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Streptozotocin Diabetes-Induced Hyperadrenal Corticoidism: Central Mechanisms

Abstract

Streptozotocin (STZ)-induced diabetic rats were used to evaluate possible central mechanisms responsible for the chronic hyperadrenalcorticoidism present in diabetics. To achieve this goal, both basal levels and K+-stimulated release of the adrenocorticotropin (ACTH) secretagogues, corticotropin releasing factor (CRF) and arginine vasopressin (AVP), were measured from mediobasal hypothalamus (MBH) or median eminence (ME) tissues, respectively, obtained from diabetic or control animals. MBH epinephrine (EPI) concentrations were measured to evaluate whether changes in CRF might be mediated through central EPI neurons. The ability of insulin replacement therapy to reverse any diabetic-related changes was also evaluated. STZ caused a severe state of diabetes as evidenced by large increases in daily water intake, decreased body weight and increased blood glucose levels compared to vehicle-treated control animals. The concentration of CRF, but not AVP or EPI, was decreased in diabetic animals. Although the basal release of CRF or AVP was not altered by diabetes, the K+-stimulated release of CRF was enhanced in diabetic animals compared to that seen in vehicle treated animals. The K+-stimulated release of AVP from ME tissues was similar in both STZ- and vehicle-treated animals. Insulin replacement therapy successfully reversed the polydipsia and hyperglycemia resulting from STZ injection, and completely reversed the diabetes-induced changes in CRF content and release. The data provide evidence that diabetes results in a selective hypersecretion of central CRF in response to normal stimulatory inputs which may be responsible, in part, for the hyperadrenalcorticoidism observed in diabetics.

Key words: Corticotropin Releasing Factor, Arginine Vasopressin, Epinephrine, Streptozotocin, Adrenocorticotropin, Insulin Replacement

Introduction

Rats with streptozotocin-induced diabetes demonstrate a hyperfunction of the pituitary-adrenal axis including a hypersecretion of adrenocorticotropin (ACTH) from the pituitary, adrenal hypertrophy and an elevated mean plasma corticosterone concentration (L'Age et al. 1974, DeNicola et al. 1976, Rhees et al. 1983). Hyperadrenal-corticoidism can affect nearly every system in the body; resulting in weight gain, hypertension, impaired glucose tolerance (which would exacerbate the diabetes already present), polyuria, osteoporosis, hyperpigmentation, increased susceptibility to infection, depression and reproductive dysfunctions like oligomenorrhea or amenorrhea in women and decreased libido and possibly impotence in men (Luckman and Sorensen1980). Despite the general acceptance that a hyperfunction of the pituitary-adrenal axis exists in the diabetic, the central and pituitary mechanisms of action responsible for the altered secretion of ACTH and the functional status of central neurotransmitter/neuropeptide factors governing the secretion of ACTH in the diabetic rat are not fully

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elucidated. The major ACTH secretagogues involved in that neuroendocrine regulation are the neuropeptides corticotropin releasing factor (CRF) and arginine vasopressin (AVP) (Negro-Vilar et al. 1987). The present studies examined changes in central CRF and AVP neurons associated with diabetes induction with streptozotocin to determine the site(s) of action and central mechanisms responsible for the diabetes-induced hyperadrenalcorticoidism. Furthermore, because of the recently described influence of central epinephrine (EPI) on CRF neurons (Spinedi et al. 1988), the effect of diabetes on central concentration of EPI were also examined. Finally, the ability of insulin replacement therapy to reverse the alterations associated with diabetes induction was also examined.

Methods and Materials

Animals and Diabetes Induction

Adult male Sprague-Dawley rats (280-320 g BW; LARC, Pullman, WA) were housed in groups of four until injected with streptozotocin (STZ, 65 mg/kg BW, Calbiochem-Behring, San Diego, CA) or vehicle (O.1 M citrate buffer pH 4.5, 0.3 ml) via the right external jugular vein exposed under ether anesthesia. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and protocols were approved by the Institutional Animal Care and Use Committee. STZ was made fresh just prior to injection. Following intravenous injections, animals were housed individually in temperature - (22 ± 1° C) and light - (12 hr light : 12 hr dark; lights on from 700 - 1900 hr) controlled rooms and were supplied food and water *ad libitum*. Day of intravenous injection was designated Day 0. Water intake and body weight were monitored daily to confirm diabetes induction (development of polydipsia and weight loss); and blood glucose levels were determined using a glucometer (One Touch II, Lifescan) on Day 3 and Day 18 to assess the influence of STZ or vehicle on blood glucose levels.

Content studies - Rats were decapitated 21 days following STZ or vehicle injection, their brains were rapidly removed and mediobasal hypothalamus (MBH) was dissected. For the dissection, the posterior border of the optic chiasma, anterior border of the mammillary bodies and lateral borders of the hypothalamus were used; and a depth of approximately 2 mm was chosen. In content studies, MBH tissues were homogenized in 300 µl of 0.2 N HCL, boiled for 10 min and centrifuged at 10,000 g for 8 min at 4° C. Supernatants were lyophilized and frozen at -80° C until analyzed by radioimmunoassay for CRF and AVP content. Pellets were dissolved in 1 N NaOH and protein content was determined by the method of Lowry et al. (1951).

In vitro release studies - Rats were decapitated 21 days following STZ or vehicle injection, their brains were rapidly removed and the ME or MBH was dissected. Tissues were placed in Krebs-Ringer bicarbonate buffer (KRBG), pH 7.4, containing l mg/ml glucose, 10 µg/ml ascorbic acid and a protease inhibitor cocktail consisting of 4-2-aminoethylbenzenesulfonylfluride (0.2 mM), aprotinin (1 µg/ml), benzamidine (1 mM), EDTA (1 mM), leupeptin (10 µg/ml) and pepstatin (10 µg/ml). Two ME or MBH tissues were transferred to each plastic flask containing 1.0 ml KRBG (pregassed for 20 min with 95% O_2 - 5% CO_2) and preincubated by shaking for 15 min at 37°C under a 95% O_2 - 5% CO_2 atmosphere. The time of 15 min for preincubation was determined empirically as the minimum time required for the 5 min samples to reach a stable, non-fluctuating baseline

secretion. At the end of the preincubation, the medium was decanted and the tissues were resuspended in 1.0 ml KRBG and incubated for six 5 min periods as described above (basal secretion incubation). The tissues were then incubated for another six 5 min periods under the same conditions except for the addition of 1.0 ml 28 mM K+ - KRBG solution. At the end of each incubation, media was decanted and frozen at -80° C until AVP and CRF content could be measured by radioimmunoassay.

Epinephrine assay - MBH tissues obtained at Day 21 from STZ - or vehicle-treated rats, were homogenized directly into 0.5 ml of mobile phase [0.05 M NaH_2PO_4, pH 2.8, 8% methanol (v/v), 0.032% sodium octyl sulfate (w/v) and 0.1 mM EDTA] used for HPLC analysis. Homogenates were then centrifuged at 10,000 g for 8 min and the resulting supernatants were frozen at - 80° C until analyzed for EPI content as previously described (Spinedi et al. 1988) by HPLC with electrochemical detection. During analysis, 0.2 ml of the supernatants was injected directly onto the HPLC column. An Environmental Sciences Associates HPLC system (Bedford, MA) fitted with a 5100 A coulometric electrochemical detector, a 5011 high sensitivity analytical cell, A 5020 guard cell and a biophase ODS 5 µM reverse phase analytical column (Bioanalytical Systems, West Lafayette, IN) was utilized with the working electrode set at +0.45 V, guard cell at +0.50 V and the dummy cell at +0.02 V relative to a platinum reference electrode at a flow rate of 2.0 ml/min. The sensitivity of the assay for EPI was 15 pg. Extraction efficiency for EPI was determined to be 88% using duplicate samples spiked with known quantities of EPI. All values in the figures have been corrected accordingly.

Radioimmunoassays - AVP and CRF content were analyzed by specific radioimmunoassays as previously described (Negro-Vilar et al. 1979; Spinedi and Rodriguez 1986;). All samples from each experiment were assayed within the same RIA. Intra-assay coefficients of variation were 8% and 10%, respectively, with a limit of sensitivity of 0.8 pg/tube and 12.0 pg/tube, respectively.

Insulin replacement therapy - Rats on replacement therapy were administered insulin (0.6 ml regular plus 0.3 NPH Humulin U-100 insulin, S.C.) once daily at 800 hr starting 3 days after STZ injection. This dose was empirically determined in groups of diabetic rats by trial and error as the dose of insulin which maintained blood glucose levels under 120 mg/dl throughout the 24 hr period (evaluated from glucose determinations at 1800 hrs and 0700 hrs each day).

Statistical analysis - Data were analyzed by Student's t-test or by analysis of variance with Neuman-Keuls posthoc test, depending on the experimental design (Steel and Torrie 1960). The 0.05 level of probability was used as the minimum criterion of significance in all experiments.

Results

Blood Glucose and Daily Water Intake

The administration of STZ caused a severe state of diabetes compared to vehicle-treated control animals. This was evidenced by the rapid development of large increases in daily water intake and blood glucose levels (Table 1) within 3 days after the STZ injection, which remained high throughout the experimental period. In fact, the blood glucose values continued to increase from day 3 to day 18, whereas the severe polydipsia remained stable at a high level throughout this period. These increases were reversed by insulin replacement therapy (Table 1).

Table 1. *Effect of Streptozotocin-induced Diabetes with or without Insulin Replacement Therapy on Daily Water Intake and Blood Glucose*

Treatment Group	Control	Diabetic	Replacement
Water Intake (ml/day)	42 ± 2	179 ± 11*	86 ± 5*
Blood Glucose (mmol/L)	6.8 ± 0.4	25.4 ± 1.6*	5.8 ± 0.2

Values represent MEAN ± SEM as determined from n = 8 animals 18 days after diabetes induction with STZ injection (65 mg/kg,iv) or vehicle injection (control group). Replacement animals received daily insulin replacement therapy starting on day 3 following STZ injection.
* Significantly different from all other treatment groups (P < 0.01).
' Significantly different from vehicle-treated control group (P < 0.01).

Central Concentrations of CRF, AVP and EPI

The development of diabetes was accompanied by a decrease in the central concentration of the primary central ACTH secretagogue, CRF, which was also successfully reversed by insulin replacement therapy (Fig. 1). The diabetes-induced decrease in central CRF was apparently selective as the concentration of another major, physiologically relevant, ACTH secretagogue, AVP (Fig. 2), or of central EPI (Fig. 3) were not altered 21 days following the induction of diabetes. The content changes were not due to alterations in protein content within the MBH tissues as these were similar in all cases.

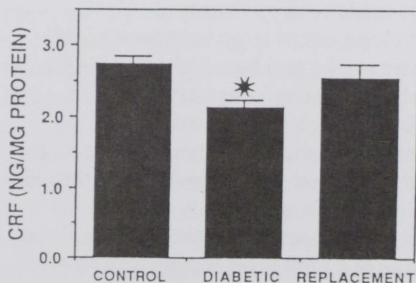

Figure 1. *Effect of streptozotocin-induced diabetes (65 mg/kg BW, iv) with (replacement group) or without (diabetic group) insulin therapy (daily injection of 0.6 ml regular plus 0.3 ml NPH Humulin U-100 insulin, s.c.) or of vehicle injection (0.3 ml citrate buffer, pH 4.5; control group) on the concentration of CRF in MBH samples three weeks after the iv injections. Column heights represent the mean CRF concentration and vertical lines represent the SEM as determined from n = 8 animals. *, significantly different from control values (P < 0.01).*

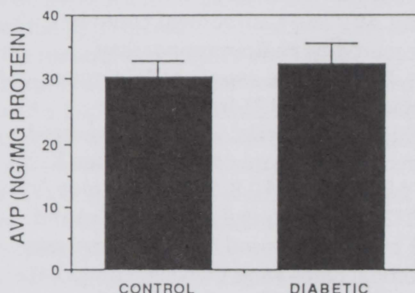

Figure 2. *Effect of streptozotocin (65 mg/kg BW, iv; diabetic group) or vehicle (0.3 ml citrate buffer, pH 4.5, iv; control group) on the concentration of AVP in MBH samples three weeks after the iv injections. Column heights represent the mean AVP content and vertical lines represent the SEM as determined from n = 8 animals.*

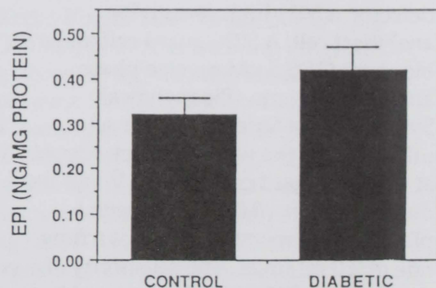

Figure 3. *Effect of streptozotocin (65 mg/kg BW, iv; diabetic group) or vehicle (0.3 ml citrate buffer, pH 4.5, iv; control group) on the concentration of epinephrine in MBH samples three weeks after the iv injections. Column heights represent the mean epinephrine concentration and vertical lines represent the SEM as determined from n = 8 animals.*

These results provide evidence that diabetes induced by STZ injection causes a selective alteration in central CRF neurons without significantly affecting central AVP or EPI neurons in the MBH. The observed differential effect of diabetes on central CRF and AVP neurons prompted the investigation of the effects of STZ-induced diabetes on AVP and CRF release from the MBH.

In Vitro AVP and CRF Release

Diabetes induction with STZ resulted in no statistically significant alteration in the basal release (solid bars) of AVP (Fig. 4) or CRF (Fig. 5) during a 5 min incubation in KRBG. The addition of 28 mM K+ (hatched bars) to the KRBG medium depolarized the nerve terminals and caused a significant increase in the release of both AVP (Fig. 4) and CRF (Fig. 5) from ME and MBH tissues, respectively. However, the K+-induced release of CRF, but not AVP, was much greater from MBH tissues obtained from diabetic animals than it was from tissues obtained from vehicle-treated animals. Insulin replacement therapy also successfully reversed the STZ-induced changes in K+-stimulated release of CRF from MBH tissues (Fig. 5; replacement groups). The results suggest that STZ-induced diabetes selectively causes alterations in the content and stimulated release of CRF from central neurons. Experiments performed with longer incubation times (30 min) provided similar results.

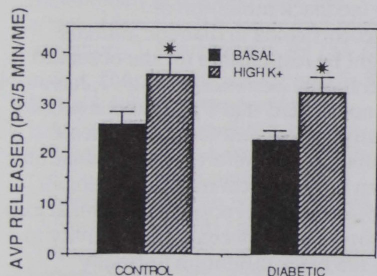

Figure 4. *Effect of streptozotocin (65 mg/kg BW, iv; diabetic group) or vehicle (0.3 ml citrate buffer, pH 4.5, iv; control group) on the in vitro release of AVP from ME tissues during a 5 min exposure to KRBG buffer (basal incubation, solid columns) or KRBG containing 28 mM K+ (high K+ incubation, hatched columns). Height of columns represents mean pg AVP released into the medium during each of six 5 min incubations per MBH tissue and vertical lines represent SEM as determined from "pooled" samples from n = 16 animals. *, significantly different from basal value in the same treatment group (P < 0.01).*

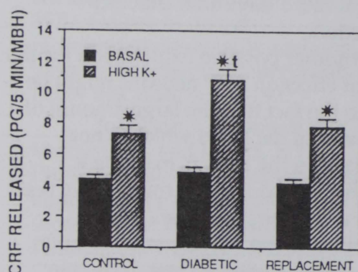

Figure 5. *Effect of streptozotocin-induced diabetes (65 mg/kg BW, iv) with (replacement group) or without (diabetic group) insulin therapy (daily injection of 0.6 ml regular plus 0.3 ml NPH Humulin U-100 insulin, S.C.) or of vehicle injection (0.3 ml citrate buffer, pH 4.5, control group) on the in vitro release of CRF from MBH tissues during a 30 min exposure to KRBG buffer (basal incubation, solid columns) or KRBG containing 28 mM K+ (high K+ incubation, hatched columns). Height of columns represents mean pg CRF released into the medium during the 30 min incubation per MBH tissue and vertical lines represent SEM as determined from n = 16 animals. *, significantly different from basal value in the same treatment group (P < 0.01). t, significantly different from control value from the same incubation (P < 0.01).*

Discussion and Conclusions

Unexpectedly, no significant changes in AVP content or release were detected despite the presence of severe hyperglycemia and large increases in water intake. Because the AVP parameters were examined 21 days following diabetes induction, it is possible that significant changes in AVP neuronal content or release may have occurred at earlier times following diabetes induction, but had normalized by 3 weeks. This possibility is less likely in light of the recent results of Scribner et al. (1991), which demonstrated similar ACTH and corticosterone responses to AVP injection in STZ-diabetic and control rats 5 days after diabetes induction.

Another possible explanation for the lack of effect on AVP neurons might be due to the fact that the large amount of AVP within the MBH which is not localized in the PVN is masking a significant effect which might have been observed if the PVN, itself, was examined alone. An examination of AVP changes within several discrete hypothalamic nuclei is now underway to evaluate this possibility. Furthermore, diabetes-induced changes in AVP release from the ME were also not observed in the present studies. In contrast, changes in CRF content and release were observed at 21 days, providing more evidence for a differential effect on central ACTH secretagogues. That the effects are due to alterations resulting from the absence of insulin is supported by the fact that insulin replacement therapy reversed all of the observed alterations.

Recent evidence from our laboratory (Johnston et al. 1989) further supports the hypothesis that STZ-induced diabetes selectively alters the release of CRF. The *in vivo* ACTH response to the injection of 0.5 μg CRF is altered 21 days following diabetes induction whereas the ACTH response to AVP or a higher dose of CRF is unaltered. At 5 days after STZ treatment the ACTH response to 0.5 μg CRF is also not altered (Scribner et al. 1991). Finally, in studies we have recently completed with Drs. Nemeroff and Owen (Emory University, Atlanta, Georgia), CRF receptors in the anterior pituitary are drastically reduced in diabetic animals (unpublished observations), which could occur in response to a chronic hypersecretion of CRF with consequent down-regulation of CRF receptors and/or glucocorticoid effects (Wynn et al. 1985).

What is currently not understood is how a chronic hypersecretion of CRF release from the MBH can be maintained in the face of hyperadrenalcorticoidism. An increase in corticosterone usually results in a rapid feedback inhibition of CRF and ACTH release (Sayers and Portanova 1974, Plotsky and Vale 1984, Levin et al. 1988). The possibility exists that changes in sensitivity of the hypothalamic-pituitary-adrenal axis to the feedback inhibition of glucocorticoids in diabetic animals might be responsible for the observed alterations. Scribner et al. 1991, have demonstrated that STZ-diabetic rats are relatively resistant to glucocorticoid inhibition. Therefore, the combination of an increased central stimulation of hypothalamic-hypophyseal-adrenal axis in the face of a decreased sensitivity to the feedback inhibition normally produced by glucocorticoids might explain the results obtained to date using this animal model. Full verification of this hypothesis requires further experimentation.

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