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Effect of insulin on brain 5-hydroxytryptamine and 5-hydroxy-indole-acetic acid of rat

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THERE is evidence that hypothalmic serotonin is involved in some autonomic regulatory mechanisms and that its turnover varies with body temperature.[1] Monoamine oxidase inhibitors prevent alloxan-induced diabetes,[2] cause hypoglycaemia and potentiate the hypoglycaemic effect of insulin.[3] We have therefore carried out experiments to see if insulin-induced hypoglycaemia affects the levels of serotonin and its major breakdown product, 5-hydroxy-indole-acetic acid in the hypothalamus and the thalamus.

Materials and methods

Acidified *n*-butanol (B.D.H.) 500 ml, was prepared by the addition of 0·425 ml HCl sp.gr. 1·18 (B.D.H.) and 50 mg dithiothreitol (DTT) (Sigma). *n*-Heptane was standard laboratory reagent. Borate buffer was prepared by dissolving 6·5 g boric acid (B.D.H.) in 250 ml H_2O and the solution then made to pH 9·3 by addition of 0·42 N sodium hydroxide (B.D.H.). The orthophthalaldehyde reagent was prepared by dissolving 6 mg orthophthalaldehyde (Koch–Light) in 25 ml methanol (B.D.H.) and the solution was made to 100 ml with 10 N HCl. Standard solutions of 5-HT (Sigma) and 5-HIAA (Sigma) were made up from stock solutions of 5-hydroxytryptamine creatinine sulphate (500 μg free base/ml H_2O) and 5-HIAA (500 μg/ml methanol), and diluted to give a solution containing 1 μg indole + 100 μg DTT/ml.

Serotonin was estimated using the following procedure which is a modification of the method of Maickel and Miller.[4]

(i) 5-HT *estimation.* Frozen brain tissue was homogenized in butanol (3·0 ml for amounts less than 300 mg with an additional 1·0 ml butanol for every 100 mg tissue over 300 mg) and centrifuged for 10 min at 2500 *g* and 4°. A portion of the supernatant solution (2·5 ml) was then added to a centrifuge tube containing 0·2 ml of 0·1 N HCl and 5·0 ml *n*-heptane. The tubes were stoppered and solutions mixed on a mechanical rotary mixer for 10 min and centrifuged at 2500 *g* for 5 min at 4°. Seven ml of the supernatant solution was pipetted off for the 5-HIAA estimation (see below) and the remaining supernatant, together with the protein disc which may appear at the interface, was drawn off with a Pasteur pipette and discarded. To an 0·1-ml aliquot from the aqueous layer 0·8-ml orthophthalaldehyde reagent was added. The test-tubes, were placed unstoppered in a boiling water-bath for 10 min, cooled to room temperature and assayed in an Aminco–Bowman spectrofluorimeter, using standard 10 mm square, tightly-stoppered cuvettes. Activation wavelength was 360 mμ, emission wavelength 475 mμ (uncorrected), slit arrangements (3, 2, 3), (3, 2, 3), 5 mm.[5]

(ii) 5-*HIAA estimation.* The 7·0 ml of organic supernatant solution obtained after the acid extraction of serotonin, was added to 0·2 ml borate buffer pH 9·3, mixed for 10 min on a mechanical rotary mixer and centrifuged for 10 min at 2500 *g* at 4°. The upper organic phase along with any further tissue residue was drawn off and discarded. An 0·1 ml aliquot taken from the aqueous phase was assayed spectrofluorimetrically using the same procedure as for 5-HT.

Standard solutions of 1 μg/ml were routinely extracted in a similar manner to the samples. Recovery for 5-HT was 95–100 per cent and for 5-HIAA 80 per cent. When standards were extracted in the presence of tissue, 5-HT recovery was unaffected, while that of 5-HIAA fell to 65–70 per cent. Percentage extractions of both indoles, were independent of either indole concentration or the volume of aqueous solvent present.

The hydroxy indole (micrograms per gram wet weight) was then determined using the following equations, which are derived from the experimental extrapolation that 2·5 ml acidified butanol and 5 ml *n*-heptane absorb 0·104 ml 0·1 N HCl and that tissue contains 80 per cent water.

5-*HT*

$$\frac{(A - \theta)(1{\cdot}16 + 8{\cdot}64\ W)}{W \times S_{\text{I}}}$$

5-*HIAA*

$$\frac{(A - \theta)(2{\cdot}608 + 0{\cdot}4571\ W)}{W \times S_{\text{II}}}$$

Where A = fluorescence reading of sample.
θ = extracted blank.
W = wet weight of tissue taken.
S_I = standard fluorescence of a solution containing 10 μg 5-HT/ml (1 μg/tube).
S_{II} = standard fluorescence of a solution containing 10 μg 5-HIAA/ml (1 μg/tube).

Experimental procedure

Male Porton strain rats weighing 150–180 *g* received either 10 i.u. insulin B.P. (Boots) or an equivalent volume (0·125 ml) of saline subcutaneously. After 1–4 hr alternate experimental and control animals were sacrificed by decapitation (between 13.30–16.00 hr). The brain was removed from the skull and the area containing the hypothalamus and thalamus, dissected out, blotted to remove blood and dropped into liquid nitrogen. To minimize postmortem changes in indoles the time from decapitation to freezing was kept under 1 min.

The dissected areas from three animals were pooled, added to 3·0 ml acidified butanol and the 5-HT and 5-HIAA content estimated. Glucose was estimated in blood collected at decapitation by the procedure of Morley *et al.*[6]

Results and discussion

Animals sacrificed at 2, 3 and 4 hr after insulin injection all showed seizure activity. Control values for 5-HT levels in the hypothalamus + thalamus were 0·917 (± 0·019 S.E.M.) μg/g wet wt. Within 1 hr of insulin injection, (see Fig. 1) 5-HT levels had risen 9 per cent ($P < 0{\cdot}001$ by analysis of variance) and this increase was still seen after 2 and 3 hr, but returned to control levels after 4 hr (blood glucose 18 mg/ml).

Control values for 5-HIAA levels in the hypothalamus and thalamus were 0·699 (± 0·018 S.E.M.) μg/g wet wt. At 1 hr after injection of insulin 5-HIAA levels remained unchanged but with more severe hypoglycaemia these levels rose by 35 per cent (see Fig. 2).

The 5-HT changes were in agreement with findings of Costa and Himwich[7] who, in the rabbit, found an initial increase in 5-HT levels in mid-brain, telencephalon and hippocampus after short periods of convulsive activity with a return to control values after convulsions of longer duration. However, they noted a 90 per cent inhibition of 5-hydroxytryptophan decarboxylase during insulin

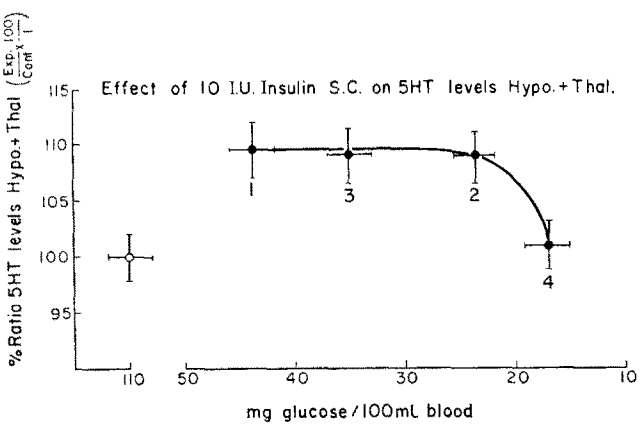

FIG. 1. Relationship of hypoglycaemia and percentage change of 5-HT levels in rat hypothalamus and thalamus after insulin injection.

○ Control glucose and 5-HT values are derived from the means of four control groups of 18 rats matched with the experimental groups.

● Values of percentage change of 5-HT are means of 18 brain samples (pooled into six groups of three) ± α where α =

$$100\left(\frac{\bar{x} + \text{S.E. of } \bar{x}}{\bar{y} - \text{S.E. of } \bar{y}}\right)$$

where $\bar{x}$ and $\bar{y}$ are the means of experimental and control groups respectively. S.E.s were obtained by analysis of variance. Experimental groups were killed at 1, 3, 2 and 4 hr after insulin injection as indicated. Glucose values are the mean ± S.E.M. (n = 18).

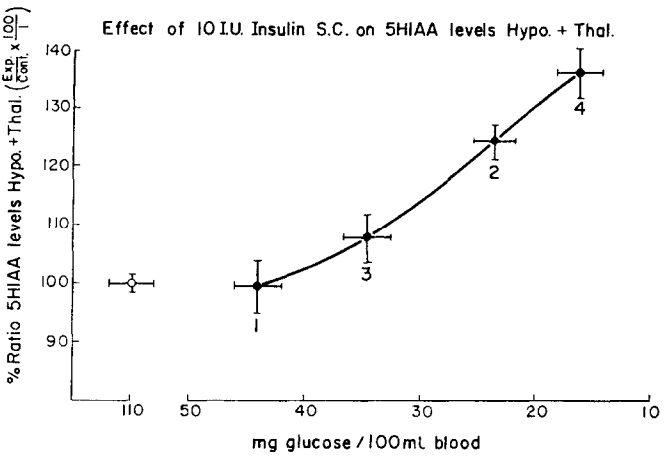

FIG. 2. Relationship of hypoglycaemia and percentage change of 5-HIAA levels in rat hypothalamus and thalamus after insulin injection in the same four control and experimental groups as Fig. 1.

○ Control glucose and 5-HIAA values are derived from the means of four control groups of 18 rats matched with the experimental groups.

● Values of percentage change of 5-HT are means of 18 brain samples (pooled in six groups of three) $\pm \alpha$ where $\alpha =$

$$100\left(\frac{\bar{x} + \text{S.E. of } \bar{x}}{\bar{y} - \text{S.E. of } \bar{y}}\right)$$

where $\bar{x}$ and $\bar{y}$ are the means of the experimental and control groups respectively. S.E.s were obtained by analysis of variance. Experimental groups were killed at 1, 3, 2 and 4 hr after insulin injection as indicated. Glucose values are the mean $\pm$ S.E.M. ($n = 18$).

hypoglycaemia and concluded that there was a decrease in the rate of synthesis of 5-HT. In contrast, the increase in 5-HIAA observed in rat brain suggests there is an increase in 5-HT synthesis. Tryptophan hydroxylation has been shown to be rate limiting in 5-HT synthesis,[8] with L aromatic amino acid decarboxylase activity some hundred times greater than hydroxylase activity.[9] Thus a 90 per cent inhibition of the former enzyme should not affect the turnover rate of serotonin (which could even be increased through tryptophan hydroxylase activation).

To what extent the increase in 5-HT turnover is due directly to insulin, to low blood glucose levels, or to the secondary effects of hypoglycaemia such as adrenaline and growth hormone release, or seizure activity, is not known.

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