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UPTAKE OF ERYTHROCYTE-ASSOCIATED COMPONENT OF BLOOD TESTOSTERONE AND CORTICOSTERONE TO RAT BRAIN

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Summary—To study transport of steroids by erythrocytes, the tissue uptake of erythrocyte-associated testosterone and corticosterone was studied *in vivo* using a single injection technique into the carotid artery of rats. A brain uptake index (BUI) was calculated by dividing the ratio of [^{3}H]steroid to [^{14}C]butanol (internal reference) in the brain tissue by that in the injection material, and multiplying by 100%. BUIs of testosterone and corticosterone in an erythrocyte suspension were $131 \pm 3\%$ (mean $\pm$ SE, $n = 6$) and $57.0 \pm 2.7\%$ ($n = 6$), respectively, which were greater than those in buffer ($100 \pm 4\%$; $n = 4$, $P < 0.01$ and $39.8 \pm 4.6\%$; $n = 4$, $P < 0.01$, respectively). The erythrocyte accounted for 83.9% and 76.7% of the total testosterone and corticosterone delivered to the tissues, respectively, when calculated on the assumption that the BUIs of steroid in buffer and in the supernatant of an erythrocyte suspension are the same. BUIs of corticosterone in hemolysate and in a suspension of erythrocyte plasma membranes ($60.8 \pm 7.0\%$; $n = 4$ and $69.5 \pm 3.7\%$; $n = 4$, respectively) were also greater than those in buffer ($P < 0.05$ and $P < 0.01$, respectively). Our results suggest that the erythrocyte-associated component of testosterone and corticosterone are delivered to the tissue of rat brain, and that their membranes may play a major role in their capacity to transport steroids to the tissues.

INTRODUCTION

Steroids that are secreted by the adrenal and gonad are transported by the bloodstream and delivered to the peripheral tissues where they exert their bioactivity. Transport of steroids by the bloodstream has been studied for decades. In general, workers have focused on the plasma components of blood steroids, such as plasma unbound, albumin-bound, and binding globulin-bound components [1]. The currently accepted concept of steroid transport is that the plasma unbound and albumin-bound components are the principal sources of the steroid that is delivered to the tissues [2, 3]. Erythrocytes, another major component of blood, have received very little attention heretofore as a component of steroid transport in blood. Recently, we have reported that the erythrocyte-associated component of blood cortisol is not only a significant component, but one which is greater than the unbound or albumin-bound components under physiological conditions [4, 5]. We have suggested that the erythrocyte-associated component of blood cortisol could be taken up by the tissues because the dissociation rate constant for the erythrocyte-associated cortisol is high, and therefore erythrocytes have ample time to supply cortisol to the tissues during capillary transit [5]. In the present study, we investigated the uptake of erythrocyte-associated steroids *in vivo* using a single injection technique into the carotid artery of rats [3]. We report that the erythrocyte-associated components of testosterone and corticosterone, which are the major respective sex steroid and glucocorticoid in male rats, are delivered to the tissues of rat brain.

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MATERIALS AND METHODS

Materials

[1α,2α-^{3}H]testosterone (55.2 Ci/mmol), [1,2-^{3}H]corticosterone (46.6 Ci/mmol), and [1-^{14}C]-butanol (1.1 mCi/mmol) were purchased from

New England Nuclear (Boston, Mass.). Soluene-350 and Hionic-Fluor were obtained from Packard Instrument Co. (Downers Grove, Ill.); Hepes buffer and bovine albumin from Sigma Chemical Co. (St Louis, Mo.); isopropyl alcohol and hydrogen peroxide from Wako Pure Chemical Industries (Osaka, Japan); pentobarbital sodium from Abbott Laboratories (Northchicago, Ill.); Centrifree tubes from Amicon Corp. (Danvers, Mass.). The radioactive steroids were used after purification by Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography [6].

Methods

(1) Brain uptake of erythrocyte-associated testosterone and corticosterone. A tissue sampling-single injection technique was performed as reported previously [3]. Radioactive steroid ([^{3}H]testosterone; 3 μCi, 55.2 Ci/mmol or [^{3}H]corticosterone; 3 μCi, 46.6 Ci/mmol) and [^{14}C]butanol (0.4 μCi; 1.1 mCi/mmol) were dissolved in 1 ml buffered Ringer's solution (pH 7.4, 5 mM Hepes) containing 0.1% bovine albumin (albumin–Ringer's solution), with or without suspended rat erythrocytes (hematocrit: *ca* 40%). Rat erythrocytes were used after being washed several times with Ringer's solution. [^{14}C]butanol has been used as an internal reference in a tissue sampling-single injection technique because the tracer is highly diffusible to the brain [3, 7, 8]. [^{14}C]butanol was applied in the present study since it was reported that even the presence of erythrocytes does not change the permeability of [^{14}C]butanol to the tissues [7, 8]. A 0.2-ml bolus of the material was injected in 1 s via the common carotid artery of a 300–400 g Sprague–Dawley male rat anesthetized with intraperitoneal pentobarbital (50 mg/kg). The animal was decapitated 15 s after injection. The cerebral hemisphere ipsilateral to the injection was removed and solubilized in duplicate in a 1.5-ml mixture (1:1) of Soluene-350 and isopropyl alcohol by heating at 50°C for 2 h; hydrogen peroxide (0.5 ml) was added to the sample and mixed gently for 30 min to reduce color-quenching of the luminescence, then 10 ml Hionic-Fluor was mixed. A 0.01-ml aliquot of the injection material was treated as for the brain tissues. A brain uptake index was calculated by dividing the ratio of ^{3}H-radioactivity to ^{14}C-radioactivity in the brain tissue by that in the injection material, and multiplying by 100%. The brain uptake index of the erythrocyte-associated steroid (BUIrbc) component was estimated based on the assumption that the brain uptake indexes of steroid in the buffer and in the supernatant of the erythrocyte suspension were the same. The brain uptake index of the erythrocyte-associated steroid (BUIrbc) was calculated by subtracting the fraction of brain uptake index of supernatant of the erythrocyte suspension (Ts × BUIb) from the total brain uptake index of the erythrocyte suspension (BUIt), divided by erythrocyte-associated steroid fraction (Trbc) according to the following formula: $BUIrbc = (BUIt - Ts \times BUIb)/Trbc$, where BUIt and BUIb are the brain uptake indexes of steroid in an erythrocyte suspension and in buffer, respectively, and Ts and Trbc are the supernatant fraction and the erythrocyte-associated fraction of the total steroid in the erythrocyte suspension, respectively. The percentage of total steroid in an erythrocyte suspension associated with erythrocytes was determined at 38°C (the physiological body temperature of rats) [9] using a microhematocrit method as reported elsewhere [5]. The part of steroid delivered to the tissues which was accounted for by erythrocytes was calculated as follows: $(Trbc \times BUIrbc \times 100\%)/(1 \times BUIt)$.

(2) Brain uptake of corticosterone in plasma and in hemolysate. In order to investigate the role of erythrocyte integrity for transport of corticosterone into rat brain tissue, brain uptake indexes of corticosterone in buffer, rat plasma which contains corticosteroid binding globulin, and hemolysate obtained by freeze–thawing an erythrocyte suspension several times were compared. The pH of hemolysate was adjusted to 7.4 with 0.1 N sodium hydroxide. The plasma unbound, albumin-bound, and corticosteroid binding globulin-bound components of corticosterone in the plasma sample were determined by ultrafiltration as reported elsewhere [10, 11].

(3) Brain uptake of erythrocyte plasma membrane-associated corticosterone. Plasma membranes of rat erythrocytes were prepared by the method of Dodge and co-workers [12], and were suspended in albumin–Ringer's solution. The volume of the plasma membrane was 30% when determined using microhematocrit tubes by centrifugation at 20,000 *g* for 40 min [12]. Brain uptake indexes of corticosterone in buffer and plasma membrane suspension were studied.

Statistical analyses

All values were given as the mean ± SE. Student's *t*-test was applied for comparison of mean values. All *P*-values greater than 0.05

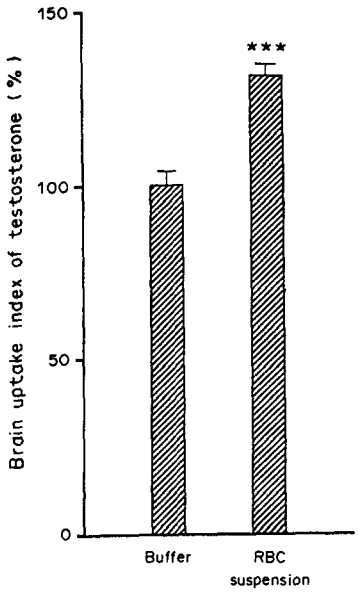

Fig. 1. The brain uptake index of testosterone dissolved in a buffered Ringer's solution containing 0.1% bovine albumin with or without suspended rat erythrocytes (hematocrit: *ca* 40%). Brackets represent SEs. The data of erythrocyte suspension ($n = 6$) were tested against those of buffer ($n = 4$); ***: $P < 0.001$.

were reported as not statistically significant (NS).

RESULTS

Brain uptake of erythrocyte-associated testosterone

The brain uptake indexes of testosterone in buffer and in an erythrocyte suspension were $100 \pm 4\%$ (mean $\pm$ SE, $n = 4$) and $131 \pm 3\%$ ($n = 6$; $P < 0.001$), respectively (Fig. 1). The mean brain uptake index of testosterone dissolved in buffer was similar to that reported previously [3]. The brain uptake index of the erythrocyte-associated testosterone was calculated as described in Materials and Methods section to be $137 \pm 4\%$ ($n = 6$). The percentage of the total testosterone in the erythrocyte suspension associated with erythrocyte was $79.2 \pm 2.1\%$ ($n = 6$). Therefore, the erythrocytes accounted for 83.9% of the total testosterone delivered to the tissues.

Brain uptake of erythrocyte-associated corticosterone

The brain uptake index of corticosterone was studied using the same experimental techniques as for testosterone (Fig. 2). The brain uptake index of corticosterone in an erythrocyte suspension was $57.0 \pm 2.7\%$ ($n = 6$), considerably greater than that of corticosterone in buffer ($39.8 \pm 4.6\%$; $n = 4$, $P < 0.01$). The brain uptake index of the erythrocyte-associated corticosterone was calculated to be $65.7 \pm 4.1\%$. Since the erythrocyte-associated component was $66.6 \pm 0.7\%$ ($n = 6$) of the total corticosterone in the erythrocyte suspension (microhematocrit method), the erythrocyte-associated component accounted for 76.7% of the total corticosterone delivered to rat brain tissues.

Brain uptake of corticosterone in plasma and in hemolysate

The brain uptake index of corticosterone in a hemolysate was greater than that of corticosterone in buffer ($P < 0.05$), while, as expected, the brain uptake index of corticosterone in plasma was smaller ($P < 0.01$) (Fig. 3). The plasma unbound, albumin-bound, and corticosteroid binding globulin-bound corticosterone in the plasma sample were determined by ultrafiltration, and represented 0.18, 0.35, and 0.47 of the total, respectively. Our result that the brain uptake index of plasma corticosterone is nearly a half of that of buffer corticosterone is consistent with the previous report that the unbound and albumin-bound corticosterone are bioavailable to rat brain tissues while the corticosteroid binding globulin-bound component is not [13].

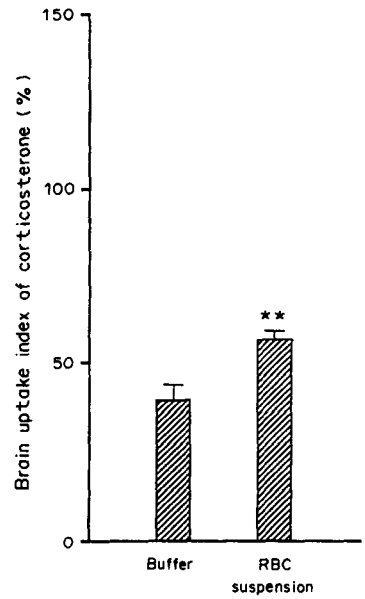

Fig. 2. The brain uptake index of corticosterone dissolved in a buffered Ringer's solution containing 0.1% bovine albumin with or without suspended rat erythrocytes (hematocrit: *ca* 40%). Brackets represent SEs. The data of erythrocyte suspension ($n = 6$) were tested against those of buffer ($n = 4$); **: $P < 0.01$.

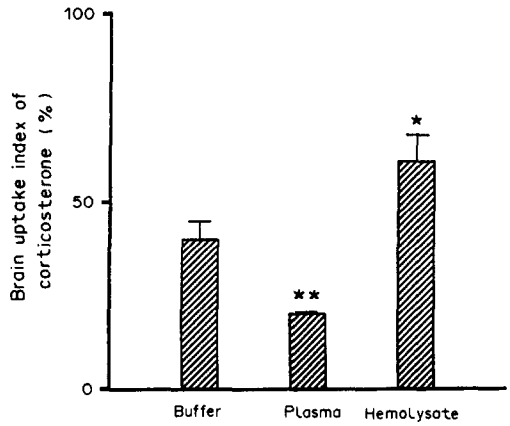

Fig. 3. The brain uptake index of corticosterone in buffered albumin–Ringer's solution, rat plasma, and rat hemolysate. Rat hemolysate was obtained by freeze–thawing an erythrocyte suspension several times. Brackets represent SEs. The data of plasma and hemolysate were compared to those of buffer ($n = 4$); *: $P < 0.05$, **: $P < 0.01$, $P < 0.01$ by ANOVA.

Brain uptake of erythrocyte plasma membrane-associated corticosterone

The brain uptake indexes of corticosterone in albumin–Ringer's solution and in a suspension of albumin–Ringer's solution containing erythrocyte plasma membranes were $39.8 \pm 5.9\%$ ($n = 4$) and $69.5 \pm 3.7\%$ ($n = 4$; $P < 0.01$), respectively.

DISCUSSION

Erythrocytes have received very little attention heretofore as a component of steroid transport in blood. In the present study, the bioavailability of erythrocyte-associated testosterone and corticosterone was assessed *in vivo* using a single injection technique into the carotid artery of rats. Brain uptake indexes of the steroids in buffer with or without suspended rat erythrocytes were studied. The presence of erythrocytes did not restrict, but rather enhanced the uptake of steroids to the rat brain; indeed, erythrocytes accounted for a large proportion of the steroids delivered to the brain. Clearly, these results indicate that erythrocytes transport a significant amount of the testosterone and corticosterone that is taken up by rat brain.

Brain uptake indexes of corticosterone in buffer, rat plasma, and hemolysate were studied to investigate the role of erythrocyte integrity for transport of corticosterone to rat brain tissues. The presence of hemolysate increased the brain uptake index of corticosterone to about the same degree as the presence of intact erythrocytes. Thus, the physical integrity of erythrocytes is not necessary for the enhanced bioavailability of erythrocyte-associated corticosterone to the tissues. In other experiments, the uptake of erythrocyte plasma membrane-associated corticosterone was studied. The brain uptake index of corticosterone in plasma membrane suspension was much greater than that in buffer. Thus, even erythrocyte plasma membranes themselves could facilitate the uptake of corticosterone into the brain. Our present results with a physiological model confirm the notion that erythrocyte-associated steroid is bioavailable. Further, our results suggest that the bioavailability of testosterone and corticosterone could be enhanced by their association with erythrocyte membranes. The mechanism by which erythrocyte membranes increase brain uptake well above that of control buffer solution is unknown. One might speculate that it may involve inter-membranous steroid flux during transit of the material injected through the capillaries. Regardless of mechanism, the current study gives indication that erythrocytes serve as a major conduit for steroid transport to the tissues.

REFERENCES

1. Westphal U.: *Steroid–Protein Interactions*. Springer-Verlag, New York (1971).
2. Albertini A. and Ekins R. P.: *Free Hormones in Blood*. Elsevier, New York (1982).
3. Pardridge W. M.: Transport of protein-bound hormones into tissues *in vivo*. *Endocr. Rev.* **2** (1981) 103–123.
4. Hiramatsu R. and Nisula B. C.: Erythrocyte-associated component of blood cortisol. *Ann. New York Acad. Sci.* **538** (1988) 159–166.
5. Hiramatsu R. and Nisula B. C.: Erythrocyte-associated cortisol: measurement, kinetics of dissociation, and potential physiological significance. *J. Clin. Endocr. Metab.* **64** (1987) 1224–1232.
6. Murphy B. E. P.: "Sephadex" column chromatography as an adjunct to competitive protein binding assays of steroids. *Nature New Biol.* **232** (1971) 21–24.
7. Crone C.: The permeability of brain capillaries to non-electrolytes. *Acta Physiol. Scand.* **64** (1965) 407–417.
8. Raichle M. E., Eichling J. O., Straatmann M. G., Welch M. J., Larson K. B. and Ter-Pogossian M. M.: Blood–brain barrier permeability of ^{11}C-labeled alcohols and ^{15}O-labeled water. *Am. J. Physiol.* **230** (1976) 543–552.
9. Waynforth H. B.: *Experimental and Surgical Technique in the Rat*. Academic Press, New York (1980) 245 pp.
10. Hiramatsu R. and Nisula B. C.: Preparation of steroid radioligands for ultrafiltration assays by a solid-phase transport globulin method using Concanavalin A-Sepharose. *Steroids* **45** (1985) 73–89.

11. Hiramatsu R. and Nisula B. C.: Effect of alcohol on the interaction of cortisol with plasma proteins, glucocorticoid receptors, and erythrocytes. *J. Steroid Biochem.* **33** (1989) 65–70.
12. Dodge J. T., Mitchell C. and Hanahan D. J.: The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Archs Biochem. Biophys.* **100** (1963) 119–130.
13. Pardridge W. M. and Mietus L. J.: Transport of steroid hormones through the rat blood–brain barrier. *J. Clin. Invest.* **64** (1979) 145–154.