Cell-specific expression of X-linked inhibitor of apoptosis in the anterior pituitary of streptozotocin-induced diabetic rats

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Abstract

Cell death is increased in the anterior pituitary of poorly controlled diabetic rats, but anti-apoptotic mechanisms are also activated. We hypothesized that specific cell types are selectively protected against diabetes-induced cell death. To determine when anti-apoptotic mechanisms are activated, streptozotocin-induced diabetic rats were killed after 1, 4, 6 and 8 weeks of evolution. Anterior pituitaries were processed for western blot analysis to determine changes in the intrinsic cell death pathway and upstream kinases involved in cell protection mechanisms. An increase in cell death was detected by ELISA at 4 weeks of diabetes. TUNEL labelling demonstrated that this corresponded to death of primarily lactotrophs, a few somatotrophs, and no thyrotrophs, corticotrophs or gonadotrophs. Levels of phosphorylated (p) Akt were increased at 1 week of diabetes, while pERK1/2 levels increased at 4 weeks and pJNK at 6 weeks. Activation of

caspase 3 decreased and anti-apoptotic members of the Bcl-2 protein family increased as early as 1 week after diabetes onset. These changes were coincident with increased IGF-I receptor levels. Levels of X-linked inhibitor of apoptosis protein (XIAP) increased significantly after 6 weeks of diabetes, as did activation of nuclear factor (NF)κB. Double immunohistochemistry indicated that XIAP was expressed in less than 1% of lactotrophs and gonadotrophs, approximately 50% of somatotrophs and more than 90% of corticotrophs and thyrotrophs. These results suggest that some cell survival mechanisms are rapidly activated in the anterior pituitary, even before increased cell death can be detected, while others are more delayed. Furthermore, both pituitary cell death and expression of protective mechanisms such as XIAP are cell-type specific.

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Introduction

Poorly controlled diabetes mellitus type 1 results in numerous secondary complications, including disruption of all endocrine axes (Bestetti *et al.* 1985, Valimaki *et al.* 1999). Modifications in the cellular composition of the anterior pituitary could be involved in these endocrine changes, as this gland undergoes increased cell death in poorly controlled diabetes mellitus (Arroba *et al.* 2003, 2005). Furthermore, this increased cell death is cell-type specific, with lactotrophs being one of the most highly affected cell types (Arroba *et al.* 2003). Indeed, turnover of lactotrophs and somatotrophs has been reported to be more susceptible than that of other pituitary cell types to changes in the hormonal environment (Oishi *et al.* 1993).

There are two major apoptosis signalling pathways (Gupta 2001), the death receptor or extrinsic pathway (Ashkanazi & Dixit 1998) and the mitochondrial or intrinsic pathway (Zamzami & Kroemer 2001). At 8 weeks of poorly controlled diabetes, caspase 8, the prototypical caspase of the extrinsic cell death pathway (Boldin *et al.* 1996, Wajant 2002), is

activated and colocalizes to lactotrophs (Arroba *et al.* 2005), possibly explaining their increased death rate. However, pituitary levels of proteins involved in the intrinsic cell death pathway, including members of the Bcl-2 family and the effector caspases 3, 6 and 7, are either unchanged or balanced towards cell survival (Arroba *et al.* 2005). Likewise, levels of X-linked inhibitor of apoptosis (XIAP) are increased in the anterior pituitary, but not in lactotrophs (Arroba *et al.* 2005), suggesting that it may be involved in the protection of other cell types.

At least two intracellular signalling pathways, orchestrated by phosphatidylinositol-3 kinase/Akt and mitogen-activated protein kinases (MAPKs), are involved in cell survival mechanisms. Extracellular signal-regulated kinase (ERK) is a member of the MAPK family and in general is activated by growth factors and transduces signals to promote cell proliferation and survival (Yoon & Seger 2006). Indeed, both MAPKs and Akt have been implicated in the secondary effects of diabetes, with inhibition of Akt and ERKs promoting cell death in different tissues (Allen *et al.* 2005, Zdychova & Komers 2005). Conversely, JNK, another

MAPK family member, becomes activated by a variety of stress signals and is involved in the induction of apoptotic cell death (Bonny *et al.* 2005). However this kinase is also associated with activation of inhibitors of caspases (IAPs), including XIAP, through NF κ B (Sanna *et al.* 1998, Stehlik 1998, Xiao *et al.* 2002, Zou *et al.* 2004).

Insulin-like growth factor (IGF)-I modulates pituitary cell turnover and function (Oomizu *et al.* 1998, Fernandez *et al.* 2004, 2005) and acts through the Akt and MAPK signalling pathways. As circulating IGF-I levels are reduced in diabetes (Olchovsky *et al.* 1991, Busiguina *et al.* 1996) and decreased IGF-I signalling has been implicated in increased apoptosis in various tissues in response to diabetes (Russell *et al.* 1999, Gerhardinger *et al.* 2001), it is conceivable that it plays an important role in the modification of cell turnover in the pituitary of diabetic rats.

Activation of cell protection mechanisms in the anterior pituitary may occur in response to the increase in cell death or they could be rapidly activated in the diabetic process in an attempt to inhibit or delay cell death. Furthermore, although the intrinsic cell death pathway is not activated at 8 weeks of diabetes, it is conceivable that it is activated earlier and is then inhibited. Therefore, the aim of this study was to determine the time course of activation of the intracellular signalling pathways associated with cell survival mechanisms and of the intrinsic cell death pathway in the pituitary of rats with poorly controlled diabetes mellitus. To determine which cell types may be selectively protected against diabetes-induced cell death, colocalization studies of anterior pituitary hormones and XIAP were performed.

Materials and Methods

Material

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

Antibodies to caspase 7, Bcl-2 α and Bax were purchased from NeoMarkers (Fremont, CA, USA). Antibodies for XIAP and caspase 3 were from BD Biosciences (San Diego, CA, USA). Antibodies for cleaved caspases 7 and 9 were obtained from MBL (Watertown, MA, USA). The antibodies for phosphorylated (p) I κ B α , pAkt, pERK1/2 and pBad were from Cell Signaling (Beverly, MA, USA). The antibodies for actin, Akt, Bad and Bcl-X_L were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody for pJNK was from Promega and ERK1/2 was from Upstate (Lake Placid, NY, USA). The IGF-I β receptor antibody was from Chemicon (Temecula, CA, USA). The antibody for flice-like inhibitory protein (FLIP) was from Sigma. The antibodies for prolactin, growth hormone (GH), thyroid-stimulating hormone (TSH), adrenal cortical-stimulating hormone (ACTH) and luteinizing hormone (LH) were from the National Hormone and Peptide Program (Torrance, CA, USA).

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) to confirm the diabetic state and rats were considered to be diabetic if they maintained mean glucose levels >300 mg/dl. Rats were killed in the morning by decapitation at 1, 4, 6 and 8 weeks and the pituitaries were immediately removed and frozen on dry ice. 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. For immunohistochemistry studies, three rats per group were analysed and for western blot analysis, four rats/each diabetic group and eight controls were used for each analysis.

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 as no differences were found between the control rats at different time-points in any of the studied variables (data not shown), control data were pooled for statistical analysis.

Insulin ELISA

This assay was performed according to the manufacturer's instructions (Linco Research, Inc., St Charles, MO, USA). Briefly, microtitre plates coated with pre-titred monoclonal mouse anti-rat insulin antibody were washed three times with wash buffer (50 mM Tris-buffered saline (TBS) containing Tween 20). Ten microlitres of prediluted standards, quality control samples and serum samples were added to wells in duplicate. Detection antibody (biotinylated anti-insulin, 80 μ l) was added and the plate sealed and incubated at RT for 2 h while shaking. The wells were then washed and 100 μ l enzyme solution (streptavidin-horseradish peroxidase conjugate) added and incubated (30 min). After washing, 100 μ l substrate solution (3,3',5,5'-tetramethylbenzidine) were added. After colour development (approximately 15 min), 100 μ l stop solution (0.3 M HCl) were added and the plates read at 450 and 590 nm on an automatic plate reader (Tecan infinite M200, Grödig Austria). The intra- and inter-assay coefficients of variation were 1.9 and 7.6 respectively. The limit of sensitivity of this assay is 0.2 ng/ml. For those diabetic samples below the limit of detection, 0.2 ng/ml was used for statistical analysis.

Cell death detection ELISA

This photometric enzyme immunoassay, for the *in vitro* quantification of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) found after

induced cell death, was performed according to the manufacturer's (Cell Death Detection ELISA, Roche) instructions and with the following modifications. Tissue 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. The microtitre plates were prepared by adding 100 µl coating solution (anti-histone antibody) to each well and incubating for 1 h at RT. The coating solution was removed and 200 µl incubation buffer added to each well, covered and incubated for 30 min at RT. The wells were then rinsed three times and the samples (25 µl sample + 75 µl incubation buffer) added and incubated for 90 min at RT. This dilution was chosen after preliminary assays showed it to be the most adequate for detecting changes. After washing, 100 µl conjugate solution (anti-DNA-peroxidase) was added. The wells were covered and incubated at RT for 90 min. After washing, 100 µl substrate solution were added, mixed and incubated for 15 min. The resulting colour was then measured at 405 nm on an automatic microplate analyser (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. This assay has a detection limit of approximately 50 dead cells/well and 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 were 8.5 and 4.3% respectively.

Immunoblotting

For western blotting, the anterior pituitary was homogenized on ice in 300 µl 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 and protein content measured using Bio-Rad Laboratories, Inc Protein Assay. Protein (30–60 µg) was resolved using 10 or 12% SDS-PAGE and then transferred onto PVDF membranes (Bio-Rad). Membranes were blocked with Tris-buffered saline containing 5% (w/v) non-fat dried milk, except for phosphorylated proteins where TBS containing 5% BSA was used, 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 Vallee, France). For each assay, the results were normalized to control levels and protein loading variability normalized by rehybridizing the membrane with antibody to actin.

Immunohistochemistry

Immunohistochemistry was performed on frozen 12 µm cryostat sections of pituitaries of rats that were diabetic for 8 weeks and controls mounted on positively charged slides. Sections were fixed in 4% paraformaldehyde (w/v) for 20 min at RT, 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. Sections were left overnight in a humid chamber at 4 °C with the primary antibodies, prolactin (1:2000), GH (1:1000), TSH (1:1000), LH (1:1000) or ACTH (1:1000) plus XIAP (1:500) in blocking solution. Then, sections were washed and incubated with an anti-rabbit antibody conjugated to Alexa Fluor 633 (Molecular Probes, Eugene, OR, USA; 1:2000) and anti-guinea pig antibody conjugated to Alexa Fluor 488 (Molecular Probes; 1:2000) for 90 min. Slides were incubated in the dark after the addition of the fluorochromes. The resulting signal was visualized using a confocal microscope (Leica, Madrid, Spain). Concentration curves were performed to determine the optimal concentration of primary antibody. No specific labelling was observed when the primary antibody was omitted.

To determine the approximate number of double-labelled cells, images were captured using a 63× objective and stored. In each field, the number of cells immunopositive for each hormone and those positive for hormone and XIAP were determined. Six sections per animal were analysed and approximately four fields per section. The mean percentage of double-labelled lactotrophs was then determined.

Terminal dUTP nick-end labelling (TUNEL) plus immunohistochemistry

Cell death detection assays were performed following the manufacturer's instructions (Roche). Briefly, after fixation in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), sections of pituitaries of rats which were diabetic for 8 weeks and controls were washed three times in buffer and incubated for 30 min with a 0.1% sodium citrate, 0.1% Triton X-100 solution to increase tissue permeability. Slides were again washed three times with buffer, and incubated with TUNEL solution for 90 min at 37 °C in a humid chamber in the dark. After washing, the slides were incubated with antibody towards prolactin (1:2000), GH (1:1000), TSH (1:1000), LH (1:1000), ACTH (1:1000) or caspase 8 (1:250) in TBS containing 3% BSA and 1% Triton X-100 and left for 48 h at 4 °C. The slides were incubated with Alexa Fluor anti-fluorescein-488 and -633-conjugated goat anti-guinea pig IgG (Molecular Probes) in blocking buffer both at a dilution of 1:1000. Finally, the slides were again washed three times before mounting in glycerol. Results were visualized with a confocal microscope and colocalization calculated as described earlier.

Figure 1 Photomicrographs of terminal dUTP nick-end labelling (TUNEL) and immunohistochemistry for (A) prolactin (PRL), (B) growth hormone (GH), (C) thyroid-stimulating hormone (TSH), (D) luteinizing hormone (LH) and (E) adrenocorticotrophin-stimulating hormone (ACTH). In the anterior pituitary of rats that were diabetic for 8 weeks TUNEL labelling (F) and caspase 8 (Casp-8) immunolabelling could be found to colocalize in the nucleus (G). The majority of the cells that were TUNEL positive expressed PRL, with a few expressing GH. No TSH, LH or ACTH immunopositive cells were found to be TUNEL positive. Closed arrows indicate TUNEL- and hormone-positive cells. Open arrows indicate cells that are only TUNEL positive.

Statistical analysis

The mean value of each animal was used for statistical analysis and 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 *F* test. Significance was chosen as $P < 0.05$.

Results

Serum glucose and insulin levels

At the time of death, glucose levels in all STZ-treated groups were higher than in the control group ($P<0.001$) with no difference between the diabetic groups. Insulin levels remained decreased throughout the study (ANOVA $P<0.0001$) and no significant differences were found between the diabetic groups (Table 1).

Cell death

Cell death in the pituitary increased significantly at 4 weeks of diabetes and remained increased throughout the rest of the study with maximum cell death being detected after 8 weeks of diabetes (control 100 ± 9 ; DB1W 123 ± 15 ; DB4W 248 ± 22 ; DB6W 283 ± 17 ; DB8W 336 ± 24 ; ANOVA $P<0.001$).

At 8 weeks of diabetes, lactotrophs were the most affected cell type (Fig. 1A), as previously reported (Arroba *et al.* 2003, 2005). In the pituitaries of diabetic rats, less than 10% of TUNEL-positive cells corresponded to GH-producing cells (Fig. 1B). No TSH-, ACTH- or LH-producing cells were found to colocalize with TUNEL labelling (Fig. 1C–E).

We previously reported that at 8 weeks of diabetes, no activation of the effector caspases 3, 6 or 7 was found (Arroba *et al.* 2005). As caspase 8 can also act as an effector caspase (Benchoua *et al.* 2002), we analysed whether this caspase was localized in the nucleus of apoptotic cells. As seen in Fig. 1H, TUNEL-labelled nuclei could be found to colocalize caspase 8.

Protein kinases

The levels of pAkt in the pituitaries of diabetic rats increased significantly at 1 week and increased further at 6 and 8 weeks, such that the highest levels were found at 8 weeks (Fig. 2A; ANOVA $P<0.05$). No differences in non-phosphorylated Akt levels were found.

The levels of pERK1/2 were significantly increased in the pituitary of diabetic rats beginning at 4 weeks and remained so throughout the rest of the study (Fig. 2B; ANOVA

Table 1 Glucose and insulin concentrations in control and diabetic rats. $n=4$ for each diabetic group and $n=8$ for controls.

	Glucose concentration (mg/dl)	Insulin concentration (ng/ml)
Control	72.4 ± 2.0	1.75 ± 0.18
DB1W	$515.6 \pm 31.2^*$	$0.23 \pm 0.03^{\dagger}$
DB4W	$519.6 \pm 36.6^*$	$0.35 \pm 0.13^{\dagger}$
DB6W	$538.1 \pm 31.8^*$	$0.28 \pm 0.06^{\dagger}$
DB8W	$482.6 \pm 46.4^*$	$0.33 \pm 0.05^{\dagger}$

*ANOVA $P<0.001$ and † ANOVA $P<0.0001$ compared with control. DB1W, 1 week of diabetes; DB4W, 4 weeks of diabetes; DB6W, 6 weeks of diabetes; DB8W, 8 weeks of diabetes.

Figure 2 (A) Relative mean levels of phosphorylated (p) Akt; *ANOVA $P<0.05$ compared with control. (B) p-ERK1/2; † ANOVA $P<0.001$ compared with control. (C) p-JNK; *ANOVA $P<0.05$ compared with control, in the pituitary of diabetic rats as determined by western blot. C, control; DB1W, 1 week of diabetes; DB4W, 4 weeks of diabetes; DB6W, 6 weeks of diabetes; DB8W, 8 weeks of diabetes. $n=4$ for each diabetic group and $n=8$ for controls.

$P < 0.001$). Non-phosphorylated ERK1/2 levels did not differ between groups.

After 6 weeks of diabetes, pJNK levels increased significantly in the anterior pituitary (Fig. 2C; ANOVA $P < 0.05$).

Activation of caspases of the mitochondrial pathway

Levels of the proform of caspase 9 were significantly higher at 4 and 8 weeks of diabetes (Fig. 3A; ANOVA $P < 0.01$), while the fragmented form (35 kDa) was significantly higher only in the pituitaries of DB8W (Fig. 3B; ANOVA $P < 0.001$).

Levels of the proform of caspase 3 were significantly higher at 8 weeks of diabetes (Fig. 3C; ANOVA $P < 0.05$), while levels of fragmented caspase 3 (17 kDa) were lower in the pituitaries of groups DB1W, DB4W and DB8W compared with control rats (Fig. 3D; ANOVA $P < 0.01$).

Procaspase 7 levels increased significantly at 1 week of diabetes and remained so throughout the study (ANOVA $P < 0.01$). No significant differences in the fragmented form of caspase 7 (20 kDa) were found between groups (Fig. 3E and F respectively).

Bcl-2 protein family

Mean concentrations of the anti-apoptotic protein, Bcl-2 α , were significantly higher in diabetic rats at 4 and 8 weeks, while Bcl-X_L, also anti-apoptotic, increased at 8 weeks (Table 2; ANOVA $P < 0.01$). Mean levels of Bax, a pro-apoptotic protein, were not significantly different between the groups at any time-point. Mean levels of BAD, also pro-apoptotic, were lower in the pituitary of diabetic rats at 1 week compared with controls and remained so throughout the study (ANOVA $P < 0.01$). The phosphorylated or inactivated form of this protein was higher in the pituitaries of diabetic rats at 6 and 8 weeks compared with controls (Table 2; ANOVA $P < 0.01$).

IGF-I receptor

The concentration of IGF-I receptor increased in the pituitary of diabetic rats at 1 week of diabetes and remained so throughout the study (Fig. 4A; $P < 0.01$).

Apoptosis inhibitors

No significant difference in the mean concentration of the protein FLIP was found between control and diabetic rats at any

time-point (controls 100 ± 13.5 vs DB1W 75.3 ± 16.1 ; DB4W 104.3 ± 15.1 ; DB6W 85.6 ± 18.6 ; DB8W 65.0 ± 10.5).

The concentration of the apoptosis inhibitor XIAP was significantly higher in the pituitaries of diabetic rats at 6 and 8 weeks after diabetes compared with controls (Fig. 4B; ANOVA $P < 0.05$). The fragmented form (30 kDa) of this protein was detected in the pituitaries of diabetic rats as early as the first week of diabetes.

NF κ B

Activation of NF κ B is directly related to the liberation of phosphorylated I κ B α . Concentrations of pI κ B α were 1.6-fold higher in the pituitaries of diabetic rats at 6 and 8 weeks compared with controls (ANOVA $P < 0.01$; Fig. 4C).

Colocalization of XIAP with caspases

Activation of caspases 8 and 9 was increased in the pituitaries of diabetic rats (Fig. 5E and K) compared with controls (Fig. 5B and H). However, while XIAP was found to colocalize in caspase 9-expressing cells in pituitaries of both control and diabetic rats (Fig. 5C and F respectively), no colocalization of XIAP with caspase 8 was found in either group (Fig. 5I and L). Activation of both caspases 3 and 7 decreased in the pituitaries of diabetic rats (Fig. 5Q and W respectively) compared with controls (Fig. 5N and T). XIAP colocalized with caspase 3 in both control and diabetic pituitaries (Fig. 5O and R), but not with caspase 7 in either group (Fig. 5U and X).

As XIAP is not coexpressed with caspase 7, we analysed whether prolactin-producing cells express the activated form of this protease. As shown in Fig. 5Y and Z, no colocalization of prolactin and caspase 7 was found in the pituitaries of either control or diabetic rats.

Cell-type-specific localization of XIAP

As previously reported (Arroba *et al.* 2005), in diabetic rats, XIAP colocalized to $< 1\%$ of prolactin-containing cells (Fig. 6C). Likewise, XIAP was found to colocalize to $< 1\%$ of LH-producing cells (Fig. 6F). However, this caspase inhibitor was found to colocalize to $45 \pm 11\%$ of GH-producing cells (Fig. 6I). The majority of cells expressing TSH ($91 \pm 6\%$) or ACTH ($93 \pm 7\%$) coexpressed XIAP (Fig. 6L and P respectively) in the pituitary of diabetic rats. In control animals, XIAP immunoreactivity was diffuse and it was found

Figure 3 (A) Relative mean levels of the intact 55 kDa form of caspase 9 in the pituitary of control and diabetic rats; † ANOVA $P < 0.01$. (B) Relative mean levels of the cleaved 35 kDa form of caspase 9 in the pituitaries of control and diabetic rats; † ANOVA $P < 0.001$. (C) Relative mean levels of the intact 32 kDa form of caspase 3 in the pituitary of control and diabetic rats; † ANOVA $P < 0.05$. (D) Relative mean levels of the cleaved 17 kDa form of caspase 3 in the pituitary of control and diabetic rats; † ANOVA $P < 0.01$. (E) Relative mean levels of the intact 33 kDa form of caspase 7 in the pituitary of control and diabetic rats; † ANOVA $P < 0.01$. (F) Relative mean levels of the cleaved 20 kDa form of caspase 7 in the pituitary of control and diabetic rats. NS, not significant; C, control; DB1W, 1 week of diabetes; DB4W, 4 weeks of diabetes; DB6W, 6 weeks of diabetes; DB8W, 8 weeks of diabetes. $n = 4$ for each diabetic group and $n = 8$ for controls.

Table 2 Mean relative protein levels of members of the Bcl-2 super family in the pituitaries of diabetic rats at different stages of evolution and control rats

	BCL-2 family				
	Bcl-2α	Bcl-X _L	p-Bad	Bad	Bax
Control	100±9.4	100±9.9	100±3	100±6.8	100±16.3
DB1W	148.2±29.9	76.1±15.4	153.3±24.3	73.1±6.4 [†]	108.5±24.4
DB4W	177.9±20.1*	147.4±18.9	161.3±55.5	65.5±5.2 [†]	101.3±10.4
DB6W	135.8±18.6	130.6±8.3	193.9±4.9 [‡]	60.2±2.6 [†]	109.0±12.6
DB8W	193.4±38.8*	177.5±2.9 [‡]	189.2±2.4 [‡]	52±8.4 [†]	104.7±15.3

p-Bad, phosphorylated Bad; *ANOVA $P<0.1$; [†]ANOVA $P<0.01$; [‡]ANOVA $P<0.001$. DB1W, 1 week of diabetes; DB4W, 4 weeks of diabetes; DB6W, 6 weeks of diabetes; DB8W, 8 weeks of diabetes. $n=4$ for each diabetic group and $n=8$ for controls.

to colocalize in approximately 10–15% of each hormone-producing cell type, with no significant difference between cell types (not shown).

Discussion

We previously reported that cell death is increased in the anterior pituitary at 8 weeks of diabetes, but that mechanisms of cell survival and proliferation are also activated (Arroba *et al.* 2003, 2005). Here, we demonstrate that some pathways involved in cell survival are activated as early as 1 week after the onset of diabetes, thus occurring before an increase in cell death is detected at 4 weeks. However, other anti-apoptotic mechanisms such as increased XIAP expression are augmented after cell death increases. Indeed, in the pituitary of diabetic rats, there are temporally specific changes in cell death, cell proliferation, caspase activation and anti-apoptotic mechanisms and some of these changes are cell-type specific.

Caspases 9, 8, 7 and 3 are all expressed and activated in the normal anterior pituitary and may be involved in basal cell death in this gland. However, caspases are also reported to be involved in non-apoptotic actions, such as proliferation, differentiation and receptor internalization (Algeciras-Schimmich *et al.* 2002, Schwerk & Schulze-Osthoff 2003), especially in tissues with a high rate of proliferation (Gupta 2000, Sordet *et al.* 2002, Huh *et al.* 2004, Oomman *et al.* 2004). Hence, these caspases could play an important role in determining the cellular composition of the pituitary in response to physiological and pathophysiological changes in the hormonal milieu (Oishi *et al.* 1993, Ahlbom *et al.* 1998, Nolan *et al.* 1998), such as occurs in diabetes.

Upstream mechanisms involved in cell survival and proliferation, such as phosphorylation of Akt and ERKs, are activated in the pituitary as early as 1 and 4 weeks after the onset of diabetes and this in turn could inhibit the intrinsic cell death pathway, including a decrease in cleaved caspase 3 and modifications in proteins of the Bcl-2 family (Kim & Chung 2002, Spencer *et al.* 2003, Lee *et al.* 2005, Subramaniam *et al.* 2005, Yang *et al.* 2005). One week after diabetes onset, protein levels of Bcl-2 family members began to shift towards an anti-apoptotic ratio, with levels of the non-phosphorylated pro-apoptotic form of Bad decreasing and the

anti-apoptotic proteins Bcl-2α and Bcl-X_L increasing, and continued to do so throughout the study. Although these changes may be cell-type specific, the overall shift in Bcl-2 family members to an anti-apoptotic balance in addition to a decrease in caspase 3 cleavage suggests that the intrinsic cell death pathway is most likely not involved in diabetes-induced pituitary cell death. As caspase 3 is expressed throughout the pituitary, identification of the cell types in which this decrease in caspase cleavage occurs was not possible in these studies.

Cleavage of caspase 9, which activates caspases 3 and 7 (Slee *et al.* 1999, Gupta 2001), increased significantly at 8 weeks of diabetes, but no activation of the effector caspases was detected. This could be due to the increase in XIAP expression since, although XIAP may not inhibit cleavage of caspase 9, it can bind to this protease and block its activity (Datta *et al.* 2000) and XIAP was expressed in the majority of caspase 9-expressing cells. In addition, differential cleavage of XIAP results in selective inhibition of caspases (Deveraux *et al.* 1999) and the 30 kDa fragment, which contains the Bir(1–2) domain and can inhibit caspases 3 and 7 (Deveraux *et al.* 1999), became detectable at 1 week of diabetes, coincident with the decrease in caspase 3 activation.

XIAP is expressed in the majority of caspase 9-expressing pituitary cells, suggesting that it could block caspase 9 activation of downstream processes in these cells. However, activation of caspase 3 is not totally blocked by XIAP as cleaved caspase 3 also colocalizes with this inhibitor; although XIAP could be involved in the reduction of caspase 3 activation reported here. XIAP did not colocalize with caspase 8 or cleaved caspase 7. As caspase 7 is an effector caspase and does not colocalize with XIAP and most lactotrophs do not express XIAP, we analysed whether lactotrophs express activated caspase 7. Activated caspase 7 was not expressed in lactotrophs of either normal or diabetic rats, indicating that it is not involved in the turnover of this cell type.

We previously reported that the diabetes-induced increase in lactotroph apoptosis is most likely due to activation of caspase 8 and the extrinsic cell death pathway (Arroba *et al.* 2005). Although XIAP can inhibit caspase 8 activation (McManus *et al.* 2004, Straszewski-Chavez *et al.* 2004), very little colocalization of this inhibitor with caspase 8 was found

Figure 4 (A) Relative mean levels of the insulin-like growth factor (IGF)-I receptor in control and diabetic rat pituitaries; [†]ANOVA $P < 0.001$ compared with control. (B) Relative mean levels of the 55 kDa form of X-linked inhibitor of apoptosis (XIAP) in control and diabetic rat pituitaries; ^{*}ANOVA $P < 0.05$ compared with control. The 30 kDa fragment was only detected in one control (shown), but became apparent as early as 1 week after the onset of diabetes in all rats. (C) Relative levels of p-IkBα in control and diabetic rat pituitaries; [†]ANOVA $P < 0.01$ compared with control. C, Control; DB1W, 1 week of diabetes; DB4W, 4 weeks of diabetes; DB6W, 6 weeks of diabetes; DB8W, 8 weeks of diabetes. $n = 4$ for each diabetic group and $n = 8$ for control.

in the pituitary. The fact that less than 1% of lactotrophs express XIAP and levels of the caspase 8-specific inhibitor FLIP (Xiao *et al.* 2002) did not change suggests that lactotrophs are not protected from caspase 8-induced cell death in poorly controlled diabetes.

No increase in apoptosis of thyrotrophs, corticotrophs or gonadotrophs was observed in the pituitary of diabetic rats, while some somatotrophs were found to be TUNEL positive. In diabetic rats, virtually all thyrotrophs and corticotrophs expressed XIAP, suggesting that they may be selectively protected from cell death by this inhibitor. However, XIAP was expressed in only 50% of somatotrophs and cell death of this cell type was only slightly increased. Furthermore, less than 1% of gonadotrophs expressed XIAP and this cell type did not undergo increased death. This suggests that gonadotrophs, and possibly somatotrophs, may be protected from diabetes-induced death through other mechanisms.

Although NFκB is associated with the extrinsic cell death pathway and can be activated by pro-apoptotic signals, including activation of death receptors and tumour necrosis factor (TNF)-α (Xiao *et al.* 2002), it can also activate IAPs (Zou *et al.* 2004). Specifically, activation of NFκB up-regulates transcription of the XIAP gene (Stehlik 1998) and this may occur by selective activation of JNK1 (Sanna *et al.* 1998). Indeed, the increase in pituitary XIAP levels at 6 weeks is coincident with increased NFκB and JNK activation.

The underlying factors involved in activation or inhibition of these pathways remain to be determined. One plausible candidate for the activation of Akt and MAPK pathways, which in turn could explain the changes in Bcl-2 proteins and inactivation of caspase 3, is IGF-I. Although circulating levels of IGF-I are reduced in this diabetic rat model (Olchovsky *et al.* 1991, Busiguina *et al.* 1996), pituitary IGF-I content increases (Olchovsky *et al.* 1991). Furthermore, we found pituitary IGF-I receptor levels to be increased at 1 week of diabetes, suggesting that the sensitivity to this cell survival factor may possibly be increased. IGF-I has been shown to inhibit high glucose-induced activation of caspase 3 and apoptosis (Russell *et al.* 1999). However, while rapid responses could be directly related to changes in glucose or insulin levels, some of the observed changes are delayed up to 8 weeks, while others are dynamic, increasing and decreasing at various time-points. These dynamic or delayed changes cannot be explained by the increase in glucose or decrease in insulin levels as these changes are rapid and are not modified during the study period. Likewise, circulating levels of TNF-α, which is involved in diabetes-induced cell death (Chen & Goeddel 2002) and apoptosis of lactotrophs (Candolfi *et al.* 2002), are increased by the first week of diabetes and remain elevated (Arroba *et al.* 2006). However, TNF-α concentrations in the anterior pituitary do not increase until the eighth week of diabetes (Arroba *et al.* 2006) and this could be involved in the delayed changes observed in this gland. It is highly conceivable that more than one factor is involved in the numerous modifications observed here and that each

Figure 5 Photomicrographs of immunohistochemistry for X-linked inhibitor of apoptosis (XIAP) and caspases in the anterior pituitary of rats that were diabetic for 8 weeks and controls. XIAP and caspase 9 (Casp9) in a control (A–C) and diabetic rat (D–F). XIAP and caspase 8 (Casp8) in a control (G–I) and diabetic rat (J–L). XIAP and caspase 3 (Casp3) in a control (M–O) and diabetic rat (P–R). XIAP and caspase 7 (Casp7) in a control (S–U) and diabetic rat (V–X). Immunohistochemistry for caspase 7 and prolactin in the pituitary of a control (Y) and diabetic rat (Z). Closed arrows indicate cells that are double labelled for both XIAP and caspase. Open arrowheads indicate cells that are only expressing caspase.

Figure 6 Photomicrographs of immunohistochemistry for X-linked inhibitor of apoptosis (XIAP; B,E,H,K,N) and prolactin (PRL, A), growth hormone (GH; G), luteinizing hormone (LH; D), thyroid-stimulating hormone (TSH; J) or adrenocorticotrophin-stimulating hormone (ACTH; M) in the anterior pituitary of rats that were diabetic for 8 weeks. There was very little colocalization (<1% of cells) of PRL or LH with XIAP (C and F respectively) in the anterior pituitary of diabetic rats. However, XIAP was found to colocalize in approximately 50% of GH (I)- and > 90% of TSH (L)- and ACTH (P)-producing cells. Open arrows indicate cells that are only expressing hormone and closed arrows indicate cells that are only expressing XIAP. Arrowheads indicate examples of double-labelled cells.

pituitary cell type may be responding differently to each factor.

One might speculate that rapid activation of cell survival mechanisms may function to protect this gland from short-term or transient events, while other mechanisms are invoked to confront prolonged pathophysiological situations. Furthermore, at least some of these mechanisms are cell-type specific and could explain why some pituitary cell types do not undergo increased apoptosis in poorly controlled diabetes. Indeed, thyrotrophs, gonadotrophs and corticotrophs are more resistant to diabetes-induced cell death. While increased expression of XIAP may be involved in the protection of thyrotrophs and corticotrophs, gonadotrophs do not express this caspase inhibitor, supporting the hypothesis of cell-specific mechanisms.

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