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A Role for Serotonin in the Hypothalamic-Pituitary-Adrenal Response to Insulin Stress[1]

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Abstract. Controversy exists concerning the possible involvement of serotonin (5-HT) in the pituitary-adrenocortical response to stress. In the present research, a variety of pharmacological and physiological manipulations were used in male rats to study the role of this neurotransmitter in the adrenocortical response to insulin-induced hypoglycemia. We first examined the effect of insulin stress on hypothalamic 5-HT metabolism and found increased turnover as determined by an enhanced accumulation of 5-HT following monoamine oxidase inhibition. Brain 5-HT depletion by intraventricular injection of 5,7-dihydroxytryptamine significantly attenuated the corticosterone response to insulin, while treatment with the 5-HT receptor blocker methysergide tended to have the same effects. The corticosterone response to insulin was potentiated by prior administration of *L*-tryptophan, and blocked by pretreatment with valine, an amino acid that competes with tryptophan for transport across the blood-brain barrier. It therefore appears that the pituitary-adrenal response to insulin is mediated at least in part by 5-HT, and may be dependent on increased uptake of tryptophan by the brain.

Although many different studies have investigated the role of serotonin (5-HT) in the regulation of hypothalamic-pituitary-adrenal hormone secretion, there is still debate over whether 5-HT acts to stimulate or to suppress this axis. Studies of the pituitary-adrenal system in the absence of stress have generally suggested an excitatory role for 5-HT. 5-HT and 5-HT agonists have produced increases in glucocorticoids in both humans [25] and animals [19–21, 35, 40, 43, 44, 49] that can be prevented by 5-HT receptor blockers. These findings are consistent with in vitro studies showing that 5-HT stimulates the secretion of corticotrophin releasing hormone (CRH) from the hypothalamus [7, 24, 27].

The results of studies using stressful manipulations, or other situations in which baseline pituitary-adrenal activity is elevated, have been less clear. In humans, the 5-HT receptor blocker metergoline reduced ACTH levels in response to insulin stress [9]. Other 5-HT antagonists in addition to metergoline, namely methysergide [8] and cyproheptadine [48], were effective in lowering ACTH levels following metyrapone administration. Cyproheptadine was also found to be effective in reducing symptoms of two clinical endocrinopathies, Cushing's disease, which is characterized by adrenocortical hypersecretion [34], and Nelson's syndrome, in which excessive ACTH secretion occurs as a result of bilateral adrenalectomy in Cushing's patients [36]. While these studies suggest that the role of 5-HT in the adrenocortical response to stress is also stimulatory, conflicting results have been obtained from various experiments using animals. Some investigators have reported inhibitory effects of 5-HT on the pituitary-adrenal stress response. In rats, for example, the increase in circulating ACTH levels produced by ether was enhanced by *p*-chlorophenylalanine (PCPA) and blocked by pretreatment with tryptophan, the dietary precursor of 5-HT [53]. Intraventricular injection of 5-HT similarly prevented the corticosterone response to surgical stress [52]. Other investigators, however, have failed to alter the adrenocortical response to stress using serotonergic drugs. The 5-HT reuptake inhibitor fluoxetine did not influence plasma corticosterone levels following either swim stress or insulin stress [18]. PCPA had no effect on increased plasma corticosterone levels seen with hypoxia or hypercapnia [38], while 5-HT depletion produced by the neuro-

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toxin 5,7-dihydroxytryptamine (5,7-DHT) or electrolytic lesions of the midbrain raphe did not alter the ACTH response to ether [29].

Further confusion about the role of 5-HT in stress stems from studies of 5-HT metabolism. Increased rates of 5-HT turnover in particular brain regions have been observed during restraint stress [6, 30, 42] as well as during foot shock, swim stress and psychosocial stress [6]. These results have been challenged by *Telegdy and Vermes* [51], who report decreased 5-HT turnover with a variety of stressors.

In view of the controversial results cited above, the following experiments were performed in an attempt to clarify the role of 5-HT in the pituitary-adrenal response to stress. We chose the stress of insulin-induced hypoglycemia because this stressor is easily quantified and manipulated, it represents a physiological challenge to the organism, and the animal's response can (through measurement of blood glucose) be assessed independently of pituitary-adrenal activity.

Materials and Methods

Animals

Adult male Wistar rats were purchased from Charles River or bred in our laboratory. Animals were housed singly in a colony room under a 14:10 light-dark cycle (lights on at 06.00 h) and were fed Purina lab chow and tap water ad libitum. The rats weighed 225–350 g at the time of experimentation.

Experimental Design

All experiments took place between 08.00 and 11.00 h when the serum corticosterone concentration is low in the normal diurnal rhythm. At the appropriate time after drug administration, animals were quietly removed from the colony room and killed by decapitation at intervals of at least 2 min. Trunk bloods were collected and allowed to clot on ice. Bloods were then centrifuged and the sera aspirated and frozen at −40 °C for corticosterone determination by radioimmunoassay. In some cases, serum glucose analyses or hypothalamic 5-HT determinations were also performed.

All animals were fasted approximately 16 h before receiving insulin or vehicle. Dose-response and time-course studies were carried out in order to determine the parameters of both the corticosterone response to insulin and the corresponding glucose levels. In the first experiment, rats were killed 40 min after the subcutaneous (s.c.) injection of 0.125–0.250 U/kg insulin or saline. For the time-course study, rats were given 0.20 U/kg insulin or saline, and then killed 0, 20, 40, 60 and 80 min later.

We then examined 5-HT turnover after insulin or saline by measuring pargyline-induced accumulation of this neurotransmitter. Animals were injected intraperitoneally (i.p.) with 75 mg/kg of pargyline and then 5 min later either killed immediately or injected with 0.20 U/kg insulin or saline. The latter two groups were killed 45 min after pargyline administration. Hypothalamic 5-HT concentrations were determined in all animals by the methods described below.

The remaining studies examined the effects of both pharmacologic and physiologic serotonergic manipulation on the corticosterone response to insulin stress. In one experiment brain 5-HT was depleted using the potent 5-HT neurotoxin 5,7-dihydroxtryptamine (5,7-DHT). 5,7-DHT decreases brain 5-HT by about 70–80% when measured 8–12 days after administration [3]. Rats were anesthetized with Equithesin, placed in a stereotaxic instrument, and then injected with 150 µg of 5,7-DHT (free base) in 20 µl of 0.1% ascorbic acid. Control animals received an equal volume of the ascorbic acid alone. Because 5,7-DHT can also produce a moderate depletion of brain norepinephrine, animals were pretreated i.p. with 25 mg/kg desipramine 45 min before drug or vehicle injections [5]. 10 days later, animals were killed 40 min following the injection of 0.20 U/kg insulin or saline. Blood samples were collected for subsequent corticosterone determination and hypothalami dissected and assayed for their 5-HT content. A further study attempted to determine whether the insulin-induced rise in corticosterone could be attenuated with a drug that blocks serotonergic action. Animals were pretreated i.p. with 5 mg/kg methysergide, a serotonergic receptor blocker, 1 h before receiving 0.20 U/kg insulin or saline. Animals were killed 40 min following this latter injection.

In the next experiment, the 5-HT precursor *L*-tryptophan was tested for its ability to potentiate the effect of a submaximal dose of insulin on circulating corticosterone levels. Fasted animals were pretreated s.c. with 200 mg/kg tryptophan 20 min before receiving 0.150 U/kg insulin. Although this was a relatively large dose of tryptophan, other studies have shown that (in the absence of a monoamine oxidase inhibitor) doses of this magnitude are generally required in order to produce any behavioral or physiological effects [17, 22, 50]. One of possible reasons for this finding is that only a small portion of a peripherally administered tryptophan dose is actually converted to brain 5-HT [13]. The final study attempted to determine whether the rise in circulating corticosterone associated with insulin stress is a result of increased availability of tryptophan to the brain. Animals were pretreated s.c. with 200 mg/kg *L*-valine 20 min before receiving 0.20 U/kg insulin. Valine is a neutral amino acid that has previously been shown to compete with tryptophan for transport across the blood-brain barrier. Blood samples were obtained in the usual manner 40 min following insulin treatment.

Serotonin Determination

Following decapitation, hypothalami were rapidly dissected over ice and homogenized in a total volume of 2.0 ml of 0.40 *N* $HClO_4$ plus 50 µl 1% ascorbic acid and 50 µl 10% EDTA. Samples were centrifuged at −5 °C for 20 min at 30,000 *g*. Supernatants were decanted and were then brought up to a pH of 5.5–6.0 using KOH. One drop of 0.04% bromphenol blue in absolute ethanol was added to each tube to aid in the pH adjustment. Samples were recentrifuged for 5 min to remove the $KClO_4$ precipitate. Supernatants were decanted, brought to room temperature, and then applied to 3×20 mm columns of a weak cation exchange resin (Bio-Rex 70). The columns were eluted according to *Holman* et al. [23] except that 2.5 *N* HCl was used to recover the 5-HT. The 5-HT eluates were reacted with *o*-phthalaldehyde (OPT) [37] and then read on a Perkin-Elmer No. 1000 spectrofluorometer at (uncorrected) excitation and emission wavelengths of 364 and 480 nm, respectively.

Radioimmunoassay

Procedure for corticosterone determination was followed according to *Meyer* [in press, 1983].

Glucose Analysis

Serum glucose was analyzed by the glucose oxidase method using kit No. 510A purchased from Sigma Co.

Drugs and Materials

Methysergide was generously donated by Sandoz Inc., and desipramine was a gift from Merrel Dow Research. *L*-Tryptophan, *L*-valine, and 5,7-dihydroxytryptamine creatinine sulfate were purchased from Sigma Chemical Co., and insulin (U40, Squibb) came from a local pharmacy. All drugs were dissolved in 0.9% saline solution, except where otherwise specified. Tryptophan was dissolved in saline containing a few drops of 10 *N* NaOH and then adjusted to a pH of 8.5.

Antiserum B3-163 for the corticosterone radioimmunoassay was purchased from Endocrine Sciences. ^{3}H-corticosterone (92.0 Ci/mmol) was from New England Nuclear, and unlabeled corticosterone, used for standards, came from Steraloids, Inc.

Bio-Rex 70 for 5-HT determination was purchased from Bio-Rad Laboratories, and 5-HT creatinine sulfate was from Regis Chemical Co. OPT was purchased from Sigma and was repurified according to *Jacobowitz and Richardson* [26].

Data Analysis

All data were subjected to analysis of variance (ANOVA) followed by individual mean comparisons using Fisher's Least Significant Difference test [32]. Data from the turnover study were evaluated using a Student's t test. Results occurring with a chance probability of less than 0.05 were considered statistically significant.

Results

The effect of differing doses of insulin on serum corticosterone and glucose concentrations is shown in figure 1. Insulin significantly elevated serum corticosterone ($F_{5,24}$ = 28.51) and lowered glucose levels ($F_{5,24}$ = 4.49) in a dose-dependent manner at all doses except the lowest one, 0.125 U/kg. The time-course of these responses to insulin is shown in figure 2. Serum corticosterone reached a maximum at 40 min and began to decrease by 60 min (fig. 2a). Post hoc testing revealed that all groups of insulin-treated animals were significantly higher than saline controls ($F_{1,40}$ = 64.04) except at the 20 min time point. Glucose levels in the insulin treated animals were found to be different than saline controls at all times ($F_{1,40}$ = 151.61; fig. 2b).

The effect of insulin on hypothalamic 5-HT turnover can be seen in figure 3. The pargyline-induced accumulation of 5-HT 40 min following insulin administration was significantly higher than its accumulation following saline (t_{18} = 2.67). This indicates an increased rate of hypothalamic 5-HT synthesis (turnover) as a result of insulin stress.

Hypothalamic 5-HT was depleted by an average of 75% when measured 10 days after treatment with 5,7-DHT

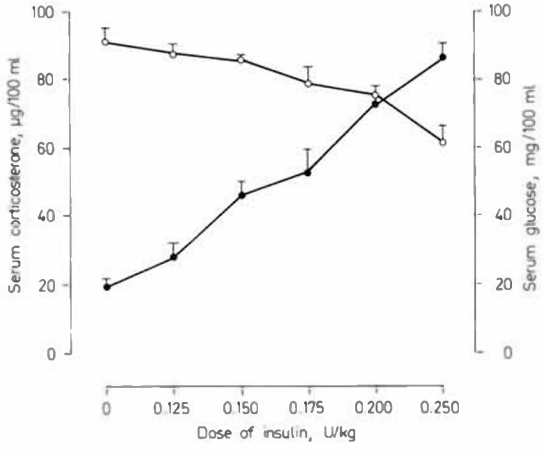

Fig. 1. Dose-dependent changes in serum corticosterone (●) and glucose (○) concentrations following insulin. Insulin was injected s.c. at the doses indicated, 40 min before the animals were decapitated. Each value shown is the mean ± SEM for 5 rats per group.

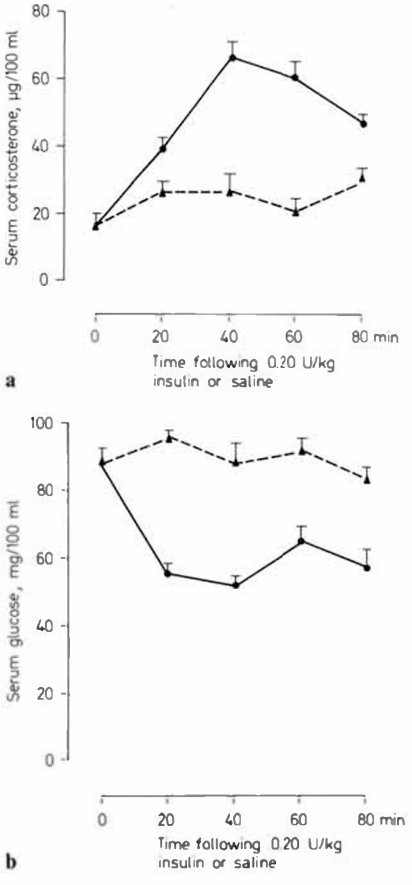

Fig. 2. **a** Time-course effect of insulin (●) on serum corticosterone. 0.20 U/kg insulin or saline (▲) was injected at time zero. Each value shown is the mean ± SEM for 6 rats per group. **b** Time-course effect of insulin on serum glucose in the same animals shown in **a**.

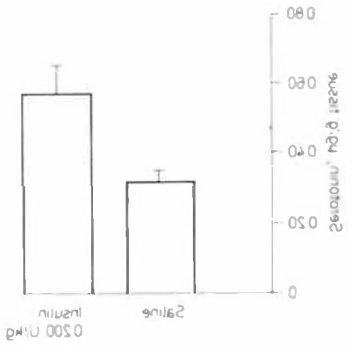

Fig. 3. Net hypothalamic 5-HT accumulation following pargyline. Animals were injected i.p. with 75 mg/kg pargyline and then 5 min later either killed, or injected with 0.20 U/kg insulin or saline. The latter two groups were killed 45 min after the second injection. Data represent the amount of 5-HT above that of the 5 min controls, which had a mean hypothalamic 5-HT concentration of 0.78 μg/g tissue. Mean hypothalamic weight for all rats was 32.7 ± 0.1 mg. Values represent the mean ± SEM for 10 rats per group.

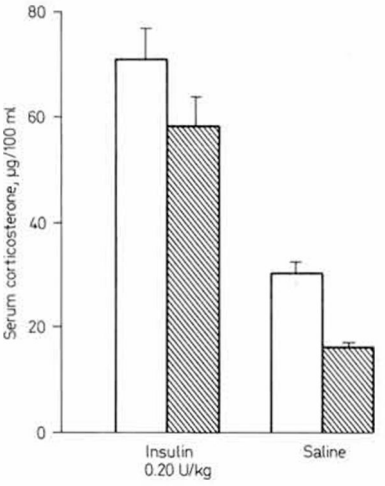

Fig. 5. Effect of methysergide (▨) on the serum corticosterone response to insulin stress. Rats were pretreated with 5 mg/kg methysergide i.p. 1 h before receiving 0.20 U/kg insulin or saline (□) and killed 40 min later. Values represent the mean ± SEM for 8 rats in all groups except the methysergide-insulin group, in which n = 7.

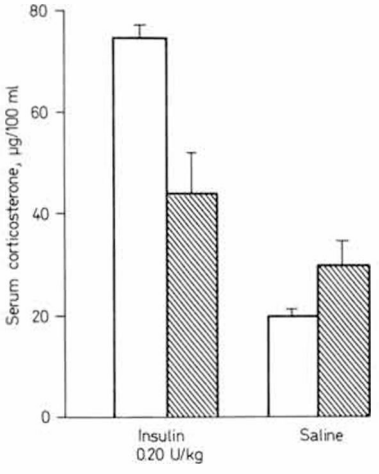

Fig. 4. Effect of 5,7-DHT on the serum corticosterone response to insulin. 150 μg 5,7-DHT in 0.1% ascorbic acid or vehicle alone was injected intraventricularly 45 min after pretreatment with 25 mg/kg desipramine. 0.20 U/kg insulin or saline were injected 10 days later and the rats killed 40 min following this injection. Values represent the mean ± SEM for 6 rats per group. □ = Sham; ▨ = 5,7-DHT.

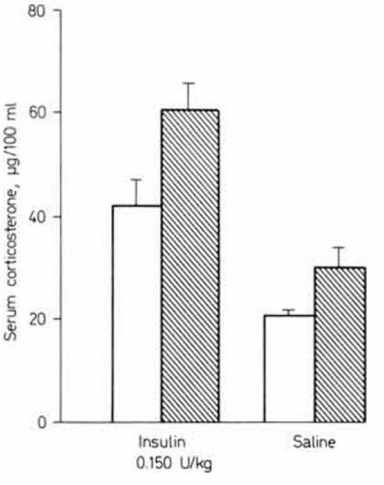

Fig. 6. Effect of tryptophan pretreatment on serum corticosterone response to insulin stress. A dose of 0.15 U/kg insulin or saline (□) was injected s.c. 40 min before rats were killed and 20 min after the s.c. injection of 200 mg/kg *L*-tryptophan (▨). Values represent the mean ± SEM for 9 rats per group.

(mean hypothalamic 5-HT content = 0.19 μg/g tissue compared to 0.76 μg/g in sham-operated animals). Analysis of the corticosterone data revealed a main effect for insulin treatment ($F_{1,20}$ = 36.25) and a significant interaction between insulin and 5,7-DHT ($F_{1,20}$ = 11.62). Post hoc testing showed that 5,7-DHT significantly lowered the insulin-induced elevation of serum corticosterone (fig. 4).

Treatment with methysergide reduced levels of circulating corticosterone in both insulin and saline treated rats ($F_{1,27}$ = 7.28). As illustrated in figure 5 the effect of methysergide was of greater magnitude in the saline-treated controls. It should be noted that these controls have been subjected to two mild stressors, namely i.p. injection and 16-hour food deprivation. Thus, methysergide (at the present dose) was

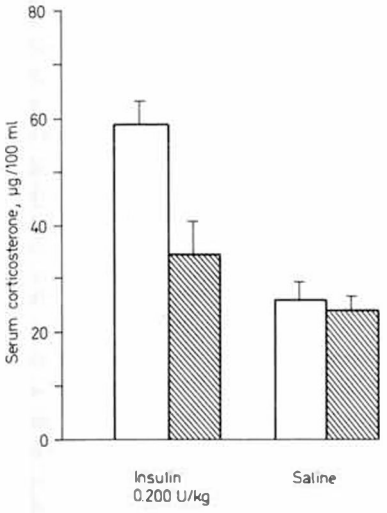

Fig. 7. Effect of valine pretreatment on serum corticosterone response to insulin stress. A dose of 0.20 U/kg insulin or saline (□) was injected s.c. 40 min before rats were killed and 20 min after the s.c. injection of 200 mg/kg valine. (▨) Values represent the mean ± SEM for 6 rats per group.

capable of inhibiting pituitary-adrenal responses to stress, but a smaller degree of inhibition was observed when the stress was more potent.

Altering plasma amino acid concentrations produced substantial changes in the corticosterone response to insulin stress. The effect of tryptophan pretreatment of animals given a submaximal dose of insulin is summarized in figure 6. An initial ANOVA yielded significant main effects for both the treatment ($F_{1,32}$ = 42.43) and pretreatment ($F_{1,32}$ = 13.08) variables. Rats given 0.150 U/kg of insulin had higher overall corticosterone levels than their saline-injected counterparts, while tryptophan pretreatment potentiated the responses to both insulin and saline.

In the case of valine pretreatment, an overall analysis of the corticosterone data revealed a significant interaction between treatment with insulin and pretreatment with valine ($F_{1,20}$ = 7.70). It can be seen (fig. 7) that although 200 mg/kg valine did not alter serum corticosterone in saline-injected rats, the same dose almost completely prevented the stress-induced increase in corticosterone observed following insulin. Post hoc testing confirmed that the valine-insulin animals were significantly different from the saline-insulin animals, but did not differ from either control group. These findings are consistent with the idea that tryptophan availability plays an important role in the pituitary-adrenal response to insulin-induced hypoglycemia.

Discussion

The results of these studies support our hypothesis that 5-HT mediates, at least in part, the effects of insulin stress on the hypothalamic-pituitary-adrenal system. This conclusion is based on the ability of various serotonergic manipulations to appropriately alter these effects, and on the increase in hypothalamic 5-HT turnover observed following insulin administration. The present findings are not in accord with those of *Fuller and Snoddy* [18], however these investigators used only one pharmacological agent, namely fluoxetine (a 5-HT reuptake inhibitor), in their studies. It should also be mentioned that because serum glucose concentrations were not measured in all animals, we cannot rule out the possibility that our experimental treatments were effective not because of their actions on 5-HT but because they directly modified the degree of hypoglycemia produced in each case. Nevertheless, it seems unlikely that all of these treatments could have influenced the hypoglycemic response in such a manner as to have generated the precise pattern of results reported above.

Treatment with 5,7-DHT significantly reduced the pituitary-adrenal response to insulin, despite causing slightly increased levels of circulating corticosterone in the saline treated controls. The latter results could be secondary to the well-known anorectic effects of this drug, rather than a direct action on serotonergic neurons involved in pituitary-adrenal regulation. It is important to note that the effectiveness of 5,7-DHT in blunting the corticosterone response to insulin specifically implicates brain 5-HT in mediating this response. However, it is not yet clear which of the currently identified serotonergic pathways might be the critical one(s). For example. *Karteszi* et al. [28] reported that anterolateral deafferentation of the hypothalamus greatly reduced the corticosterone rise seen following insulin. This finding would be consistent with a role for ascending fibers rising from the classic brainstem serotonergic cell groups [41]. On the other hand, other researchers [2] claim that complete hypothalamic isolation did not prevent the corticosterone response to insulin, a result that might suggest an involvement of the more recently discovered serotonergic neurons within the hypothalamus itself [4, 31]. More detailed mapping studies will be necessary before this issue can be resolved.

Studies with the 5-HT receptor blocker methysergide provide more general support for a role for 5-HT in the pituitary-adrenal response to stress. The overall effect of methysergide was to diminish the corticosterone elevations observed following both the mild stress of fasting and saline injection as well as the more severe stress of insulin-induced hypoglycemia. Clearly, though, the drug was more robust in blocking the hormonal response to the milder stress. The inability of methysergide to prevent more completely the reaction to insulin may be related to its relatively weak potency at type 1 as opposed to type 2 5-HT receptors [47]. Neuro-

physiologically, type 1 receptors have been found to mediate postsynaptic inhibition by 5-HT [1] and appear to be linked to adenylate cyclase [46], but the possible endocrine function of these receptors is still unknown.

If insulin stimulates the pituitary-adrenal system partly via a central serotonergic mechanism, it is important to consider how 5-HT neurotransmission might be enhanced following insulin administration. This requires an understanding of the relationship between brain 5-HT and the state of tryptophan, the dietary precursor of this neurotransmitter, in the circulation. Tryptophan hydroxylase, the rate-limiting enzyme in 5-HT synthesis, is not saturated in brain with its substrate tryptophan [16]. Thus, 5-HT synthesis can be altered by either increasing or decreasing the availability of tryptophan with respect to the enzyme. One of the factors that influence tryptophan availability to the brain is plasma concentrations of large, neutral amino acids that compete for transport across the blood-brain barrier [11, 12, 45]. Treatments that increase the ratio between tryptophan and its competitors generally increase the relative entry of tryptophan into the brain, while treatments that reduce this ratio produce the opposite effect.

Another major factor that can potentially affect tryptophan availability to the brain is the degree of tryptophan binding in the blood. Tryptophan is the only amino acid that is bound to plasma albumin. Normally, about 80–90% of total tryptophan is bound while 10–20% circulates free [39]. Nonesterified fatty acids (NEFA) share these albumin binding sites with tryptophan. Thus, if concentrations of NEFA increase, the percentage of free tryptophan in the plasma also increases. Fasting, for example, increases NEFA, thereby causing increased levels of free tryptophan [10, 11]. Based on the methysergide results discussed above, it is possible that this effect of fasting plays a significant role in the elevated corticosterone concentrations observed in fasted animals. Insulin, on the other hand, reduces the levels of NEFA by stimulating their uptake into various peripheral tissues. It might be expected that this would result in decreased tryptophan availability to the brain. However, *Fernstrom and Wurtman* [14] found an increase in both whole-brain tryptophan levels and 5-HT synthesis when carbohydrate ingestion was used to stimulate insulin secretion. Fortunately, this apparent inconsistency can readily be explained when we consider two other factors. First, insulin stimulates the peripheral uptake of amino acids as well as NEFA. Tryptophan, however, does not appear to be affected in this manner by insulin, perhaps because of its association with albumin. The concentration of total plasma tryptophan following insulin administration therefore remains unchanged or is even elevated (depending on the insulin dose) in contrast to the reduced concentrations of competing amino acids [15]. Second, *Yuwiler* et al. [54] found that tryptophan has a considerably higher affinity for the brain capillary amino acid transport carrier than it does for albumin. As a result, much of the bound tryptophan may actually be available for entry into the brain, particularly when large numbers of the appropriate carrier sites are unoccupied. In light of these arguments, it seems likely that the most important factor regulating changes in brain tryptophan following insulin administration is the reduction in plasma concentration of other amino acids that compete with tryptophan for transport across the blood-brain barrier.

The model being developed here proposes that insulin increases brain tryptophan, which then results in enhanced serotonergic transmission and (among other things) a stimulation of the pituitary-adrenal system. This model is supported not only by the results of other investigators reported above, but also by our own ability to modify the corticosterone response to insulin by directly manipulating tryptophan availability. Pretreatment with tryptophan itself significantly enhanced the insulin response, while pretreatment with valine (which would be expected to occupy most of the brain capillary neutral amino acid transport sites) attenuated this response. Although we did not actually determine brain tryptophan and 5-HT concentrations following tryptophan or valine administraton, the expected changes in these parameters have previously been reported by other investigators [12, 13, 30]. It is interesting to note that tryptophan also increased serum corticosterone concentrations in the noninsulin injected animals. Once again, this result may be due to the fasted state of the subjects, as we have previously observed no response to tryptophan alone (i.e., in the absence of a monoamine oxidase inhibitor) in either nonfasted rats [*Meyer, Yehuda*, unpubl. observations] or mice [40].

Finally, our findings can be related to recent research on immobilization stress, a treatment that has been associated with increased plasma levels of NEFA [33] and therefore an increased ratio of free to bound tryptophan in the circulation. Several experiments have demonstrated a positive correlation between pituitary-adrenal stimulation and increased brain tryptophan levels following immobilization [10, 30, 42]. In one case, valine pretreatment was shown to significantly inhibit both of these effects [30]. These findings suggest that changes in brain 5-HT mediated by altered tryptophan availability may be important in the pituitary-adrenal response to a variety of stressors.

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