PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 155, 377-381 (1977)

Effect of a Pituitary Peptide on Liver Tissue Respiration[1] (39811)

RICARDO H. LANDABURU AND RAQUEL DE LAMO

Department of Physiology, Wayne State University, School of Medicine, Detroit, Michigan 48201

With the observation of Cannon *et al.* (1, 2) that physical stress or epinephrine administration induces a hypercoagulant effect in normal animals, a series of studies was carried out for the purpose of identifying and characterizing a substance from the anterior pituitary which is capable of mediating the release of vitamin-K-dependent coagulation factors from the liver (3-5). We named this substance liver trophic or hepatotrophic hormone (HTH) (6). Studies performed on the hypercoagulation produced by corticoids or ACTH administration gave results similar to those obtained with HTH administration. The response, however, was not present in hypophysectomized animals (7). Consequently, we proposed that corticoids produced the release of HTH, which, in turn, stimulated the release of coagulation factors by the liver. In pursuit of this thesis, we performed experiments reported in this communication.

Methods. Sprague-Dawley male rats, 2 months old (180-200 g), were used. No special diet was used and, in every case, the animals were fasted 24 hr before the experiments. HTH concentrates were obtained from bovine hypophysis as described previously (4), and further purified as follows: The solution dialyzed out of the anterior pituitary acid extract was concentrated in a film evaporator to one-tenth the original volume and filtered through an ultrafiltèr membrane with a general molecular mass retention of 500 daltons (Diaflo UM05-Amicon, Lexington, Mass.). The solution was then further purified by preparative thin-layer chromatography in MN300 F cellulose plates of 500 μm thickness, using butanol-acetic acid-water (40:10:50). The ninhydrin negative component with R_f = 0.51 was eluted out of the plates with acidified distilled water (pH 2.0 with HCl), and, after concentration under vacuum in a film evaporator, was filtered through a Sephadex G-10, 1.8 × 100-cm column (Pharmacia Fine Chemicals, Uppsala, Sweden) at a filtration rate of 6 ml/hr. The effluent was monitored at 206 nm and collected in 2-ml fractions. Usually fractions 34 to 38 contained the activity.

The preparation was further purified by thin-layer electrophoresis using the same cellulose plates as for TLC. Pyridine-acetic acid buffer pH 4.8 was used. The preparation migrated toward the anode as a single component that was detected by staining with t-butyl hypochloride and starch (8). The substance was then eluted with acidified distilled water and dried. Usually 300 μg was obtained from 100 g of anterior pituitary. The HTH preparation was free of any other peptide structure as determined by standard techniques of thin-layer chromatography and electrophoresis.

HTH administration to rats or dogs produces increase in coagulation factors II, VII, IX, and X, as well as fibrinogen. The overall changes in blood coagulation are better assessed by thrombin generation test or plasma clotting time (PCT). We have standardized the PCT assay and defined HTH activity in units. One unit is the activity capable of shortening PCT in rats by 1 sec. Usually 1000 units are obtained from 1 g of anterior pituitary tissue. HTH used in this work was diluted in Krebs-Ringer's solution to contain 20 units/ml.

Hydrocortisone acetate (Merck, Sharp & Dohme, West Pointe, Pa.) and epinephrine (Sigma Chemical Co., St. Louis, Mo.) were dissolved in Krebs-Ringer's phosphate solution and used in concentrations determined to elicit an optimal response. Epinephrine was diluted in Krebs-Ringer's phosphate solution (9) immediately before the experi-

[1] This research was partially supported by General Research Grant RR 05384 from the National Institutes of Health, U.S. Public Health Service, and was partially performed at the New York Blood Center.

ISSN 0037-9727

ments. No preservatives were used. Hydrocortisone acetate in concentrations higher than 25×10^{-6} *M* was prepared by dissolving an aliquot of ethanol solution of the drug (4 mg/ml) in Krebs-Ringer's solution. The alcohol content of this solution did not modify tissue respiration in control determinations.

The organs for oxygen uptake studies were obtained from rats under ether anesthesia. The liver, spleen, and kidney were perfused *in situ* with cold Krebs-Ringer's phosphate solution to wash out most of the blood, and then were excised. They were placed immediately in the same solution at 4°. Tissue slices about 0.5 mm thick were cut with a Stadie Riggs blade. After washing in chilled Krebs-Ringer's phosphate solution, the tissue slices were drained and weighed.

Bovine pituitary glands and hypothalamus were collected at the slaughterhouse and brought to the laboratory on ice. The tissue from the anterior hypophysis and anterior hypothalamus was separated as previously described (4), sliced, and treated as described above for rat tissue.

Oxygen uptake was measured by standard procedures at 37° with air as the gas phase. Warburg vessels (18 ml) with sidearms were used and were agitated at 100 strokes/min in a Warburg apparatus. The tissue was suspended in 2 ml of Krebs-Ringer's phosphate solution in the main vessel. The sidearm contained the substance under study (HTH, epinephrine, or hydrocortisone), diluted in 0.5 ml of incubation medium. In some experiments, pituitary or hypothalamus slices were placed in the sidearm. In these experiments, the proportion of wet tissue to Krebs-Ringer's phosphate solution was kept constant at 100 mg/ml in both the flask and sidearm. Although the tissue slices consumed oxygen at a constant rate over a period of at least 2 hr, the onset of rate change after HTH or drug addition was delayed after long incubation. Consequently, after 15 min of equilibration, the oxygen consumed was measured, and, after a uniform oxygen uptake-time relationship was obtained and recorded, the contents of the sidearm were carefully poured into the main vessel and measurement was continued. Using this technique, the experiment itself provided the control data.

MH_1C_1 rat hepatoma cells were kindly provided by the Department of Virology (New York Blood Center, N.Y.). Cells collected from F-12 (10) growth media were counted and separated by centrifugation. The pellet was gently suspended in Krebs-Ringer's solution and counted for viable cells after suspension using trypan blue stain, 0.4% (1:6). In experiments with cultured cells, it was necessary to use an agitation rate of 80 strokes/min because at 100 strokes/min there was a continuous drop in oxygen consumption due to mechanical injuries to the cell.

Results. The primary evidence of HTH specificity for the liver comes from the observation that the only animal experimental model that is not responsive to HTH administration is the hepatectomized one (4). In microrespiratory experiments the peptide affected rat liver slices, enhancing oxygen consumption between 20 and 40% (Fig. 1). Cultured rat hepatoma cells responded in the same way as liver slices. HTH preparations had no effect on tissue slices of spleen or kidneys.

Respiration increases in liver slices served as a measurable index of HTH release. The participation of hydrocortisone in HTH release from the pituitary gland was explored using this technique. Hydrocortisone, at a final concentration of 5×10^{-6} *M*, increased the respiration rate of a mixture of 200 mg of rat liver slices plus 50 mg of bovine pituitary slices. Hydrocortisone at 5×10^{-5} through 5×10^{-7} *M* concentrations also gave positive results. The liver and pituitary slices must function together. Hydrocortisone had no effect on oxygen consumption of either tissue incubated independently (Fig. 2), or when liver slices were replaced by spleen or kidney slices.

In previous work the administration of epinephrine produced an increase in the concentration of coagulation factor in plasma with accelerated coagulation in normal animals, but no changes in coagulation were detected in hypophysectomized animals (3), presumably because HTH is needed. Another approach was arranged.

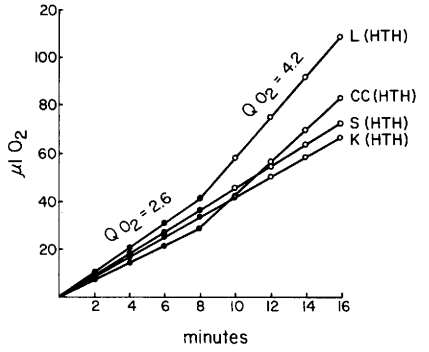

FIG. 1. Tissue oxygen uptake in the presence of HTH preparation as measured by Warburg's direct method. The oxygen consumed by 200 mg of rat liver (L), kidney (K), or spleen slices (S), or 28×10^7 cultured rat hepatoma cells (CC), placed in a Warburg flask, was recorded before and after addition of hepatotrophic hormone (HTH) contained in the sidearm (10 units in 0.5 ml). All tissues were in Krebs-Ringer's phosphate solution (100 mg/ml). HTH, dissolved in the same solution, was poured after 8 min of uniform recording. The temperature was 37°, and the stroke rate for tissue was 100/min and for rat hepatoma cultured cells was 80/min. Q_{O_2} = μl of O_2/100 mg of tissue/min. The liver and rat hepatoma cultured cells responded to HTH, while kidney and spleen did not respond. The figure was composed with data from a single set of experiments. Statistical significance of the difference in respiration rate between L and L (HTH), as determined from data of seven experiments, was: L, Q_{O_2} = 2.6 ± 0.48; L (HTH), Q_{O_2} = 4.2 ± 1.49; $P < 0.01$ (Student's t test).

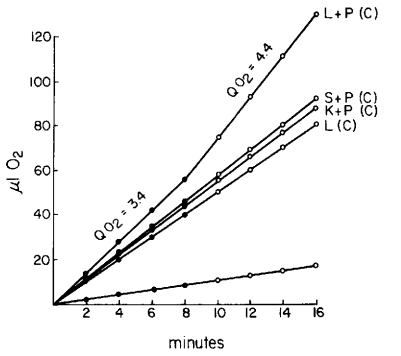

FIG. 2. Tissue oxygen uptake in the presence of hydrocortisone acetate as measured by Warburg's direct method. The oxygen consumed by 200 mg of rat liver (L), kidney (K), or spleen slices (S) plus 50 mg of bovine anterior pituitary slices (P), placed in a Warburg Flask, was recorded before and after addition of hydrocortisone contained in the sidearm (25×10^{-6} M). All tissues were in Krebs-Ringer's phosphate solution (100 mg/ml), and hydrocortisone, dissolved in the same solution, was poured after 8 min of uniform recording. The temperature was 37° and the stroke rate was 100/min. Q_{O_2} = μl of O_2/100 mg of tissue/min. Lowest data line represents oxygen uptake by 50 mg of pituitary tissue alone. Liver plus pituitary slices responded to the cortisol addition. The figure was composed with data from a single set of experiments. The significance of the increase in oxygen consumption after addition of cortisol to the combination L + P in six experiments was: L + P, Q_{O_2} = 3.2 ± 0.62; L + P (C), Q_{O_2} = 4.4 ± 1.1; $P < 0.02$ (Student's t test).

HTH release by epinephrine was studied using respiration techniques. The addition of epinephrine to a mixture of 200 mg of rat liver slices together with 50 mg of bovine pituitary tissue and 50 mg of bovine anterior hypothalamus produced an increase in oxygen uptake. The final concentration of epinephrine was 2×10^{-7} M. Epinephrine gave negative results when added to any combination of two of the component tissues of the mixture (Fig. 3) or mixtures of pituitary slices (50 mg) with spleen or with kidney slices (200 mg).

The addition of hypothalamus tissue slices (50 mg) to the combination of liver (200 mg) and pituitary (50 mg) slices stimulated oxygen consumption by liver tissue. The combination of hypothalamus with either pituitary or liver alone produced no changes in respiration quotient (Fig. 4).

Although every experiment gave different values due to slight variations in the weight of the tissue, the results are comparable and differences are significant when they are expressed in microliters of O_2 consumed per unit weight and time (see legends of Figs. 1–4).

Discussion. The results reported in this presentation support the conclusion that HTH is a specific distinct substance that can be released from the hypophysis by cortisol or as a consequence of hypothalamus stimulation by epinephrine. However, the information available does not permit speculation about a relation between the *in vivo* increase of coagulation factors after HTH administration and the *in vitro* results on oxygen uptake by liver slices reported here. It has been reported that respiration of rat liver slices is in-

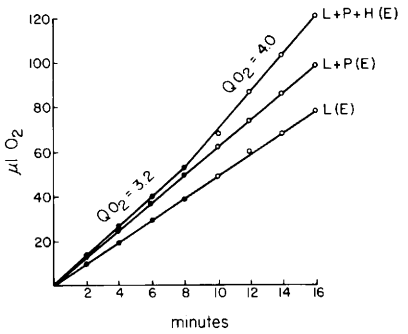

FIG. 3. Tissue oxygen uptake in the presence of epinephrine as measured by Warburg's direct method. The oxygen consumed by 200 mg of rat liver slices (L), alone and in the presence of bovine pituitary (P) and bovine anterior hypothalamus tissue slices (H), placed in a Warburg flask, was recorded before and after addition of epinephrine (E) contained in the sidearm (1×10^{-6} *M*). All tissues were in Krebs-Ringer's phosphate solution (100 mg/ml), and epinephrine dissolved in the same solution was poured after 8 min of uniform recording. The temperature was 37° and the stroke rate was 100/min. Q_{O_2} = μl of O_2/100 mg of tissue/min. The combination L + P + H responded to epinephrine addition. The figure was composed with data from a single set of experiments. The significance of the increase in oxygen uptake after epinephrine addition to L + P + H in six experiments was: L + P + H, Q_{O_2} = 3.8 ± 0.66; L + P + H (E), Q_{O_2} = 4.4 ± 0.40, $P < 0.05$ (Student's *t* test).

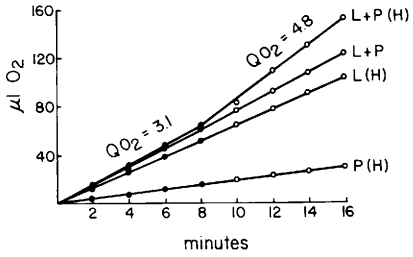

FIG. 4. Tissue oxygen uptake in the presence of hypothalamus tissue as measured by Warburg's direct method. The oxygen consumed by 200 mg of rat liver tissue slices (L) in the presence of bovine pituitary tissue slices (P) (50 mg), placed in a Warburg flask, was recorded before and after addition of bovine anterior hypothalamus slices (H) (50 mg in 0.5 ml) contained in the sidearm. All tissues were in Krebs-Ringer's phosphate solution (100 mg/ml). Hypothalamus tissue was poured from the sidearm after 8 min of uniform recording. The temperature was 37° and the stroke rate was 100/min. Q_{O_2} = μl of O_2/100 mg of tissue/min. The addition of hypothalamus tissue to the combination L + P stimulates oxygen uptake. There was no stimulation when hypothalamus tissue was added to 200 mg of liver or pituitary slices alone. The figure was composed with data from a single set of experiments.

hibited by oral anticoagulants *in vitro*. This was partially due to vitamin K reductase inhibition (11). Under special conditions HTH is able to counteract, to a certain extent, the hypoprothrombinemic effect of dicumarol (5). This effect of HTH is dependent on the presence of adequate quantities of vitamin K, because prothrombin concentration did not increase when animals maintained on a vitamin-K-free diet were treated with HTH administration (5). Probably there is a causal relationship between the effect of dicumarol on blood coagulation and liver slice metabolism and the opposite results obtained with HTH under the same conditions.

The administration of corticoids to rats produces a clear state of hypercoagulation as manifested by a shortening of the clotting time and a rise in concentration of the vitamin-K-dependent coagulation factors in plasma (5). This response with corticoids, which is similar to the one obtained with HTH administration, does not occur in hypophysectomized animals. We now find that stimulation of oxygen uptake by liver slices with corticoids occurs only in the presence of pituitary tissue. The corticoid concentration necessary for optimal *in vitro* response is higher than that normally present in the animal. The corticoid concentration, however, is similar to the doses necessary to elicit hypercoagulation *in vivo* (prednisolone, 0.01 to 0.1 mg/kg; hydrocortisone, 0.05 to 0.5 mg/kg; and cortisone, 0.5 mg/kg (7).

Similar to corticoids, epinephrine administration induces blood hypercoagulation, and this response is not observed in hypophysectomized rats (3). The results with epinephrine could be due to the liberation of one, or several, of the hypothalamic releasing factors, which, in turn, could stimulate the secretion of hypophyseal hormones. However, none of the well-recognized hypophyseal principles is known to produce hypercoagulation, or to stimulate liver metabolism specifically. HTH is obtained through a procedure which excludes a molecular mass greater

than 500 daltons and, consequently, all other identified hypophyseal hormones. Since respiration of liver slices is stimulated only in the presence of pituitary tissue or purified HTH, we assume that all respiration increases were due to HTH release.

Summary. The capacity of a pituitary peptide to stimulate the liver is further supported by tissue respiration studies. Peptide concentrates stimulate oxygen uptake of rat liver slices. The stimulating effect appears to be specific for the liver. The response of liver tissue was used to study the releasing mechanism of the peptide from hypophysis using Warburg's direct procedure. Cortisol (5×10^{-6} *M*) increased liver slice oxygen uptake only when pituitary tissue was included in the mixture. Epinephrine (2×10^{-7} *M*) produced the same results only when pituitary and hypothalamus tissue were included. A possible relation between the effect of HTH, cortisol, and epinephrine on *in vitro* liver tissue metabolism and the *in vivo* release of vitamin-K-dependent coagulation factors was discussed.

1. Cannon, W. B., and Gray, H., Amer. J. Physiol. **34,** 232 (1914).
2. Cannon, W. B., and Mendenhall, W. B., Amer. J. Physiol. **34** 251 (1914).
3. Landaburu, R. H., Castellanos, D. E., Giavedoni, E., and Lo Presti, C. A., Acta Physiol. Lat. Amer. **21,** 64 (1971).
4. Landaburu, R. H., Lo Presti, C. A., Hillard, M. A., and Castellanos, D. E., Acta Physiol. Lat. Amer. **21,** 74 (1971).
5. Landaburu, R. H., Quintana, C. M., Landaburu, C. A., and Castellanos, D. E., Acta Physiol. Lat. Amer. **21,** 339 (1971).
6. Landaburu, R. H., and de Lamo, R., IRCS Med. Sci. **3,** 68 (1975).
7. Landaburu, R. H., and D. E. Castellanos, New Istanb. Contrib. Clin. Sci. **10,** 113 (1972).
8. Mazur, R. H., Ellis, B. W., and Cammarata, P. S., J. Biol. Chem. **237,** 1619 (1962).
9. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., "Manometric Techniques." Burgess, Minneapolis (1957).
10. Hani, R. G., Proc. N. A. S. **53,** 288, (1965).
11. Wosilait, W. D., J. Pharmacol. Exp. Ther. **132,** 212 (1961).

Received September 1, 1976. P.S.E.B.M. 1977, Vol. 155.