EFFECTS OF CHRONIC DIABETES ON *IN VITRO* GnRH RELEASE FROM THE HYPOTHALAMUS OF THE MALE RAT

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ABSTRACT

Infertility is a common sequela to chronic diabetes in animal models as well as in humans. Because the reduced or absent spermatogenesis typically seen in these males is thought to be due to disruption of the reproductive neuroendocrine axis, we sought to determine the effects of streptozotocin (STZ)-induced diabetes on *in vitro* hypothalamic gonadotropin releasing hormone (GnRH) release, relative to the effects of STZ-induced diabetes on circulating LH and FSH levels. Young adult male (Sprague-Dawley) rats were injected intraperitoneally either with 65 mg/kg of STZ or with vehicle only. Diabetic rats were hyperglycemic throughout the following 8 month period [478.7 ± 67.5 mg/dl in diabetics ($n=24$) vs. 122.9 ± 30.7 mg/dl in controls ($n=24$)]. At the end of the 8 months, serum LH and FSH levels were decreased in diabetics vs. controls. Hypothalamic slices were collected and perfused with a modified Krebs buffer (pH 7.2) using a programmable perfusion system. In each of the three perfusion studies conducted, basal GnRH release in the diabetic groups ($n=6$) was comparable to that of controls. In the first of these *in vitro* studies, 5 min pulses of 60 mM KCl induced significant increases in GnRH release in control tissues. However, GnRH release from the diabetic tissue groups was consistently attenuated at each of the three KCl pulses. In the second and third *in vitro* studies, 10 min pulses either of 0.1 μ M norepinephrine (NE) or 0.5 μ M phorbol 12,13-dibutyrate (PDBu) significantly increased GnRH release in control groups ($n=6$). Response to the initial pulse either of NE or PDBu was significantly blunted in the diabetic groups ($n=6$). Control and diabetic group responses to the second of two pulses either of NE or PDBu were not significantly different. These results suggest that decreased circulating gonadotropin levels and reduced reproductive function in diabetics is, at least in part, a result of reduced responsiveness of the GnRH neuron to stimulatory factors. In addition to detrimental effects on hypothalamic neurotransmitters controlling pulsatile GnRH release, chronic diabetes may exert direct negative effects on intracellular regulatory mechanisms governing GnRH release, especially those related to activation of protein kinase C.

Secretion of the decapeptide gonadotropin-releasing hormone (GnRH) from specialized hypothalamic neurons into the pituitary portal plexus regulates LH and FSH secretion

from gonadotrophs found in the anterior lobe of the pituitary gland. Pulse frequency, and possibly, amplitude of GnRH release closely regulate secretory patterns of the two gonado-

tropins, these patterns in turn being responsible for normal male reproductive functions (12). On the other hand, chronic diabetes mellitus is commonly associated with infertility attributed both to alterations in hypothalamo-pituitary (4, 5, 11, 13) as well as pituitary-gonadal activity (17, 18). Typically, circulating LH and testosterone levels are decreased which undoubtedly account for the reduction in or absence of spermatogenesis in males (11, 17, 18).

Previously, we have shown that experimentally-induced diabetes of up to 16 weeks duration in the male rat is associated with deficiencies in the activities of several hypothalamic neurotransmitters directly involved in the regulation of pulsatile GnRH release (13). Circulating LH (but not FSH) levels in these diabetic rats were also significantly diminished. It has been suggested by several groups that diabetes adversely affects the hypothalamo-pituitary axis, presumably by altering normal secretory patterns of hypothalamic GnRH (4, 11, 13). Accordingly, we sought to examine the effects of long-term diabetes on *in vitro* hypothalamic GnRH release stimulated non-selectively by high KCl. Because norepinephrine (NE) represents a necessary component of the central mechanism regulating GnRH release (12), we also sought to examine the effects of long-term diabetes on *in vitro* NE-stimulated GnRH release.

Diabetes-related peripheral neuropathy is associated with decreased turnover of polyphosphoinositides and reduced protein phosphorylation (3, 16). Polyphosphoinositide turnover results in the formation of inositol phosphates important in Ca^{2+} flux and diacylglycerol (DAG) which, in conjunction with phospholipids and Ca^{2+} , increases protein kinase C activity (19). While representing a known and important regulatory component of CNS neuronal secretory processes (19), it is not known whether CNS, as well as peripheral nerve, polyphosphoinositide turnover is decreased in the diabetic individual. Because of recent evidence suggesting that protein kinase C plays a role in the intracellular regulation of hypothalamic GnRH release (22), we also included studies to examine the effects of long-term diabetes on *in vitro* GnRH release stimulated by a protein kinase C agonist, phorbol 12,13-dibutyrate (PDBu).

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (160–180 g at the start of the studies) were obtained from Charles River Laboratories (Wilmington, MA). The rats were housed in clear-plastic cages with free access to food (Wayne Lab-Blox) and tap water. They were kept on a light/dark cycle consisting of 14 h : 10 h L : D (lights on: 0600 daily). Within days of their arrival at this institution, the rats were divided into two groups. Both groups were taken off food, but not water, overnight. The following morning, one group was injected intraperitoneally with 65 mg/kg of streptozotocin (Sigma Chemical, St. Louis, MO). Streptozotocin was prepared immediately prior to injection using cold citrate buffer (pH 4.5) as the diluent. The other group was injected intraperitoneally with diluent only. Three days later, the streptozotocin-treated group was hyperglycemic with blood glucose levels well in excess of 300 mg/dl and exhibiting polyuria. Only streptozotocin-treated rats which remained hyperglycemic throughout the next 8 months were used (approximately 69% of those injected). Both control and diabetic rats were maintained on normal diets for the 8 month study period.

At the end of the 8 months, the rats were decapitated between 1300–1330. The brain of each rat was quickly removed. Sagittal slices of the hypothalamus approximately 0.4 mm thick and consisting of the preoptic area and mediobasal areas (POA-MBH) were quickly placed in a 0.1 ml microchamber. A modified Krebs medium was perfused through each of these tissue chambers at a flow rate of 6.12 ml/h via a programmable perfusion system (Endotronics, Coon Rapids, MN). The medium consisted of 118 mM NaCl, 11 mM glucose, 5.4 mM KCl, 25 mM NaHCO_3 , 1 mM NaH_2PO_4 , 1.2 mM MgCl_2 , 0.004 mM EDTA, 2.5 mM CaCl_2 and 0.11 nM Bacitracin. Microchambers were maintained at 37°C by an automatically controlled, circulating water bath. Media were bubbled with 95% air: 5% CO_2 which provided gas saturations of 180–210 mmHg (pO_2) and 28–46 mmHg (pCO_2), respectively. A preincubation perfusion of media only was maintained for 40 min to allow the tissue slices to equilibrate to the *in vitro* conditions. In the first study of stimulat-

ed GnRH release, POA-MBH slices were exposed to three 5 min pulses of 60 mM KCl over a 100 min incubation period (*i.e.*, 5 min on; 25 min off). In the second and third studies, the tissue slices were exposed to either to two 10 min pulses of 0.1 μ M NE or 0.5 μ M PDBu, respectively, over a 95 min incubation period (*i.e.*, 10 min on; 20 min off). In each study, the pulses were controlled automatically, assuring consistent periods and pulsed substance durations. Samples of perfusion effluent were collected at 5 min intervals during the incubation period. Samples were 'snap-frozen' in an acetone-dry ice bath and stored at -70°C until assayed for GnRH.

Gonadotropin Radioimmunoassays

Measurement of serum LH concentrations was done in duplicate using a rabbit anti-ovine LH antibody (No. 15; from Dr G. Niswender, Colorado State University, Ft. Collins, CO) at a final dilution of 1/120,000. Purified ovine LH was iodinated with I^{125} to a specific activity of 304 $\mu\text{Ci}/\text{mg}$ according to the method of Hunter and Greenwood (9). The standard was rat LH (NIDDK-LH-RP1) provided by Dr A. Parlow (NIDDK). Serum samples were diluted to volume of 300 μl with 0.5% BSA in 0.05 M phosphate buffer. One hundred microliters of primary antibody were added and the samples were then incubated overnight at 4°C . One hundred microliters of labelled LH were added to each sample and incubated overnight. Five hundred microliters of a suspension of iron-containing polymer beads coated with donkey anti-rabbit γ -globulin (1:3 dilution) was added to each sample and vortexed for 5 min. The incubation was continued for another hour at room temperature. The samples were then placed on a magnetic base for 15 min, separating bound from free hormone (Amerlex-M Magnetic Particle Separations, System: Amersham Corporation, Arlington Heights, IL). Supernatants were discarded and the radioactivity in the magnetically-retrieved pellet was measured in a gamma counter. Maximum binding and nonspecific binding were 26.5% and 1.8%, respectively. Assay sensitivity was 0.87 ng per tube. Intra- and interassay coefficients of variation were 5.2% and 7.1%, respectively.

Serum FSH levels were determined using a

specific hFSH antiserum provided by Dr Parlow (NIDDK) at a final dilution of 1/25,000. Purified rat FSH was iodinated with I^{125} to a specific activity of 365 $\mu\text{Ci}/\text{mg}$ again according to the method of Hunter and Greenwood (9). The standard was rat FSH (NIDDK-FSH-RP1), again provided by Dr Parlow (NIDDK). Serum samples were diluted to a total volume of 300 μl with 0.5% BSA in 0.05 M phosphate buffer, to which was added 100 μl of primary antibody. The samples were then preincubated for 48 h at 4°C . One hundred μl of iodinated FSH was then added and the incubation continued for an additional 72 h at 4°C . At that time, 500 μl of a suspension of iron-containing polymer beads coated with donkey anti-rabbit γ -globulin (1:2 dilution) was added to each sample. Bound and free hormone were separated using the Amerlex-M Magnetic Separations System as described above. After discarding supernatants, radioactivity was measured in a gamma counter. Maximum binding and nonspecific binding were 35.0% and 1.7%, respectively. Assay sensitivity was 28.9 ng per tube. Intra- and interassay coefficients of variation were 4.4% and 5.2%, respectively.

GnRH Radioimmunoassay

Sample aliquots were incubated for 48 h at 4°C with antiserum (EL-14 antibody) at a final dilution of 1/750,000 (6). Monoiodinated ^{125}I -GnRH, prepared by the chloramine T method, was then added for an additional 48 h at 4°C . Bound and free hormone were separated by addition of ice-cold ethanol followed by centrifugation at 4,000 g for 25 min. Supernatants were decanted and radioactivity measured in a gamma counter. Sensitivity of the RIA was 0.6 pg per tube. Intraassay coefficients of variation were between 5–7%, and interassay coefficients of variation less than 10%, for a 2 pg concentration of synthetic GnRH. Neither 60 mM KCl nor 0.5 μ M PDBu interfered with the assay. Data are expressed as pg of GnRH per mg of hypothalamic tissue slices (wet weight) released into each 5 min media fraction.

Data Analysis

All data are expressed as mean $\pm$ standard error.

Fig. 1 Serum LH (left panel) and FSH (right panel) concentrations from trunk blood samples in control rats (n=9) and long-term diabetic rats (n=8). Diabetes was induced by intraperitoneal injection of 65 mg/kg of streptozotocin (STZ). Control (non-STZ injected) and diabetic rats were sacrificed 8 months later. Half-brackets denote the standard error of the mean.

Fig. 2 Effect of 60 mM KCl pulses (5 min on; 25 min off) on *in vitro* GnRH release from hypothalamic slices of normal (open circles; n=6) and long-term diabetic (closed circles; n= 6) males. The 40 min preincubation period is not shown. Half-brackets denote the standard error of the mean. * $P < 0.03$ vs. non-diabetic group at that time point; ** $P < 0.06$ vs. non-diabetic group at that time point

ror of each mean. Differences in serum gonadotropin levels in control *versus* diabetic groups were analyzed by Student's *t* test. Stimulated release of GnRH and amines from control *versus* diabetic groups was analyzed using the nonparametric Wilcoxon test for paired samples.

RESULTS

Throughout the 8 month period of study, diabetic rats remained hyperglycemic [478.7±

Fig. 3 Effect of 0.1 μM norepinephrine (NE) pulses (10 min on; 20 min off) on *in vitro* GnRH release from hypothalamic slices of normal (open circles; n=6) and long-term diabetic (closed circles; n=6) males. The 40 min pre-incubation period is not shown. Half-brackets indicate the standard error of the mean. * $P < 0.03$ vs. non-diabetic at that time point

Fig. 4 Effect of 0.5 μM phorbol 12,13-dibutyrate (PDBu) pulses (10 min on; 20 min off) on *in vitro* GnRH release from hypothalamic slices of normal (open circles; n=6) and long-term diabetic (closed circles; n=6) males. The 40 min pre-incubation period is not shown. Half-brackets denote the standard error of the mean. * $P < 0.03$ vs. non-diabetic at that time point; ** $P < 0.06$ vs. non-diabetic group at that time point

67.5 mg/dl (n=18) vs. 122.9±30.7 mg/dl for control rats (n=18); measured at the end of the study]. Body weights were not significantly different between non-diabetic rats (587.5±29.2 g) and diabetic rats (531.7±32.6 g) measured at the end of the study period. Approximately 70% of the diabetic rats exhibited well-developed, bilateral cataracts. Serum LH and FSH levels in diabetic rats were significantly reduced (Fig. 1).

Data for KCl-induced *in vitro* GnRH release are shown in Fig. 2. Basal GnRH release in the

diabetic group was not significantly different from that in the control group. Pulses of 60 mM KCl increased GnRH release in both control ($n=6$) and diabetic groups ($n=6$); however, KCl-induced GnRH release was significantly attenuated in the diabetic group. This diabetes-associated attenuation of GnRH release was consistent among three successive KCl pulses.

While 10 min pulses either of 0.1 μ M NE (Fig. 3) or 0.5 μ M PDBu (Fig. 4) increased GnRH release in the control group ($n=6$), response to the initial pulse of either GnRH secretagogue was significantly diminished in the diabetic groups ($n=6$ for both). Response to the second pulse either of NE or PDBu was not significantly different in non-diabetic *versus* diabetic groups. Basal GnRH release in the diabetic groups was not significantly different from that seen in control groups.

DISCUSSION

Our studies suggest that hypothalamic GnRH release is inhibited in the chronically diabetic animal. We have shown previously that selected hypothalamic neurotransmitter activities are decreased in diabetic male rats which could explain, in part, the decreases in circulating gonadotropin levels which we (13) and others (4, 5, 11, 17, 18) have seen in these animals. However, our present results showing that either high KCl or noradrenergic stimulated *in vitro* GnRH release from the hypothalamus of chronically diabetic rats were also attenuated would suggest that this disease in some way directly affects the GnRH neuron. Our studies further demonstrate that diabetes is associated with inhibition of phorbol ester stimulated GnRH release *in vitro*, suggesting that the GnRH regulating activity of protein kinase C or related intracellular event has been altered in the diabetic male rat. The detrimental effects of long-term diabetes on circulating gonadotropin levels (and thus normal reproductive function) may be a result of the altered GnRH release. However, our studies do not rule out the possibility that diabetes may also directly affect the activity of pituitary gonadotrophs.

Diabetes is often associated with reproductive neuroendocrinopathy. Sexual impotence (10), decreased libido (1) and impaired sper-

matogenesis leading to infertility (1, 2, 10, 14, 15, 21) are commonly seen with the clinical onset of diabetes in the human male. 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 diabetic men (20). Infertility is also a common sequela of chemically induced (8, 18) and genetic (17, 23) animal models of diabetes. Diabetes-associated reproductive dysfunction has been attributed both to alterations in hypothalamo-pituitary activity (5, 11, 13) and to alterations in pituitary-gonadal activity (17, 18, 20, 23). Common to most studies of experimentally-induced diabetes in the male rat is a decrease in circulating LH (4, 5, 8, 11, 13, 18) and testosterone (11, 18) levels which would account for the reported reduction in or absence of spermatogenesis (23) and reduction in accessory sex organ weights (8). The reductions in serum LH and testosterone could be restored to normal, or near normal, levels by appropriate insulin administration (11).

If diabetes-related reproductive dysfunction were solely a problem of Leydig cell failure with resultant low serum testosterone levels, then the characteristic 'postcastration rise in serum gonadotropins' should become evident in such diabetics. We (13) and others (4, 8, 17, 18) have seen, instead, a consistent decrease in serum LH levels after up to several months of diabetes and, in the present studies, a consistent decrease in both serum LH and FSH levels after 8 months of diabetes. Further, the present studies suggest that diabetes is associated with impaired hypothalamic GnRH secretion. Bestetti and colleagues (4) have demonstrated GnRH axonal degeneration in the arcuate nucleus and median eminence of the diabetic male rat. This degeneration was not related to undernourishment in the diabetic rats. Again, while not ruling out possible deleterious effects of diabetes on pituitary or gonadal functions, these studies emphasize that hypothalamic (*i.e.*, GnRH) impairment is a major factor in diabetes-related reproductive dysfunctions.

Our results suggest that chronic diabetes adversely affects pulsatile GnRH release, and thereby normal reproductive activities, at several regulatory levels. These levels include

neuronal systems regulating or modulating changes in GnRH release. In fact, we have shown previously that aminergic turnover in the hypothalamus, critical to normal patterns of GnRH release, is diminished significantly in the diabetic animal (13). Our present results (*i.e.*, using nonselective KCl stimulation and selective NE stimulation) suggest further that the GnRH neuron itself is also adversely affected in the long-term diabetic animal.

It has been reported that peripheral neuropathy is associated with reduced turnover of polyphosphoinositides and diminished protein phosphorylation in both genetic (db/db) and chemically (streptozotocin) induced animal models for diabetes (3, 16). Based on studies involving the development of diabetes-related peripheral neuropathy in db/db mice, Berti-Mattera and Eichberg (3) suggested that such changes were a consequence of hyperglycemia rather than abnormal insulin levels. In addition to reduced formation of inositol phosphates with associated changes in Ca^{2+} flux, changes in polyphosphoinositol metabolism would also presumably lead to reduced diacylglycerol formation and protein kinase C activity. Protein kinase C, a Ca^{2+} -activated, phospholipid-dependent enzyme found throughout most of the CNS is essential to the secretion of numerous hormones and neurotransmitters (19). Valenca and colleagues (22) recently demonstrated that *in vitro* hypothalamic GnRH release could be stimulated by a phorbol ester (*i.e.*, PDBu). This suggests that protein kinase C plays a role in the intracellular regulation of hypothalamic GnRH release. Our results not only confirm their observations that phorbol esters stimulate *in vitro* hypothalamic GnRH release but also show that chronic diabetes suppresses PDBu-induced GnRH release. This would then imply that long-term diabetes adversely affects protein kinase C activation or some, as yet undefined, intracellular event 'downstream' from protein kinase C activation important to hypothalamic GnRH release.

In summary, our results demonstrate that reduced serum gonadotropin levels and diminished reproductive function in the chronically diabetic male is, at least in part, a result of altered hypothalamic GnRH release. Altered GnRH release in such males may be a consequence of changes in neurotransmitters

(*e.g.*, NE) regulating GnRH release, of disrupted intraneuronal regulation (*e.g.*, protein kinase C) of GnRH release or a combination of such factors. Future studies will attempt to determine more precisely both the role of protein kinase C in regulating GnRH release as well as the effect(s) of diabetes to alter that regulatory mechanism.

The excellent technical assistance of Diana Frasier throughout these studies is gratefully acknowledged. This research was supported by a grant from the American Diabetes Association and NIH grant HD 10202 (Neuroendocrine Core).

Received for publication 10 July 1989

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