ABSTRACT

Infertility is a common sequela to experimentally-induced diabetes mellitus in the male rat as well as to diabetes in the human male. This has been attributed both to alterations in hypothalamo-pituitary and pituitary-testicular activity. In view of the role of serotonin in the hypothalamic regulation of luteinizing hormone secretion, we determined the effects of streptozotocin-induced diabetes on hypothalamic serotonin synthesis relative to circulating levels of luteinizing hormone, follicle-stimulating hormone and prolactin in these animals. Half of a group of adult male Sprague-Dawley rats were injected intraperitoneally with 16 mg·kg⁻¹ of streptozotocin. The other half were injected with vehicle only. The diabetic group was hyperglycemic throughout the study period (488.7±21.2 mg·dl⁻¹ in diabetic rats vs. 125.1±39.3 mg·dl⁻¹ in control rats). A third group of rats served as semi-starved controls, weight-matched by total calorie restriction diet to diabetic rats. All of the rats were injected intraperitoneally with 200 mg·kg⁻¹ of NSD-1015 30 min prior to sacrifice. Accumulation of 5-hydroxytryptophan in various hypothalamic areas was then assayed by liquid chromatography with electrochemical detection as a relative index for the rate of serotonin synthesis. Serum radioimmunoassayable luteinizing hormone, follicle-stimulating hormone and prolactin were also assayed in these animals. Our results show a 50% decrease in serotonin synthesis in the preoptic area-anterior hypothalamus and mediobasal hypothalamus-median eminence 16 weeks after streptozotocin treatment. We also found that serotonin synthesis was inhibited 4 weeks after streptozotocin treatment in the preoptic area-anterior hypothalamus, but not in the mediobasal hypothalamus-median eminence, indicating region-specificity in the early hypothalamic response to streptozotocin-induced diabetes. Serum luteinizing hormone levels were decreased both 4 and 16 weeks after streptozotocin treatment. No changes either in hypothalamic serotonin synthesis or in serum luteinizing hormone levels were observed 1 week after streptozotocin treatment. Serum follicle-stimulating hormone and prolactin levels remained unaffected by streptozotocin treatment throughout the study period. The differential effect of diabetes on the gonadotropins may represent further evidence for the hypothesis of differential regulatory mechanisms governing the secretion of these two hormones and further emphasizes the pathophysiological specificity (i.e., not a generalized metabolic disturbance effect) of diabetes-induced male reproductive neuroendocrinopathy. Our study suggests, at least in part, an explanation for such pathological interaction, namely, diabetic inhibition of a region-specific neurotransmitter system integral to hypothalamic regulation of luteinizing hormone secretion.
Diabetes mellitus is often associated with human neuroendocrinopathy. Sexual impotence (10, 19), decreased libido (15) and impaired spermatogenesis leading to decreased semen quality (1, 10, 24) can be the first symptoms heralding the onset of diabetes. Although some of these problems undoubtedly derive from the peripheral neuropathy and angiopathy associated with diabetes, it has also been demonstrated that serum testosterone levels are reduced in the male diabetic (23).

Experimentally-induced diabetes (e.g., using alloxan or streptozotocin (STZ)) is associated with decreased serum gonadotrophin (luteinizing hormone: LH and follicle-stimulating hormone: FSH) (7, 22) and serum testosterone (20, 22) levels, reduced or absent spermatogenesis (28) and reductions in accessory sex organ weights (7, 9, 21). Reduction in serum LH levels has been correlated with apparent alterations in the regulation of hypothalamic gonadotrophin releasing hormone (GnRH) in male diabetic rats (3).

Hypothalamic monoaminergic systems are thought to exert integrated control over the secretion of several anterior pituitary hormones including the gonadotrophins as well as prolactin (PRL) (12, 18). The potential effect of diabetes to alter the activities of such monoaminergic systems could explain, at least in part, the relationship between diabetes and reproductive dysfunction.

Given the numerous published reports relating hypothalamic serotonin (5-hydroxytryptamine: 5HT) mechanisms to hypothalamic regulation of gonadotrophin secretion, we sought to determine the effects of STZ-induced diabetes on hypothalamic 5HT synthesis in male rats relative to the effects of STZ-induced diabetes on circulating levels of LH, FSH and PRL.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley (CH:CD (SD) BR; outbred) rats (120–140 g) were obtained from Charles River Breeding Laboratories, Inc. The rats were housed 3–4 per cage in an air-conditioned (22±2°C), windowless room. A light:dark cycle consisting of 14L:10D (lights on at 0600 daily) was controlled automatically. The rats were provided with food (Wayne Lab-Blox) and fresh tap water ad libitum.

Induction of Diabetes

Within a few days of their arrival, half of the shipment of rats were injected with STZ (16 mg·kg⁻¹ in 0.01 mM citrate buffer, pH 4.5, intraperitoneally). The other half received vehicle injection only. Induction of diabetes was confirmed within 3 days after STZ injection by blood glucose determination. Diabetic rats were hyperglycemic throughout the duration of the study (488.7±21.2 mg·dl⁻¹ vs. 125.1±39.3 mg·dl⁻¹ in control rats; mean±SEM).

Tissue Collection

One, 4 and 16 weeks after STZ (or vehicle) injection, the rats were injected intraperitoneally with 200 mg·kg⁻¹ of NSD-1015 (3-hydroxybenzylhydrazine) (Sigma Chemical Co., St. Louis, MO) dissolved in 0.9% saline (pH 7.4). The rats were then killed by decapitation 30 min after NSD-1015 injection. Trunk blood was collected for determination of LH, FSH and PRL concentrations. The brains were removed rapidly and frozen on dry ice. Within 2-3 days, the brains were allowed to thaw partially for dissection of the mediobasal hypothalamus–median eminence (MBH-ME) and preoptic area–anterior hypothalamus (POA-AH) as described elsewhere (30).

Hormone Radioimmunoassay

Measurement of serum LH, FSH and PRL concentrations was performed in duplicate using the ovine-ovine LH and FSH radioimmunoassay and the NIAMDD rat PRL kit as described elsewhere (26). Results are expressed relative to rat reference preparation RP-1. Samples from each experiment (i.e., 1 week, 4 weeks or 16 weeks post-STZ treatment) were run in a single assay. Intra-assay coefficients of variation were 5.8 to 6.8% for LH, 4.9 to 5.8% for FSH and 6.8 to 7.3% for PRL.

Indolic Measurements

Indole determinations were done using liquid chromatography with electrochemical detection (LCED) as previously described (13, 14). The tissues were homogenized individually in 50 µl (ME) or 200 µl (MBH and POA-AH) of chilled 0.4 N perchloric acid containing 10⁻³ M sodium bisulfite. N-Methyltryptamine was added to each sample as well as the assay.
standard in order to estimate procedural losses.

The homogenates were centrifuged for 1 min at 12,000 g. 5-Hydroxytryptophan (5HTP) was separated from 5HT, 5-hydroxyindole acetic acid (SHIAA) and the internal standard by LCED on a C-18 reverse phase column using a mobile phase consisting of 0.1 M sodium acetate, 0.1 M citric acid and 10% (v/v) methanol at a flow rate of 1.0 ml·min⁻¹. The indoles were detected using a glassy carbon electrode at a potential of 0.71 V versus an Ag/AgCl reference electrode as each indole was eluted from the column. The working electrode was coupled to a detector set at 1 nA (ME) or 5 nA (MBH or POA-AH) range of sensitivity. Indole peaks were identified by relative retention times compared to those of the standards; concentrations were determined by comparing peak heights of the unknown samples with those of standards using a programmed integrator interfaced with the detector unit. Values were corrected for the recovery of internal standard which averaged 98.6±1.0%. Intra-assay variation was ±4.1% for 5HTP, ±4.9% for 5HT and ±6.8% for 5-HIAA.

**Catecholamine Measurements**

L-Dihydroxyphenylalanine (L-DOPA), norepinephrine (NE) and dopamine (DA) in aliquots of the previously described tissue homogenates were adsorbed onto activated alumina, washed and extracted back into perchloric acid prior to separation by LCED on a C-18 reverse phase column. Dihydroxybenzylamine (DHBA) was used as an internal standard to estimate procedural losses. Catecholamines were eluted at a flow rate of 1.5 ml·min⁻¹ with a mobile phase consisting of two volumes of 0.2 M citric acid to one volume of 0.2 M Na₂HPO₄, containing 0.05 mM EDTA, 0.1 mM octanyl sulfonate and 1.5% methanol (pH 2.75). Eluted catecholamines were measured at a potential of 0.65 V versus an Ag/AgCl reference electrode. Subsequent analyses were performed as described previously for indolic measurements. Recovery averaged 89.5%. Intra-assay variation was ±5.1% for L-DOPA, ±5.2% for NE and ±4.6% for DA.

**Data Analysis**

Since the enzyme for decarboxylation of 5HTP to form 5HT has a low $K_m$ and high velocity relative to the hydroxylation of tryptophan to form 5HTP, the rate of 5HT synthesis may be considered equal to the function of 5HTP formed per unit of tissue per unit of time following decarboxylase inhibition with NSD-1015. Since 5HTP concentrations are normally immeasurable, accumulation of 5HTP at a single time point (i.e., 30 min after NSD-1015 injection) was used as an index for the rate of 5HT synthesis. Because L-DOPA serves as precursor both for NE and DA, this pharmacological technique does not provide specific rates of NE synthesis versus DA synthesis in the tissues studied. Therefore, the rate of L-DOPA accumulation is described only as an index for 'catecholamine' synthesis in this study.

The data are expressed as means±standard errors of those means. The data were analyzed initially by one-way analysis of variance among multiple means. This was followed by analysis of the statistical significance of differences between specific means using the Neuman-Keuls multiple range test (Zar, 1974).

**RESULTS**

**One Week**

One week after STZ-induction of diabetes no significant alterations in the estimated rates of 5HT synthesis in the MBH-ME or POA-AH regions were observed (Fig. 1). However, estimated rates of catecholamine synthesis were significantly reduced in the POA-AH but not MBH-ME (Fig. 2). Nor were serum levels of LH, FSH and PRL in these rats significantly different from those of control rats (Figs. 2 and 3).

**Four Weeks**

Four weeks after STZ-induction of diabetes, the estimated rate of 5HT synthesis in the POA-AH, but not in the MBH-ME, was significantly decreased (Fig. 1). No changes were seen in catecholamine synthesis in either MBH-ME or POA-AH (Fig. 2). Serum LH levels were significantly suppressed in this STZ-treated group of rats (Fig. 2). However, neither serum FSH nor PRL levels were changed in STZ-treated rats (Figs. 2 and 3).

**Sixteen Weeks**

Sixteen weeks after STZ-induction of dia-
Fig. 1 5HTP accumulation (ng per mg of hypothalamic tissue) after NSD-1015 administration as a relative index for the rate of 5HT synthesis in the POA-AH (preoptic area-anterior hypothalamus) and MBH-ME (mediobasal hypothalamus-median eminence) 1, 4 and 16 weeks after STZ (streptozotocin) (hatched bars) or non-STZ (solid bars) treatment. Half-brackets refer to the standard error of the mean. Sample number indicated in parentheses. N.S., nonsignificant.

Fig. 2 Catecholamine (NE and/or DA) synthesis in the POA-AH and MBH-ME, estimated by the accumulation of L-DOPA following NSD-1015 treatment. Measurements were made 1, 4 and 16 weeks after STZ (hatched bars) or non-STZ (solid bars) treatment. Half-brackets refer to the standard error of the mean. Sample number indicated in parentheses. N.S., nonsignificant.

betes, the estimated rates of 5HT synthesis in both MBH-ME and POA-AH were significantly reduced (Fig. 1). Catecholamine synthesis in the MBH-ME and POA-AH appeared to remain unaltered in these animals (Fig. 2). Serum LH levels were depressed in the diabetic rats (Fig. 2). Neither serum FSH nor PRL levels were significantly altered in the diabetic rats (Figs. 2 and 3).

**Semi-Starved Controls**

Non-diabetic rats with unrestricted diet demonstrated a +42.1% weight gain. Diabetic and semi-starved groups of rats exhibited -7.6% and -6.2% weight losses, respectively, after 4 weeks and -11.3% and -10.3% weight losses, respectively, after 16 weeks. However, neither hypothalamic 5HT synthesis nor serum gonadotropin levels were altered significantly after 4 or 16 weeks of total calorie-restriction diet. Semi-starved rats weight-matched to 16 week (but not 4 week) diabetic rats did show a slight but significant increase in serum PRL concentrations versus non-diabetic rats of normal weight (38.7±4.1 ng·ml⁻¹ vs. 26.5±4.2 ng·ml⁻¹; P<0.05).

**DISCUSSION**

Infertility is a common sequel to experimentally-induced diabetes in the male rat (7, 20-22, 25) as well as in the human male (1, 2, 6, 15, 16, 23, 24). Diabetes-associated reproductive dysfunction has been attributed both to alternations in hypothalamo-pituitary and pituitary gonadal activity. Typically, diabetes in the male rat is associated with a decrease in circulating LH (3, 8, 26) and testosterone (24, 26) levels which undoubtedly account for the
reported reduction in or absence of spermatogenesis (28, 31) and reduction in accessory sex organ weights (7, 9, 21).

A 5HT mechanism within the POA-AH has been implicated as a major regulatory factor in the control of LH secretion in the male rat (17). Our results demonstrate both decreased SHT synthesis in the POA-AH and decreased serum LH levels of the male rat 4 and 16 weeks after induction of diabetes by STZ. These observations not only add further support for the hypothesis that SHT plays a significant role in the central control of LH secretion but also suggest a mechanism for diabetes suppression of LH secretion in the male rat.

An earlier report (5) also found that STZ-induced diabetes led to a reduction in brain SHT synthesis in the male rat. An approximately 50% decrease in SHT synthesis was common to both their and our studies. However, our study found that SHT synthesis was inhibited in the POA-AH but not in the MBH-ME 4 weeks after STZ treatment, indicating region-specificity in the early hypothalamic response to the onset of experimentally-induced diabetes.

In the diabetic female rat, hypothalamic GnRH activity appears to be unaltered since hypothalamic levels of this peptide and veratrine-induced release of this peptide remained unchanged following STZ treatment to induce diabetes (29). Because LH secretion would not respond to the negative feedback effects of testosterone implants in diabetic male rats, Jackson and Hutson (14) suggested that diabetes inhibits the reproductive neuroendocrine axis. They were unable to determine whether the locus for the androgen non-responsiveness in these diabetic animals is the pituitary gland or hypothalamus. However, other investigators have reported that pituitary gonadotrophin secretion in STZ-induced diabetic rats responds normally to GnRH stimulation (8, 22) suggesting that diabetes adversely affects hypothalamic GnRH dis-
Our results may lend further support for this idea, indicating that diabetes can affect at least one neural mechanism involved in the control of GnRH release.

Interestingly, FSH levels remain unchanged both by hypothalamic 5HT lesioning (17) as well as STZ-diabetes, the latter as shown by our study. The lack of an effect of diabetes on FSH in contrast to LH secretion may not be surprising in consideration of the hypothesis of differential regulatory mechanisms governing the secretion of gonadotropins (4) and further emphasizes the pathophysiological selectivity of diabetes-induced male reproductive neuroendocrinopathy.

Stress is known to increase endogenous opioid activity (11). Increased opioid activity can then both stimulate PRL secretion (9) and inhibit LH secretion (5). However, recent studies have shown either normal or decreased circulating PRL levels in diabetic rats (32, 16). Although finding no changes in serum PRL levels, Yohev and colleagues (32) did observe decreased serum LH levels. Our study confirms those findings, namely that serum PRL levels remained unchanged throughout the course of our study while serum LH levels were decreased 4 and 16 weeks after diabetes induction. These findings tend to argue against the possibility that diabetes-induced stress could account for decreases in serum LH levels seen in the diabetic animal.

Diabetic rats fail to gain, and may even lose, body weight. Thus, many studies involving diabetes include semi-starved controls weight-matched to the diabetic animals. However, within limits, weight loss as a result of semistarvation does not seem to alter circulating or pituitary LH levels, hypothalamic GnRH levels (3, 9, 27), LH response to GnRH challenge (3), the postcastration LH response to androgen (9) or the response of hypothalamic GnRH to catecholaminergic stimulation (R. W. Steger, personal communication). We also found that semi-starved rats, weight-matched by total calorie-restriction diet to 4 and 16 week diabetic rats, exhibited no significant changes either in hypothalamic 5HT synthesis or serum gonadotropin levels. Accordingly, we do not believe that the change in hypothalamic 5HT synthesis and serum LH levels seen in diabetic animals is a general response to a catabolic state in these animals.

In summary, previous reports not only suggest a site of action for diabetes-induced reproductive neuroendocrinopathy but, with the results of our study, indicate a potential mechanism for such pathological interaction, namely, diabetic alteration of the function of at least one specific neurotransmitter system active in the regulation of hypothalamic GnRH activity. Although not discounting evidence for direct diabetes-induced changes in pituitary-gonadal activity, our study represents the first such evidence that diabetes may affect reproductive function through its antagonism of a specific neuroendocrine mechanism.

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