PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 163, 64–68 (1980)

Inactivation of Thyrotropin-Releasing Hormone by Plasma of Adult Male, Spayed Female, Pregnant, and Fetal Rhesus Macaques (*Macaca mulatta*) (40724)

MARY ROSENBLUM AND GEORGE W. KITTINGER

Department of Perinatal Physiology, Oregon Regional Primate Research Center, Beaverton, Oregon 97005

In the rhesus macaque, as in other species, the endocrine function of the thyroid is critical to normal growth and development (1, 2). Maintenance of normal thyroid function depends on the basal or stimulated secretion of thyrotropin (TSH) by the pituitary gland. In turn, a hypothalamic hormone, thyrotropin-releasing hormone (TRH), acts directly upon the pituitary to stimulate secretion of TSH and prolactin. Both hypothalamic lesions and deafferentation of the basal hypothalamus interfere with the ability of the pituitary to produce and release TSH. Normal secretory activity of the thyroid gland depends on TSH and the integrity of the hypothalamic–pituitary axis (1).

Recently, evidence has been reported that tends to indicate a possible independence of fetal thyroid function from fetal hypothalamic–pituitary secretions. Goiters have been induced experimentally in fetal rats in which encephalectomy and destruction of the hypothalamus were induced by hypervitaminosis A (3). In studies in our laboratory, fetuses that experienced surgical hypophysectomy midway through gestation had negligible plasma thyroxine levels and greatly reduced thyroid organ weights. These phenomena were entirely compatible with the associated retarded cytologic development of the thyroid (4). Fetuses whose hypothalami had been surgically ablated midway through gestation but whose pituitaries had been left intact exhibited quite different results: their thyroid hormone levels, skeletal ontogeny, cytologic development, and thyroid organ weights closely approximated those of intact control animals (2, 4).

It had been previously determined that neither TSH nor the thyroid hormones thyroxine and triiodothyronine cross the placenta in physiologically effective quantities in either direction (5, 6). The thyroid hormone levels and thyroid weights in hypophysectomized animals further supported the conclusion that maternal TSH had no direct effect on the fetal thyroid (4). It therefore seems possible that a maternal source of hypothalamic TRH might act directly upon the pituitaries of fetuses lacking functional hypothalami. Azukazawa (7) demonstrated in rhesus macaques that an injection of TRH into the maternal compartment causes a precipitous rise in the level of TSH in the fetal plasma. He also demonstrated that TRH can pass from the fetal to the maternal circulatory system.

In any investigation of a direct maternal TRH effect on the pituitary, the difference between TRH levels in the hypothalamic–pituitary portal and peripheral circulation as well as the inactivation of TRH by both fetal and maternal plasma must be taken into consideration. But the rapid inactivation of TRH makes estimates of physiological activities difficult (8). Neary's recent research with rat and human neonatal plasma has indicated that TRH is inactivated at a much slower rate in the neonatal plasma than in that of adults (9, 10). A significantly slower fetal TRH inactivation rate would suggest that TRH might persist in physiologically effective amounts in fetal plasma. Furthermore, response of the fetal pituitary to TRH may be greater than that of the adult pituitary (7).

The object of our experiments was to determine the rate of inactivation of TRH in fetal rhesus plasma and to compare this to the rate of TRH inactivation in plasma of normal adult male, pregnant, and spayed rhesus macaques. In addition, we wished to determine the rate of inactivation of TRH in pregnant humans and to compare this to the inactivation rate in plasma of pregnant rhesus macaques. The feasibility of using

0037-9727/80/010064-05$01.00/0

Macaca mulatta as a human model of TRH influence on fetal development was investigated by comparison of the TRH inactivation rate in plasma of pregnant rhesus macaques and in plasma of pregnant women.

Materials and Methods. Experimental animals and sample collection. Adult male rhesus macaques (*M. mulatta*) were maintained under colony conditions on a normal diet of commercial monkey chow and tap water *ad lib.* Blood was withdrawn from their femoral veins into heparinized syringes. No anesthetics were used, and animals were minimally restrained at the time of venipuncture. The blood samples were collected at approximately 9 AM each day to minimize diurnal variation in hormone and enzyme levels. The blood was immediately centrifuged at 4°C for 10 min to remove cells, and the plasma was separated and held in ice until the actual inactivation was begun. A maximum of 3 hr elapsed between blood collection and the beginning of incubation. Plasma samples showing evidence of hemolysis were not used.

On Day 145 of gestation, blood samples were obtained from pregnant monkeys under sedation at the time of cesarean (C) section. Samples were withdrawn from the femoral veins into heparinized syringes. These samples were processed as described above.

Adult rhesus females were bilaterally oophorectomized (spayed) and maintained in the same way as the adult males. No estrogen supplement was given. The blood samples were obtained in the manner described above. Each animal had fully recovered from the operation at the time of venipuncture.

The fetuses were delivered by C-section between Days 75 and 140 of gestation. Cord blood was withdrawn into a heparinized syringe immediately upon removal of the fetus from the uterus, prior to the severing of the umbilical cord. The blood was centrifuged immediately and used for the experimental incubation within 3 hr, as previously described by Neary (9). Human blood samples were obtained from pregnant women at term during elective C-section. The blood was heparinized and immediately transported on ice to the laboratory for centrifugation.

Incubation of thyrotropin-releasing hormone with plasma. A 400-μl volume of either fetal or adult plasma was added to 1600 μl of an incubation medium containing 0.01 *M* phosphate buffer, 0.15 *M* sodium chloride, and 0.25% bovine serum albumin (pH 7.5). This incubation mixture was preincubated in a water bath at 37°C for 10 min in the absence of added TRH, at which time 100 μl was removed for measurement of endogenous TRH in the plasma. At this time, 4 μg of synthetic TRH (Sigma P9012 L-pyroglytamyl-L-prolyl amide) were added to the preincubated plasma buffer mixture, and the "zero" aliquot of 100 μl was removed. The incubation mixture, including the TRH, was then returned to the 37°C water bath for 1 hr. During this time period, 100-μl aliquots were removed at intervals of 5, 10, 20, 30, 40, 50, and 60 min. At the time of sampling, the portions, including the preincubation and "zero" samples, were diluted to 1 ml with the incubation buffer and immediately immersed in a boiling water bath for 3 min to stop the inactivation of TRH. Upon completion of the incubation, samples were stored at −20°C until assayed for TRH.

Assay for thyrotropin-releasing hormone. A modification of the radioimmunoassay procedure of Bassiri and Utiger for TRH was used to determine the TRH levels in the incubated samples (11). The hormone used for the standards and for iodination was obtained from Sigma Chemical Company. The iodination was carried out with high specific activity ^{125}I purchased from Amersham/Searle (100 mCi/ml) and the chloramine-T iodination procedure reported by Greenwood *et al.* (12). The freshly iodinated TRH was first purified on a Sephadex G-10 column, then repurified on the column immediately prior to use in all assays.

The antiserum to TRH used in these experiments was obtained through the generosity of Dr. Wylie Vale. The antiserum was used in a final dilution of 1:3000. This was sufficient to bind 75% of 7500–8000 dpm of iodinated TRH with a 1–2% nonspecific binding.

For the immunoassay, 100 μl of unlabeled standard TRH, or the inactivated samples, diluted in an assay buffer (0.01 *M* phosphate-buffered saline and 0.25% bovine serum albumin [pH 7.5]), were pipetted into 10 × 75-mm polystyrene test tubes in duplicate. To each test tube was added 100 μl of the anti-TRH antiserum diluted to result in approximately 50% binding to the radioisotopically labeled hormone, and 100 μl of ^{125}I-TRH containing 7000 to 8500 dpm. The tubes were allowed to incubate for 72 hr at 4°C. At this time, anti-rabbit gamma globulin and diluted normal rabbit serum were added to precipitate and antibody-bound hormone. The tubes were then incubated at 4°C for an additional 24 hr. Upon completion of the incubation period, 1 ml of 0.01 *M* phosphate-buffered saline (pH 7.5) was added to each of the tubes, which were then spun in a refrigerated centrifuge at 13,000*g* for 30 min. The supernatant was decanted and the radioactivity of the precipitate was determined in a gamma counter. A standard curve was constructed with a log–logit plot, and the TRH level of each sample was determined from the standard curve.

Results and discussion. The change in immunoreactivity of TRH incubated with rhesus plasma at 37°C is shown in Fig. 1. A comparision was made of the average rates of disappearance of TRH from fetal rhesus plasma, pregnant rhesus plasma, spayed rhesus plasma, and adult male rhesus plasma. After 30 min of incubation, the males' plasma showed a 0.42-μg drop in TRH per milliliter of diluted plasma. In comparison, the fetal plasma showed a decrease of 0.07 μg; the pregnant rhesus plasma decreased by 0.20 μg and the spayed rhesus plasma by 0.31 μg. At the end of the

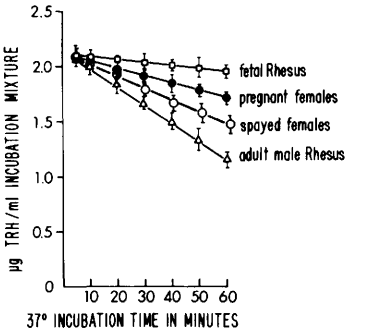

FIG. 1. The disappearance of TRH from the plasma of adult male (n = 8), pregnant (n = 8), spayed female (n = 6), and fetal rhesus monkeys (n = 6) as a function of time.

60-min incubation period, the difference between adult male and fetal plasma TRH levels was highly significant ($P << 0.01$), according to the Newman–Keuls method of analysis of variance (13–15). The TRH degradation rates of pregnant and spayed rhesus plasma samples also were extremely different from the adult male rhesus values ($P < 0.001$). The TRH concentration in diluted adult male rhesus plasma decreased by 44%, and that in pregnant rhesus plasma decreased by 10%. In fetal rhesus plasma there was a 7% decrease, and in spayed rhesus plasma a 32% decrease.

The TRH inactivation rates were linear during the 60-min incubation. Analysis by linear regression yielded half-life values ($t_{1/2}$) for the TRH in the adult male, fetal, and pregnant rhesus plasma (Table I). Statistically significant differences existed between the half-lives of these three groups. In adult male rhesus plasma, TRH had a $t_{1/2}$ of 70 ± 4 min (mean ± SE), and in pregnant rhesus plasma it had a $t_{1/2}$ of 142 ± 16 min. In spayed rhesus plasma it was 94 ±

TABLE I. HALF-LIFE OF THYROTROPIN-RELEASING HORMONE IN ADULT MALE, PREGNANT, SPAYED, AND FETAL RHESUS PLASMA AS DETERMINED BY THE METHOD OF LINEAR REGRESSION

Rhesus group	Slope (± SE)	Rate of disappearance (μg TRH/min/100 ml plasma; ± SE)[a]	Half-life (min; ± SE)
Fetal	0.0020 ± 0.0001	1.03 ± 0.21	409 ± 91
Adult male	0.0180 ± 0.0020	9.06 ± 0.74	70 ± 4
Pregnant (at term)	0.0073 ± 0.0010	3.64 ± 0.71	142 ± 16
Spayed	0.0010 ± 0.0009	4.83 ± 0.46	94 ± 8

[a] TRH, Thyrotropin-releasing hormone.

TABLE II. RATES OF DISAPPEARANCE OF THYROTROPIN-RELEASING HORMONE FROM THE PLASMA OF ADULT MALE, PREGNANT, SPAYED, AND FETAL RHESUS MACAQUES[a]

Adult male	Pregnant	Spayed female	Fetal	Day of gestation
7.5	4.6	6.0	0.9	75
7.0	2.9	4.0	0.9	140
13.0	3.5	4.0	0.5	102
8.5	2.2	4.5	0.9	75
9.0	2.5	4.0	1.0	102
8.0	6.4	6.5	2.0	107
8.0	4.6			
11.5	2.5			
9.06 ± 0.74[b]	3.64 ± 0.71	4.83 ± 0.46	1.03 ± 0.21	

[a] Results are given in μg thyrotropin-releasing hormone/min/100 ml plasma.
[b] Mean ± SE.

8 min, and in fetal rhesus plasma 409 ± 16 min.

Table II shows the individual rates of TRH degradation for the four groups of adult male, pregnant, spayed, and fetal rhesus macaques. The rates of disappearance of TRH from the plasma of eight adult males were compared to the disappearance rates of TRH from the plasma of six fetuses of varying ages. In the adult male group, a mean (± SE) rate of 9.06 ± 0 74 μg/min/100 ml of plasma was observed; the fetal rate was 1.03 ± 0.21 μg/min/100 ml. The difference between these rates was highly significant ($P << 0.01$). The rate of disappearance of TRH from the plasma of the pregnant monkeys was compared to the rates for the adult male, fetal, and spayed groups. The rate in pregnant rhesus plasma was significantly slower than that in adult male plasma ($P << 0.01$). The rate of disappearance of TRH from the pregnant rhesus plasma also was significantly different from that of fetal plasma ($P < 0.01$). We had thought that the age or sex of the fetus might have an effect on the rate of TRH degradation. In the fetuses we examined, gestational age ranged from 75 to 140 days; the inactivation rates of the more mature fetuses did not differ significantly from those of the less mature fetuses, nor was there any rate difference between male and female fetuses.

In Table III the TRH inactivation rate of pregnant human plasma is compared to that of pregnant rhesus plasma. Human plasma inactivated TRH at a rate of 3.46 ± 0.54 μg/min/100 ml of plasma, and rhesus plasma inactivated TRH at a rate of 3.64 ± 0.71 μg/min/100 ml. There was no significant difference between these two rates. Even though the rates of TRH inactivation were substantially lower in the plasma of the rhesus fetuses, it has not yet been established that maternal TRH stimulates the release of physiologically effective quantities of TSH by fetal pituitaries.

Summary. The results of our experiments indicate a significant difference between the rate of disappearance of TRH from plasma of adult male, pregnant female, and fetal rhesus macaques. In fetal rhesus plasma, TRH exists in an immunoreactive state for a significantly longer period of time than in either pregnant, spayed female, or adult male plasma. If TRH of maternal origin

TABLE III. RATES OF DISAPPEARANCE OF THYROTROPIN-RELEASING HORMONE FROM THE PLASMA OF PREGNANT HUMANS AND PREGNANT RHESUS MACAQUES[a]

Pregnant human	Pregnant rhesus
4.55	5.65
2.95	4.35
3.45	3.10
2.20	4.15
2.50	2.35
6.40	2.55
4.60	2.70
2.50	2.85
	3.45
3.64 ± 0.71[b]	3.46 ± 0.54

[a] Results are given in μg thyrotropin-releasing hormone/min/100 ml plasma.
[b] Mean ± SE.

does cross the placenta in physiologically effective quantities, the slower degradation rate does increase the potential effectiveness of the hormone, since it would remain active in the circulation for a longer period of time.

Our evidence also suggests that the estrogen level is not a determining factor in the enzymatic inactivation of TRH. We found no significant difference between inactivation rates in plasma from spayed and pregnant rhesus macaques. Neither did we find a significant difference in the rate of TRH degradation in pregnant rhesus macaques and pregnant women. Therefore, the rhesus macaque should prove to be an acceptable model of TRH influence on fetal development.

The authors wish to thank Dr. John C. Parker and Dr. Wylie Vale for their generous gifts of TRH antisera.

The work described in this article, Publication No. 1042 of the Oregon Regional Primate Research Center, was supported by Grants RR00163 and HD09820 of the National Institutes of Health.

1. Fisher D. A., and Dussalt, J. H., *in* "Handbook of Physiology" (M. A. Greer and D. H. Soloman, eds.), Vol. III, Sec. 7, p. 21. Amer. Physiol. Soc., Washington, D.C. (1974).
2. Kerr, G. R., Tyson, I. B., Allen, J. R., Wallace, J. H., and Scheffler, G., Biol. Neonate **21**, 282 (1972).
3. Eguchi, Y., Suzuki, S., Morikawa, Y., and Hashimoto, Y., Endocrinology **88**, 261 (1971).
4. Kittinger, G. W., *in* "The Fetus and Birth" (M. O'Connor and J. Knight, eds.), p. 235, Elsevier/Exerpta Medica, Amsterdam/New York (1977).
5. Knobil, E., and Josimovich, J. B., Ann. N.Y. Acad. Sci. **75**, 895 (1958).
6. Dussalt, J. H., Hobel, C. H., and Fisher, D. A., Endocrinology **88**, 47 (1971).
7. Azukizawa, M., Murata, Y., Ikenove, T., Martin, C. B., Jr., and Hershman, J. M., J. Clin. Endocrinol. Metab. **43**, 1020 (1976).
8. Bassiri, R. M., and Utiger, R. D., Endocrinology **91**, 657 (1972).
9. Neary, J. T., Keiffer, J., Federico, P., Mover, H., Maloof, F., and Soodak, M., Science **193**, 403 (1976).
10. Neary, J. T., Nakamura, C., Daives, I. J., Soodak, M., and Maloof, F., J. Clin. Invest. **62**, 1 (1978).
11. Bassiri, R. M., and Utiger, R. D., Endocrinology **90**, 722 (1972).
12. Greenwood, F. C., Hunter, W. H., and Glover, J. S., Biochem. J. **89**, 114 (1963).
13. Newman, D., Biometrika **31**, 20 (1939).
14. Keuls, M., Euphytica **1**, 112 (1952).
15. Snedecor, G. W., and Cochran, W. G. (eds), *in* "Statistical Methods," p. 273. Iowa State Univ. Press, Ames (1973).

Received April 27, 1979. P.S.E.B.M. 1980, Vol. 163.