Effects of a high-glucose environment on the pituitary growth hormone-releasing hormone receptor: type 1 diabetes compared with in vitro glucotoxicity

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Bédard K, Strecko J, Thériault K, Bédard J, Veyerat-Durebex C, Gaudreau P. Effects of a high-glucose environment on the pituitary growth hormone-releasing hormone receptor: type 1 diabetes compared with in vitro glucotoxicity. Am J Physiol Endocrinol Metab 294: E740–E751, 2008. First published February 19, 2008; doi:10.1152/ajpendo.00141.2007.—The present study investigated the effects of diabetes and high glucose on GHRH receptor (GHRH-R) mRNA and protein levels in the pituitary of diabetic rats 2, 21, and 60 days post-streptozotocin (post-STZ) administration. Two days post-STZ, the 2.5-kb GHRH-R mRNA transcript was increased. Twenty-one days post-STZ, both the 2.5- and 4-kb transcripts and a 72-kDa 125I-GHRH-GHRH-R complex were elevated. Sixty days post-STZ, the 4-kb transcript remained increased and the 45-kDa 125I-GHRH-GHRH-R complex (functional receptor) was decreased. Hypothalamic GHRH mRNA and serum total IGF-I levels were reduced at all three time points. To better understand the role of high glucose in the anterior pituitary of several mammalian species (14, 17, 18, 19, 26, 30, 52). The presence of ~2.5- and ~4-kb GHRH-R mRNA transcripts has been reported (26, 30) in the rat and mouse pituitary. Although the short and most abundant transcript generates the functional 423-amino acid GHRH-R (30, 33), the structure of the 4-kb transcript remains to be elucidated. Correlative evidence (15) suggests that an increased ratio of 4/2.5-kb transcript levels may reflect a shift of high- to low-affinity GHRH binding sites. Chemical cross-linking with 125I-GHRH(1–44)NH2 identified the presence of specific ~27- and ~42-kDa (16) or ~28-, ~47-, and ~65-kDa-labeled entities in the rat pituitary (6). The 47-kDa entity corresponds to the functional GHRH-R (30). The lowest molecular weight entity has been proposed to represent a degradation product of the 47-kDa entity, and the highest a glycosylated or dimeric form of GHRH-R (6, 7). The presence of GHRH and GHRH-R mRNAs has also been demonstrated in extrapituitary rat tissues (29), and the Henle’s loop of the renal medulla contains the highest GHRH-R mRNA levels (5). Pituitary and renal GHRH-R mRNA levels are regulated in a complex manner according to physiological and hormonal status (5, 15, 20, 21, 23, 32, 34, 37, 42, 51). Changes occurring in chronic pathologies, such as diabetes, induce disturbances of the somatotroph axis. In spontaneous diabetic rats and in the streptozotocin (STZ)-induced diabetic rat (STZ rat), a widely used model of type 1 diabetes, pulsatile GH secretion and GHRH-R mRNA levels have not yet been investigated. Therefore, the aims of the present study were to assess the dynamics of these changes in the anterior pituitary of 2-mo-old male rats 2, 21,
and 60 days post-STZ administration and to determine whether or not glucotoxicity exerts direct effects on GHRH-R levels and functionality using cultured anterior pituitary cells from 2-mo-old healthy male rats.

**MATERIALS AND METHODS**

**Animal handling and STZ administration.** Two-mo-old male Sprague Dawley rats (Charles River Canada, St. Constant, QC, Canada; Tables 1 and 2) were kept in temperature- (22°C), humidity- (65%), and lighting-controlled (12:12-h light-dark cycle, lights on at 0700) rooms. They had free access to standard rat chow (2018-Teklad Global, 18% protein rodent diet) and water. They were either housed individually in metabolic cages for the duration of the short-term studies (2 and 21 days) or maintained in metabolic cages for the first 2 wk of the long-term study (60 days) and 1 day/wk subsequently. The rats were randomly assigned to either control or diabetic groups. Body weight (BW), food and water intakes, and urine volume were assessed before STZ administration to ensure that both groups were identical.

Diabetes was induced by a single intraperitoneal injection of STZ (Sigma-Aldrich Canada, Oakville, ON, Canada) freshly solubilized in 0.5 ml of citrate buffer, pH 4.5 (Sigma-Aldrich Canada) (8). A dosage of 100 and 65 mg STZ/kg BW was administered for the short- and long-term studies, respectively, in the same buffer at a concentration of 0.5–0.8 mM. STZ was injected in cold Tris-acetate buffer containing 50 mM sucrose, and suspended by gentle manual scraping. They were centrifuged (800 × g), rinsed in 25 mM HEPES collecting buffer (57). The protocol was repeated by enzymatic digestion (57), centrifuged (800 × g); isolated pituitaries were rinsed and homogenized as previously described (51). Isolation and culture of anterior pituitary cells. Anterior pituitaries and livers were snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction (Table 1). For cross-linking, anterior pituitaries were rinsed and homogenized as previously described (Table 2) (6, 7). For cell culture, anterior pituitaries from 2-mo-old healthy male Sprague Dawley rats were cut into four to six pieces and rinsed in 25 mM HEPES collecting buffer (57). The protocol was modified by the Animal Care Committee of the Centre Hospitalier de l’Université de Montréal Research Center, in compliance with guidelines of the Canadian Council on Animal Care.

**Isolation and culture of anterior pituitary cells.** Cells were isolated by enzymatic digestion (57), centrifuged (800 g, 4 min, 4°C), and washed three times in DMEM supplemented with 6 mM d-glucose (DG), 10% fetal bovine serum, 1% penicillin [50 U/ml streptomycin (1.25 μg/ml); Invitrogen Canada, Burlington, ON, Canada], and 0.1% amphotericin (Sigma-Aldrich Canada). They were cultured for 16 h in this medium and subsequently for 1, 4, 24, or 48 h in new medium containing 6 or 33 mM DG or l-glucose (37°C, 95% air-5% CO2; ≈1 × 106 cells/ml). The culture media were changed after 24 h. The experimental concentrations of glucose did not vary significantly over time (10 ± 2), as determined by the hexokinase assay (Sigma-Aldrich Canada). In some internalization experiments, cells were cultured for 4 h in 6 mM DG and 16 h in the medium containing 6 or 33 mM DG or l-glucose and detached by gentle manual scraping. They were centrifuged (800 g, 5 min, 4°C), washed 3 times in cold Tris-acetate buffer containing 50 mM sucrose, and suspended in the same buffer at a concentration of 0.5–0.8 × 106 cells/ml (57). Cell viability, assessed by the trypan blue exclusion method, was ≥91% after harvesting.

**Detection of apoptosis and necrosis in cultured anterior pituitary cells.** Cells were plated on sterile glass coverslips in multiwell dishes at a density of ≈1 × 106 cells/ml culture medium, containing 6 mM DG, and cultured for 16 h. They were subsequently incubated for 4, 24, or 48 h in new medium containing 6 or 33 mM DG followed by a 20-min incubation at 4°C with 100 μl of YO-PRO-1 or propidium iode solution to label apoptotic and necrotic cells, respectively

**Table 1.** *BW, kidney wet weight, food and water intakes, and biochemical parameters of short- (2 and 21 days) and long-term (60 days) STZ rats used for GHRH-R mRNA study*

<table>
<thead>
<tr>
<th></th>
<th>2 Days Postinjection</th>
<th>21 Days Postinjection</th>
<th>60 Days Postinjection</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
<td>Control</td>
</tr>
<tr>
<td><strong>BW, g</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Day 0</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euthanasia</td>
<td>324 ± 5</td>
<td>331 ± 3</td>
<td>298 ± 8</td>
</tr>
<tr>
<td>Right kidney weight, g (euthanasia)</td>
<td>338 ± 3*</td>
<td>302 ± 4.9 ± c</td>
<td>397 ± 12*</td>
</tr>
<tr>
<td>Right kidney weight/100 g BW, g (euthanasia)</td>
<td>0.34 ± 0.01</td>
<td>0.41 ± 0.01*</td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td>Food intake, g/24 h</td>
<td>30 ± 2</td>
<td>28 ± 2</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>Euthanasia</td>
<td>31 ± 3</td>
<td>27 ± 5</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>Water intake, g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Day 0</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euthanasia</td>
<td>33 ± 2</td>
<td>39 ± 2</td>
<td>35 ± 2</td>
</tr>
<tr>
<td>Blood glucose, mg/dl (euthanasia)</td>
<td>33 ± 1</td>
<td>127 ± 10 ± c</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>Glucosuria, mg/dl (euthanasia)</td>
<td>98 ± 3</td>
<td>&gt;600F</td>
<td>125 ± 6</td>
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<tr>
<td>Urine volume, ml/24 h</td>
<td>12 ± 1</td>
<td>14 ± 1</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Euthanasia</td>
<td>9 ± 1</td>
<td>82 ± 8.3 ± c</td>
<td>16 ± 2</td>
</tr>
</tbody>
</table>

Values represent the mean ± SE of 7–9 rats/group. BW, body weight; STZ, streptozotocin; GHRH-R, growth hormone-releasing hormone receptor; ND, not determined. *Day 0 = STZ injection. *P < 0.05 compared with controls at day 0; †P < 0.01 compared with STZ rats at day 0; ‡P < 0.001 compared with controls at euthanasia; §P < 0.05 compared with controls at day 0; ¶P < 0.001 compared with controls at day 0; †P < 0.05 compared with STZ rats at day 0; ‡P < 0.01 compared with controls at day 0.*
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Table 2. BW, kidney wet weight, food and water intakes, and biochemical parameters of short- (2 and 21 days) and long-term (60 days) STZ rats used for \(^{125}\text{I}\)-GHRH cross-linking study

<table>
<thead>
<tr>
<th></th>
<th>Control 2 Days Postinjection</th>
<th>Diabetic 2 Days Postinjection</th>
<th>Control 21 Days Postinjection</th>
<th>Diabetic 21 Days Postinjection</th>
<th>Control 60 Days Postinjection</th>
<th>Diabetic 60 Days Postinjection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BW, g</strong></td>
<td>248 ± 3</td>
<td>245 ± 3</td>
<td>257 ± 4</td>
<td>260 ± 4</td>
<td>252 ± 4</td>
<td>250 ± 4</td>
</tr>
<tr>
<td><strong>Euthanasia</strong></td>
<td>259 ± 12</td>
<td>221 ± 3e</td>
<td>391 ± 7d</td>
<td>275 ± 9c</td>
<td>524 ± 14d</td>
<td>380 ± 17c</td>
</tr>
<tr>
<td><strong>Right kidney wet weight, g (euthanasia)</strong></td>
<td>0.97 ± 0.02</td>
<td>0.98 ± 0.03</td>
<td>1.28 ± 0.02</td>
<td>1.37 ± 0.05</td>
<td>1.48 ± 0.05</td>
<td>1.75 ± 1.01</td>
</tr>
<tr>
<td><strong>Right kidney wet weight/100 g BW, g (euthanasia)</strong></td>
<td>0.37 ± 0.01</td>
<td>0.44 ± 0.01c</td>
<td>0.33 ± 0.01</td>
<td>0.50 ± 0.06b</td>
<td>0.28 ± 0.01</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td><strong>Food intake, g/24 h</strong></td>
<td>26 ± 1</td>
<td>26 ± 1</td>
<td>28 ± 1</td>
<td>27 ± 1</td>
<td>27 ± 1</td>
<td>26 ± 1</td>
</tr>
<tr>
<td><strong>Water intake, g</strong></td>
<td>27 ± 1</td>
<td>26 ± 2</td>
<td>28 ± 1</td>
<td>44 ± 2ac</td>
<td>26 ± 1</td>
<td>47 ± 2ac</td>
</tr>
<tr>
<td><strong>Urine volume, ml/24 h</strong></td>
<td>9 ± 1</td>
<td>8 ± 1</td>
<td>7 ± 1</td>
<td>8 ± 1</td>
<td>9 ± 1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td><strong>Blood glucose, mg/dl (euthanasia)</strong></td>
<td>105 ± 3</td>
<td>&lt;400p</td>
<td>108 ± 4</td>
<td>&gt;500p</td>
<td>93.5 ± 2.45</td>
<td>&gt;400p</td>
</tr>
<tr>
<td><strong>Glucosuria, mg/dl (euthanasia)</strong></td>
<td>&lt;60</td>
<td>&gt;2,000p</td>
<td>&lt;60</td>
<td>&gt;2,000p</td>
<td>&lt;60</td>
<td>&gt;2,000p</td>
</tr>
</tbody>
</table>

Values represent the mean ± SE of 9 rats/group. 2 Days Postinjection: a = 0.001 compared with controls at euthanasia; b = 0.01 compared with controls at euthanasia; c = P < 0.001 compared with controls at euthanasia; d = P < 0.01 compared with controls at euthanasia. n = 2, membranes washed in standard saline citrate (SSC), pH 7.0 (2 × SSC/0.1% SDS, 23°C, 2 × 10 min; 1 × SSC/0.1% SDS, 65°C, 1–2 × 10 min; 0.5 × SSC/0.1% SDS, 65°C, 1–2 × 10 min). Stripped membranes were rehydrated with rat GAPDH and 28S rRNA probes (American Type Culture Collection, Rockville, MD). Amounts of GHRH-R mRNA were normalized in each lane with GAPDH, after assessment of the stability of GAPDH mRNA compared with 28S rRNA, in our experimental conditions. Specificity of the \(^{[32]}\)P RPR64 cDNA probe and linearity of the reaction were verified in each experiment’s (5) GHRH-R mRNA transcripts, and GAPDH mRNA and 28S rRNA levels were quantified by densitometry using an IS1000 digital imaging system (Alpha InfoTech, Montreal, QC, Canada). The intra-assay coefficient of variation of normalized GHRH-R mRNA signals was ≤10% in all experiments. The results were expressed as percentages of relative densities to those of control groups, using a fixed amount of total RNA as well as total RNA from each anterior pituitary.

Analysis of GHRH mRNA levels in the hypothalamus by real-time RT-PCR. Total RNA from the hypothalamus of control and STZ rats was extracted with TRIzol and resuspended in nuclease-free water (Ambion, Austin, TX). Quality control of RNA samples was assessed in a 2100 Bioanalyzer using the RNA 6000 Nano LabChip kit (Agilent Technologies, Mississauga, ON, Canada). The RNA integrity number of all samples was >9.0. Reverse transcription (RT) of 2 μg total RNA was performed with the SuperScript II RT kit and oligo(dT)\(_{12-18}\) primers according to the manufacturer’s protocol (Invitrogen Canada), including RNase H treatment. Rat GHRH and GAPDH (internal control) mRNA levels were determined by real-time PCR in separate tubes at 1:50 (GHRH) and 1:100 (GAPDH) dilution of the RT product and reagents from the Quantitect SYBR Green PCR kit. Reactions were performed in triplicate (final volume 25 μl), in the presence of 300 nM sense and antisense primers, in a Rotor Gene 3000 real-time thermal cycler (Montreal Biotech, Montreal, QC, Canada). No template and no amplification controls were included in each experiment to confirm the specificity of the reactions. The parameters included a single cycle at 95°C for 15 min followed by 45 cycles at 94°C for 15 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and a melting step from 72 to 99°C (ramping at 1°C/s). Specificity of the PCR products [GHRH forward: 5'-CATG-GACAGCAGCATCCAC-3'; GHRH reverse: 5'-AACCTTGATCTTGTGCTCGG-3' (nt 286-410, Genbank NM_031577); GAPDH forward: 5'-AGGGCTGCTTCCCTGTGAC-3'; GAPDH reverse: 5'-TGGGATGAACTCATACTGGAACATGTA-3' (nt 82-182, Genbank M17701)] was established by melting curve analysis and elec-
trophoresis on 2% agarose gel containing 0.5 μg/ml of ethidium bromide, with a 100-bp molecular weight standard (Invitrogen Canada). The results were analyzed with the Rotor-Gene application software. A five-point standard curve was performed for each gene tested, using 1:5 serial dilutions (1:5 to 1:3,125) of hypothalamus total RNA from 2-mo-old male rats. Hypothalamic GHRH mRNA relative levels were obtained in STZ rats by comparing with those of control rats, according to the Pfaffl (43) equation and using GAPDH as reference gene. The intra-assay coefficient of variation of GHRH and GAPDH cycle threshold (Ct) values was ≤2.5% in all experiments.

Analysis of GHRH-R mRNA levels in cultured anterior pituitary cells by real-time RT-PCR. Total RNA was extracted with TRIzol, and the samples were resuspended in nuclease-free water. Quality control of the RNA samples was assessed as above. The RNA integrity number of all samples was ≥9.8. RT of 2 μg total RNA and real-time PCR were also performed as in Analysis of GHRH mRNA levels in the hypothalamus by real-time RT-PCR, except that annealing was at 52°C for 30 s. Specificity of the PCR products [GHRH-R forward: 5′-CGGTTGTTCCCAAGGTGTT-3′; GHRH-R reverse: 5′-TAGGAGATGTGAGGCGCAAC-3′ (nt 578-736, GenBank NM_012850); GAPDH forward: 5′-GGGTTGTAACCCAGGAATAT-3′; GAPDH reverse: 5′-ACTGGTTGCTAGACGCCTC-3′ (nt 1242-1376, GenBank NM_017008)] was also determined as in Analysis of GHRH mRNA levels in the hypothalamus by real-time RT-PCR. A standard curve was performed for each gene tested using anterior pituitary total RNA from 2-mo-old male rats. Quantification of GHRH-R mRNA relative levels in cells cultured with 33 mM DG was performed as above. The intra-assay coefficient of variation of GHRH-R and GAPDH Ct values was ≤2.5% in all experiments.

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SE.

Internalization studies with Fluoro-GHRH in cultured anterior pituitary cells. Cells were incubated in Tris-acetate buffer containing 50 mM sucrose and 10 nM of the fluorescent GHRH agonist [Nps-carboxyfluoresceinyl-d-Ala²,Ala⁶,Lys²²]hGHRH (1-29)NH₂ (Fluoro-GHRH) (58) at 4°C for 40 min. Nonspecific binding was determined in the presence of 1 μM GHRH(1-29)NH₂. In all experiments, the final incubation volume with Fluoro-GHRH was 200 μl/assay point. After removal of the supernatant, the cells were washed once in cold Tris-sucrose buffer and either prepared for immediate visualization or warmed at 37°C for 90 min to allow Fluoro-GHRH internalization. This reaction was stopped by washing in cold Tris-sucrose buffer and rapid cooling on ice (57). Cells cultured in petri dishes were cytocentrifuged onto glass slides, and coverslips were apposed for microscopic visualization (57), and those cultured in multwell dishes were mounted onto glass slides. Slides were kept on ice in the dark for immediate fluorescence microscopy analysis. Total fluorescence was quantified using the pseudo color/segmented histogram function of MetaMorph, and the results were expressed in intensity units (57).

Determination of immunoreactive cAMP levels in cultured anterior pituitary cells. Anterior pituitary cells were cultured in multwell plates (~1.2 × 10⁷ cells·well⁻¹·150 μl⁻¹) in 6 mM DG for 16 h and subsequently for 4, 24, or 48 h in new medium containing 6 or 33 mM DG. At the end of the culture period, the cells were washed twice with DMEM-0.2% BSA and equilibrated 30 min with this medium before a 10-min stimulation (37°C) with rGHRH(1-29)NH₂ (0.1-100 nM) and 1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich Canada). Basal levels were determined in DMEM-0.2% BSA-1 mM 3-isobutyl-1-methylxanthine alone, which was used for GHRH serial dilutions. Forskolin (10 μM, <0.01% vol/vol DMSO; Sigma-Aldrich Canada) was the positive control in each experiment. Reactions were stopped by adding the lysis buffer supplied with the enzyme immunoassay kit. Total cAMP was measured using the nonacetylation procedure of the cAMP Direct Biotrak enzyme immunoassay kit (Amersham Biosciences). Optical densities were measured at 450 nm in a Bio-Rad model 3550 microplate reader. The intra-assay coefficient of variation was ≤10% in all experiments. Total basal and GHRH-stimulated levels of immunoreactive cAMP were expressed in picomoles per well (36). Net GHRH-induced cAMP levels (minus basal cAMP levels) were expressed as percentages of relative levels compared with those obtained in the presence of 10 nM rGHRH for each culture period.

Serum IGF-I immunooassay. Total IGF-I serum levels were determined with a commercial RIA kit (R&D Systems, Minneapolis, MN). All samples from each group were analyzed in duplicate in a single assay. The intra-assay coefficient of variation was 5.6%, and the lower sensitivity limit was 3.5 pg/ml.

Statistical analysis. The results were expressed as means ± SE. Data from experimental (diabetes, deglycosylation, radioligand, and glucose concentrations) and control conditions were compared by unpaired Student’s t-test, with Welch correction, when variances were significantly different. Changes in the level of lipoperoxidation over time in cells cultured with 33 mM DG as well as the combined effects of glucose concentration and Trolox or CHP on cell membrane lipoperoxidation were analyzed by ANOVA, followed by Dunnett’s multiple comparison test.

RESULTS

Characteristics of STZ rats. As shown in Tables 1 and 2, BW loss, hyperglycemia (>400 mg/dl), hyperglucosuria (>2,000 mg/dl), and polyuria characterized both short- and long-term diabetic status. At 2 days post-STZ administration, a few rats...
exhibited ketoneuria (40 mg/dl); however, at 21 and 60 days post-STZ, urinary ketone levels exceeded 60 mg/dl in all diabetic rats (data not shown). At 2, 21, and 60 days post-STZ, the BW of diabetic rats was decreased to 85–89 (P < 0.01 to P < 0.001), 67–70 (P < 0.001), and 60–73% (P < 0.001), respectively, of age-matched controls, and their right kidney wet weight/100 g BW was increased to 119–121, 132–151, and 170–171% (P < 0.01 to P < 0.001), respectively, of age-matched controls. Food intake per 24 h was unchanged at 2 days post-STZ but increased to 157–184% (P < 0.001) by 21 and 60 days postdiabetes induction compared with age-matched controls. Water intake and urine volume/24 h increased three to six times (P < 0.001) and to seven to 20 (P < 0.001) times, respectively, by 2–60 days post-STZ. No deaths occurred over the 2- to 60-day experimentation periods.

**GHRH mRNA levels in the hypothalamus of STZ rats.** As shown in Fig. 1, hypothalamic GHRH mRNA levels decreased to 60, 49, and 67% of age-matched controls (P < 0.05 to P < 0.01) by 2, 21, and 60 days post-STZ, respectively.

**GHRH-R mRNA transcript levels in the anterior pituitary of STZ rats.** GHRH-R mRNA levels were analyzed by Northern blotting, allowing the quantification of 2.5- and 4-kb transcripts. Both were present in the gland of control and diabetic rats 2, 21, and 60 days post-STZ, as reported previously in healthy young rats (15, 30). Levels of GHRH-R mRNA transcripts showed several changes according to the length and severity of diabetes (Fig. 2). When expressed per 12 μg total RNA, 2.5-kb GHRH-R mRNA transcript levels increased to 140 and 150% of age-matched controls by 2 (Fig. 2, A and B) and 21 (Fig. 2, A and C) days post-STZ, respectively (P < 0.05). They returned to control levels by 60 days post-STZ (Fig. 2, A and D), indicating a decrease compared with shorter periods of diabetes. A significant increase of 4-kb transcript levels was observed at 21 and 60 days post-STZ compared with age-matched controls [280 (P < 0.001) and 170% (P < 0.05), respectively; Fig. 2, A, C, and D]. Combined levels of 2.5- and 4-kb transcripts were increased to 150 and 180% (P < 0.01) by 2 and 21 days post-STZ and returned to normal by 60 days postdiabetes induction (P < 0.01). Finally, the 4/2.5-kb ratio of transcripts was not modified by 2 days post-STZ but increased to 220 (P < 0.001) and 170% (P < 0.05) at 21 and 60 days post-STZ, respectively. Anterior pituitary total RNA content did not change by 2 days post-STZ but decreased to 40–43% of controls (P < 0.001) at 21 and 60 days postdiabetes, confirming our visual observation of a diminished anterior pituitary size at these time points. Therefore, when GHRH-R mRNA levels were expressed as a function of total RNA content per anterior pituitary, differences were seen mainly at the 2.5-kb GHRH-R mRNA transcript levels, which decreased to 63 and 52% (P < 0.05) at 21 and 60 days post-STZ, respectively, compared with age-matched controls (data not shown).

**125I-GHRH-GHRH-R complex levels in the anterior pituitary of STZ rats.** The presence of GHRH-R labeled with 125I (125I-GHRH) was determined in the presence of 1 μM rGHRH(1-29)NH2, representing 60–75% of total binding for the 72- and 45-kDa entities and 40–55% of total binding for the 24-kDa entity. The level of complexes showed a number of changes during the evolution of diabetes (Fig. 3, A–D). The density of the 72-kDa complex increased to 260% (P < 0.05) by 21 days post-STZ (Fig. 3C). This increase did not remain significant (160%) at 60 days post-STZ (Fig. 3D) compared with age-matched controls. The density of the 45-kDa complex decreased to 45% of that in age-matched controls (P < 0.05) by 60 days post-STZ (Fig. 3, A and D). The level of the 24-kDa complex did not change in the course of diabetes. A low level of specific ~150-kDa complex (3–7% of total densities) was also detected, but it did not change with diabetes. When complex levels were expressed as a function of total protein content per anterior pituitary, differences were seen mainly at the level of the 45-kDa complex. A decrease to 40–45% of age-matched control levels was observed at 21 and 60 days post-STZ (P < 0.05; data not shown).

**Serum IGF-I levels in STZ rats.** Immunoreactive IGF-I levels declined to 71, 24, and 63% of age-matched controls 2 [STZ: 1,004 ± 99 ng/ml; controls: 1,410 ± 95 ng/ml (P < 0.05)], 21 [STZ: 539 ± 54 ng/ml; controls: 2,231 ± 135 (P < 0.001)], and 60 days [STZ: 1,339 ± 139 ng/ml; controls: 2,098 ± 146 (P < 0.01)] post-STZ, respectively.

**Deglycosylated 125I-GHRH-GHRH-R complex levels in the anterior pituitary of normal rats.** Using a panel of enzymes, including N-glycosidase F, endo-α,β-N-acetylglactosaminidase, and α2,3,6,8,9-neuraminidase, deglycosylation experiments in tissue preparations from 2-mo-old healthy rats showed that the low abundance of 153 ± 5-kDa 125I-GHRH-GHRH-R-specific complexes was reduced to 105 ± 5 kDa (P < 0.05; Fig. 4). No other change was seen in the profile of 125I-GHRH-GHRH-R cross-linked complexes in our experimental conditions, even when optimal enzyme concentrations were increased by twofold.

**Effect of radioligand concentration on the density of 125I-GHRH-GHRH-R complexes in the anterior pituitary of normal rats.** Our preliminary data in baby hamster kidney cells, stably transfected with human or rat GHRH-R, revealed significant increases of ~100 and ~150-kDa-specific 125I-GHRH-GHRH-R complexes when 500 pM 125I-GHRH was used instead of 100 pM in cross-linking experiments (56). This approach was taken to induce changes of rat anterior pituitary 125I-GHRH-GHRH-R complexes, compatible with receptor dimerization/oligomer-
Cross-linking experiments, performed with 100 or 500 pM $^{125}$I-GHRH, revealed changes in the relative intensity of 25- and 69-kDa-specific complexes compared with the 48-kDa complex (Fig. 5, A and B). A decrease in the intensity of the 25 ± 1-kDa band to 63% ($P < 0.02$) and an increase in the density of the 69 ± 2-kDa band to 160% ($P < 0.01$) were observed without any significant change in the level of other molecular weight entities, suggesting a dimerization of the functional GHRH-R with a truncated form.

Effect of glucose concentration on apoptosis, necrosis, and membrane lipoperoxidation in cultured anterior pituitary cells from normal rats. The percentages of fluorescent apoptotic cells after a 16- to 24-h culture period were 13 ± 1 and 9 ± 4% with 6 and 33 mM DG, respectively, whereas those of necrotic cells were 12 ± 1 and 10 ± 1%, respectively. The percentages of fluorescent apoptotic cells, after a 48-h culture period, were 5 ± 3 and 6 ± 1% with 6 and 33 mM DG, respectively, and that of necrotic cells was 6 ± 1% with both glucose concent-

**Fig. 2.** GHRH receptor (GHRH-R) mRNA transcript levels in the anterior pituitary of diabetic (Dia) and control (C) rats at 2, 21, and 60 days post-STZ administration. Samples of 12 µg total RNA from each rat pituitary were analyzed by Northern blotting. A: autoradiographic representation of GHRH-R mRNA transcripts and GAPDH mRNA levels from a representative Dia and C rat at 2, 21, and 60 days post-STZ administration. B, C, and D: Dia GHRH-R mRNA normalized levels were expressed as %relative density compared with the age-matched control group established at 100%. Results represent the mean ± SE of 1 experiment performed once, using 8 STZ and 8 C rats. GHRH-R mRNA levels were normalized with GAPDH. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with respective control levels.

**Fig. 3.** $^{125}$I-GHRH-GHRH-R complex levels in anterior pituitary homogenates of Dia and C rats at 2, 21, and 60 days post-STZ administration. Samples of 375 µg of protein from each rat anterior pituitary were analyzed after cross-linking with 5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and SDS-PAGE. A: autoradiographic representation of $^{125}$I-GHRH-labeled complexes from a representative C and Dia rat at 21 and 60 days post-STZ administration. T, total binding; NS, nonspecific binding assessed in the presence of 1 µM rat (r)GHRH(1–29)NH$_2$. B, C, and D: Dia levels of $^{125}$I-GHRH-labeled complexes were expressed as %relative density compared with the age-matched C group established at 100%. Results represent the mean ± SE of 3 independent experiments performed in duplicate, using 2 C and 3 STZ rats per experiment. *$P < 0.05$ compared with respective control levels.
Effect of glucose concentration on GHRH-induced cAMP levels in cultured anterior pituitary cells from normal rat. Neither basal nor forskolin-stimulated levels of immunoreactive cAMP were altered after 4-, 24-, and 48-h culture in the presence of 33 mM DG compared with those in 6 mM DG (data not shown). The rGHRH(1-29)NH₂-induced response was not affected after a 4-h culture in the presence of 33 mM DG (Fig. 9A). However, after 24-h culture in 33 mM DG, cAMP levels induced by 0.1, 1, 10, and 100 nM rGHRH(1-29)NH₂ decreased to 47 (P < 0.05), 63 (P < 0.05), 59 (P < 0.01), and 55% (P < 0.01), respectively (Fig. 9B), compared with cells incubated with 6 mM DG. Similarly, after 48-h culture in 33 mM DG, cAMP levels induced by 1, 10, and 100 nM rGHRH(1-29)NH₂ declined to 67 (P < 0.05), 63 (P < 0.01), and 48% (P < 0.01) of control values, respectively (Fig. 9C).

Effect of glucose concentration on GHRH-R mRNA levels in cultured anterior pituitary cells from normal rats. GHRH-R mRNA levels were quantified by real-time RT-PCR, allowing for high sensitivity with small amounts of cells. Normalized GHRH-R mRNA levels were not changed in cells incubated with 33 mM DG for 4 h (Fig. 7) compared with GHRH-R mRNA levels in cells incubated with 6 mM DG. They were decreased to 77 and 91% after 24- and 48-h incubation in the presence of 33 mM DG and returned to control levels after 72-h incubation (Fig. 7).

Effect of glucose concentration on Fluo-GHRH internalization in cultured anterior pituitary cells from normal rats. After 40-min incubation at 4°C with Fluo-GHRH, a low level of fluorescence was observed at the surface of cells incubated with 6 or 33 mM DG (Fig. 8A). Because Fluo-GHRH internalization is time and temperature dependent, the incubation time of 90 min at 37°C was selected as optimal on the basis of our previous results in freshly dispersed anterior pituitary cells (57). When pituitary cells are warmed at 37°C for 90 min, a punctate pattern appears with moderate to high levels of fluorescence due to internalization and concentration of Fluo-GHRH in organelles (57). In pituitary cells cultured for 16–24 h in the presence of 6 mM DG, these changes were correlated with an increase in total fluorescence to 160% (P < 0.001) and Fluo-GHRH internalization [Fig. 8A (a) and B]. In cells cultured in the presence of 33 mM DG, no significant increase of fluorescence and internalization was observed [Fig. 8A (b)], and the total fluorescence level was not different from that with 6 mM DG at 4°C (Fig. 8B).

Fig. 5. Effect of radioligand concentration on 125I-GHRH-GHRH-R complex levels in normal rat anterior pituitary. Cross-linking and SDS-PAGE were performed as described in MATERIALS AND METHODS and Fig. 3. A: densities of 125I-GHRH-labeled complexes are expressed per 200 μg of total protein as %density relative to that obtained for the 48-kDa band. B: autoradiographic representation of 125I-GHRH-labeled complexes from 2-mo-old rat anterior pituitary. Results represent the mean ± SE of 2 independent experiments performed in duplicate (interassay apparent molecular weight variation <6%). *P < 0.05; **P < 0.01 for the 69- and 25-kDa bands, respectively, when homogenates were incubated with 500 pM radioligand compared with 100 pM.
DISCUSSION

Type 1 diabetes, induced in rats by a single injection or multiple injections of STZ, has been employed in numerous studies to identify the in vivo effects of uncontrolled hyperglycemic states. Several perturbations of the GHRH-GH-IGF-I somatotroph axis have been identified in STZ rats. However, the regulation of anterior pituitary GHRH-R at both the mRNA and protein levels has not been investigated in relation to the duration and severity of diabetes and hyperglycemia. Moreover, these changes have not been compared with those obtained after the direct exposure of normal anterior pituitary cells to high glucose.

The hypothalamo-pituitary GHRH-GHRH-R system plays an important role in stimulating GH secretion and synthesis (2, 54) as well as somatotroph proliferation (4). In the pituitary of STZ rats, GHRH-R mRNA levels were submitted to a time-dependent regulation. At 2 days postdiabetes induction, a time at which no change in daily food intake was observed and a slight decrease of BW was present, hypothalamic GHRH mRNA levels were already greatly decreased. In parallel, the 2.5-kb anterior pituitary GHRH-R mRNA transcript, coding for the functional GHRH-R, was increased, but pituitary total RNA content was unchanged. Levels of the 45-kDa 125I-GHRH-GHRH-R complex, representing the functional GHRH-R (6, 7) and that of other 125I-GHRH-GHRH-R complexes, were also unchanged. Since hypothalamic GHRH content has been reported to increase significantly only during the early stage of diabetes (2–4 days post-STZ) (26), enhanced GHRH tone could initially contribute to upregulate the 2.5-kb pituitary GHRH-R mRNA transcript (17). Alternatively, since hypothalamic functions are perturbed in STZ rats (20, 47), increased GHRH content could result from a decreased capacity of hypothalamic neurons to release the peptide, leading to compensatory up-regulation of the 2.5-kb transcript. Because serum total IGF-I was already decreased, by 2 days post-STZ it may be proposed to result primarily from dampening of pituitary GHRH-R activation by GHRH.
decreased, as reported previously by Busiguina et al. (8). Under the same conditions of STZ administration, we observed that pituitary GHRH-R mRNA transcripts and the 4/2.5-kb ratio were increased, suggesting a decrease in the number of high-affinity GHRH binding sites, as reported in aged rats (15). Although the structure of the 4-kb transcript is currently unknown, our preliminary evidence (56) suggests that it could represent a 2.5-kb GHRH-R mRNA transcript bound to an adapter protein, thus preventing optimal translation. In parallel, a trend toward a lower reduction than at 21 days post-STZ was observed. The pituitary 2.5-kb GHRH-R mRNA transcript was decreased and the 4-kb transcript remained elevated, resulting in an increase of 24/45-kDa dimer formation in such a perturbed cellular environment. In the present study, we have shown that dimerization of GHRH-R entities can occur in the anterior pituitary cells of healthy rats. In addition, recent data from McElvaine and Mayo (31), showing a dimerization of the human GHRH-R with a COOH-terminal truncated variant, strengthen this hypothesis. Therefore, in the anterior pituitary of diabetic rats, dimerization of GHRH-R species could participate in the decreased responsiveness to GHRH, leading to a reduction of anterior pituitary size and a substantial decline of total IGF-I circulating levels.

Sixty days post-STZ, BW and food intakes remained altered, and hypothalamic GHRH mRNA levels were still reduced, but a trend toward a lower reduction than at 21 days post-STZ was observed. The pituitary 2.5-kb GHRH-R mRNA transcript was decreased and the 4-kb transcript remained elevated, resulting in an increase of the 4/2.5-kb ratio and a substantial diminution of the 45-kDa 125I-GHRH-GHRH-R complex. Interestingly, total IGF-I circulating levels were partly restored compared with those measured at 21 days post-STZ.

Our present results in a rat model of STZ-induced diabetes and weight loss do not entirely support the hypothesis that changes in anterior pituitary GHRH-R arise exclusively from nonfasting weight loss. Rather, it suggests that GHRH-R mRNA transcripts and functional protein are regulated in a complex manner in models of STZ-diabetic rats and that hyperglycemia plays a role in addition to that of other metabolic and hormonal factors. Apart from GHRH, a number of hormonal regulators could participate in the changes of GHRH-R levels in the pituitary. Thyroid hormones and glucocorticoids have been shown to increase GHRH-R mRNA levels (23, 32, 34, 37, 42, 51). In STZ rats, circulating levels of glucocorticoids and thyroid hormones are increased (9) and decreased (39, 46), respectively. Since physiological levels of both are required for optimal GHRH-R expression in the pituitary (23, 32, 34, 37, 42, 51), changes in their circulating levels with diabetes could contribute to some of the alterations observed in GHRH-R mRNA levels. However, the dynamics of changes in the concentration of these serum hormones will have to be correlated with binding proteins and nuclear receptors to clearly establish a causal role in pituitary GHRH-R dysregulation. Increased IGF-I (50) and insulin levels (28) have been shown to negatively regulate pituitary GHRH-R mRNA, and a diminution of their circulating levels in STZ rats could also be involved. However, Kim et al. (20) have proposed that the catabolic effect of weight loss, but not insulopenia, reduced circulating levels of IGF-I, or hyperglycemia, is the primary regulator of pituitary GHRH-R mRNA levels in STZ-induced diabetes.

To more directly study the effect of a high-glucose environment on pituitary GHRH-R and exclude the confounding
actions of regulators of the receptor (GHRH, steroid and thyroid hormones, IGF-I, insulin) present in vivo in STZ rats, studies in cultured rat anterior pituitary cells were performed. Exposure to a high glucose level did not promote the necrosis or apoptosis of anterior pituitary cells but increased oxidative stress, as measured by membrane lipoperoxidation. In these conditions, GHRH-R mRNA levels were decreased particularly after 24-h culture, Fluo-GHRH internalization was drastically reduced, and GHRH-induced cAMP production was diminished, indicating lowered levels of functional GHRH-R as seen in diabetic rats 60 days post-STZ. This could be related in part to a transient modification of GHRH-R mRNA levels and concurrent changes in functional receptor levels due to dimerization and/or other structural alterations occurring in a glucotoxic/prooxidant environment. These in vitro results are consistent with those of Renier and Serri (45) showing that GHRH-induced GH release was decreased after exposure of cultured normal rat anterior pituitary cells to high glucose (22 mM, 72 h).

Our study is the first report to show a direct effect of high glucose on GHRH-R, indicating that it can contribute to a regulation of GHRH-R expression and functionality and contribute to decrease GHRH sensitivity in somatotrophs, leading to a decline of GH secretion in vitro (45) and in vivo (35, 38, 53, 55). These results may reconcile some of the divergent reports on in vivo and in vitro somatotroph sensitivity to GHRH from a large variety of single time point evaluations, ranging from 2- to 60-day post-STZ administration and exclusively analyzing GHRH-R mRNA but not functional protein levels. The molecular and cellular events by which glucotoxicity-induced oxidative stress affects pituitary GHRH-R will now deserve investigation. Interestingly, in primary-cultured rabbit proximal tubule cells, the angiotensin type 1 receptor, another member of the G protein-coupled receptor family, is downregulated (mRNA and protein levels) by high glucose (25 mM) via a PKC-oxidative stress-transforming growth factor-

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