Differential responses of the growth hormone axis in two rat models of streptozotocin-induced insulinopenic diabetes

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Abstract

The impact of streptozotocin (STZ)-induced, insulinopenic diabetes on the GH axis of rats and mice differs from study to study, where this variation may be related to the induction scheme, severity of the diabetes and/or the genetic background of the animal model used. In order to begin differentiate between these possibilities, we compared the effects of two different STZ induction schemes on the GH axis of male Sprague–Dawley rats: (1) a single high-dose injection of STZ (HI STZ, 80 mg/kg, i.p.), which results in rapid chemical destruction of the pancreatic β-cells, and (2) multiple low-dose injections of STZ (LO STZ, 20 mg/kg for 5 consecutive days, i.p.), which results in a gradual, autoimmune destruction of β-cells. STZ-treated animals were killed after 3 weeks of hyperglycemia (>400 mg/dl), and in both paradigms circulating insulin levels were reduced to <40% of vehicle-treated controls. HI STZ-treated rats lost weight, while body weights of LO STZ-treated animals gradually increased over time, similar to vehicle-treated controls. As previously reported, HI STZ resulted in a decrease in circulating GH and IGF-I levels which was associated with a rise in hypothalamic neuropeptide Y (NPY) mRNA (355% of vehicle-treated controls) and a fall in GH-releasing hormone (GHRH) mRNA (45% of vehicle-treated controls) levels. Changes in hypothalamic neuropeptide expression were reflected by an increase in immunoreactive NPY within the arcuate and paraventricular nuclei and a decrease in GHRH immunoreactivity in the arcuate nucleus, as assessed by immunohistochemistry. Consistent with the decline in circulating GH and hypothalamic GHRH, pituitary GH mRNA levels of HI STZ-treated rats were 58% of controls. However, pituitary receptor mRNA levels for GHRH and ghrelin increased and those for somatostatin (sst2, sst3 and sst5) decreased following HI STZ treatment. The impact of LO STZ treatment on the GH axis differed from that observed following HI STZ treatment, despite comparable changes in circulating glucose and insulin. Specifically, LO STZ treatment did suppress circulating IGF-I levels to the same extent as HI STZ treatment; however, the impact on hypothalamic NPY mRNA levels was less dramatic (158% of vehicle-treated controls) where NPY immunoreactivity was increased only within the paraventricular nucleus. Also, there were no changes in circulating GH, hypothalamic GHRH or pituitary receptor expression following LO STZ treatment, with the exception that pituitary sst3 mRNA levels were suppressed compared with vehicle-treated controls. Taken together these results clearly demonstrate that insulinopenia, hyperglycemia and reduced circulating IGF-I levels are not the primary mediators of hypothalamic and pituitary changes in the GH axis of rats following HI STZ treatment. Changes in the GH axis of HI STZ-treated rats were accompanied by weight loss, and these changes are strikingly similar to those observed in the fasted rat, which suggests that factors associated with the catabolic state are critical in modifying the GH axis following STZ-induced diabetes.
Wistar), these metabolic changes have been associated with suppression of the growth hormone (GH) axis, which includes a decrease in hypothalamic GH-releasing hormone (GHRH) and pituitary GH mRNA levels and a reduction in pulsatile GH release, and mean circulating insulin-like growth factor (IGF)-I levels (Busiguina et al. 1989a, 1989b). Despite these changes, the pituitary of the HI STZ-treated rat is more sensitive to the stimulatory actions of GHRH (Sheppard et al. 1989a, 1989b) and GH secretagogue (GHS) receptor (GHS-R) agonists (ipamorelin; Johansen et al. 2003) and less sensitive to the inhibitory actions of somatostatin (SRIH; Bruno et al. 1994, Sheppard et al. 1989b).

Mice (ICR background) treated with HI STZ (200 mg/kg, i.p. injection) also lose weight and exhibit a dramatic reduction in circulating GH and IGF-I and a decrease in pituitary GH and hypothalamic GHRH expression, which is associated with pituitary GHRH hypersensitivity, in vitro (Murao et al. 1995). It should be noted that these changes in the GH axis are strikingly similar to those observed in the fasted rat (Park et al. 2004, Tannenbaum et al. 1979) and therefore might be related to the catabolic condition and not to the absolute circulating levels of insulin and glucose. This hypothesis is consistent with a report showing BALB/c mice, when treated with a single i.v. injection of HI STZ (250–300 mg/kg), have elevated circulating GH levels, which was associated with hypoinsulinemia and hyperglycemia without dramatic weight loss or ketosis (Flyvbjerg et al. 1999), a response similar to that reported in poorly treated animals with diabetes (Li et al. 2000a, Like & Rossini 1976). Body weight and blood glucose levels were measured at day −4 (time of first STZ treatment), day 0 (time of last STZ treatment) and on days 2, 4, 7, 14, 21 and 28 following the last day of STZ injection. Body weights were not suppressed with treatment (Fig. 1A) and blood glucose levels began to rise to 262 mg/dl at 2 days after the last injection. All LO STZ–treated animals showed hyperglycemia (>22·2 nmol/l or 400 mg/dl) 7 days after last injection and remained hyperglycemic until their death (Fig. 1B).

HI STZ–induced diabetic animals were killed 21 days following bolus STZ treatment, and LO STZ animals were killed 4 weeks after the last STZ injection. Therefore both groups of animals were exposed to hyperglycemia for 3 weeks. Blood, pituitaries and hypothalami were collected and stored at −70 °C until further analysis.

Measurement of glucose, GH, IGF-I and insulin concentrations

Glucose levels were measured using blood from the tail vein by GlucoDr Blood Glucose Meter (Allmedicus, Korea; maximal reading 600 mg/dl). Serum GH concentrations were measured by rat GH RIA kit (Amersham Biosciences Co.). Total serum IGF-I levels were assayed using a rat IGF-I RIA kit (Amersham) after acid/ethanol extraction according to the manufacturer’s instructions. Serum insulin concentrations were assessed using the rat insulin RIA kit (Amersham).

RNA isolation

Total hypothalamic and pituitary RNA were recovered using standard procedure reported previously (Kamegai et al. 1998b, 1998c). RNA was then precipitated with isopropanol, and the pellet was washed with 70% ethanol, air dried, and dissolved in sterile DEPC.

Materials and Methods

Animals

Male Sprague–Dawley rats (7–8 weeks; 220–250 g) were housed under controlled environmental conditions (12 h:12 h light/dark). Food and tap water were available ad libitum. Experiments were conducted according to the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

STZ-induced diabetes

HI STZ In order to induce rapid-onset diabetes, rats were treated with vehicle (citrate buffer, pH 4.5; n=5) or STZ (80 mg/kg, i.p.; n=7) between 14:00 and 16:00 h. Body weights and blood glucose levels were determined on day 0 (time of STZ treatment) and on days 2, 4, 7, 14, 21 and 21. HI STZ–treated animals lost weight (Fig. 1A) and all treated animals displayed hyperglycemia (>22·2 nmol/l or 400 mg/dl) 2 days after STZ injection and remained hyperglycemic until their death (Fig. 1B).

LO STZ To generate a model resembling a more gradual onset of diabetes, rats received vehicle (n=5) or STZ (20 mg/kg, i.p.; n=7) for 5 consecutive days as previously described (Li et al. 2000a, Like & Rossini 1976). Body weight and blood glucose levels were measured at day −4 (time of first STZ treatment), day 0 (time of last STZ treatment) and on days 2, 4, 7, 14, 21 and 28 following the last day of STZ injection. Body weights were not suppressed with treatment (Fig. 1A) and blood glucose levels began to rise to 262 mg/dl at 2 days after the last injection. All LO STZ–treated animals showed hyperglycemia (>22·2 nmol/l or 400 mg/dl) 7 days after last injection and remained hyperglycemic until their death (Fig. 1B).

HI STZ-induced diabetic animals were killed 21 days following bolus STZ treatment, and LO STZ animals were killed 4 weeks after the last STZ injection. Therefore both groups of animals were exposed to hyperglycemia for 3 weeks. Blood, pituitaries and hypothalami were collected and stored at −70 °C until further analysis.

Measurement of glucose, GH, IGF-I and insulin concentrations

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RNA isolation

Total hypothalamic and pituitary RNA were recovered using standard procedure reported previously (Kamegai et al. 1998b, 1998c). RNA was then precipitated with isopropanol, and the pellet was washed with 70% ethanol, air dried, and dissolved in sterile DEPC.
The concentration and purity of RNA were determined by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at wavelengths of 260/280 nm.

RNase protection assay (RPA) of hypothalamic GHRH, SRIH and NPY mRNA

Hypothalamic GHRH, SRIH and NPY mRNA levels were measured by RPA using the HybSpeed RPA kit (Ambion, Austin, TX, USA) as previously described (Park et al. 2004). Briefly, in a single reaction, probes for GHRH, SRIH, NPY and β-actin were incubated for 20 min at 68 °C in 10 µl HybSpeed Hybridization Buffer containing 50% total RNA isolated from a single hypothalamus or 50 µg yeast RNA. Unhybridized probes were removed by treating the reactions with RNase A/T1 mix for 1 h at 37 °C. Protected fragments were separated on a 5% polyacrylamide/8 M urea gel. The gel was dried on chromatography paper and exposed to a phosphorimager screen (Packard Instruments, Fallbrook, CA, USA). Band intensity was evaluated by image-analysis software (Non-linear Dynamics, Newcastle upon Tyne, UK).

Real-time reverse transcriptase (RT)-PCR of pituitary GH, SRIH receptor subtypes, GHRH receptor (GHRH-R) and GHS-R mRNA

Total pituitary RNA (1 µg) was used as a template to generate cDNA by RT with random hexamer priming. The resultant cDNA was amplified using the LightCycler. Real-time PCR analysis was carried out with SYBR Green I and primers (for GH, sst3, sst4, sst5, GHRH-R and β-actin) or hybridization probes and primers (for sst1, sst2 and GHS-R). The sequences of primers for GH (GenBank accession no. V01237) were as follows: sense, 5'-CTG GCT GCT GAC ACC TAC AAA-3'; antisense, 5'-CAG GAG AGC AGC CCA TAG TTT-3'. Details of the procedure of the real-time PCR for the SRIH receptor subtypes, GHRH-R and GHS-R mRNA levels have been described previously (Park et al. 2004).

Statistical analysis

All data are expressed as means ± s.e.m. Comparisons between groups were made by Student’s t-test or ANOVA, and P<0.05 was considered significant. All comparisons were made between samples electrophoresed on the same gel (for RPAs) or real-time PCR run.

Results

HI STZ treatment resulted in hyperglycemia, hypo-insulinemia and significant weight loss (~27%), consistent with previous reports (Busiguna et al. 2000b, Marks et al. 1993, Olchovsky et al. 1990; Fig. 1). Circulating GH and IGF-I levels were also significantly decreased with HI STZ treatment and these changes were associated with a suppression of pituitary GH mRNA and hepatic IGF-I mRNA levels (Fig. 2). LO STZ-treated diabetic animals showed hyperglycemia, hypoinsulinemia (Fig. 1) and decreased circulating IGF-I levels (Fig. 2) similar to that observed in HI STZ-treated rats. In contrast, LO STZ-treated rats did not lose weight (Fig. 1) and circulating GH levels and pituitary GH and (diethyl pyrocarbonate-treated) water. The concentration and purity of RNA were determined by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at wavelengths of 260/280 nm.

**Figure 1** Effect of a single high dose of STZ (80 mg/kg, i.p.; n=5 vehicle, n=7 STZ) and multiple low doses of STZ (20 mg/kg, i.p., for 5 consecutive days; n=5 vehicle, n=7 STZ) on body weight (A), blood glucose (B) and serum insulin levels (C). Glucose and body weight were measured at the indicated day and insulin concentrations were assessed after animals were killed. Values are the means ± s.e.m. Values with a different subscript letter are significantly different (P<0.05).
hepatic IGF-I expression did not differ when compared with vehicle-treated controls (Fig. 2).

In HI STZ-treated diabetic animals, hypothalamic NPY mRNA levels were dramatically increased (355% of vehicle-treated controls; \(P < 0.01\)), while GHRH mRNA levels were decreased to 50% of controls (\(P < 0.01\); Fig. 3). In contrast, in LO STZ-treated animals, hypothalamic NPY mRNA levels were increased to only 158% of vehicle-treated controls (\(P < 0.05\); Fig. 3). Under these conditions, STZ failed to alter GHRH mRNA expression. SRIH mRNA levels in HI and LO STZ-treated animals did not differ from controls.

At the pituitary level HI STZ treatment resulted in an increase in GHRH-R and GHS-R mRNA levels (Fig. 4) and a decrease in mRNA levels of the SRIH receptor subtypes sst2, sst3 and sst5 (Fig. 5). In contrast, LO STZ treatment only suppressed sst3 mRNA levels compared with vehicle-treated control. In both induction schemes pituitary sst1 and sst4 mRNA levels were not altered.

**Discussion**

The results of the present study clearly demonstrate that changes in the GH axis following STZ-induced diabetes are dependent on the induction scheme used. HI STZ treatment led to a significant weight loss which was associated with changes in the GH axis similar to that...
Changes in GH axis in two models of STZ-induced diabetes

Effect of a single high dose of STZ (80 mg/kg, i.p., n=5 vehicle, n=7 STZ) and multiple low doses of STZ (20 mg/kg, i.p., for 5 consecutive days, n=5 vehicle, n=7 STZ) on pituitary GHRH-R and GHS-R mRNA levels. GHRH-R and GHS-R mRNA levels were measured by real-time RT-PCR and adjusted by β-actin. Data are expressed as percentage of respective vehicle-treated controls and are shown as the means ± S.E.M. **P<0.01.

Figure 4

Effect of a single high dose of STZ (80 mg/kg, i.p., n=5 vehicle, n=7 STZ) and multiple low doses of STZ (20 mg/kg, i.p., for 5 consecutive days, n=5 vehicle, n=7 STZ) on pituitary SRIF receptor subtype (sst1–sst5) mRNA levels. sst1–sst5 mRNA levels were measured by real-time RT-PCR and adjusted by β-actin. Data are expressed as percentage of respective vehicle-treated controls and are shown as the means ± S.E.M. *P<0.05, **P<0.01.

Figure 5

observed in the fasted rat (Park et al. 2004, Tannenbaum et al. 1979), which included suppression of hypothalamic GHRH, pituitary GH and hepatic IGF-I mRNA levels and decreased circulating GH and IGF-I, accompanied by a reciprocal shift in the expression of GH stimulatory and inhibitory receptors. In contrast, LO STZ treatment, which did not alter body weight, had little impact on the GH axis, with the exception that circulating IGF-I levels and pituitary sst3 mRNA levels were reduced compared with controls. Since both HI STZ and LO STZ treatment regimens resulted in insulinopenia, hyperglycemia and suppressed circulating IGF-I, we can conclude that these factors are not the primary modulators of the central and pituitary GH axis following STZ treatment. Given the striking similarity between the GH axis of rats treated with HI STZ and those that have been fasted, it is likely that changes associated with the catabolic condition play a key role in precipitating events leading to many of the changes in the GH axis following HI STZ treatment.

One event that may be central to HI STZ-induced changes in the GH axis is the dramatic rise in hypothalamic NPY. In the rat and mouse, food deprivation and HI STZ-induced diabetes lead to increased activity of NPY neurons within the arcuale nucleus (ARC) of the hypothalamus (Marks et al. 1993, Mizuno et al. 1999, Shimizu-Albergine et al. 2001, Vuagnat et al. 1998, White et al. 1990). In the rat, the fasting- and STZ-induced increases in NPY neuronal activity are associated with a decline in hypothalamic GHRH expression and suppression of pulsatile GH release (Busiguina et al. 2000a, Park et al. 2004, Tannenbaum 1981, Tannenbaum et al. 1979, 1986, 1989, White et al. 1990). NPY maybe a key inhibitor of the GH axis in that intracerebroventricular administration of NPY inhibits pulsatile GH release in rats and decreases GHRH mRNA levels in both rats and mice (Pierroz et al. 1996, Raposinho et al. 2000, 2001, Sainsbury & Herzog 2001, Suzuki et al. 1996). The significance of endogenous NPY in regulation of hypothalamic GHRH expression in catabolic states is supported by a recent report from our laboratory demonstrating that NPY-knockout mice do not exhibit fasting-induced suppression of GHRH mRNA (Park et al. 2005). In that NPY levels did not rise as dramatically LO STZ-treated rats, compared with HI STZ rats, we might speculate that these changes were not adequate to suppress GHRH expression.

Despite the reduction in GHRH expression following HI STZ treatment, we observed a reciprocal shift in the expression pattern of GH inhibitory and GH stimulatory receptors that would favor GH release and synthesis, similar to that observed following fasting (Park et al. 2004). As previously reported by Bruno et al. (1994), we also observed that HI STZ treatment resulted in a decline in pituitary sst2, sst3 and sst5 mRNA levels. In addition, we report for the first time that HI STZ treatment enhances pituitary GHRH-R and GHS-R mRNA levels. It is possible that a reduction in GHRH input to the pituitary is required for some of the changes in pituitary receptor expression following HI STZ treatment in that we have previously reported that GHRH acutely inhibits GHRH-R expression and stimulates sst2 expression in vitro (Kamegai et al. 1998a, Park et al. 2000).

Changes in pituitary expression of GH regulatory receptors in the HI STZ-treated rats are in line with reports demonstrating changes in pituitary sensitivity to their respective ligands. Specifically, pituitaries of HI
STZ-treated rats are more sensitive to the stimulatory actions of GHRH (Sheppard et al. 1989a, 1989b) and less sensitive to the inhibitory actions of SRIH (Bruno et al. 1994, Sheppard et al. 1989b) in vitro. Also, in vivo sensitivity to GHRH and a GHS-R ligand, GHRP-6, was negatively correlated with body weight in HI STZ-treated rats (Diz et al. 2003). In addition, STZ-treated mice have been reported to display enhanced GH responses to the GHS-R ligand ipamorelin (Johansen et al. 2003). Comparable changes in pituitary sensitivity to GH secretagogues are observed in patients with uncontrolled insulin-dependent diabetes (Catalina et al. 1998, Krassowski et al. 1988). Therefore, it is possible that the enhanced GH output observed in the insulin-dependent diabetic human (Catalina et al. 1998, Krassowski et al. 1988) may be related, at least in part, to changes in pituitary receptor expression that would favor GH release. It has also been hypothesized that the characteristic reduction in circulating IGF-I and insulin, both known inhibitors of GH synthesis and release (Yamashita & Melmed 1986a, 1986b), could enhance GH output in insulinopenic diabetes (Bereket et al. 1999). However, it should be noted that pulsatile GH release is blocked in the HI STZ-induced diabetic rat (Tannenbaum 1981), suggesting that the enhanced sensitivity to GH secretagogues is not sufficient to override metabolic changes in hypothalamic input in this animal model.

In the current study, circulating IGF-I levels were reduced in both HI STZ- and LO STZ-treated rats despite differential effects on pituitary GH synthesis and circulating GH levels, clearly demonstrating that a decrease in GH output is not required for the reduction in IGF-I output observed in the diabetic state. These observations are consistent with a previous report where circulating IGF-I levels are reduced in LO STZ-treated rats, without significant changes in circulating GH levels (Khamaisi et al. 2002). The liver is the primary source of IGF-I (Sjogren et al. 1999) and in the HI STZ-treated rat the fall in circulating IGF-I was reflected in a decrease in hepatic IGF-I mRNA levels. However, this relationship was not observed in the LO STZ-treated rat, where hepatic IGF-I mRNA levels did not differ from vehicle-treated controls, suggesting that a decrease in IGF-I gene expression is not the only component in modulating circulating IGF-I levels in diabetes. It has been reported that the clearance rate of IGF-I is increased in diabetic rats which may be due to a reduction in circulating IGF-I binding proteins (IGFBP3 and IGFBP4; Higaki et al. 1997, Khamaisi et al. 2002).

Studies using STZ, to induce diabetes in rodents, have provided a plethora of valuable information regarding the impact of insulinopenia and hyperglycemia on various physiologic endpoints. However, caution should be exercised when interpreting these results because STZ is a potent toxin that has been shown to damage multiple tissue types, in addition to its experimentally relevant effect on pancreatic β-cells. These include toxic effects on the neuroendocrine gastrointestinal tract which results in a decrease in gastric motility (Brenna et al. 2003), direct toxic effects on hepatocyte function which inhibits biliary excretions (Carnovale & Rodriguez Garay 1984) and toxic effects on the kidneys leading to urinary protein leakage (Palm et al. 2004). All of these effects could contribute to the acute weight loss observed in HI STZ-treated rats. Finally, the toxic effects of STZ can extend to the pituitary. Liu et al. (2002) have shown HI STZ (100–200 mg/kg) results in the blockade of GH secretory vesicle release and somatotrope rupture in rats suggesting that some of the reduction in pituitary GH output in HI STZ-treated rats could be due to toxic destruction of the somatotropes. Therefore, the time after STZ treatment and STZ dose are critical in differentiating between toxic and metabolic effects of STZ treatment on GH release, and LO STZ-induced diabetes may be a more suitable model.

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