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DOWN-REGULATION OF PROLACTIN RECEPTORS IN THE LIVER, MAMMARY GLAND AND KIDNEY OF FEMALE VIRGIN RAT, INFUSED WITH OVINE PROLACTIN OR HUMAN GROWTH HORMONE

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SUMMARY: Down regulation of prolactin (PRL) receptors resulting from i.v. infusion of oPRL or human growth hormone (hGH) into female virgin rats was demonstrated. A decrease of over 85% in the number of free receptors was achieved within 15 - 30 min using infusion of oPRL or hGH at 25 µg/h and remained at this level until the end of infusion. Ovine growth hormone or recombinant bovine growth hormone at ten-fold higher concentration had no effect at all. The decrease in the specific binding resulted from a lower number of binding sites and not from change in the dissociation constants. The decrease in the total receptors in the liver was more gradual and leveled off at 40 - 50% of the initial value. Our results suggest that a change in blood PRL or hGH level may lead to a new steady state in the number, occupancy and distribution of prolactin receptors.

Down regulation of polypeptide hormone receptors resulting from exposure to exogenous hormone is widely accepted as a general phenomenon. However, most of the studies concerning short-term regulation of PRL receptors were performed in *in vitro* systems (1 - 5) whereas *in vivo* studies were scarce. In one of these studies, the combined effects of injection and infusion of prolactin on PRL binding to liver and mammary gland tumor slices (6) was assayed while in another, the effect of single injections of large doses of PRL on its free and total receptors in rat liver and rabbit mammary gland (7) were assayed. In the present study, a different approach was undertaken. The animals were anaesthetized with ketamine, which unlike ether or barbituates, does not cause a fast secretion of endogenous PRL (8) and were continuously infused with various hormones to achieve a new steady-state in the respective hormone level. The effect of hormone infusion was tested by measuring the binding of ^{125}[I]-oPRL to microsomal fractions obtained from the mammary gland, kidney and liver. In the latter case, the total number of receptors was also estimated after desaturation with 4M $MgCl_2$ (9).

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MATERIALS AND METHODS

Ovine prolactin (NIH-oPRL-S14), ovine growth hormone (oGH) NIH-GH-I1) and and RIA kits were obtained from the National Hormone and Pituitary Program, National Institutes of Health. Recombinant bovine growth hormone (bGH) was donated by Dr. A. Nimrod of Biotechnology General Israel, LTD., Nes Ziona, Israel. Pure human growth hormone (hGH) was a gift of Dr. A. Havron of Interpharm Laboratories LTD., Rehovot, Israel. Other sources of materials were: Bovine serum albumin (BSA) RIA-grade and Chloramine-T from Sigma (St. Louis, MO.); carrier-free sodium ^{125}[I] from New England Nuclear Corp. (Boston, MA.), Ketamine from Parke-Davis, (Pontypool, Gwent, U.K.) and Protein Assay Kit I for protein determination from Bio-Rad (Richmond, CA.). All other reagents were of analytical grade. Sabra virgin female rats (Hebrew University strain) 180 - 200 g were maintained in 12 h light-dark cycles with water and food available *ad libitum*.

Infusion procedure and preparation of the microsomal fraction: Rats were anaesthetized i.p. with Ketamine-Ketalar (125 mg/100 g body weight), and the right jugular vein was cannulated with polyethylene tube (PE-50 I.D. = 0.58 mm; O.D. = 0.065 mm). Hormones solubilized in 0.9% NaCl were infused via the jugular vein by a constant infusion (0.25 ml/h) using a Harvard infusion pump. At the termination of the infusion, the animals were quickly decapitated, blood was collected and the liver, mammary gland and kidney were immediately removed. The respective tissues were cut into small pieces and homogenized in Ultraturrax (1 min) with 5 vol. of 25 mM Tris-HCl buffer pH 7.5, containing 0.01 M $MgCl_2$ and 0.25 M sucrose. The homogenate was filtered through four layers of gauze and spun for 20 min at 11000 x g. The membrane fractions were recovered by centrifugation at 100000 x g for 1 h. The precipitates were suspended in 25 mM Tris-HCl buffer pH 7.5 containing 10 mM $MgCl_2$ (reaction buffer) and kept frozen at -20°. The entire preparation was carried out at 4°. Protein was determined according to Bradford (10) using a Biorad Commercial Kit. Blood oPRL and hGH were determined in plasma by RIA using respective antisera.

Preparation of iodinated hormones: oPRL and hGH were iodinated to a specific activity of 30 - 60 µCi/µg with Chloramine-T at low concentrations (1.5 µg/5 µg hormone as previously described (11).

Binding assays: Binding assays of ^{125}[I]-oPRL to liver or mammary gland microsomal fractions or ^{125}[I]-hGH to kidney membranes were performed to determine the free-binding sites as previously described (11). The membranes were desaturated with 4 M $MgCl_2$ (9) for estimation of the total number of PRL receptors. Scatchard analysis was performed using variable amounts (0 - 60 ng/tube) of unlabeled oPRL. In all cases, the specific binding was calculated as a percentage of tracer that could be displaced by 1 µg unlabeled oPRL/tube.

RESULTS

The effect of 4 h infusion with various hormones on ^{125}[I]-oPRL binding to liver membranes is summarized in Fig. 1. As can be seen, infusion with oPRL (Exps. 1 and 2) or hGH (Exp. 3) resulted in a dramatic decrease ($p<0.001$) in specific binding to the free receptors while infusion with oGH (Exps. 1 and 2) or recombinant bGH (Exp. 3) had no significant effect compared to the control rats infused with saline. In all cases, Scatchard analyses were also performed but no statistically significant differences in the Kd values between various treatments were found. It was therefore assumed that the decrease in specific binding resulted entirely from the decreased number of binding sites and not from the

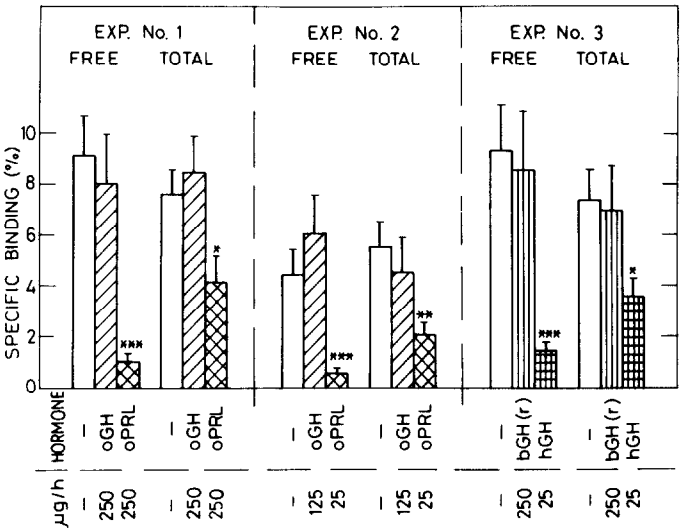

Figure 1 Specific binding of ^{125}[I]-oPRL to the liver microsomal fraction prepared from female virgin rats infused for 4 h with oPRL, oGH, hGH, recombinant bGH or saline. Each bar represents an average ± SEM, of six animals. Treatments statistically different from the respective controls are marked: * - $p<0.05$; ** -$p<0.01$; *** - $p<0.001$. Total receptors were determined after desaturation with M $MgCl_2$ (9). The assays were performed with 100 µg microsomal protein/tube.

change in affinity. The average Kd value for all treatments was 0.225 nM while the B_{max} values varied from 145 in the control to 16 - 19 f-moles oPRL bound per 1 mg membrane protein in the oPRL or hGH infused animals. Desaturation with 4 M $MgCl_2$ restored about 50% of the binding capacity without affecting the Kd. However, the total specific binding was still significantly lower ($p<0.05$ in Exps. 1 and 3, and $p<0.01$ in Exp. 2) than the respective controls. The limited amount of tissue from the mammary gland and the kidneys did not allow us to perform Scatchard analyses or desaturation experiments, thus the results in Table 1 summarize the specific binding to free-receptors only. Since the binding of ^{125}[I]-oPRL to kidney membranes was very low, ^{125}[I]-hGH was used as a labeled ligand. Infusion with oGH (Exps. 1 and 2) or recombinant bGH (Exp. 3) did not significantly change the specific binding (compared to control) while infusion with oPRL or with hGH caused a significant decrease. Specific binding to the membranes from all three organs of rats anaesthetized but not infused, or rats killed without treatment was almost identical to that of the rats infused with saline for 4 h. This indicates that the anaesthetic agent, ketamine, had no direct or indirect effect on the receptor level.

Table I Specific binding of ^{125}[I]-oPRL to the mammary gland and ^{125}[I]-oPRL to the kidney microsomal fractions prepared from female virgin rats infused with oPRL, oGH, hGH, recombinant bGH or saline for 4 h

Exp. no.	Hormone infused	µg/h	Specific binding (%)[1] Mammary gland[2]	Kidney[3]
1	None	-	1.03 ± 0.65	7.17 ± 1.46
	oGH	250	0.89 ± 0.03	N.D.
	oPRL	250	0.14 ± 0.01*	2.20 ± 0.87*
2	None	-	4.40 ± 0.49	12.90 ± 3.08
	oGH	125	3.91 ± 1.57	12.00 ± 3.08
	oPRL	25	2.20 ± 0.28**	5.77 ± 1.09*
3	None	-	1.99 ± 0.57	10.60 ± 2.34
	bGH[4]	250	1.64 ± 0.64	10.20 ± 2.89
	hGH	25	0.89 ± 0.24*	1.65 ± 0.48**

[1]Av. ± SEM, n = 6. All results statistically different from the respective controls are marked with asterisk ($p<0.05$) or two asterisks ($p<0.01$).

[2]70 µg microsomal protein/tube

[3]250 µg microsomal protein/tube

[4]recombinant

In the second set of experiments, rats were infused with 25 µg/h of oPRL or hGH for 0 - 180 min. As shown in Fig. 2, a drastic decrease in the specific binding to the free receptors from liver and kidneys was observed within 15 - 30 min in both hormones and remained at that level throughout 180 min. The decrease was somewhat slower in the mammary gland, but reached a similar level after 30 min. Each time point represents an average of 2 - 4 different animals which significantly contributes to the experimental variability as exemplified by the relatively large SEMs values. As in former experiments, total receptors were measured only in liver membranes. The decrease in the total receptors was generally less drastic, becoming significantly different from zero time only after 120 min and 60 min in the animal infused respectively with oPRL or hGH. Blood levels of oPRL or hGH rose sharply (see Table 2) and a new steady-state was reached within 15 - 30 min of infusion. Those results also indicate that the half-life of the infused hGH is considerably shorter (~7 min) than that of infused oPRL (~11 min). Similar results were obtained after 4 hours of infusion (Exps. 1 - 3). Infusion with 250 µg/h resulted in an oPRL blood level that was 10-fold higher than with 25 µg/h.

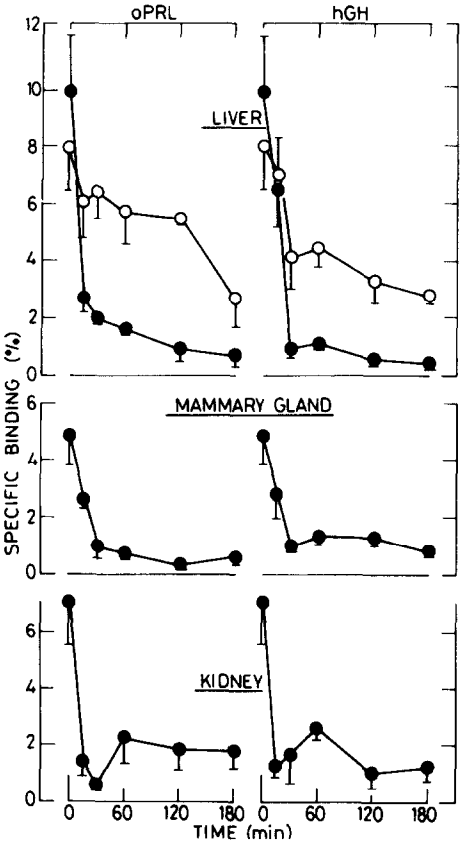

Figure 2 Time course of change in the specific binding of ^{125}[I]-oPRL to the liver and mammary gland and of ^{125}[I]-hGH to kidney microsomal fractions prepared from female virgin rats infused for 0 - 180 min with oPRL or hGH (25 µg/h at 0.25 ml/h). Each point is an average ± SEM of 2 - 4 animals. Full circles represent free receptors and empty circles, total receptors as determined after desaturation with 4 M $MgCl_2$ (9). The assays were performed with 100 µg microsomal protein in the liver, 70 µg in the mammary gland and 250 - 350 µg in the kidney.

Table 2 Plasma levels of oPRL and hGH in female virgin rats infused with these hormones for 0 - 180 min at 25 µg/h

	Plasma level of the respective hormone in ng/ml (av ± SEM) after:					
Hormone infused	0 min	15 min	30 min	60 min	120 min	180 min
oPRL	14 ± 3	538 ± 338 (426)[1]	506 ± 98 (535)	372 ± 103 (570)	673 ± 195 (573)	592 ± 132 (573)
n [2]	7	2	4	2	3	4
hGH	27 ± 5	365 ± 257 (322)[1]	373 ± 105 (360)	352 ± 109 (364)	461 ± 121 (365)	298 ± 72 (365)
n[2]	6	2	3	4	3	4

[1]The numbers in parentheses are the predicted values that were calculated assuming plasma volume of 8 ml/rat, half-lives of 11 min and 7 min for oPRL and hGH, respectively and using the following equation:
Hormone level in plasma at t' = (Rate of infusion) (Half-life) $(1 - e^{-t'/\text{half-life}})$.

[2]Number of animals tested at each time point.

DISCUSSION

The present work demonstrated the infusion technique of ketamine anaesthetized rats to be an appropriate tool for studying short-term *in vivo* prolactin receptors regulation under controlled steady-state conditions of hormone concentration. The results clearly revealed that oPRL and hGH, unlike oGH or recombinant bGH, down-regulated prolactin receptors, indicating that hGH binds to rat prolactin receptors not only *in vitro* (12 - 15) but also *in vivo*. A decrease in 85 - 90% of the binding capacity of free-receptors was achieved in 15 - 30 min in the liver, kidney and mammary gland at the infusion rate of 25 µg/h in parallel to a formation of a new steady-state in the blood level of the infused hormone. A more gradual decrease in the total prolactin liver receptors was exhibited after desaturation with 4 M $MgCl_2$, leveling off (see Figs. 1 and 2) at ~40% of the initial value. This finding may be interpreted as (a) part of the receptors binding the ligand were not internalized or (b) the receptors were internalized but not degraded. In view of the fast uptake of ^{125}[I]-oPRL injected into rabbits and rats (16) and the rapid accumulation of intact iodinated hormone in the intracellular Golgi heavy fraction (16, 17), the second possibility is the more plausible. This conclusion is also supported by (a) the findings of Djiane *et al.* (18) who demonstrated that after intravenous injection of prolactin, the disappearance of the binding activity from the rabbit mammary gland plasma membrane-rich fractionation was faster than that of the Golgi-rich fraction; (b) 50 - 70% of the binding capacity of ^{125}[I]-oPRL in the rabbit liver is of intracellular origin (19).

It has been suggested that prolactin or other polypeptide hormone receptors are internalized along with the hormone molecule and recently, a direct evidence of this hypothesis concerning insulin receptors was published (20). Our results support this concept as well. In conclusion, since it is rather improbable that the synthesis rate of the receptors was changed in our experiments, we suggest that the increased prolactin blood level in the course of infusion is followed by a formation of a new steady-state in the receptor level - namely, a decrease to 40 - 50% of the initial level in total receptors and 80% occupancy,

mostly in the intracellular microsomal fraction. Further experiments are now in progress to determine the intracellular distribution of total and free receptors before and after infusion.

Lactogenic activity of hGH was observed *in vitro* in primate (21), mice (22) and most recently, in cows (23). Our *in vivo* results bring additional proof of the mediation of this activity through interaction with prolactin receptors.

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