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Pancreatic islets from dexamethasone-treated rats show alterations in global gene expression and mitochondrial pathways

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Abstract. Chronic administration of glucocorticoids (GC) leads to characteristic features of type 2 diabetes in mammals. The main action of dexamethasone in target cells occurs through modulation of gene expression, although the exact mechanisms are still unknown. We therefore investigated the gene expression profile of pancreatic islets from rats treated with dexamethasone using a cDNA array screening analysis. The expression of selected genes and proteins involved in mitochondrial apoptosis was further analyzed by PCR and immunoblotting. Insulin, triglyceride and free fatty acid plasma levels, as well as glucose-induced insulin secretion, were significantly higher in dexamethasone-treated rats compared with controls. Out of 1176 genes, 60 were up-regulated and 28 were down-regulated by dexamethasone treatment. Some of the modulated genes are involved in apoptosis, stress response, and proliferation pathways. RT-PCR confirmed the cDNA array results for 6 selected genes. Bax α protein expression was increased, while Bcl-2 was decreased. *In vivo* dexamethasone treatment decreased the mitochondrial production of NAD(P)H, and increased ROS production. Concluding, our data indicate that dexamethasone modulates the expression of genes and proteins involved in several pathways of pancreatic-islet cells, and mitochondria dysfunction might be involved in the deleterious effects after long-term GC treatment.

Key words: Gene expression — Cell viability — Insulin secretion — Diabetes

Abbreviations: FFA, free fatty acid; GC, glucocorticoids; JNK, c-Jun N-terminal kinases; ROS, reactive oxygen species.

Introduction

Glucocorticoids (GCs) induce insulin resistance (Hoogwerf and Danese 1999; Rafacho et al. 2007) and impair insulin secretion from pancreatic β-cells (Kawai and Kuzuya 1977; Delaunay et al. 1997; Patel et al. 2006). Additionally, high levels of GC also lead to an increase in hepatic gluconeogenesis, (Lenzen and Bailey 1984) which contributes to the diabetic status. On the other hand, despite extensive studies, the molecular mechanisms involved in GC-induced diabetes are only partially known.

GC action on target cells is primarily driven by activation of cytoplasmic GC receptors (GCR). The complex GC-GCR is translocated to the nucleus and regulates gene expression (Beato 1991; Auphan et al. 1995; Schaaf and Cidlowski 2002) through either direct binding of GCR homodimers to DNA enhancer sequences – known as glucocorticoid response elements (Beato et al. 1995) – or

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indirectly through DNA binding-independent mechanisms involving protein to protein interactions (Beato 1991; Auphan et al. 1995). Chronic administration of GC increases plasma insulin levels (Lenzen and Bailey 1984; Rafacho et al. 2007). GC can directly affect insulin secretion through binding to GCR present in β-cells (Fischer et al. 1990). This GC action may lead to a stimulatory (Nicod et al. 2003) or inhibitory effect (Lambillotte et al. 1997; Jeong et al. 2001) on insulin release, depending on the concentration, time of exposure (Lambillotte et al. 1997) and experimental procedures used.

GCs are used in clinical practice as anti-inflammatory and immunosuppressor agents (Auphan et al. 1995; Schmidt et al. 2004). The immunusuppressor effects are mediated by the induction of apoptosis in cells of the immune system and other cell types (Schmidt et al. 2004). A number of genes, including cellular oncogenes, Bcl-2, transcription factors, and the tumor suppressor gene p53, have been implicated in the control of apoptosis in mammalians (Zhang et al. 1999; Eizirik and Mandrup-Poulsen 2001; Kutlu et al. 2003; Souza et al. 2004). In fact, proteins from the Bcl-2 family are crucial for the activation of apoptosis by the intrinsic pathway (mitochondrial) (Cory and Adams 2002). Pro- and anti-apoptotic Bcl-2 protein family members located on the surface of mitochondrial membrane compete to regulate the membrane integrity and cytochrome *c* release. After its release by activated pro-apoptotic proteins (Bax, Bik, Bak) (Adams and Cory 2001), cytochrome *c* binds to Apaf-1 forming the apoptosome, which leads to cleavage of procaspase-9 to form activated caspase-9 (Kaufmann and Hengartner 2001). Apoptosis is achieved through cleavage of specific substrates such as laminin, cytoskeletal proteins, poly-ADP ribose polymerase, and downstream caspases (Cory and Adams 2002). Anti-apoptotic proteins, including Bcl-2 and Bcl-xl , protect cells mainly by modulating the activity of channels, such as the voltage-dependent anion channels (VDAC), and inhibiting pore formation on the mitochondrial surface.

In this study, we performed gene expression screening by cDNA array to identify potential pathways and functionally relevant gene clusters in pancreatic islets from dexamethasone-treated animals at an early time point when β-cell function is altered but cell apoptosis is still undetected (Rafacho et al. 2009). Using this approach, we found evidence that dexamethasone treatment modulates gene expression of several pathways, reducing the expression of anti-apoptotic and increasing the expression of pro-apoptotic proteins, namely the Bcl-2 members. We also found that dexamethasone decreases mitochondrial metabolism and increased ROS production indicating that β-cell death after long-term exposure to GC may start with modulation of mitochondrial pathways leading to mitochondrial dysfunction and diabetes.

Materials and Methods

Chemicals

Dexamethasone phosphate (Dexanil®) was purchased from Acta (Campinas, Sao Paulo, Brazil). Non-esterified-fatty-acids (NEFA) were from Wako Chemicals USA, Inc. (Richmond, USA). Triglycerides and total cholesterol were from Roche Diagnostics (Mannheim, German). Dextrose, NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, KOH and Na_2SO_4 were from Mallinckrodt Baker, Inc. (Paris, France). Collagenase, Hepes, albumin, activated charcoal and dextran were from Sigma (St. Louis, USA). Ethanol, methanol, chloroform and phenol were from Synth (Diadema, Sao Paulo, Brazil). SDS-PAGE and immunoblotting were performed using Bio-Rad systems (Richmond, USA). All chemicals used for immunoblotting were from Sigma (St. Louis, USA) and all reagents used in the experiments for RT-PCR were from Invitrogen (Carlsbad, USA). Nitrocellulose membranes (Hybond N, 0.45 μm) were from Amersham (Buckinghamshire, United Kingdom). Anti-Bcl2 (rabbit polyclonal, sc-7382, dilution 1 : 500), anti-Bax α (rabbit polyclonal, sc-493, dilution 1:500) and anti-Bcl-xl (rabbit polyclonal, sc-7195, dilution 1 : 500) antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA).

Animals, dexamethasone treatment and islet isolation

Ninety-day-old Wistar rats were provided by the Animal Breeding Center of the University of Campinas (Campinas, SP, Brazil). All experimental procedures conformed to The Guiding Principles for the Care and Use of Animals (DHEW Publication, NIH 80-23) and were approved by the Committee for Ethics in Animal Experimentation of the University of Campinas (Brazil). Dexamethasone group received daily dexamethasone injections at a concentration of 1 mg/kg/day (i.p.), for 5 consecutive days, dissolved in saline. The control group received an equal amount of vehicle. For each set of experiments, islets from five adult male Wistar rats were obtained by collagenase (1 mg/ml) digestion of pancreata and isolated by centrifugation (2000 rpm for 10 minutes) on Ficoll gradients.

Insulin secretion

Groups of five islets from dexamethasone-treated and from control animals were first incubated for 45 min at 37°C in Krebs-bicarbonate buffer (containing 5.6 mmol/l glucose and equilibrated with 95% O_2 – 5% CO_2, pH 7.4). The solution was then replaced with fresh Krebs-bicarbonate buffer and the islets were incubated for 1 h with medium containing 2.8, 8.3 or 22 mmol/l of glucose. Insulin secretion was measured by radioimmunoassay (RIA) using a guinea-pig anti-rat insulin antibody and rat insulin as standard.

Metabolic, hormonal and biochemical measurements

On the day following the last dexamethasone administration, fasted (12 h) rats were killed and blood samples were collected. Blood glucose levels were measured with a glucometer ("one touch" – Johnson & Johnson, São Paulo, Brazil). Serum insulin levels were detected by RIA. Triglycerides, NEFA, and total cholesterol levels were determined by ELISA, according to the manufacturer's instructions.

cDNA array analyses

The gene expression profile was analyzed by a cDNA expression array (Clontech Laboratories, Atlas^{TM} rat cDNA array II) representing 1176 genes. Total RNA was extracted from approximately 1000 islets using Trizol reagent (InVitrogen). After extraction, the samples were treated with DNAse I and RNAse Out (Invitrogen). The quality and purity of the RNA were checked by agarose gel electrophoresis and by PCR. Radiolabeled ([^{33}P] dATP) cDNAs were prepared using 5–10 μg of total RNA and a gene-specific CDS primer mix (Clontech Labs, Palo Alto, USA), and processed as recommended by the manufacturer. Following hybridization and washing, the membranes were exposed to a phosphoimager screen (Molecular Dynamics, San José, USA) and scanned using a Storm 840 Scanner (Molecular Dynamics). The images were analyzed by QuantityOne (Bio-rad) and normalized using three internal control genes. Only changes in gene expression that were reproducible and higher than the cut-off of two-fold or lower than half in two independent experiments were considered valid. The evaluation of fold changes was performed using a Microcal Origin program, version 4.10 (Microcal Software, Inc., Northampton, USA).

Semi-quantitative analysis of mRNA by RT-PCR

Total cellular RNA was extracted from groups of 500 islets using Trizol reagent. Reverse transcription was carried out with 2 μg of total RNA using a reverse transcriptase (Superscript II, InVitrogen) and random hexamer primers, according to the manufacturer's instructions. RT-PCR assays were done using recombinant Taq DNA polymerase with 10 pmol of each primer in a final volume of 50 μl. The primers were designed and synthesized based on the published gene sequence, as shown in Table 1. The PCR was carried out in a thermal cycler (model 9700, Applied Biosystems, Foster City, USA) with an initial denaturation step at 94°C for 3 min, subject to variable number of cycles of denaturation at 94°C for 30 s, annealing for 30 s, elongation at 72°C for 45 s and a final elongation step at 72°C for 7 min. The melting temperature and number of cycles were 61°C and 34 cycles for Bcl-2, 60°C and 35 cycles for Bcl-xl , 59°C and 34 cycles for Bax α, 60°C and 37 cycles for Bad, 59°C and 32 cycles for JNK, 59°C and 32 cycles for Fas, 59°C and 29 cycles for RPS-29. The cycle numbers were defined after titration between 20 and 45 cycles and were within the logarithmic phase of amplification. PCR products were run on 1% agarose gels, and the DNA was visualized by ethidium bromide staining. The band intensities were determined by digital scanning followed by quantification using Scion Image analysis software (Scion Corp., Frederick, USA).

Tissue extracts and immunoblotting

After isolation, the islets were homogenized by sonication in ice cold buffer (10% Triton-X 100, 100 mmol/l Tris (pH 7.4), 10 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride,

Table 1. Primers for selected genes used in the RT-PCR

Gene	Primer	Primer sequence (5`- 3`)	Gene Bank accession number	Product sizes
Bcl-2	F	GTATGATAACCGGGAGATCG	NM016993	611
	R	AGCCAGGAGAAATCAAACAG		
Bcl-xl	F	GTGGCTGGTGTAGTTCTGCTGG	U72350	314
	R	AACAAGGCAGGCTCTTCTCCC		
Bad	F	CAGTGATCTGCTCCACATTC	NM022698	330
	R	ATGATAGGACAGCACCCAGT		
Bax α	F	AAGAAGCTGAGCGAGTGTCT	U49729	360
	R	CAAAGATGGTCACTGTCTGC		
FasR	F	GACTTTAGCTGGGCAGATGT	D26112	692
	R	CAGAAGAGAGCATGGGAAAT		
JNK	F	GCCATCATGAGCAGAAGTAA	NML27129	210
	R	GCTTAGCATGGGTCTGATTC		
RPS-29	F	AGGCAAGATGGGTCACCAGC	NM012876	202
	R	AGTCGAATCATCCATTCAGGTCG		

F, forward primer; R, reverse primer; RPS-29, ribosomal protein S29; JNK, c-Jun N-terminal kinase; FasR, Fas receptor.

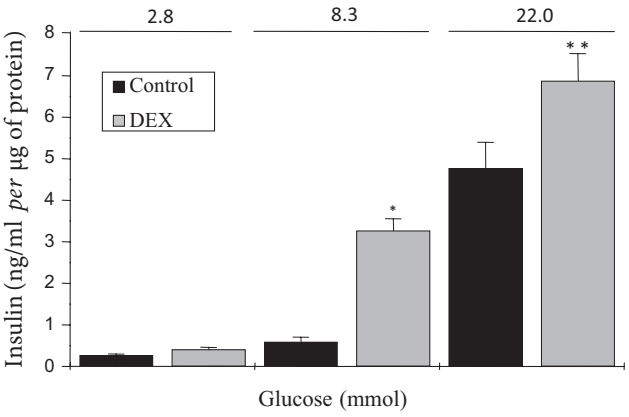

Figure 1. Effect of dexamethasone on insulin secretion in isolated rat islets. Rats were treated with dexamethasone (1 mg/kg/day) for 5 days. After isolation, groups of 5 islets were incubated in HEPES-bicarbonate buffer containing 2.8, 8.3 or 22 mM glucose. Columns represent the cumulative 1 h insulin secretion and are means ± SEM of 3 independent experiments ($n = 18$). The insulin secretion was normalized by total islet protein content. Means with a different letter above columns are significantly different ($p < 0.05$). DEX, dexamethasone group.

10 mmol/l EDTA, 10 mmol/l sodium vanadate, and 2 mmol/l PMSF) and centrifuged at 15,000 × *g* at 4°C for 20 min. After protein measurement, 70 µg of protein were separated by SDS-PAGE on 10% polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes at 120 V for 2 h. Non-specific binding to nitrocellulose was reduced by preincubating the filter in blocking buffer (5% BSA, 10 mmol/l Tris, 150 mmol/l NaCl, and 0.02% Tween 20) overnight at 4°C. The nitrocellulose membranes were then incubated for 4 h at 22°C with anti-Bcl-2, anti-Bax α, or anti-Bcl-xl antibodies in incubating buffer (3% BSA, 10 mmol/l Tris, 150 mmol/l NaCl, and 0.02% Tween 20). The blots were subsequently incubated with 150 ng/ml of secondary antibody HRP conjugated (Zymed, San Francisco, USA) for 2 h at 22°C. Finally, the blots were incubated in SuperSignal solution (Pierce, Boston, USA) and exposed to autoradiography film (Kodak, São Paulo, Brazil).

Table 2. Effects of dexamethasone treatment on lipids, insulin and glucose plasma levels

	Control	DEX
FFA (mg/dl)	96.3 ± 7.3	187.6 ± 8.7 *
Triglycerides (mg/dl)	76.5 ± 8.5	116.1 ± 13.5 *
Cholesterol (mg/dl)	80.5 ± 3.7	78.4 ± 7.1
Insulin (ng/ml)	0.14 ± 0.20	2.1 ± 0.4 *
Glucose (mg/dl)	69.3 ± 1.7	137.3 ± 8.8 *

Values are means ± SEM.* significantly different DEX *vs.* Control group ($n = 9$). * $p < 0.05$, with p values calculated from t test on the average difference between Control and DEX. FFA, free fatty acids.

Band intensities were measured by digital scanning followed by quantification using Scion Image analysis software (Scion Corp. Frederick, MD, USA).

MTS cell viability assay

In all sets of experiments, the viability of the cells was determined after 3 h incubation period using a microplate-based MTS kinetic assay, following the instructions contained in the manual (CellTiter 96 Non-Radioactive Cell Proliferation Assays, Promega Corporation, Madison, WI).

Determination of intracellular ROS by H_2DCFH-DA fluorescence

Islets from control and dexamethasone-treated animals were isolated as described before and incubated for 45 min in Krebs-Hepes buffer containing 5.6 mmol/l of glucose. Thereafter islets were placed in black coloured Costar plates and incubated in Krebs-Hepes buffer containing 25 mmol/l of glucose and 10 µmol/l of DCFH-DA (2',7'-dichlorfluorescein-diacetate) for 30 min at 37°C. Fluorescence emission from DCF was detected at 460/530 nm excitation/emission using a fluorometer (Fusion, PerkinElmer Life Science, USA). Protein concentration was measured by Bradford. The resulting fluorescence was expressed as ΔUF normalized to µg/ml of protein.

Statistical analysis

The results were expressed as mean ± SEM for the indicated number of experiments (n). Statistical comparisons were made using unpaired Student's t-test or ANOVA followed by the Bonferroni's post-test when appropriate. $p < 0.05$ was considered statistically significant.

Results

Effects of dexamethasone treatment on lipids, insulin and glucose plasma levels

Table 2 shows the mean values for insulin, glucose, triglycerides and free fatty acid (FFA) levels in the plasma of fasted rats. Dexamethasone-treated animals (DEX) demonstrated a significant increase in all biochemical parameters compared to control group ($n = 9$, $p < 0.05$) except for the cholesterol plasma levels that were similar between groups after the dexamethasone treatment.

Effects of dexamethasone treatment on insulin release

Figure 1 shows the amount of insulin secreted normalized to the total protein content in the islets. At a non-stimulatory

concentration of glucose (2.8 mM), there were no differences between DEX and control group ($n = 6$). Using a glucose concentration that matched post-prandial levels *in vivo* (8.3 mmol/l), the insulin secretion was significantly higher in DEX compared to control islets (17.98 ± 1.6 and 8.85 ± 1.5 ng/ml/μg protein, respectively). Finally, at supra-stimulatory concentrations (22 mmol/l glucose), the insulin secretion in DEX was also significantly higher than in control islets (68.8 ± 6.5 and 47.8 ± 6.2 ng/ml/μg protein, respectively, $p < 0.05$).

c-DNA array screening

Out of the 1176 genes represented in the cDNA array membrane, 60 were up-regulated and 28 down-regulated in islets from dexamethasone-treated rats. A complete list of all dexamethasone-modulated genes is given in Table 3. Interestingly, islets from dexamethasone-treated animals showed up-regulation of several pro-apoptotic genes, including Bax α and JNK, oncogenes like H-Ras a, Ras p21 protein activator and growth factors: fibroblast growth factors, like fibroblast, TGF-β. Among the down-regulated genes are those involved in antioxidant response (gluthathione peroxidase 4) proliferation pathways (MAPKK1, fibroblast growth factor 10) and heat-shock proteins.

RT-PCR and Western blotting analysis

RT-PCR analysis showed that Bax α, Fas and JNK genes expression was higher in DEX islets compared with control islets (1.95-, 1.5- and 1.65-fold, respectively; $p < 0.05$, $n = 6$) (Fig. 2). Dexamethasone treatment did not alter the Bcl-2, Bcl-xl and Bad gene expression. Western-blotting analysis indicated that the expression of pro-apoptotic Bax α protein was increased (2.3-fold) whereas the anti-apoptotic protein Bcl-2 was decreased (1.5-fold) after dexamethasone treatment, when compared to control. No differences in the expression of Bcl-xl were noticed after GC exposure (Fig. 3). Although no differences in Bcl-2 gene expression were observed, Bcl-2 protein expression was 1.5-fold lower in islets from dexamethasone-treated rats compared with control islets ($p < 0.05$, $n = 4$).

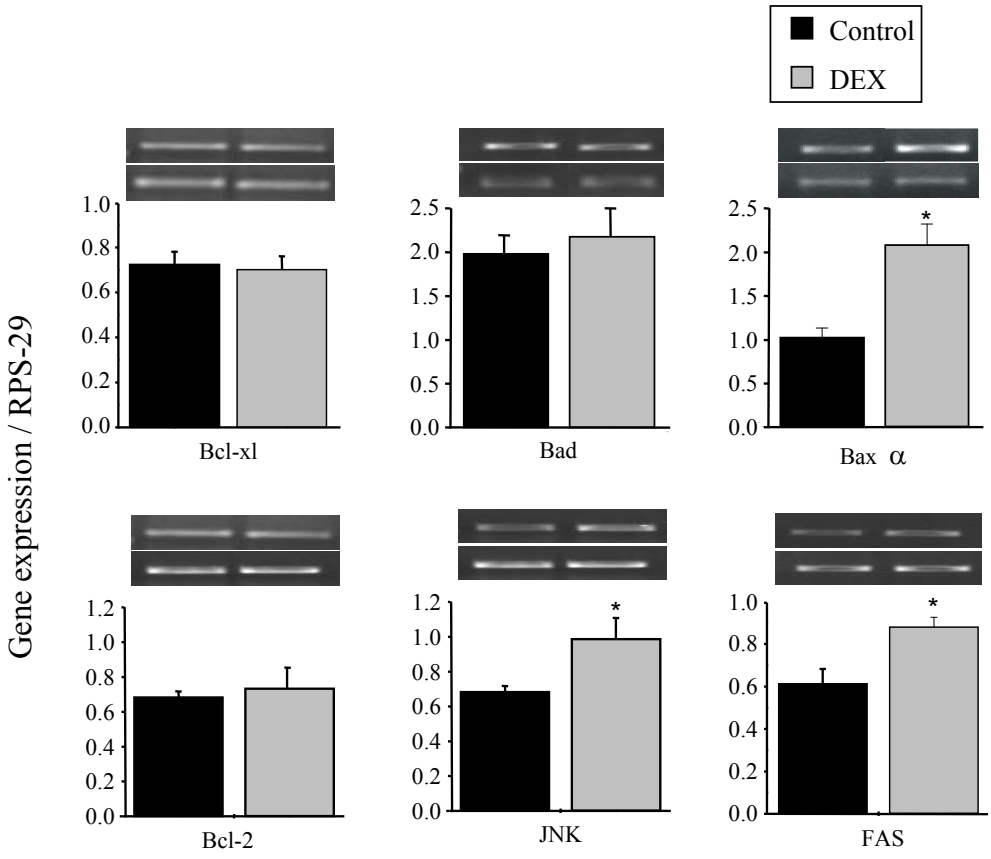

Figure 2. Confirmation by RT-PCR of 6 selected genes that were significantly up- or down-regulated in pancreatic islets after dexamethasone treatment. The bars represent the means ± SEM of 6 experiments performed with specific primer sets (Table 1) and normalized against the ribosomal protein S29 (RPS-29). * $p < 0.05$, with p values calculated from t test on the average difference between control and dexamethasone group (DEX).

Effects of dexamethasone treatment on mitochondrial activity

Islet-cells from DEX rats showed a decrease of 23.35% mitochondrial NAD(P)H formation compared to controls, as measured by the MTS assays ($n = 3$, $p < 0.05$). The results were normalized to total protein content and indicated as percentage of control islets.

Effects of dexamethasone on ROS production

Experiments using H_2DCF-DA fluorescence as an indicator of oxidative stress showed that dexamethasone induce small increase (19%) in total reactive oxygen species in islets from rats treated with dexamethasone for 5 days ($n = 8$, $p < 0.05$).

Table 3. Changes in pancreatic islet-related mRNA modulation by dexamethasone treatment

Gene name	GenBank accession	Fold modulation
Up-regulated genes		
Neuropeptide Y	M20373	15.20
Cytochrome P450 IIA1 (hepatic steroid hydroxylase IIA1) gene	J02669	11.30
Clusterin	M64723	8.48
Interleukin 13	L26913	7.05
Fibroblast growth factor receptor 1	D12498	6.90
trk precursor	M85214	6.37
GAP-associated protein (p190).	M94721	5.04
p21, cdk interacting ptn 1	L41275	5.02
Thyroid hormone receptor ErbA-β -2, pituitary specific	M25071	4.95
Secretogranin III	U02983	4.94
Arginine vasopressin receptor 1B	D45400	4.63
Cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase)	J02657	4.38
Interleukin 7	AF010464	4.24
Transforming growth factor beta (TGF-β)	M55431	4.08
Somatostatin receptor 4	U04738	4.05
Colony stimulating factor 1 receptor	X61479	4.00
Insulin-like growth factor binding protein 1	M89791	3.99
Adrenergic receptor, α 1a	U13368	3.98
Phospholipase A2, group 2C	U07798	3.82
Complement component 5, receptor 1	AB003042	3.69
Parathyroid hormone receptor	L19475	3.67
Bax-α	U49729	3.50
Endothelin 1	M64711	3.48
Phosphatidate phosphohydrolase type 2a	U90556	3.42
Interleukin 1 β	M98820	3.33
ATPase, Na+K+ transporting, α 1	M28647	3.25
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c	D13123	3.21
Apolipoprotein D	X55572	3.21
Calcineurin	L03554	3.16
Transforming growth factor, β 1	X52498	3.13
Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator)	D12771	3.08
c-jun Nh2-terminal kinase 1, JNK-1- mapk8	L27129	3.02
Interleukin 18	U77776	2.99
Platelet-derived growth factor receptor, β	Z14119	2.98
Inositol 1, 4, 5-triphosphate receptor 3	L06096	2.97
ATPase, Ca++ transporting, plasma membrane 2	J03754	2.93
Cytochrome P450 4F6	U39208	2.87
Pancreatic polypeptide receptor 1	U42388	2.77
Insulin receptor-related receptor	M90661	2.77
Cathepsin L	Y00697	2.73

Table 3. Continued

Gene name	GenBank accession	Fold modulation
Interferon γ -induced protein 10	U17035	2.73
N-ras proto-oncogene, transforming protein p21	X68394	2.66
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b,	M35052	2.65
Arrestin-D	U03629	2.59
Cytokine-induced neutrophil chemoattractant-2	D21095	2.55
v-crk proto-oncogene	D44481	2.49
Arachidonate 12-lipoxygenase	L06040	2.46
Low-density lipoprotein receptor	X13722	2.43
Adrenergic receptor kinase, β 2	M87855	2.39
Platelet-derived growth factor	Z14117	2.29
Protein tyrosine phosphatase 2E	U18293	2.27
Protein tyrosine phosphatase 4a1	L27843	2.20
Granzyme M	L05175	2.16
Protein convertase 1, prohormone convertase 1	M76705	2.13
cAMP-specific 3,5-cyclic phosphodiesterase 4C	M25347	2.01
v-crk-associated tyrosine kinase substrate	D29766	2.01
H-ras proto-oncogene; transforming protein p21	M13011	2.00
RAS p21 protein activator 1	L13151	2.00
Arrestin, β 1	M91589	2.00
Adenosine A1 receptor	M64299	2.00
Adenosine A2B receptor	M91466	2.00
Down-regulated genes		
Glutathione peroxidase 4	X82679	0.48
Inhibitor of DNA binding 1, helix-loop-helix protein (splice variation)	D10862	0.47
Interleukin 8 receptor, β	X77797	0.47
Colony stimulating factor 3	U37101	0.43
Heat shock 90-kDa protein β (HSP90-β); HSP84; HSPCB	S45392	0.40
Mitogen activated protein kinase kinase 1	Z16415	0.38
Glutathione peroxidase 5	X62404	0.37
beta-nerve growth factor precursor (β-NGF)	M36589	0.37
Cadherin 22	D83349	0.36
Heat shock 70kD protein 5	M14050	0.36
Cyclin D1	AF148946	0.35
Jun D proto-oncogene	D26307	0.33
CXC chemokine LIX	U90448	0.33
Glucagon receptor	L04796	0.31
Cholinergic receptor, nicotinic, alpha polypeptide 4	L31620	0.30
ERK2, mitogen-activated protein kinase 1	M64300	0.30
Glutathione synthetase	L38615	0.28
Cholinergic receptor, nicotinic, β polypeptide 2	L31622	0.28
ATP receptor, P2X purinoceptor 1	X80477	0.27
Fibroblast growth factor 10	D79215	0.27
Glutathione reductase	U73174	0.20
Cytochrome c oxidase, subunit Va	X15030	0.18
Integrin, β 4	U60096	0.17
Heat shock 70kD protein 1A	Z27118	0.12
Corticotrophin releasing hormone receptor 2	U16253	0.12
Transcription factor AP-1	X17163	0.11
Glucose-6-phosphate dehydrogenase	X07467	0.10
Cadherin 6	D25290	0.08

Genes were screening using the Atlas Rat 1.2 Array. Values represent -fold modulation, as compared to expression in control islets. $n = 3$ independent experiments.

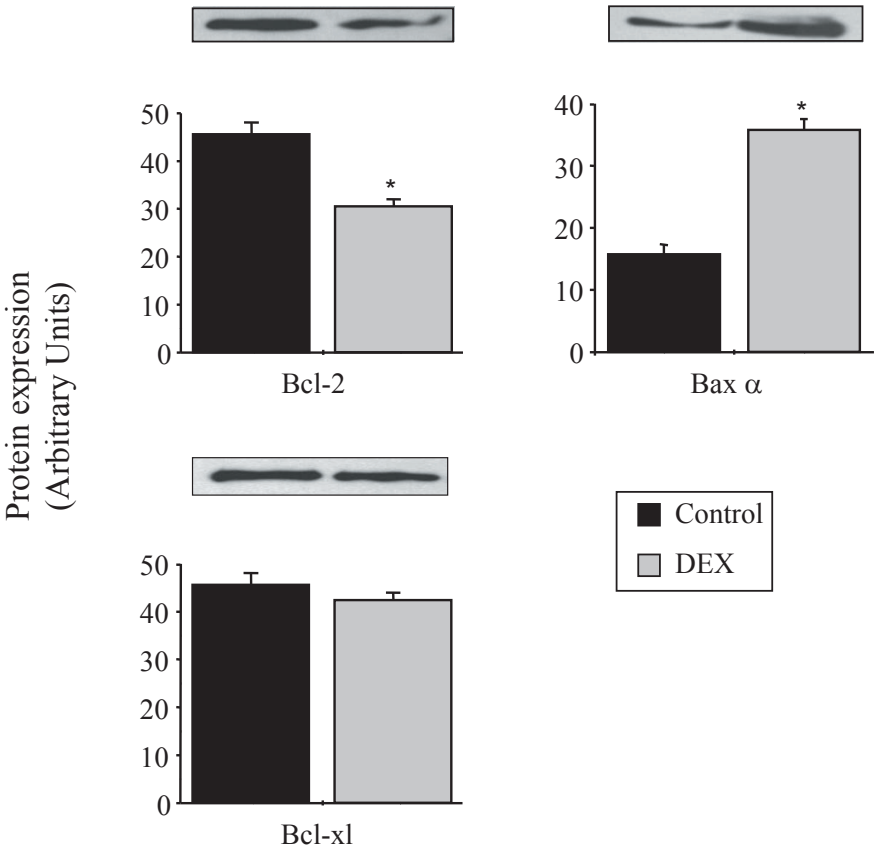

Figure 3. Protein expression of three transcripts to validate the cDNA array analysis. The islet proteins were extracted (see Materials and Methods) and equal amounts of protein from control and dexamethasone-treated (DEX) islets were resolved by SDS-PAGE on 10% gels and transferred to a nitrocellulose membrane. The proteins were identified with anti-Bax α, anti-Bcl-2, and anti-Bcl-xl antibodies. The values are the means ± SEM of 4 experiments. * $p < 0.05$ for control *vs.* DEX islets.

Discussion

GCs administration has been used experimentally as a model of insulin resistance. In this work, we used an *in vivo* model were the deleterious effects of GC on insulin secretion and apoptosis were still compensate by other factors such as hyperglycemia, hyperinsulinemia and hyperlipidemia (Rafacho et al. 2007, 2009). The objectives were to indentify the early changes in gene and protein expression that could lead to β-cell failure and diabetes after long-term exposure to GC.

When in excess, GCs can induce hyperglycemia and type 2 diabetes (Lenzen and Bailey 1984; Ranta et al. 2006). Dexamethasone also induces hyperlipidemia, which is associated with the increased insulin and glucose levels (Nicod et al. 2003). Increases in triglycerides, FFA, glucose and insulin plasma levels (Table 1) definitely contribute to the cellular dysfunctions that lead to diabetes. The high insulin blood levels in dexamethasone-treated animals are probably an adaptation of pancreatic β-cell to the insulin resistance caused by the steroid in liver, muscle, adipose tissues and in addition to increased rates of gluconeogenesis (Lenzen and Bailey 1984; Nicod et al. 2003). These characteristics (high level of insulin and glucose in plasma) are typical features of the type 2 diabetes, showing that dexamethasone treatment may be an interesting animal model for studying several factors at the onset of type 2 diabetes.

Whether *in vivo* or *in vitro*, GCs can either decrease or increase insulin release, depending on the dose and period of exposure (Jeong et al. 2001). Several studies suggest an inhibitory effect of *in vitro* administration of dexamethasone on insulin release from pancreatic islets (Barseghian and Levine 1980; Pierluissi et al. 1986; Lambillotte et al. 1997; Jeong et al. 2001). On the other hand, a clear increase in potassium- and glucose-stimulated insulin secretion from isolated pancreatic islets is observed after *in vivo* administration of dexamethasone to rats (Rafacho et al. 2009) (Fig. 1). These data are in accordance with previous studies using similar approaches (Kawai and Kuzuya 1977; Brunstedt and Nielsen 1981) and with clinical observations in patients (Cushing's syndrome) with high levels of GC (Schafroth et

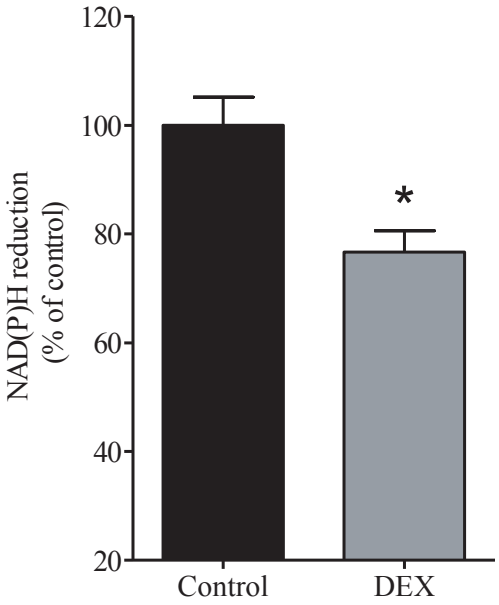

Figure 4. Rats were treated with dexamethasone (1 mg/kg/day) during 5 days. Groups of 100 islets were collected for each group and MTS experiments were performed according to instructions. Dexamethasone decreases NAD(P)H reduction as showed in Figure 4. The values are the means ± SEM of 3 experiments, normalized by control values . * $p < 0.05$ for control *vs.* DEX islets. DEX, dexamethasone group.

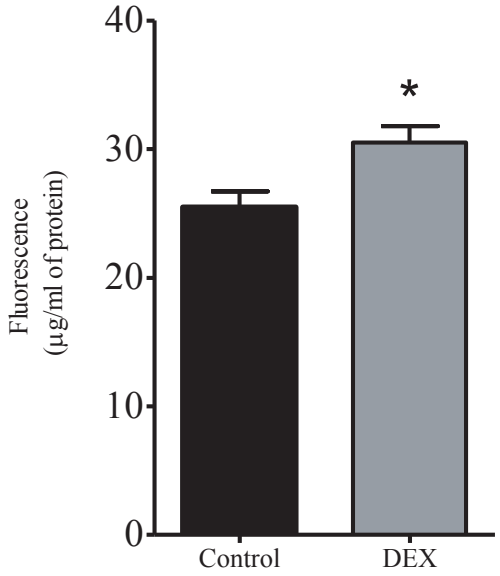

Figure 5. Reactive oxygen species were measured by H_2DCF-DA fluorescence. After isolation, islet from dexamethasone-treated rats and control animals were incubated for 45 minutes in Kreb's solution and for 30 minutes with H_2DCF-DA as described in Materials and Methods. The values are the means ± SEM of 4 experiments performed in duplicate. * $p < 0.05$ for control *vs.* DEX islets. DEX, dexamethasone group.

al. 2000). The rat islets used in our study for the 1-h culture had been preconditioned to the 5-day *in vivo* dexamethasone treatment, which might cause a compensatory insulin release in response to increased insulin resistance and elevated glucose production by the liver. The hyper-triglyceridemia may be a consequence of an increase hepatic production and secretion of very low density lipoprotein, and/or a direct effect on enzymes involved with triglyceride catabolism, leading to the impairment of lipoprotein lipase activity in adipose tissue (Cole et al. 1982). These changes, in the long term, may permanently damage the β-cell. An indicative of this harm is the impairment in NAD(P)H reduction rate in islets from dexamethasone-treated rats (Fig. 4). In view of the fact that β-cells are essentially a sensor for metabolic energy changes, a decrease in NAD(P)H production may indicate an impairment of mitochondrial activity and loss of viability of these cells.

The apoptosis in type 2 diabetes has been the matter of recent studies aiming to decipher the molecular mechanisms that leads to β-cell failure (Porte and Kahn 2001; Ranta et al. 2006). The decrease in β-cell mass is possibly a result of an increase in apoptosis rather than a decrease in proliferation (Pick et al. 1998), and apoptosis may therefore be one of the causes of the type 2 diabetes progression. The β-cell undergoes apoptosis when exposed to dexamethasone *in vitro* (Weinhaus et al. 2000; Ranta et al. 2006; Roma et al. 2009). Reactive oxygen species are involved in dexamethasone-induced apoptosis and either catalase overexpression or antioxidant treatment (N-acetyl-L-cysteine) can counteract *in vitro* GC effects in pancreatic cell lines and islets, respectively (Roma et al. 2009, 2011). On the other hand, i*n vivo* GC treatment leads to increase in β-cell mass. Proliferation may be due to increase proliferation rate of β-cell and ductal cells (Jorns et al. 2010) and might be triggered by high glucose and/or insulin levels (Rafacho et al. 2009). In animals treated with hydrocortisone (25 mg/kg/day) for 5 days, apoptosis and proliferation are present (Jorns et al. 2010). Here we showed that even in a situation where proliferation has shown to prevail over apoptosis (dexamethasone 1 mg/kg/day during 5 days) mitochondrial apoptotic pathway is early modulated by the GC and can contribute to the further decompensation and β-cell death. The mitochondrial dysfunction might result from oxidative stress induced by dexamethasone (Fig. 5) (Roma et al. 2009, 2011) or by other deleterious effects from elevated levels of glucose and lipids (El-Assaad et al. 2010).

The modulation of gene and protein expression in pancreatic islets by dexamethasone treatment is only partially understood. Interestingly, Bax α, an important pro-apoptotic protein, is up-regulated in pancreatic islets by *in vivo*

dexamethasone treatment (Fig. 3). There is already concrete evidences supporting a role for Bax α in the induction of apoptosis in pancreatic islets (Oltvai et al. 1993; Mizuno et al. 1998) and other cell types (Schmidt et al. 2004). Additionally to the genes that might directly act in apoptosis by the mitochondrial pathway, we found several other genes of interest in pancreatic islets from rats exposed to dexamethasone, which directly and/or indirectly mediates GC effects on beta cell. Some of these genes are stress response like JNK, which is one of the hallmarks of oxidative stress and JNK signaling activation leads to apoptosis and impaired insulin secretion (Kaneto et al. 2005). We also observed up-regulation of some growth factors and oncogenes, in agreement with previous results where proliferation pathways are activated and lead to increase proliferation rates in this model (Rafacho et al. 2009). On the other hand, mRNA expression of antioxidant proteins were down-regulated, indicating the possible link between these early changes in gene expression and the involvement of oxidative stress in late alterations of β-cell function and survival. Indeed, GCs can modulate these pathways in many cell types such as lymphoid cells (Schmidt et al. 2004), NIH-373 fibroblast cells (Kassel et al. 2001), chondrocytes (Chrysis et al. 2005), pancreatic islet-cells (Weinhaus et al. 2000; Ranta et al. 2006) and others. Nevertheless, more experiments are necessary for the better understanding of the role of all these genes in cellular protection and apoptosis after GC exposure. These data need to be extended to measurements of protein expression and phosphorylation and reporter genes in dependence upon exposure to GC.

In conclusion, our results show that dexamethasone modulates the expression of several genes in pancreatic islets. The deleterious effects of dexamethasone on β-cell possibly involve the production of ROS and modulation of mitochondrial pathway, principally the Bcl-2 proteins. This may contribute to the shift from proliferation observed in the early stages of GC treatment, to apoptosis after long-term exposure to GC and ultimately GC-induced type 2 diabetes.

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