Increased apoptosis of lactotrophs in streptozotocin-induced diabetic rats is followed by increased proliferation

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Abstract

Poorly controlled diabetes mellitus can result in decreased prolactin production and thus problems with lactation, reproduction, and other physiological processes. This may be due to a loss of lactotrophs, as we have previously shown that long-term (8 weeks) poorly controlled streptozotocin-induced diabetes results in increased death of lactotrophs and that this most likely occurs through the activation of caspase-8 and the extrinsic cell death cascade. However, cell proliferation is also increased in the anterior pituitary at this time, although the cell type undergoing this proliferation and whether it is a response to the increased cell death remains unknown. In order to determine the time-course of increased cell death and proliferation in the anterior pituitary and if this is related to changes in tumor necrosis factor (TNF)- α , a cytokine involved in the activation of the extrinsic cell death

pathway, rats were killed at 1, 4, 6, and 8 weeks after the induction of diabetes. Cell death was significantly increased after 4 weeks, as was caspase-8 activation, although circulating levels of TNF- α were increased as early as 1 week. Pituitary levels of TNF- α did not change significantly until 8 weeks after diabetes onset. Similarly, Western-blot analysis of proliferating cell nuclear antigen showed that anterior pituitary cell proliferation increased significantly 8 weeks after diabetes onset, with the majority of proliferating cells, as detected by BrdU incorporation, corresponding to lactotrophs. These results suggest that the increased death of lactotrophs in poorly controlled diabetic rats is followed by increased proliferation of this cell type, even when no treatment is given.

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Introduction

It is well known that prolactin is important for lactation and reproductive functions; however, it is also involved in the regulation of growth, development and metabolism, electrolyte balance, immune responses, behavior, and stress responses (Bole-Feyso *et al.* 1998). Hence, modifications in prolactin secretion may have wide-ranging effects in both males and females. Circulating prolactin levels have been shown to be reduced in poorly controlled diabetes mellitus (Ikawa *et al.* 1992, Montelongo *et al.* 1992, Ostrom & Ferris 1993, Valimaki *et al.* 1991) and diabetes can result in reduced lactating capabilities in both women and experimental animals (Ikawa *et al.* 1992, Lau *et al.* 1993, Ostrom & Ferris 1993, Neville & Morton 2001). In addition, decreased prolactin levels in diabetic animals have been associated with steroidogenic defects (Sudha *et al.* 1999) and a decreased stress response (Ratner *et al.* 1991), including the response to hypoglycemia (Kinsley *et al.* 1996).

We have previously demonstrated that there is a reduction in the number of lactotrophs in the rat pituitary after 8 weeks of poorly controlled diabetes (Arroba *et al.* 2003), which could explain the decrease in circulating prolactin levels in

these animals. This loss of lactotrophs is due, at least in part, to increased apoptosis of this cell type involving activation of caspase-8 (Arroba *et al.* 2005). Caspase-8 is the prototypical caspase of the extrinsic or death receptor pathway of apoptosis that is activated by an extracellular insult or ligand binding to members of the tumor necrosis factor (TNF)- α receptor superfamily (Boldin *et al.* 1996, Wajant 2002). Indeed, circulating TNF- α levels are reported to be elevated in diabetic patients, as well as in streptozotocin-induced diabetic rats (El-Seweid *et al.* 2002, Jain *et al.* 2002), and this cytokine is implicated in apoptosis during diabetes (Chen & Goeddel 2002) and in lactotrophs (Candolfi *et al.* 2002), suggesting its possible involvement in the loss of this cell type in diabetes.

The number of cells for any specific cell type in a tissue is the result of the balance between proliferation and death. Hence, the reduction in the number of lactotrophs in the anterior pituitary of diabetic rats could also involve a decrease in proliferation. However, at 8 weeks of diabetes, pituitary levels of proliferating cell nuclear antigen (PCNA) are increased (Arroba *et al.* 2003). It is possible that this increased proliferation is in response to the increased cell death and hence, be specific to the dying cell type or types. Thus,

the aims of this study were to: (1) determine when programmed cell death begins in the pituitary of rats with poorly controlled diabetes mellitus; (2) associate this increase in cell death with temporal changes in circulating and pituitary TNF- α levels and caspase-8 activation; and (3) analyze when the increase in cell proliferation begins and determine if lactotrophs are affected.

Materials and Methods

Materials

Electrophoresis reagents were from Bio-Rad. All chemicals were purchased from Sigma or Merck unless otherwise noted.

The antibody for prolactin was from the National Hormone and Peptide Program (Cat. No. AFP65191, Torrance, CA, USA). The antibody to caspase-8 or Fas-associated death domain (FADD)-like ICE/MORT-1-associated Ced3 homologue (FLICE) was purchased from NeoMarkers (Cat. No. RB-1200, Fremont, CA, USA), PCNA from Signet Laboratories (Cat. No. 523-01, Dedham, MA, USA), phosphorylated-histone-H3 (phospho-H3) from Upstate Biologicals (Cat. No. 06-570, Lake Placid, NY, USA), and actin from Santa Cruz Biotechnology (Cat. No. sc-1616, Santa Cruz, CA, USA). BrdU was purchased from Sigma, and the antibody toward BrdU was from DAKO (Cat. No. M0744, Glostrup, Denmark).

Animals

Adult male Wistar rats were injected (i.p.) with 70 mg/kg streptozotocin (STZ; Sigma). Control rats received vehicle only. Blood glucose concentrations were measured in the morning via tail puncture (Glucocard Memory 2; Menarini Diagnostic, Florence, Italy) and the animals were considered to be diabetic if they maintained mean glucose levels > 300 mg/dl. The rats were killed in the morning by decapitation at 1, 4, 6, and 8 weeks after diabetes onset, and the pituitaries were immediately removed and frozen on dry ice. Trunk blood was collected, allowed to clot, and centrifuged. The serum was separated and stored at -20°C . The anterior pituitary was processed for protein extraction as indicated later and the samples were stored at -80°C . The rats were treated according to the European Community laws for animal care, and the studies were approved by the ethics committee of the institution. All the experiments were repeated at least twice. In all ELISA and western analyses, four diabetic rats/group and eight control rats were used. In immunohistochemistry studies, three rats per group were used.

The following groups of rats were established: diabetic for 1 week (DB1W), diabetic for 4 weeks (DB4W), diabetic for 6 weeks (DB6W), and diabetic for 8 weeks (DB8W). Control rats were killed at each time-point, but because no differences were found between control rats at the different time-points

in any of the studied variables (data not shown), control data were pooled for statistical analysis.

BrdU injection

In a separate experiment to label proliferating cells, diabetic and control rats were prepared as above and BrdU was injected (100 mg/kg per day; i.p.) on the 4 consecutive days prior to killing at 8 weeks after the induction of diabetes ($n=4$ per group).

ELISA cell death detection

This assay was carried out according to the manufacturer's (Cat. No. 1544676, Roche Diagnostics) instructions. Briefly, each anterior pituitary was homogenized in 300 μl incubation buffer, placed on ice for 1 h, centrifuged at 1200 g for 5 min at 4°C , and the supernatant collected. Microtiter plates were prepared by adding 100 μl coating solution (anti-histone antibody) to each well and incubated for 1 h at room temperature. The coating solution was removed and 200 μl incubation buffer added and incubated for 30 min at room temperature. The wells were then rinsed twice and the samples (25 μl sample + 75 μl buffer) added and incubated for 90 min at room temperature. After washing, 100 μl conjugate solution (anti-DNA-peroxidase) were added and incubated at room temperature for 90 min. After washing, 100 μl substrate solution were added and mixed until the color developed adequately (approximately 15 min). The samples were measured at 405 nm on an automatic microplate analyzer (Biotek Instruments, Inc., Winooski, VT, USA). Each sample was measured in duplicate in each assay. Background measurements were made and this value subtracted from the mean value of each sample. Induced cell death in cultured cells (GH3) was used as positive control. This assay had a detection limit of approximately 50 dead cells/well and the results were normalized to protein levels in each sample and are reported as relative levels of cell death compared with controls. The inter- and intra-assay coefficients of variation (CV values), as determined by repeated assay of test samples with low, intermediate and high levels, were 8.5 and 4.3% respectively.

Immunoblotting

For western blotting, tissue was homogenized on ice in 300 μl radio-immunoprecipitation assay buffer (RIPA) lysis buffer with an EDTA-free protease inhibitor cocktail (Roche Diagnostics) and then centrifuged at 12 000 g for 5 min at 4°C to remove the insoluble material. Clear supernatants were transferred to a new tube to measure protein content by using Bio-Rad Laboratories, Inc. protein assay. Protein (30–60 μg) was resolved using 12% SDS-PAGE and then transferred onto PVDF membranes (Bio-Rad). Membranes were blocked with Tris-buffered saline containing 5% (w/v) nonfat dried milk and incubated with the primary antibody

at a dilution of 1:1000. Membranes were subsequently washed and incubated with the corresponding secondary antibody conjugated with peroxidase at a dilution of 1:2000 (Pierce, Rockford, IL, USA). Bound peroxidase activity was visualized by chemiluminescence (Perkin-Elmer Life Sciences, Boston, MA, USA) and quantified by densitometry using Bio-1D (Vilber Lourmat, Marne La Vallée, France). For each assay, the results were normalized to control levels and protein-loading variability normalized by rehybridizing the membrane with antibody to actin (Sigma).

TNF- α ELISA

This assay was carried out according to the manufacturer's (Cat. No. HK102, HyCult Biotechnology b.v., The Netherlands) instructions. Briefly, serum was diluted 1:4 in dilution buffer as recommended by the manufacturer. For pituitary protein extracts (in RIPA as described earlier), samples from each experimental group were diluted 1:1, 1:2, 1:4, and 1:8 and assayed in duplicate to determine the linearity of this assay for tissue extracts, as well as the optimal dilution factor. For the results reported here, all pituitary extracts were measured at a dilution of 1:4 in dilution buffer. Duplicate 100 μ l aliquots of each standard and sample were transferred to assigned wells and incubated for 2 h at 37 °C. Wells were washed four times with 200 μ l wash buffer and 100 μ l tracer added to each well and incubated for 1 h at room temperature. Wells were washed four times and 100 μ l streptavidin-peroxidase conjugate added and incubated for 1 h at room temperature. After washing, 100 μ l substrate solution were added and the samples incubated for 30 min in the dark at room temperature. The reaction was stopped by adding 100 μ l stop solution. The absorbance was measured at 450 nm. Each sample was measured in duplicate in each assay. Background measurements were made and this value subtracted from the mean value of each sample. Serum levels are reported as picogram per milliliter and pituitary values were normalized to picogram per milligram of protein. This assay had a minimum detection level of 10 pg/ml and a measurable concentration range up to 2000 pg/ml. Assay of individual pituitary extracts at different dilutions showed less than 15% variability between those results that were within the measurable concentration range. When assayed at the above-mentioned dilution (1:4), all samples were within the measurable range of the standard curve. The inter- and intra-assay coefficient of variation values for repeated measure of samples were 7.6 and 5.3% respectively.

Immunohistochemistry

Immunohistochemistry was performed on frozen 12 μ m cryostat sections, fixed in 4% paraformaldehyde (20 min, room temperature), washed in TBS+0.1% BSA and 0.1% Triton X-100 (this buffer was used for all subsequent washes), and blocked in TBS containing 3% BSA and 1%

Triton X-100 for 2 h. For immunofluorescence, sections were left overnight in a humid chamber at 4 °C with the primary antibodies for prolactin (1:2000) plus H3 (1:100) in blocking solution. Afterwards, the sections were washed and incubated with a biotin-conjugated anti-rabbit (Pierce; 1:1000) and an anti-guinea pig antibody conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA; 1:2000) for 90 min. After washing (3 $\times$ 20 min each), sections were then incubated in streptavidin-Alexa Fluor 633 conjugate (Molecular Probes, 1:2000) for 1 h. After the addition of the Alexa fluorochromes, all incubations were carried out in the dark. The resulting signal was visualized by using a confocal microscope (Leica, Madrid, Spain).

For double labeling of BrdU and prolactin, the sections were fixed in 4% paraformaldehyde (in 0.1 M PBS) for 20 min at room temperature and then washed in PBS (3 $\times$ 15 min each). The sections were incubated with 1% hydrogen peroxide in 100% methanol for 20 min, washed twice for 20 min in PBS, incubated in 2 M HCl for 30 min, and washed in PBS twice for 20 min each. Afterwards, sections were blocked in TBS buffer with 3% BSA and 1% Triton X-100. This buffer was used in the following washes and incubations. The sections were incubated overnight at 4 °C with the primary antibodies, PRL (1:2000) and BrdU (1:100). The following day, sections were washed and incubated with a biotin-conjugated anti-mouse antibody and an anti-guinea pig antibody conjugated to HRP (Pierce, 1:1000) for 90 min. Peroxidase and biotin activities were revealed, using 3,3'-diaminobenzidine with 0.01% hydrogen peroxide and 4-nitroblue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl-phosphate toluidine (Roche Diagnostic) as chromogens. Immunostained sections were observed by optical microscopy (Zeiss Axioplan, Germany).

Concentration curves were performed to determine the appropriate concentration of primary antibodies. In all immunohistochemistry assays, omission of primary antibody resulted in no specific labeling.

Statistical analysis

All results were performed at least two times. All results were reported as mean $\pm$ S.E.M. All western-blot results were normalized to actin levels in the same sample and then to mean control levels in each assay (control=100%). One-way ANOVA was used for comparisons between groups, followed by a Scheffé's *F*-test. Significance was chosen as $P < 0.05$.

Results

Serum glucose concentrations

At the time of death, glucose concentrations in all STZ-treated groups were higher than in the control group

Table 1 Plasma glucose concentrations in control and diabetic rats. Results are mean ± s.e.m. *n*=4 for each diabetic group and *n*=8 for controls

	Glucose concentrations (mg/dl)
Control	69.95 ± 1.9
DB1W	520.5 ± 30.9*
DB4W	524.6 ± 37.3*
DB6W	541.1 ± 21.4*
DB8W	478.6 ± 41.6*

*ANOVA: *P*<0.001. DB1W, 1 week of diabetes; DB4W, 4 weeks of diabetes; DB6W, 6 weeks of diabetes; DB8W, 8 weeks of diabetes.

(*P*<0.001) with no difference between the diabetic groups (Table 1).

Pituitary cell death

Mean cell death, as determined by ELISA for histones, was significantly increased in the pituitary of diabetic rats compared with controls 4 weeks after the onset of diabetes and increased further during weeks 6 and 8 (Fig. 1A; ANOVA: *P*<0.001).

Figure 1 (A) Relative levels of cell death in the pituitary of diabetic rats as determined by ELISA. *ANOVA: *P*<0.001. (B) Mean total prolactin levels per pituitary in control and diabetic rats. *ANOVA: *P*<0.001. C, control; DB1W, 1 week of diabetes; DB4W, 4 weeks of diabetes; DB6W, 6 weeks of diabetes; DB8W, 8 weeks of diabetes. Results are mean ± s.e.m. *n*=4 for each diabetic group and *n*=8 for controls.

Intracellular prolactin levels

Total prolactin levels per pituitary decreased throughout the study, becoming significantly lower than controls at 6 weeks (Fig. 1B; ANOVA: *P*<0.01).

Caspase-8 activation in the anterior pituitary

The fragmented form of caspase-8 was significantly higher in pituitaries of DB4W and DB8W compared with controls (Fig. 2; ANOVA: *P*<0.01), while the proform increased significantly at 8 weeks of diabetes (ANOVA: *P*<0.002).

TNF-α levels

Serum TNF-α levels were significantly elevated compared with controls throughout the study. However, at 6 weeks there was a significant decrease in circulating TNF-α levels, which rose again at 8 weeks of diabetes evolution (Fig. 3A; ANOVA: *P*<0.0001).

TNF-α protein levels in the anterior pituitary were not significantly modified until after 8 weeks of diabetes, when there was a significant increase (Fig. 3B; ANOVA: *P*<0.001).

Proliferation

Western-blot analysis showed a significant decrease in relative PCNA levels 1 week after streptozotocin treatment that returned to control levels at 4 and 6 weeks and increased significantly at 8 weeks of diabetes (Fig. 4; ANOVA: *P*<0.01).

This increase in proliferation was confirmed by immunostaining for phospho-H3. The number of phospho-H3 positive cells was greater in the anterior pituitary of diabetic animals compared with controls after 8 weeks of diabetes, but not at 6 weeks (Fig. 5A–C). No colocalization of H3 with prolactin, growth hormone, thyroid stimulating hormone, luteinizing hormone, or adrenocorticotrophic hormone was found (data not shown).

Fewer BrdU immunostained cells (dark blue) were found in control (Fig. 5D) compared with DB8W pituitaries (Fig. 5E). Cells immunostained for BrdU could be found to colocalize with prolactin immunostaining (brown; Fig. 5E, solid arrows). Although the majority of BrdU positive cells coexpressed prolactin, some did not (Fig. 5D and E, hollow arrows).

Discussion

Cell turnover in the anterior pituitary can be altered by different events, including poorly controlled diabetes mellitus. We previously reported that at 8 weeks of diabetes evolution lactotrophs undergo increased cell death, most likely through activation of caspase-8 (Arroba *et al.* 2003, 2005). Here, we show that after 1 week of diabetes pituitary cell death is not significantly modified, but by 4 weeks a significant increase in cell death is detected and this

Figure 2 (A) Relative levels of the intact 55 kDa form of caspase-8 in the pituitary of control and diabetic rats. ANOVA: $P < 0.002$. *Significant difference compared with controls. (B) Relative levels of the cleaved 25 kDa form of caspase-8 in the pituitaries of control and diabetic rats. ANOVA: $P < 0.01$. *Significant difference compared with controls. C, control; DB1W, 1 week of diabetes; DB4W, 4 weeks of diabetes; DB6W, 6 weeks of diabetes; DB8W, 8 weeks of diabetes. Results are mean $\pm$ S.E.M. $n = 4$ for each diabetic group and $n = 8$ for controls.

continued to increase throughout the 8 weeks of study. The decline in intracellular prolactin levels temporally parallels these changes, suggesting that the loss of lactotrophs is involved in the decrease in circulating prolactin levels. It is possible that there is also a decrease in prolactin synthesis per lactotroph, although previous reports suggest that this is not the case (Boujon *et al.* 1995).

The increase in cell death at 4 weeks of diabetes is coincident with increased activation of caspase-8; however, although cell death continues to be increased at 6 weeks, activation of caspase-8 decreases at this time-point, and then rises again at 8 weeks. It is possible that more than one mechanism of cell death is involved, although we are unable to detect activation of the intrinsic cell death pathway or downstream effector caspases-3, -6, or -7 after 8 weeks of diabetes (Arroba *et al.* 2005) or earlier during the evolution of diabetes (unpublished observation).

Circulating TNF- α levels have previously been reported to be elevated in diabetic patients and streptozotocin-induced diabetic rats (El-Seweid *et al.* 2002, Jain *et al.* 2002) and this cytokine is implicated in activation of the extrinsic cell death pathway (Gupta 2001), apoptosis during diabetes (Chen & Goeddel 2002), and apoptosis of lactotrophs (Candolfi *et al.* 2002). However, circulating TNF- α levels were increased as early as 1 week after the onset of diabetes, while caspase-8 activation and increased cell death was not detected until the fourth week. It is possible that there is a delayed response to TNF- α as has been shown in other paradigms (Augstein *et al.* 2004, Jaita *et al.* 2005). Circulating TNF- α levels and activation of caspase-8 both decreased significantly at 6 weeks and increased at 8 weeks, suggesting that they may be correlated, although a direct relationship cannot be established from

these studies. The underlying cause of these dynamic changes is not apparent. In the diabetic rats, there was no change in mean glucose levels at any time throughout the study, suggesting that a change in glucose metabolism is not responsible for the inflection in the elevated levels of circulating TNF- α at 6 weeks. Furthermore, although body weight and weight gain of diabetic rats were reduced compared with controls, these parameters were stabilized in the diabetic rats at this moment of the study and cannot explain the changes in circulating TNF- α levels.

In the pituitary, TNF- α is produced by macrophages, as well as by somatotrophs, while lactotrophs do not appear to synthesize this cytokine (Arras *et al.* 1996). Pituitary TNF- α levels were not increased until after 8 weeks of diabetes, and hence, cannot account for the increase in caspase-8 activation at 4 weeks, although it is possible that the second rise in caspase-8 activation at 8 weeks could be related to the increase in local TNF- α . Local production of this cytokine is suggested to be a stress response (Kariagina *et al.* 2004) and this delayed increase in local TNF- α concentrations could indicate that this gland has reached a critical moment in response to the chronic poor metabolic control.

We previously reported an increase in PCNA levels in the rat pituitary after 8 weeks of diabetes (Arroba *et al.* 2003). We hypothesized that this increase in proliferation could be in response to the increased cell death in an attempt to maintain the cell population. This would suggest that proliferation should occur either simultaneously or after cell death has increased. Indeed, all markers of cellular proliferation, PCNA, phosphorylated histone-H3 and BrdU incorporation, were increased only after 8 weeks of diabetes, while significant cell death was detected as early as 4 weeks. In addition, this increase in proliferation was found to affect

Figure 3 Mean levels of (A) TNF- α in serum (*ANOVA: $P < 0.0001$) and (B) the anterior pituitary (*ANOVA: $P < 0.001$) of control and diabetic rats. C, control; DB1W, diabetic for 1 week; DB4W, diabetic for 4 weeks; DB6W, diabetic for 6 weeks; DB8W, diabetic for 8 weeks. Results are mean $\pm$ S.E.M. $n = 4$ for each diabetic group and $n = 8$ for controls.

lactotrophs, the cell type most affected by the increase in cell death. Phosphorylated histone-H3 immunoreactivity did not colocalize with any anterior pituitary hormone. This is most likely due to the fact that this marker of mitosis labels cells that were dividing at the moment of death of the animal and at this stage of the cell cycle secreted hormones are not yet expressed. In contrast, BrdU is detected in cells that divided after BrdU injection, which in this study is up to 4 days prior to being processed. This would allow time for cell differentiation and hormone expression, allowing identification of proliferating cell types.

Cell turnover in the normal adult anterior pituitary is influenced by physiological signals including age, sex, and a variety of hormones (Takahashi & Kawashima 1982,

Figure 4 Mean levels of proliferating cell nuclear antigen (PCNA) in the anterior pituitary of control and diabetic rats at different times of diabetes evolution as measured by western blot. ANOVA: $P < 0.01$. C, control; DB1W, diabetic for 1 week; DB4W, diabetic for 4 weeks; DB6W, diabetic for 6 weeks; DB8W, diabetic for 8 weeks. Results are mean $\pm$ S.E.M. $n = 4$ for each diabetic group and $n = 8$ for controls.

Hoeffler & Frawley 1987, Carbajo-Pérez & Watanabe 1990, Ellerkmann *et al.* 1991, Kineman *et al.* 1992, Oishi *et al.* 1993, Kulig *et al.* 1998, Nolan *et al.* 1998). Changes in the balance of these signals can modify the cellular make-up of the anterior pituitary, with lactotrophs being one of the most highly affected cell types (Takahashi & Kawashima 1982, Hoeffler & Frawley 1987, Ellerkmann *et al.* 1991, Kineman *et al.* 1992, Oishi *et al.* 1993). One of the most clear examples is the dramatic increase in lactotroph proliferation during pregnancy and lactation (Goluboff & Ezrin 1969), followed by massive cell death at the termination of pregnancy or lactation (Haggi *et al.* 1986, Ahlbom *et al.* 1998). The increase in lactotroph cell death upon weaning has been associated with changes in Bax, Bcl-2, and p53 expression (Ahlbom *et al.* 1998). The Bcl-2 family of proteins is also involved in estrogen and thyroid hormone modulation of lactotroph turnover (Kulig *et al.* 1998), while bromocryptine, a dopamine antagonist, induced death of lactotrophs involves p53 (Aoki *et al.* 2001). Thus, physiological turnover of lactotrophs appears to involve the mitochondrial/intrinsic intracellular pathway.

However, little is known regarding pituitary cell death in pathological situations. Nitric oxide-induced pituitary cell death is reported to involve activation of caspases-3 and -9 (Velardez *et al.* 2004), but whether this involves lactotrophs

Figure 5 Immunohistochemistry for phosphorylated histone 3 (p-H3) to label mitotic cells in the anterior pituitary of (A) control rats and (B) rats that were diabetic for 6 weeks and (C) 8 weeks. Increased labeling was seen only at 8 weeks of diabetes evolution. $n=3$ in each experimental group. Immunolabeling for BrdU (blue) and prolactin (brown) in (D) control, and (E) diabetic rats that were injected with BrdU on 4 consecutive days prior to death after 8 weeks of diabetes showed that there was an increase in proliferating cells in the diabetic rats. The majority of the BrdU positive cells were immunopositive for prolactin. Solid arrows indicate cells that were positive for both BrdU and prolactin. Hollow arrows indicate BrdU positive cells. $n=4$ in each experimental group.

remains to be determined. In contrast to physiological situations of anterior pituitary cell turnover, diabetes induced lactotroph cell death appears to involve the extrinsic cell death pathway and activation of caspase-8. Increased levels of TNF- α may be involved as this cytokine has been shown to induce apoptosis of lactotrophs (Candolfi *et al.* 2002).

The results reported here demonstrate that cell death in the anterior pituitary begins as early as 1 month after the onset of diabetes, involving activation of caspase-8. A delayed increase in proliferation occurs after 8 weeks of diabetes and affects primarily lactotrophs, indicating that the rise in proliferation is specific to the cell type undergoing increased death. The underlying cause of these changes and whether insulin treatment can prevent or reverse these modifications remains unknown. However, it is clear that between 4 and 8 weeks of diabetes evolution there is a critical period when dynamic changes in anterior pituitary cell turnover occur and that lactotrophs are highly affected.

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