Original Article

Expression of Hypothalamic KiSS-1 System and Rescue of Defective Gonadotropic Responses by Kisspeptin in Streptozotocin-Induced Diabetic Male Rats

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Hypogonadotropism is a common feature of uncontrolled diabetes, for which the ultimate mechanism remains to be elucidated. Kisspeptins, ligands of G protein–coupled receptor 54 (GPR54) encoded by the KiSS-1 gene, have recently emerged as major gatekeepers of the gonadotropic axis. Alteration in the hypothalamic KiSS-1 system has been reported in adverse metabolic conditions linked to suppressed gonadotropins, such as undernutrition. However, its potential contribution to defective gonadotropin secretion in diabetes has not been evaluated. We report herein analyses of luteinizing hormone (LH) responses to kisspeptin and hypothalamic expression of the KiSS-1 gene in streptozotocin (STZ)-induced diabetic male rats. In addition, functional studies involving kisspeptin replacement or continuous administration of leptin and insulin to diabetic male rats are presented. Kisspeptin administration evoked robust LH and testosterone bursts and enhanced postgonadectomy LH concentrations, despite prevailing attenuation of gonadotropic axis in diabetic animals. In addition, hypothalamic KiSS-1 mRNA levels were unambiguously decreased in diabetic male rats, and the postorchidectomy rise in KiSS-1 mRNA was severely blunted. Repeated administration of kisspeptin to diabetic rats evoked persistent LH and testosterone responses and partially rescued prostate and testis weights. In addition, central infusion of leptin, but not insulin, was sufficient to normalize hypothalamic KiSS-1 mRNA levels, as well as LH and testosterone concentrations. In summary, we provide evidence for altered expression of the hypothalamic KiSS-1 system in a model of uncontrolled diabetes. This observation, together with the ability of exogenous kisspeptin to rescue defective LH responses in diabetic rats, unravel the physiopathological implication, and potential therapeutic intervention, of the KiSS-1 system in altered gonadotropin secretion of type 1 diabetes. *Diabetes* 55:2602–2610, 2006

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LH, luteinizing hormone; GnRH, gonadotropin-releasing hormone; GPR54, G protein–coupled receptor 54; STZ, streptozotocin.

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Reproductive function is highly sensitive to changes in the metabolic status and energy reserves of an organism, and adverse metabolic conditions are commonly associated with defective reproductive capacity (1,2). In this context, uncontrolled diabetes is often linked to sexual and reproductive dysfunction, whose underlying mechanisms clearly exceed its vascular and neurological complications (3). Thus, hypogonadotropism and hypogonadism are common features of experimental diabetes (3–6). In the diabetic male, these involve decreased basal and pulsatile luteinizing hormone (LH) secretion, altered sensitivity to negative feedback effects of androgens, reduced LH responses to gonadectomy, and insufficient testosterone secretion (3–7). The primary targets for such a plethora of manifestations are yet to be completely elucidated, but a central origin has been invoked. However, studies using streptozotocin (STZ)-administered rats demonstrated that hypothalamic gonadotropin-releasing hormone (GnRH) content and secretory capacity are conserved in diabetic animals (8,9), and pituitary responses to GnRH are roughly preserved (5,8,10). These observations suggest that the primary defect causing hypogonadotropism in the uncontrolled diabetic male lies upstream of GnRH neurons (11,12). However, the molecular conduits for such a phenomenon remain to be fully uncovered.

The precise control of pulsatile GnRH secretion is an intricate phenomenon that involves a large array of regulators of central and peripheral origin (13–15). Our understanding of this neuroendocrine network was recently revolutionized by the unraveling of the master role of the KiSS-1/G protein–coupled receptor 54 (GPR54) system in the central control of the GnRH axis (16). Such a contention originally stems from the observation that inactivating mutations of the gene encoding GPR54 are found in patients suffering idiopathic hypogonadotropic hypogonadism (17,18). These findings drew immediate attention to the previously unsuspected neuroendocrine facet of kisspeptins, ligands of GPR54, which are structurally related peptides generated by the differential proteolytic processing of a common precursor encoded by the metastasis suppressor KiSS-1 gene (19–21). Indeed, a wealth of data has now demonstrated that kisspeptins are potent elicitors of the GnRH/gonadotropin axis in a number of species, including humans (22–34), and mechanistic studies have suggested a relevant role of KiSS-1 signaling in puberty onset in rodents and primates (24,27). Moreover,

hypothalamic expression of the KiSS-1 gene appeared to be maximal at puberty and regulated by sex steroids (22–26). These observations have defined the essential role of KiSS-1 in the physiological control of reproductive axis in mammals.

Interestingly, hypothalamic KiSS-1 has been proposed as key molecular conduit for relaying a number of peripheral signals into the GnRH system. These include not only feedback actions of androgen and estrogen (22,25,26) but also metabolic cues. Thus, changes in the hypothalamic KiSS-1 system have been recently reported in situations of negative energy balance, which are also linked to suppressed gonadotropin secretion (35). Considering that diabetes is characterized by a profound metabolic disturbance frequently associated to hypogonadotropism, we hypothesized that the mechanism whereby this condition induces a state of defective gonadotropin secretion might ultimately target hypothalamic KiSS-1. To test this hypothesis, we evaluated LH responses to kisspeptin, as well as KiSS-1 gene expression, in a model of uncontrolled diabetes, the STZ-injected male rat. In addition, we conducted mechanistic studies, aiming to disclose the causative factors for altered KiSS-1 gene expression in diabetes.

RESEARCH DESIGN AND METHODS

Adult Wistar male rats bred in the vivarium of the University of Córdoba were used. The animals were maintained under constant conditions of light (14 h of light, from 0700) and temperature (22°C) and were housed in individual cages with free access to pelleted food and tap water. Experimental procedures were approved by the Córdoba University ethical committee and conducted in accordance with the European Union normative for use of experimental animals. Kisspeptin (110-119)-NH_2 (termed hereafter kisspeptin-10) was obtained from Phoenix Pharmaceuticals (Belmont, CA). STZ and insulin were purchased from Sigma (St. Louis, MO). Leptin was obtained from ProSpec-Tany TechnoGene (Rehovot, Israel).

Experiments were conducted in STZ-induced diabetic male rats, at 4 weeks after STZ injection. A protocol of two consecutive injections of STZ (50 mg/kg s.c. in 0.01 mol/l citrate buffer, pH 4.5) at a 24-h interval was selected based on previous studies (11,12). After STZ injections, body weights were recorded daily, and serum glucose levels were measured after 5 days and at the time of the experimental procedures (hormonal tests or tissue sampling) to check for diabetes status; animals showing severe hyperglycemia (>450 mg/dl) were selected for analysis (>98% in the current study). For experiments involving gonadectomy, the animals were subjected to bilateral gonadectomy via the scrotal route, under light ether anesthesia, at 1 week before hormonal tests or tissue sampling. Sham-operated males served as corresponding controls.

Experiment 1: hormonal responses to kisspeptin in diabetic male rats. Basal hormonal profiles, as well as the ability of kisspeptin to evoke LH and testosterone responses, were evaluated in groups (n = 10–12) of diabetic male rats at 4 weeks after STZ injection. For hormonal tests, a protocol of intracerebral injection of kisspeptin-10 was applied to control and STZ-injected animals. To allow delivery of kisspeptin into the lateral cerebral ventricle, the animals were implanted under ether anesthesia with intracerebroventricular cannulae lowered to a depth of 3 mm beneath the surface of the skull; the insert point was 1 mm posterior and 1.2 mm lateral to bregma, as described previously (22,29,31). Two doses of kisspeptin-10 were tested: 1 nmol (maximal) and 10 pmol (submaximal) in 10 μl. Blood samples (300 μl) were obtained by jugular venipuncture under light ether anesthesia before (0 min) and at 15 and 60 min after kisspeptin injection.

Experiment 2: hormonal responses to kisspeptin in gonadectomized diabetic male rats. In addition, LH responses to orchidectomy and kisspeptin administration were explored in diabetic males. To this end, bilateral gonadectomy was performed in diabetic rats, as described above. At 1 week after surgery, groups of gonadectomized animals (n = 10–12) were subjected to central intracerebroventricular injection of kisspeptin-10, as described in experiment 1, and blood samples (300 μl) were obtained by jugular venipuncture before (0 min) and at 15 and 60 min after kisspeptin administration.

Experiment 3: hypothalamic expression of KiSS-1 and GPR54 genes in diabetic male rats. Gene expression analysis was performed in hypothalamic samples from diabetic male rats, either at intact or postgonadectomy conditions. Protocols of STZ injections and bilateral orchidectomy were as described for experiments 1–2. For each experimental group (n = 5), the animals were killed by decapitation, and the hypothalami were immediately dissected as described in detail elsewhere (24), frozen in liquid nitrogen, and stored at −80°C until used for RNA analysis.

Experiment 4: repeated administration of kisspeptin and gonadotropic function in diabetic rats. Rescue of defective gonadotropic function in uncontrolled diabetes by sustained kisspeptin replacement was explored. Groups of male rats (n = 7–8), injected with STZ as described in experiment 1 were subjected to a protocol of repeated central administration of kisspeptin-10 or vehicle. Daily intracerebroventricular administration of kisspeptin into the lateral cerebral ventricle was conducted for 6 days at a dose of 1 nmol per rat of kisspeptin-10 every 12 h, as previously reported (29,35). Nondiabetic male rats (n = 8), centrally injected with vehicle, served as controls. Body weights were monitored daily. At the end of treatment, the animals were killed by decapitation 60 min after the last injection of kisspeptin, trunk blood was collected, and prostate and testis weights were recorded.

Experiment 5: effects of insulin and leptin on hypothalamic KiSS-1 gene expression in diabetic rats. The relative contribution of the lack of insulin or leptin to decreased hypothalamic KiSS-1 mRNA levels in diabetes was evaluated. Groups of STZ-injected males (n = 6–8) were subjected to continuous intracerebral infusion of effective doses of 1 nmol/day of insulin or leptin (36,37) for 6 days. The animals were implanted with osmotic minipumps (1 μl/h delivery rate, Alzet mini-osmotic pump model no. 2001; Durect, Cupertino, CA) containing vehicle (0.001 mol/l HCl in saline), leptin, or insulin, at a final concentration 1 nmol/24 μl. The osmotic pumps were placed intradermally between the scapulas and connected to intracerebroventricular cannulae to allow central delivery of the peptides. Nondiabetic male rats (n = 8), centrally infused with vehicle, served as controls. Body weights were daily monitored. After a 6-day infusion, the animals were killed by decapitation, trunk blood was collected for hormone determinations, and prostate and testis weights were recorded.

Hormone measurements. Serum LH levels were determined, using radioimmunoassay kits supplied by the National Institutes of Health (Dr. A.F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Peptide Program, Torrance, CA). Intra- and interassay coefficients of variation (CVs) were <8 and <10%, respectively. The sensitivity of the assay was 5 pg/tube. Serum glucose concentrations were determined using an automatic glucose analyzer (Accu-Chek; Roche Diagnostics, Barcelona, Spain). Serum leptin and testosterone levels were determined, using commercial kits from Linco Research (St. Charles, MO) and MP Biomedicals (Costa Mesa, CA), following the instructions of the manufacturer. The sensitivity of the assays was 0.05 ng/tube (leptin) and 0.1 ng/tube (testosterone); the intra-assay CVs were <5%.

RNA analysis by semiquantitative RT-PCR. Total RNA was isolated from hypothalamic samples using the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method. Hypothalamic expression of KiSS-1 and GPR54 mRNAs was assessed by final-time RT-PCR, optimized for semiquantitative detection, using previously defined primer pairs and conditions (22,35). As internal control for reverse transcription and reaction efficiency, amplification of a 240-bp fragment of S11 ribosomal protein mRNA was carried out in parallel in each sample (22). As previously optimized (22), 32 and 24 PCR cycles were chosen for analysis of specific targets (KiSS-1 and GPR54) and RP-S11, respectively. Specificity of PCR products was confirmed by direct sequencing (Central Sequencing Service, Córdoba University). Quantification of intensity of RT-PCR signals was carried out by densitometric scanning (1-D Manager; TDI, Madrid, Spain), and values of the specific targets were normalized to those of internal controls. Liquid controls and reactions without reverse transcription resulted in negative amplification.

To verify changes in KiSS-1 gene expression, real-time RT-PCR was performed using the iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). General procedures for real-time RT-PCR of KiSS-1 mRNA were as previously described (22,35). Calculation of relative expression levels of the target was conducted based on the cycle threshold (C_T) method. The C_T for each sample was calculated using the iCycler iQ real-time PCR detection system software with an automatic fluorescence threshold (R_n) setting. Fold expression of target mRNA over reference values was calculated by the equation $2^{-\Delta\Delta CT}$, where ΔC_T is determined by subtracting the corresponding RP-S11 C_T value from the specific C_T of the target, and $\Delta\Delta C_T$ was obtained by subtracting the ΔC_T of each experimental sample from that of the reference sample.

Presentation of data and statistics. Hormonal determinations (LH, testosterone, and leptin) were conducted in duplicate, with a minimal total number of 10 samples per group. When appropriate, integrated LH secretory responses were calculated as the area under the curve, using the trapezoidal rule. Semiquantitative RT-PCR analyses were carried out in duplicate from at least four independent RNA samples of each experimental group. Quantitative RNA and hormonal data are presented as the means ± SE. Results were analyzed for statistically significant differences, using unpaired Student's t test or

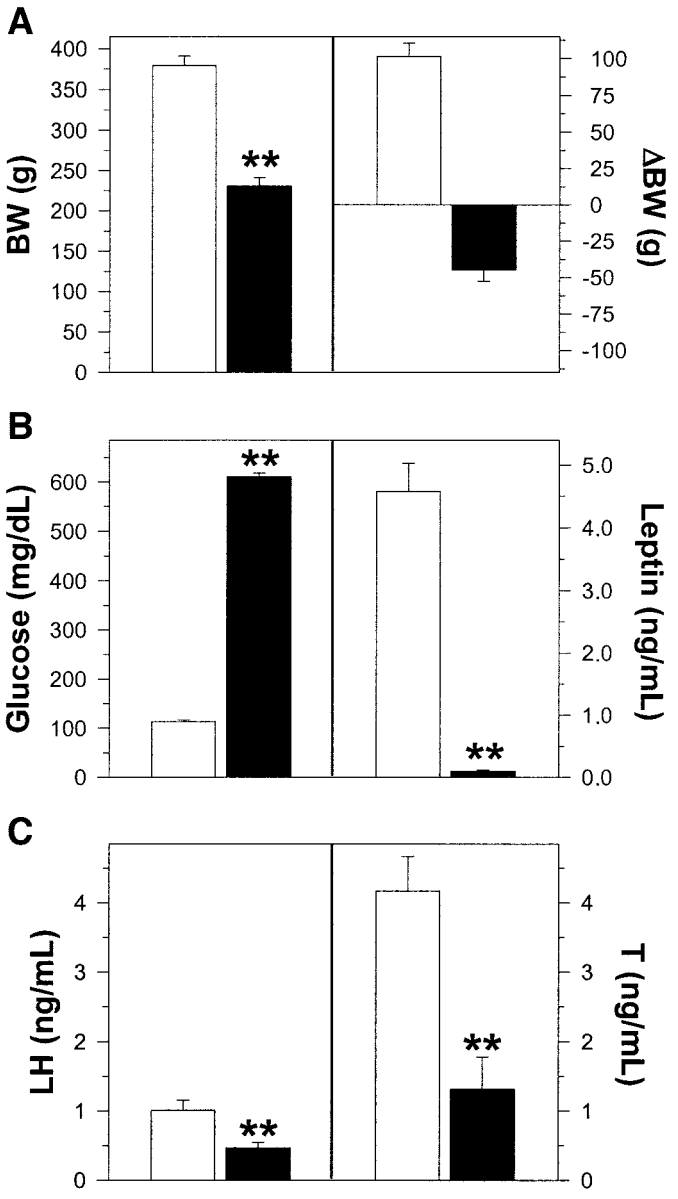

FIG. 1. Auxological data and hormone profiles of adult male rats at 4 weeks after injection of STZ. *A*: Total body weight (BW) and net body weight gain in control and STZ-injected animals. *B*: Glucose and leptin levels in long-term diabetic males and their controls. *C*: Serum LH and testosterone (T) levels in controls and 4 weeks' post-STZ animals are indicated. Data are the means ± SE of at least 10 independent determinations per group. **$P < 0.01$ vs. corresponding control values (Student's *t* test). □, control rats; ■, 4 weeks' post-STZ rats.

ANOVA followed by Student-Newman-Keuls multiple range test (SigmaStat 2.0; Jandel, San Rafael, CA). $P \leq 0.05$ was considered significant.

RESULTS

Effects of kisspeptin on LH and testosterone secretion in diabetic male rats. Auxological data and hormonal profiles of diabetic male rats 4 weeks after STZ administration are presented in Fig. 1. Diabetic males showed a significant reduction in total body weight versus controls, with a negative net body weight gain during the study period. In addition, all animals were massively hyperglycemic, and >90% showed serum glucose concentrations >600 mg/dl; normoglycemic controls had glucose levels <120 mg/dl. Moreover, STZ-injected males presented virtually negligible serum leptin levels. In terms of gonadotropic axis function, basal serum LH levels were significantly decreased in rats 4 weeks after STZ. Likewise, diabetic males showed a significant reduction of circulating testosterone concentrations that were approximately one-fourth of those in control animals.

Hormonal tests in this model revealed that despite evidence for basal suppression of the gonadotropic axis, responses to maximal and submaximal doses of kisspeptin were fully preserved in long-term diabetic males. Thus, central injection of 10 pmol kisspeptin-10 to animals 4 weeks after STZ evoked a significant increase in serum LH levels, which peaked at 15 min and was not detectable at 60 min, similar to that observed in control animals. Likewise, the profiles of LH response to 1 nmol kisspeptin, with significantly increased levels at 15 min that remained elevated at 60 min after injection, were analogous in control and diabetic male rats. In good agreement, despite differences in basal concentrations, testosterone responses to central injection of 1 nmol kisspeptin reached equal maximal levels in control and rats 4 weeks after STZ. In addition, although basal LH levels were lower in diabetic animals, the integrated LH secretory responses to kisspeptin were similar in control and diabetic animals for both doses tested (Fig. 2).

Hormonal profiles and LH responses to kisspeptin in orchidectomized diabetic male rats. Hormonal profiling and functional tests were also performed in STZ-injected male rats at 1 week after bilateral gonadectomy. In keeping with previous studies (5–7), LH hypersecretion in response to orchidectomy was partially blunted in diabetic animals, with a ~50% reduction in the postorchidectomy rise of serum LH levels (Fig. 3). However, central kisspeptin administration was able to fully reverse defective LH responses to gonadectomy in diabetic rats. Thus, central administration of 1 nmol kisspeptin to orchidectomized diabetic rats restored maximal LH hypersecretion, as observed in paired control gonadectomized animals, with significantly elevated LH levels over preinjection values at 15 and 60 min after peptide injection. Calculation of integrated LH secretory responses corroborated that net LH hypersecretion in response to orchidectomy was significantly decreased in gonadectomized diabetic animals, a status that was completely rescued by exogenous administration of kisspeptin (Fig. 4).

Hypothalamic expression of KiSS-1 gene in intact and gonadectomized diabetic male rats. Expression analyses of KiSS-1 and GPR54 genes at the hypothalamus were conducted in diabetic male rats either intact or at 1 week after gonadectomy. Long-term diabetes (4 weeks after STZ) induced a significant (>45%) decrease in KiSS-1 mRNA levels in whole hypothalamic fragments. In addition, whereas at 1 week postorchidectomy in control animals resulted in a clear-cut 2.0- to 2.5-fold increase in KiSS-1 mRNA at the hypothalamus, gonadectomy of diabetic rats only induced a modest, albeit statistically significant ($P < 0.05$), increase in hypothalamic KiSS-1 mRNA expression. In good agreement, relative levels of KiSS-1 mRNA at 1 week postorchidectomy were significantly lower in diabetic males at 4 weeks than in their corresponding controls (Fig. 5). In contrast to the KiSS-1 gene, hypothalamic expression of GPR54 mRNA remained unaltered at 4 weeks after STZ injection, and neither was it significantly modified by 1 week postgonadectomy in control or diabetic male rats (data not shown).

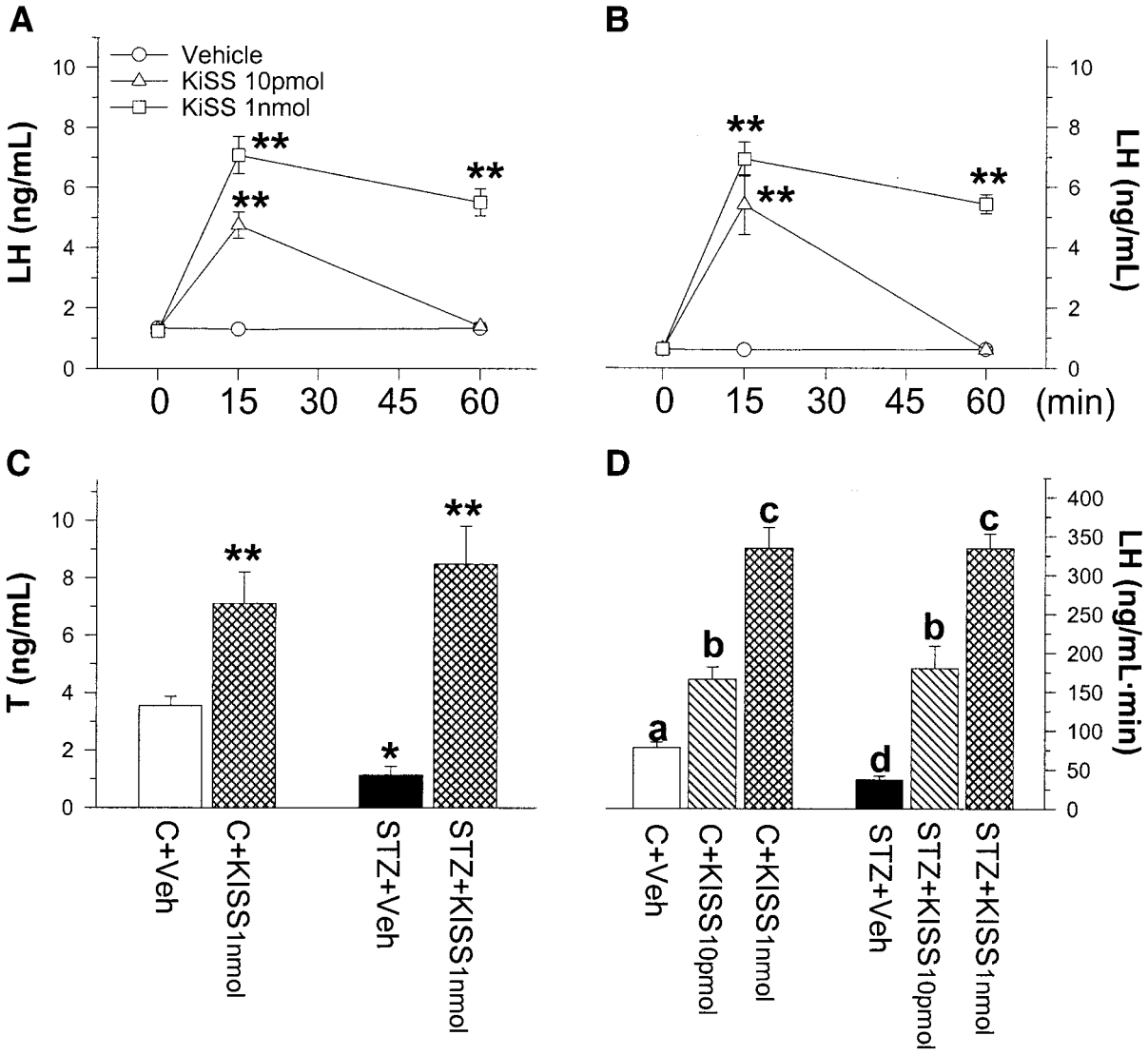

FIG. 2. LH and testosterone (T) responses to central kisspeptin administration in control and 4 weeks' post-STZ diabetic animals. *A* and *B*: The time course for the LH-releasing effects of two doses of kisspeptin-10 (1 nmol and 10 pmol i.c.v.) in control (*A*) and STZ-injected (*B*) animals is shown. *C*: Serum testosterone levels at 60 min after injection of kisspeptin-10 (1 nmol). *D*: Integrated LH secretion after central administration of kisspeptin-10 (area under the curve [AUC] during the 60-min study period) for both doses in control and diabetic animals. Data are the means ± SE of at least 10 independent determinations per group. *$P < 0.01$ vs. corresponding values in nondiabetic controls; **$P < 0.01$ vs. corresponding control values. For area under the curve data, groups with different lower case letters are statistically different (ANOVA followed by Student-Newman-Keuls multiple range test). C, control; KISS, kisspeptin-10; Veh, vehicle.

Repeated administration of kisspeptin and gonadotropic function in diabetic male rats. To provide further proof for the ability of kisspeptin to rescue diabetes-induced hypogonadotropism, at 4 weeks after STZ administration, males were subjected to a protocol of repeated intracerebral injection of kisspeptin-10 at a regimen (1 nmol/12 h for 6 days) capable of stimulating the gonadotrpic axis under adverse metabolic conditions, such as undernutrition (35). Compilation of data from this experiment is presented in Fig. 6. Long-term (4 weeks after STZ) diabetes resulted in a significant reduction in total body weight that was similar in magnitude in vehicle- and kisspeptin-administered animals. In 4 weeks' post–STZ administration males injected with vehicle, serum LH and testosterone concentrations were significantly lower than those of control animals, in keeping with results from experiment 1. Likewise, relative prostate weights were decreased in diabetic males, which also showed a reduction in absolute testis weight. Repeated injections of kisspeptin-10 evoked persistent increases in circulating LH and testosterone levels, as evidenced in terminal serum determinations (60 min after the last bolus of kisspeptin). Similarly, chronic administration of kisspeptin partially rescued the decrease in prostate weight, with a significant (~45%) increase versus vehicle-administered diabetic males. However, such an increase did not reach prostate weights in nondiabetic animals (Fig. 6). Finally, repeated kisspeptin administration induced ~20% elevation in testicular weight in diabetic rats (not shown).

Effects of insulin and leptin on hypothalamic KiSS-1 mRNA levels in diabetic male rats. To provide a mechanistic insight for the observed decrease in hypothalamic expression of the KiSS-1 gene in diabetic male rats, hormonal responses and KiSS-1 mRNA levels were monitored in this model after continuous infusion of insulin or leptin. These two factors were selected based on the

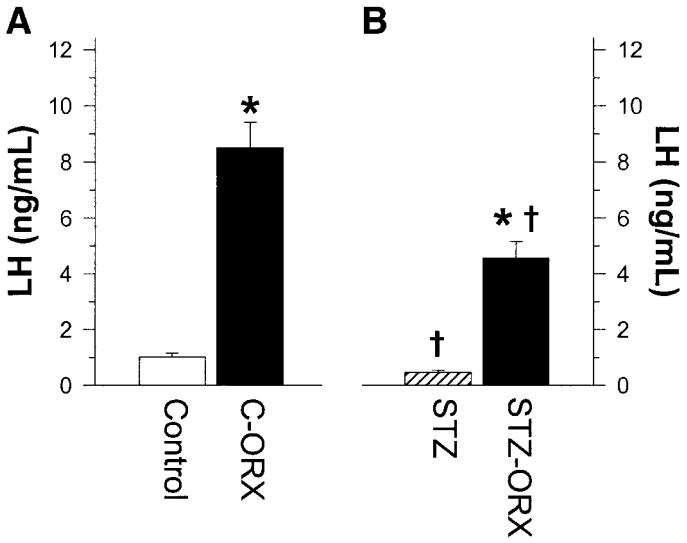

FIG. 3. LH responses to gonadectomy in control (A) and 4 weeks' post-STZ diabetic (*B*) male rats. Orchidectomy (ORX) was performed in STZ-injected males, and serum LH levels were determined 1 week after surgery. Data are the means ± SE of at least 10 independent determinations per group. **P* < 0.01 vs. corresponding controls; †*P* < 0.01 vs. corresponding values in nondiabetic animals (ANOVA followed by Student-Newman-Keuls multiple range test). C, control.

dramatic alterations in their circulating levels after STZ injection (Fig. 1) and the proven prominent roles of leptin and insulin in the control of the gonadotropic axis (1,2,38). To selectively target direct actions of these hormones at the central level, a protocol of intracerebral infusion was selected. In keeping with data from experiment 4, body and testicular weights were significantly reduced in diabetic male rats. Similarly, serum levels of LH and testosterone, as well as relative prostate weights, were decreased in those animals. Central infusion of leptin (at a rate of 1 nmol/day) for 6 days evoked a significant increase in circulating LH and testosterone levels that reached values similar to those of nondiabetic controls. Similarly, continuous leptin infusion enhanced relative prostate weights by ~40% (which, nonetheless, remained lower than control values) and moderately increased (15–20%) testicular weight (not shown). In contrast, intracerebral infusion of equimolar doses of insulin failed to significantly alter serum LH or testosterone levels, and neither did it elevate relative prostate weights in those animals (Fig. 7). Central infusion of insulin or leptin did not correct the massive hyperglycemia in diabetic rats at 4 weeks (not shown).

Regarding KiSS-1 gene expression at the hypothalamus, male rats 4 weeks after STZ injection showed significantly decreased KiSS-1 mRNA levels (~45–50% of nondiabetic controls), in keeping with results from experiment 3. Central infusion of leptin was sufficient to increase hypothalamic KiSS-1 mRNA to levels similar to those of control animals. In contrast, continuous intracerebral administration of insulin for 6 days only evoked a marginal increase in hypothalamic KiSS-1 mRNA levels that did not reach statistical significance (Fig. 7).

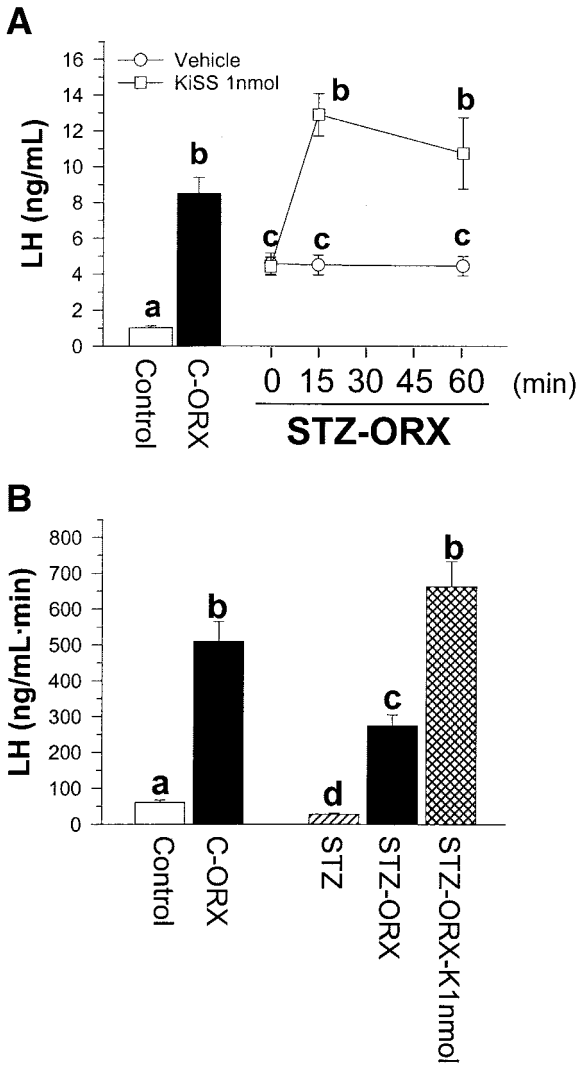

FIG. 4. LH responses to central kisspeptin administration in 4 weeks' post-STZ diabetic rats 1 week after orchidectomy (ORX). *A*: The time course for the LH releasing effects of intracerebroventricular injection of kisspeptin-10 in 4 weeks' post-STZ diabetic rats. *B*: The integrated LH secretory responses to kisspeptin-10 (area under the curve [AUC] during the 60-min study period) in orchidectomized diabetic rats are shown. For comparative purposes, the corresponding LH levels in control male rats, at 1 week after gonadectomy, are also included. Data are the means ± SE of at least 10 independent determinations. Groups with different lower case letters are statistically different (ANOVA followed by Student-Newman-Keuls multiple range test). C, control.

DISCUSSION

Type 1 diabetes is a highly prevalent metabolic disease that, if poorly controlled, is frequently linked to a plethora of reproductive deficits (3). Although it has been globally assumed that most of these manifestations are caused by the peripheral vascular and neurological complications of uncontrolled diabetes, data from models of experimental diabetes clearly indicate that variable degrees of hypogonadotropic hypogonadism are commonly observed in this condition, whose neuroendocrine substrate is yet to be fully unfolded. In the current study, we challenged the hypothesis that hypothalamic KiSS-1 is the central target whereby diabetes disrupts the function of the gonadotropic axis. Our analyses strongly suggest that altered expression and function of the hypothalamic KiSS-1 system is involved in the etiopathogenesis of diabetic hypogonadotropism, a phenomenon that is likely caused by severe hypoleptinemia of uncontrolled diabetes.

Analyses of defective LH secretion in STZ-injected rats, both in basal conditions and after gonadectomy, were exhaustively conducted in the late 1980s and early 1990s (3–7). However, those hormonal studies were carried out

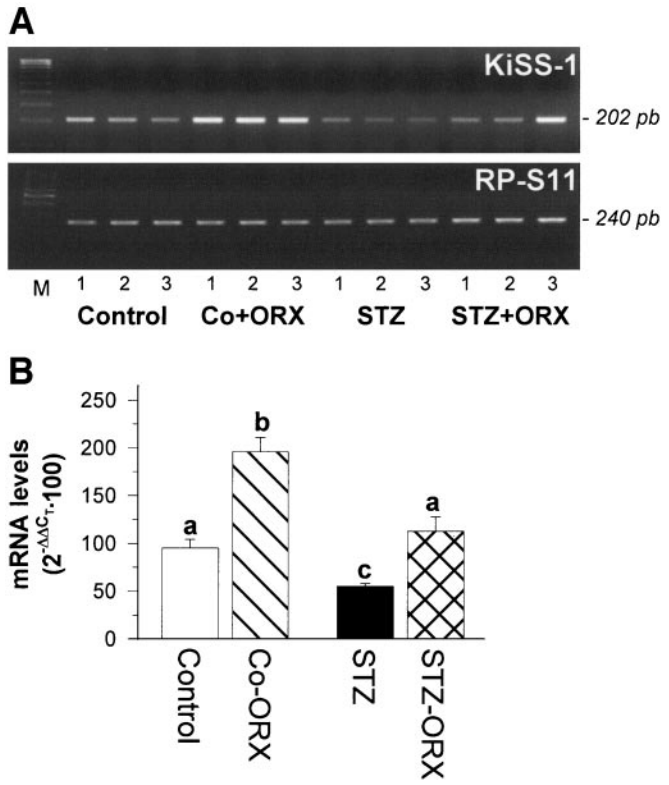

FIG. 5. ***A*** **and** ***B*****: Hypothalamic expression of KiSS-1 gene in adult male rats 4 weeks after injection of vehicle (control) or STZ (long-term diabetes), either under intact conditions or 1 week after orchidectomy (ORX). For each group, three independent samples are presented. Parallel amplification of S11 ribosomal protein (RP-S11) mRNA served as internal control.** ***B*****: KiSS-1 mRNA levels are the means ± SE of at least four independent duplicate determinations, assessed by real-time RT-PCR. Groups with different lower case letters are statistically different (*P* < 0.05, ANOVA followed by Student-Newman-Keuls multiple range test). Co, control.**

before cloning and/or characterization of key molecular signals in the integrated control of metabolism and reproductive function, such as leptin, GALP (galanin-like peptide), and, more recently, kisspeptin (2,35,39). Such recent developments force us to accommodate these new factors into the previously known hypoganodotropic phenotype of diabetes. Keeping this notion in mind, we conducted hormonal tests and expression analyses aiming to assess the function of the hypothalamic KiSS-1 system in STZ-induced diabetic rats. Hormonal profiles confirmed that diabetic animals have significantly lower concentrations of LH and testosterone, pointing to a gonadotropic suppression of central origin. Of note, relative KiSS-1 mRNA levels at the hypothalamus in diabetic animals closely paralleled the decline in serum LH, suggesting a potential causative link between these two phenomena. However, evidence for quantitative changes in expression and/or secretion of kisspeptin at the peptide level is needed to fully support this hypothesis, a possibility that is currently hampered by the lack of optimal antibodies for immunodetection of kisspeptin in rat brain tissues.

Despite evidence for basal suppression of the gonadotropic axis in diabetic rats, acute LH responses to kisspeptin were fully maintained in this model. Moreover, although absolute levels after kisspeptin were similar in diabetic and control males, given the significant reduction in basal LH concentrations in STZ-injected animals, relative responses (i.e., increases over corresponding preinjection levels) were nearly twofold higher in this group. These observations closely resemble our recent findings on LH hyperresponsiveness to exogenous kisspeptin in food-deprived rats, which is likely caused by decreased expression of hypothalamic KiSS-1 system in fasting (35). This is probably the case also in our diabetic model (Figs. 5 and 7). Whether these phenomena are causatively related is currently under investigation at our laboratory. Nonetheless, it is remarkable to note that acute kisspeptin administration was able not only to rescue defective LH

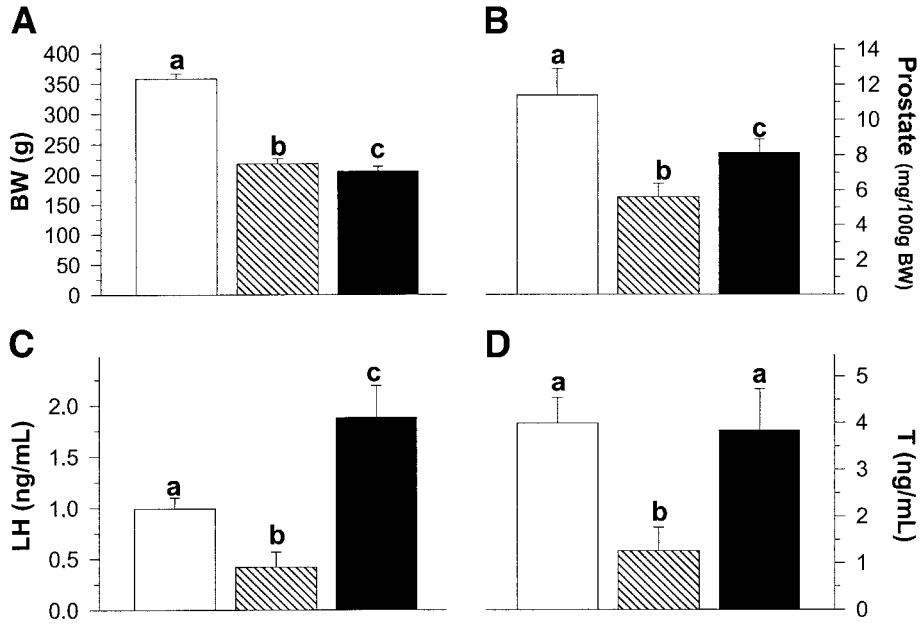

FIG. 6. Effects of repeated intracerebral administration of kisspeptin-10 (1 nmol/rat every 12 h for 6 days) on several reproductive parameters of the STZ-induced diabetic male rat. Body weight (BW) and prostate weight (*A* and *B*) were recorded at the end of the treatment period. In addition, serum LH and testosterone (T) levels in terminal blood samples (1 h after the last kisspeptin injection) are presented (*C* and *D*). Data are the means ± SE of at least 10 independent determinations per group. Groups with different lower case letters are statistically different (*P* < 0.05, ANOVA followed by Student-Newman-Keuls multiple range test). □, control; ▨, STZ plus vehicle; ■, STZ plus KiSS.

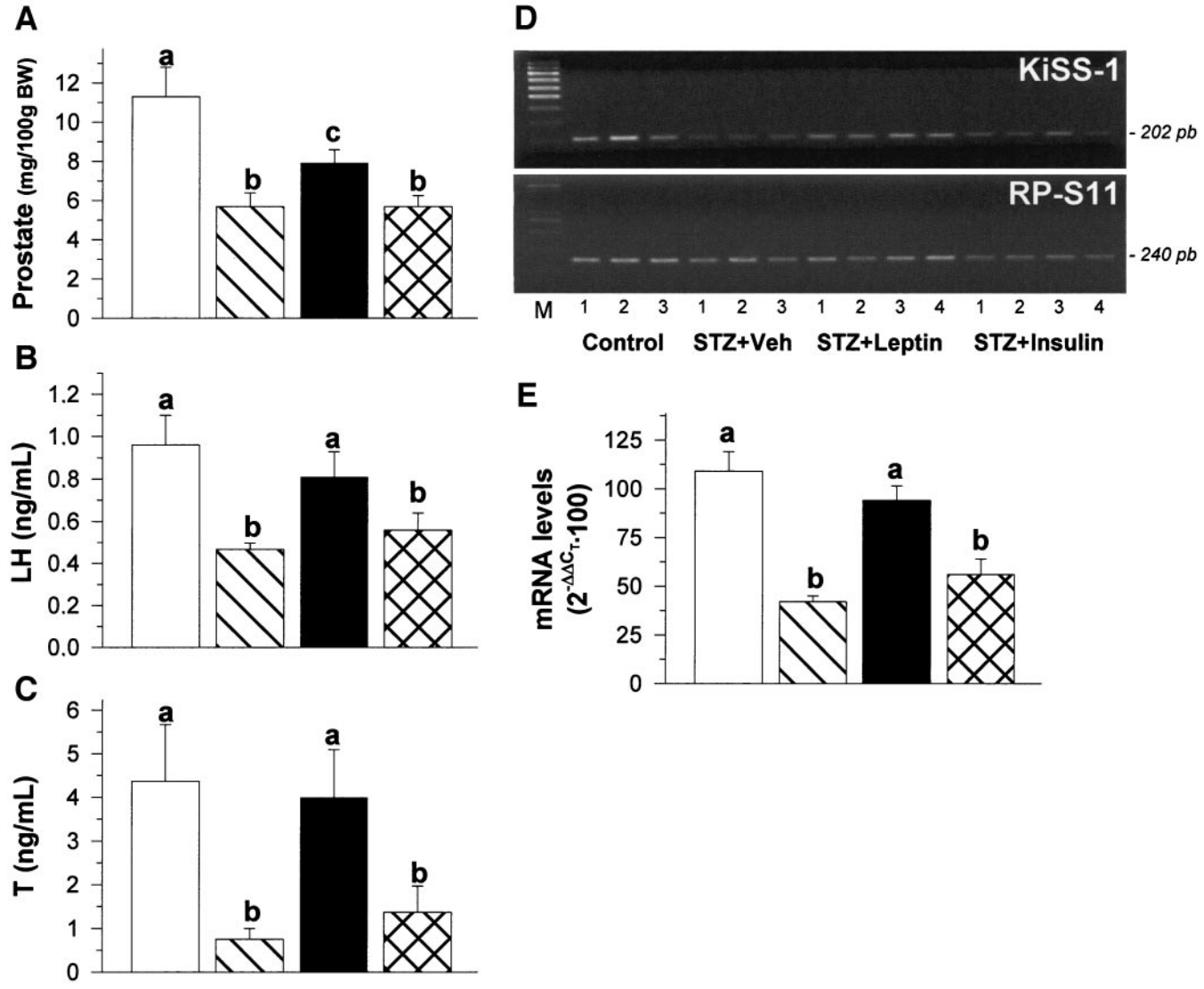

FIG. 7. Effects of continuous intracerebral infusion of leptin or insulin on several reproductive parameters and hypothalamic expression of KiSS-1 gene in diabetic male rats. *A–C*: Prostate weights and serum LH and testosterone (T) levels in terminal blood samples. Data are the means ± SE of at least 10 independent determinations per group. *D* and *E*: Expression analysis of KiSS-1 mRNA. Three representative independent samples per group are shown. Parallel amplification of S11 ribosomal protein mRNA served as internal control. Semiquantitative KiSS-1 mRNA levels are the means ± SE of at least four independent determinations in duplicate, assessed by real-time RT-PCR assays. Groups with different superscript letters are statistically different ($P < 0.05$, ANOVA followed by Student-Newman-Keuls multiple range test). □, control; ▨, STZ plus vehicle; ■, STZ plus leptin; ▩, STZ plus insulin. Veh, vehicle.

secretion but also to induce supramaximal LH responses, under conditions of severe metabolic imbalance, such as undernutrition and diabetes.

A hallmark feature of the hypogonadotropic state of uncontrolled diabetes is the defective LH response to gonadectomy (3,5–7), which was fully confirmed in our experiments. Interestingly, acute central administration of kisspeptin to orchidectomized diabetic rats was able to enhance LH hypersecretion to levels similar to those of control gonadectomized animals. In addition, expression studies evidenced a defective increase in hypothalamic KiSS-1 mRNA levels postgonadectomy in diabetic animals, which were markedly lower than those of orchidectomized controls. Collectively considered, the above observations strongly suggest that the blunted rise in hypothalamic KiSS-1 following gonadectomy is a major cause for the diminished elevation of LH levels postorchidectomy in diabetic males and that normalization of such LH responses can be achieved by solely replacing the endogenous kisspeptin tone.

Besides hormonal responses to acute administration, protocols of repeated injection of kisspeptin were implemented as a means to provide further proof for its ability to rescue the hypogonadotropic phenotype of uncontrolled diabetes. Repeated intracerebral administration of kisspeptin was able to normalize serum testosterone levels and evoke unambiguous LH responses, despite the significant decrease in prevailing concentrations. Moreover, prostate and, to a lesser extent, testis weights were partially recovered after chronic treatment with kisspeptin. Altogether, these data reinforce the contention that replacement of kisspeptin tone ameliorates the hypogonadotropic phenotype of diabetes, a finding with potential therapeutic implications. Of note, a protocol of repeated injections, rather than continuous infusion or persistent overexpression (e.g., by means of adenoviral assisted

delivery), of kisspeptin was selected to avoid potential desensitization events, as recently reported in monkeys (40). However, this procedure limited the duration of kisspeptin replacement (6 days), which may explain why prostate and testis weights were not fully recovered at the end of the treatment period.

From a mechanistic perspective, the factor(s) responsible for the observed changes in hypothalamic KiSS-1 system in diabetic animals was also explored. Diabetes is defined by a plethora of endocrine and metabolic disturbances that include overt hypoinsulinemia and hypoleptinemia, which might individually contribute to decreased KiSS-1 expression and hypogonadotropism (1,38). Protocols of intracerebral infusion in STZ-injected males revealed that leptin replacement is sufficient to restore hypothalamic KiSS-1 mRNA levels and normalize LH and testosterone concentrations in diabetic animals, whereas equimolar doses of insulin at central levels were mostly ineffective. Of note, continuous intracerebroventricular infusion was selected as experimental setup to selectively target primary actions at central levels, independent of the potential metabolic effects of insulin (or leptin) peripherally (e.g., on glucose levels). These observations underscore a pivotal role of leptin in the control of KiSS-1 expression at the hypothalamus. Indeed, during the final stage of preparation of this article, the presence of leptin receptors in kisspeptin neurons was reported in mice (41). Moreover, our data shows that hypoleptinemia is a major contributing factor for the decreased KiSS-1 expression and hypogonadotropism of diabetic rats. That intracerebral administration of insulin failed to rescue KiSS-1 expression centrally does not preclude its potential modulatory action at other levels of the gonadotropic axis (e.g., on GnRH neurons) (42), but it strongly suggests that insulin per se is not sufficient to restore normal gonadotropic function in the face of persistently decreased KiSS-1 tone.

In summary, we report herein the characterization of the LH-releasing effects of kisspeptin and the hypothalamic expression of the KiSS-1 gene in an experimental model of type 1 diabetes. Our results show that decreased expression of KiSS-1 is detected in diabetic male rats, which is caused, at least partially, by severe hypoleptinemia. Moreover, replacement of central kisspeptin tone rescues defective gonadotropic function in diabetic animals. To our knowledge, the data presented herein is the first experimental evidence for the potential contribution of disturbed KiSS-1 function to the state of hypogonadotropism frequently observed in uncontrolled diabetes, a phenomenon whose eventual therapeutic implications merit further investigation.

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