androgen receptor (**C, D**) and high capacity-low affinity estrogen binding protein (HCLA; **E, F**). ■—■ Specific bound; ●—● Nonspecific bound; O—O Control animals; ■--■ 4 day diabetic rats; ▲···▲ 10 day diabetic rats

Table 1. Effects of 4-day streptozotocin (STZ) diabetes, with and without insulin treatment, on the rat hepatic estrogen receptor (ER). Protamine zinc insulin (PZI) dose: 10 U/kg s.c. for 4 days. Values are mean ± SEM

Animal model	ER binding kinetics		
	K_d (nmol/l)	B_{max} (fmol/mg)	Number
Control	0.68 ± 0.07	121.1 ± 6.25	9
4-day STZ	0.83 ± 0.36	139.0 ± 12.4	4
4-day STZ + PZI	0.74 ± 0.23	217.0 ± 23.8[a]	4

[a] Statistically different from control at $p < 0.05$ level

HCLA and androgen receptor exhibited a greater than 50% decrease in binding capacity with no alterations in the K_d values at both 4 and 10 days of diabetes (Fig. 1, Table 2). The decrease in binding capacities was evident whether the results were calculated per mg protein as in Table 2, or per gram wet weight of liver (androgen receptor: control = 2.5 ± 0.38, 4-day streptozotocin = 0.94 ± 0.23, 10-day streptozotocin = 0.73 ± 0.1 fmol/g; HCLA: control = 5.18 ± 1.12, 4-day streptozotocin = 1.23 ± 0.28, 10-day streptozotocin = 1.41 ± 0.41 pmol/g).

Further evidence that there were no changes in the affinity of steroid binding by receptors was obtained using competitor studies. For example, concentrations of unlabelled androgenic competitor of 0.1 pmol/l to 1 μmol/l were used to generate complete competition curves for the androgen receptor. The competitor concentrations at which 50% of the radioactive ligand was displaced (IC_{50}) were calculated. The synthetic androgen, R1881, had the lowest IC_{50} (i.e. the highest affinity for the androgen receptor) with similar values in both control and 10-day diabetic rats; 5 α-dihydrotestosterone ranked next. No specific displacement occurred with 5 β-dihydrotestosterone in either case. Testosterone and 17 β-estradiol required concentrations several orders of magnitude higher in order to reach the IC_{50}, both in control and 10-day diabetic animals.

Diabetes was confirmed by the presence of glyosuria of 0.5% or greater. Administration of insulin to the diabetic animals in doses which were able to prevent glycosuria was unable to restore the decreased binding capacity of the androgen receptor and HCLA (Table 2). There were no differences in the binding capacities between 4 and 10 days post-induction of diabetes. Since insulin-reversible changes in hepatic drug and steroid metabolism are observed at 4 days of diabetes [31], we conducted the rest of the experiments at this time.

The effect of hormonal replacement in the 4-day diabetic animals

Testosterone replacement to the diabetic animal did not result in restoration of the control androgen receptor or HCLA levels (Table 2). This treatment regimen

Table 2. Effects of streptozotocin (STZ) diabetes, and various hormonal treatments on the rat hepatic androgen receptor and high capacity-low affinity estrogen binding protein: mean ± SEM. PZI - protamine zinc insulin; ToI - Toronto insulin; TES - testosterone enanthate; oGH - ovine growth hormone

Animal model	Androgen receptor			High capacity-low affinity estrogen binding protein		
	K_d (nmol/l)	B_{max} (fmol/mg)	Number	K_d ($\times 10^{-7}$ mol/l)	B_{max} (pmol/mg)	Number
Vehicle control	0.33 ± 0.04	22.51 ± 2.48	26	1.08 ± 0.34	14.83 ± 3.97	11
4-day STZ	0.31 ± 0.05	9.71 ± 1.22[a]	22	0.82 ± 0.18	4.82 ± 0.95[a]	8
4-day STZ + PZI	0.60 ± 0.19	11.56 ± 3.72[a]	4	0.79 ± 0.19	3.41 ± 0.99[a]	5
4-day STZ + ToI	0.28 ± 0.06	6.66 ± 1.30[a]	8	0.57 ± 0.06	1.41 ± 0.38[a]	7
10-day STZ	0.36 ± 0.04	8.31 ± 1.21[a]	23	0.51 ± 0.26	4.37 ± 1.59[a]	5
10-day STZ + PZI	n.d.	n.d.		1.08 ± 0.18	5.72 ± 1.23[a]	5
4-day STZ + TES	0.41 ± 0.07	10.05 ± 1.79[a]	8	0.56 ± 0.10	3.65 ± 0.82[a]	5
4-day STZ + T_3	0.12 ± 0.01	9.49 ± 1.97[a]	6	0.40 ± 0.10	1.83 ± 0.40[a]	5
4-day STZ + oGH (mp)	0.35 ± 0.11	7.16 ± 1.92[a]	6	0.56 ± 0.11	1.81 ± 0.22[a]	5
4-day STZ + oGH (sc)	0.33 ± 0.11	7.28 ± 2.88[a]	7	0.52 ± 0.06	1.54 ± 0.28[a]	7
Vehicle control[c]	0.25 ± 0.03	20.98 ± 3.20	8	0.66 ± 0.11	4.05 ± 0.47[a]	4
Vehicle + 4-day STZ[c]	0.33 ± 0.06	7.75 ± 0.75[b]	6	0.36 ± 0.03	2.07 ± 0.37[b]	4
4-day STZ + oGH[c]	0.44 ± 0.18	5.41 ± 1.93[b]	4	0.43 ± 0.04	2.25 ± 0.34[b]	4
4-day STZ + oGH + TES + PZI[c]	0.15 ± 0.06	10.12 ± 1.02[b]	4	0.54 ± 0.18	2.37 ± 0.37[b]	4

n.d., Not determined; mp = osmotic minipump; sc = subcutaneous injection. [a] Different from control at $p < 0.05$; [b] different from control and vehicle injected control at $p < 0.05$; [c] four daily intravenous injections of either saline or oGH

Table 3. Effects of streptozotocin (STZ) diabetes, with and without insulin therapy, on serum corticosterone levels and hepatic glucocorticoid receptor (GR) in male Wistar rats. Values are the mean ± SEM; PZI = protamine zinc insulin (10 μ/kg s.c. once per day)

Animal model	Serum corticosterone (μg/100 ml)	Number	GR binding kinetics		Number
			K_d (nmol/l)	B_{max} (fmol/mg protein)	
Control	2.65 ± 0.85	4	1.40 ± 0.31	88.2 ± 15.3	6
4-day STZ	3.95 ± 1.39	4	1.92 ± 0.59	114.3 ± 31.1	8
4-day STZ + PZI	2.06 ± 0.52	4	1.57 ± 0.49	107.9 ± 15.6	6

had previously been shown to restore serum testosterone levels to normal and to reverse the alterations in drug and steroid metabolism [22]. Treatment with triiodothyronine was also ineffective in increasing the binding proteins to normal. Ovine growth hormone was administered in doses calculated to restore the normal male release pattern (7 s.c. doses per day). Also, we administered intravenous tail vein injections 4 times a day at a dose of 30 μg/injection. This dosage regimen has been shown to restore drug and steroid metabolism to normal [40]. However, all growth hormone regimens were ineffective in restoring the hormone binding protein levels. Addition of protamine zinc insulin and testosterone to the treatment regimen to more closely duplicate the normal physiological situation was also ineffective in restoring the HCLA and androgen receptor levels.

Serum corticosterone and glucocorticoid receptor levels

Stress appeared to have an effect on the HCLA binding proteins; decreased levels were noted in the animals subjected to 4 daily intravenous injections, including the control group. Therefore, we measured the levels of serum corticosterone and hepatic glucocorticoid receptor to determine if changes in these parameters could be complicating our analyses (Table 3). There were no significant differences among the three experimental groups-control, 4-day diabetic and insulin-treated diabetic animals, with regard to the serum corticosterone levels or binding capacity of the glucocorticoid receptor. Therefore, there is no evidence that these animals were under any significant stress, e.g. due to the diabetic state itself.

Discussion

We found no alterations in the kinetics of the male hepatic estrogen receptor with short term (4–10 days) streptozotocin diabetes. Our results are in accord with those of Ekka et al. [41, 42] who used Scatchard analysis to compare the number of uterine estrogen receptor binding sites between control and diabetic female rats; they found no differences. There was a slight increase in estrogen receptor binding capacity in the diabetic male treated with insulin in our study. This may be due to a stabilising effect of insulin upon the hepatic estrogen receptor [43].

In contrast to the lack of effect on the estrogen receptor, we found a significant decrease in the binding capacity of both the androgen receptor and HCLA 4 days after streptozotocin-induction of diabetes. The decrease seen in the male hepatic androgen receptor and HCLA was of a similar magnitude to the decrease reported for the prostatic androgen receptor in streptozotocin-diabetic male rats [44]. However, unlike the

prostatic androgen receptor, neither testosterone nor insulin treatments were able to restore the hepatic androgen receptor or HCLA. This is in contrast to what occurs with drug and steroid metabolism. We have consistently found that control levels of metabolism are restored with treatments equal in dosage and time length to those which were used in Table 2 [1, 31]. Since insulin also prevents a generalised decrease in hepatic protein concentration which can occur in diabetic rats [45, 46], the reduction in binding capacities does not appear to be a generalised effect.

When given in the male release pattern, the ineffectiveness of ovine growth hormone in restoring the reduced binding capacities of the androgen receptor and HCLA indicates that the presence of peaks of growth hormone in the blood is not the regulatory factor in the androgen receptor and HCLA levels. Possibly the inability of the present study to obtain sufficiently low-trough plasma levels between the peaks was a factor in this lack of response. None of the treatment regimens attempted was able to restore the proteins to their control levels even when the hormones were combined. No evidence for a change in affinity of binding of steroids to the various proteins was noted. In fact, the IC_{50} values were quite similar between control and diabetic-derived fractions using a number of steroid competitors.

Basal serum corticosterone levels remained normal in both the untreated and insulin-treated diabetic animals. Also, hepatic glucocorticoid receptor capacity was not significantly changed by diabetes. Svec reported similar results with respect to the hepatic glucocorticoid receptor in 4-day streptozotocin-diabetic rats [47]. We conclude from this that the diabetic state was not excessively stressful with regard to the parameters measured.

Analysis of the nuclear receptor levels is now being carried out. These values together with the present study may provide more complete data with regard to the effects of diabetes on hormone receptors in the liver.

In conclusion, we found that 4 or 10 days' duration of streptozotocin-induced diabetes in the male Wistar rat did not affect the capacity or affinity of binding of the hepatic cytosolic-estrogen receptors. No change in binding affinity occurred in either the androgen receptor or high capacity-low affinity estrogen binding protein. However, the capacities of both these proteins were reduced to less than half of control values in the diabetic state. Hormone treatments which are known to correct diabetic-induced alterations in hepatic drug and steroid metabolism were ineffective in restoring the decreased binding capacities of the androgen receptor and estrogen binding protein. Therefore, it is concluded that these cytosolic proteins do not play a direct role in the regulation of drug and steroid metabolism in the diabetic rat.

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