

Uncoupling protein-2 increases nitric oxide production and TNFAIP3 pathway activation in pancreatic islets

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Abstract

Mutations in the uncoupling protein 2 (*Ucp2*) gene are linked to type-2 diabetes. Here, a potential mechanism by which lack of UCP2 is cytoprotective in pancreatic β -cells was investigated. Nitric oxide (NO) production was elevated in *Ucp2*^{-/-} islets. Proliferation (cyclin D2, *Ccnd2*) and anti-apoptosis (*Tnfaip3*) genes had increased expression in *Ucp2*^{-/-} islets, whereas the mRNA of pro-apoptosis genes (*Jun*, *Myc*) was reduced. TNFAIP3 cellular localization was detected in both α - and β -cells of *Ucp2*^{-/-} islets but in neither α - nor β -cells of UCP2^{+/+} islets, where it was detected in pancreatic polypeptide-expressing cells. TNFAIP3 distribution was not markedly altered 14 days after streptozotocin treatment. Basal apoptosis was attenuated in *Ucp2*^{-/-} β -cells, while the nuclear factor κ B (NF- κ B) pathway was transactivated after islet isolation. *Ucp2*^{+/+} and *Ucp2*^{-/-} islets were treated with cytokines for 24 h. Cytokines did not increase NF- κ B transactivation or apoptosis in *Ucp2*^{-/-} islets and TNFAIP3 was more strongly induced in *Ucp2*^{-/-} islets. Inhibition of NO production strongly reduced NF- κ B activation and apoptosis. These data show that null expression of *Ucp2* induces transactivation of NF- κ B in isolated islets, possibly due to NO-dependent up-regulation of inhibitor of κ B kinase β activity. NF- κ B transactivation appears to result in altered expression of genes that enhance a pro-survival phenotype basally and when β -cells are exposed to cytokines. TNFAIP3 is of particular interest because of its ability to regulate NF- κ B signaling pathways.

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Introduction

Uncoupling protein 2 (UCP2) belongs to a family of mitochondrial proteins characterized by an ability to transport ions, including protons and fatty acid anions (Esteves & Brand 2005); however, the specific physiological role of UCP2 is still unclear. In pancreatic β -cells, increased UCP2 expression is associated with suppression of glucose-stimulated insulin secretion (Chan *et al.* 1999, 2001) and mediates the negative effects of superoxide on insulin secretion (Krauss *et al.* 2003). In addition, the lack of UCP2 in mice protects them from the diabetogenic effects of a high-fat diet and also maintains glucose-stimulated insulin secretion (Joseph *et al.* 2002, 2004). Although baseline apoptosis is higher in *Ucp2*^{-/-} mouse islets, and these islets produce higher levels of reactive oxygen species, no further increase in these parameters is observed after long-term high-fat diet treatment (Joseph *et al.* 2004). The mechanistic basis for protection from diabetes may be partially related to increased capacity for fatty acid oxidation (Joseph *et al.* 2004, Fatehi-Hassanabad & Chan 2007), but other mechanisms may also be operative.

It has been proposed that UCP2 modulates the inflammatory response and regulates the production of reactive oxygen species (Brookes 2005) and nitric oxide (NO) in cells (Bai *et al.* 2005). Depending on the amount produced, reactive oxygen species may stimulate or inhibit glucose-stimulated insulin secretion from β -cells (Pi *et al.* 2007), which may account for some inconsistencies in the literature regarding UCP2 function. Likewise, in the central nervous system, induction of UCP2 protects neurons from oxidative stress (Sullivan *et al.* 2004) but not all studies show this effect (de Bilbao *et al.* 2004, Prabhakaran *et al.* 2005). In our study of autonomic dysreflexia following spinal cord injury, no differences in cell damage were detected in *Ucp2*^{-/-} versus *Ucp2*^{+/+} mice (Webb *et al.* 2006). In macrophages from *Ucp2*^{-/-} mice, cytokine, NO and reactive oxygen species (ROS) production, as well as inducible NO synthase (iNOS) expression, were elevated (Bai *et al.* 2005). This proinflammatory state was attributed to increased activation of the transcription factor nuclear factor κ B (NF- κ B). It seems clear that under some conditions, UCP2 can provide protection from oxidative stress but such protection may depend on

tissue-specific factors, such as the ability to induce other protective proteins, the overall effects on ATP production and the involvement of resident macrophages.

In β -cells, NF- κ B is generally regarded as a mediator of inflammation, leading to induction of pro-apoptotic genes and cell death (Ho & Bray 1999, Ou *et al.* 2005, Eldor *et al.* 2006, Ortis *et al.* 2006). Acutely exposing β -cells to cytokines (Ortis *et al.* 2006) induces NF- κ B nuclear translocation, whereas blocking NF- κ B activation prevents β -cell damage (Eldor *et al.* 2006, Kwon *et al.* 2006). Reduction of the β -cell mass in type 2 diabetes is among the pathological effects caused by proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin 1 β (IL1 β) and interferon- γ (IFN γ ; Eizirik & Mandrup-Poulsen 2001). In combination, IFN α and IL1 β increase NO production and iNOS expression (Thomas *et al.* 2002). Application of the NO precursor nitroprusside is lethal to β -cells (Kroncke *et al.* 1991), while inhibition of iNOS with aminoguanidine is protective (Kuttler *et al.* 1987). An alternative role for NF- κ B in regulation of β -cell function and survival has been revealed. Novel data from Hagerkvist *et al.* (2007) showed that the tyrosine kinase inhibitor imatinib mesylate protected β -cells from death in non-obese diabetic and streptozotocin (STZ)-induced diabetic mice. *In vitro*, imatinib reduced cytokine-stimulated apoptosis and this was associated with reduced association of inhibitor of κ B (I κ B α) with NF- κ B subunit RelA. In another study, non-obese diabetic mice expressing a super-repressor of NF- κ B activity had increased susceptibility to developing diabetes, which could be overcome by neutralizing TNF- α . These mice had reduced expression of anti-apoptotic proteins in β -cells (Kim *et al.* 2007).

Because we observed that lack of UCP2 protected β -cells from *UCP2*^{-/-} mice from a high-fat diet-induced cell death (Joseph *et al.* 2004, 2005), we predicted that protection from other pro-apoptotic stimuli such as cytokines might also be afforded by lack of UCP2. Thus, the objective of this study was to examine the effects of cytokines on β -cells from *Ucp2*^{+/+} and *Ucp2*^{-/-} mice, with emphasis on the role of NF- κ B pathway. It was hypothesized that *Ucp2*^{-/-} islets would have increased activation of the NF- κ B pathway, similar to previous results reported in immune tissues (Bai *et al.* 2005).

Materials and methods

Materials

Cell culture medium (DMEM), antibiotic-antimycotic solution, calf and fetal bovine serum, Griess Reagent kit, TRIzol and Cloned AMV First-Strand cDNA Synthesis kit were purchased from Invitrogen. HEPES,

BAY 11-7082, caffeic acid phenethyl ester (CAPE) and all cytokines were purchased from Sigma-Aldrich. Roche supplied BSA and the TUNEL assay kit. Nuclear extraction kits, NF- κ B transactivation and I κ B α phosphorylation ELISAs were purchased from Active Motif (Carlsbad, CA, USA). Antibodies to iNOS (BD Biosciences, Mississauga, Ontario, Canada), NF- κ B (C-20), TNFAIP3 (H-100 and A-12), JUN (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β -actin (Sigma-Aldrich) were also purchased. Insulin and somatostatin antibodies were purchased from Dako (Glostrup, Denmark), glucagon antibody from Abcam (Cambridge, UK) and pancreatic polypeptide from Thermo Fisher Scientific (Rockford, IL, USA).

Animals and pancreatic islet cultures

All procedures involving animals were approved by the respective university Animal Care Committees (Toronto and Prince Edward Island) and followed the guidelines of the Canadian Council on Animal Care. Littermate *Ucp2*^{+/+} and *Ucp2*^{-/-} mice on a mixed C57Bl6/129 background bred from heterozygous mice (Zhang *et al.* 2001), age 16 weeks old and fed *ad libitum* were used for mouse islet experiments. Mice were anesthetised with pentobarbital (65 mg/ml, i.p.) and isolated pancreatic islets were obtained by collagenase digestion as described (Zhang *et al.* 2001). After digestion, the islets were purified by filtration (Salvalaggio *et al.* 2002), with minor modifications. Islets were cultured for 24 h in DMEM, 8.3 mmol/l glucose, supplemented with 10% calf serum, 1% antibiotic-antimycotic solution, 10 mmol/l HEPES with or without NF- κ B inhibitors Bay 11-7082 (BAY, 10 μ mol/l) or CAPE (10 μ mol/l) or the iNOS inhibitor 1400W (10 μ mol/l, Sigma) plus a combination of IL1 β (50 ng/ml), IFN γ (50 ng/ml) \pm TNF- α (5 ng/ml). A density of 30 islets/well was incubated for each treatment. Mice made diabetic by STZ were treated according to Lee *et al.* (2009) and tissues were harvested 14 days later.

NF- κ B RelA activity and I κ B α phosphorylation assay

Isolated islets from *Ucp2*^{+/+} and *Ucp2*^{-/-} mice were cultured as noted above for 24 h. Nuclear proteins were extracted according to the manufacturer's instructions (Active Motif). The supernatant (nuclear fraction) was kept at -80 °C until analysis. The active NF- κ B contained in the nuclear extracts was measured by its DNA-binding activity on immobilized oligonucleotides encoding a specific consensus site using a NF- κ B RelA transcription factor ELISA kit (Active Motif). Phosphorylated I κ B α was measured in islet cellular extracts. *Ucp2*^{+/+} and *Ucp2*^{-/-} islets were pretreated with BAY (10 μ mol/l) or 1400W (10 μ mol/l) for 2 h and then

with a combination of cytokines, as described above. After 10 min incubation, islets were centrifuged and washed with PBS. Cellular extraction, protein determination and I κ B α ELISA to measure phosphorylated I κ B α were carried out according to ELISA kit instructions (Active Motif). Samples were measured by luminescence detection.

Apoptotic and necrotic cell determination

Determination of DNA strand breaks in islet cells was assessed by the TUNEL technique. After 24 h culture, islets were trypsinized (0.16 mg/ml trypsin) and the cells were fixed on precoated poly-L-lysine glass slides by cytospinning. Cells were sequentially stained for TUNEL and insulin, then the proportion of cells positive for TUNEL and insulin cells was quantified at 200 \times magnification. An average of 2000 cells/cell preparation was counted for each treatment condition and data were expressed as percentage of total β -cells. For necrosis measurements, control or treated mouse islets, or dispersed islet cells, plated on glass coverslips were incubated with propidium iodide (PI, 4 μ mol/l) for 20 min at room temperature in darkness in buffer of the following composition (in mmol/l): 130 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 5 NaHCO₃, 10 HEPES, pH 7.4. The coverslips were immediately washed in the same buffer, transferred to an open chamber, placed on the microscope stage and maintained at 36–37 °C using a Delta T4 Culture Dish Controller (Biopetechs, Butler, PA, USA). PI fluorescence was excited at 540 nm and emission measured with a 660 nm band pass filter, using a 550 nm beam splitter using an Olympus BX51W1 fluorescent microscope fitted with a 20 \times /0.95 water

immersion objective and cooled CCD camera. For excitation, a xenon lamp-based DeltaRam high-speed monochromator from Photon Technology International (PTI, Lawrenceville, NJ, USA) was used. For control of the monochromator and videocamera, as well as for fluorescent imaging and collecting of data, the ImageMaster 3.0 software (PTI) was used. The data were expressed as percent of PI positive cells.

Nitrate/nitrite production

Nitrate/nitrite derived from NO produced in 24 h from 25 islets was measured by the Griess reaction.

Quantitative real time-PCR

Total RNA was extracted from 30 isolated islets by the TRIzol method (Invitrogen). cDNA was synthesized from 1 μ g total RNA. Real-time-PCR was performed to measure changes in selected genes regulated by NF- κ B or otherwise important to β -cell function. Primer sequences are listed in Table 1.

Western blotting

Groups of cultured islets were washed with 0.1 M PBS, then lysed with Tris-EDTA-sucrose buffer (pH 7.4; containing 1% Triton X-100, 4 μ g/ml aprotinin and 5 μ g/ml bestatin), sonicated on ice for 5 min and separated by SDS-PAGE. Membranes were probed with specific primary antibodies and protein band intensities were normalized to β -actin.

Table 1 Primer sequences used for real-time PCR

Gene (alternate name)	Forward	Reverse
<i>Actb</i> (Actin, beta)	5'-CTGAATGGCCAGGTCTGA-3'	5'-CCCTGGTGCCCTCAACAC-3'
<i>Ddit3</i> (Chop)	5'-CAACAGAGGTACACGCACAT-3'	5'-CCTGGGCCATAGAAGCTGACT-3'
<i>Myc</i>	5'-CGGTTCCCTTCTGACAGAAGTGA-3'	5'-CAGCCAAGGTTGTGAGGTTAGG-3'
<i>Ptgs2</i> (Cyclo-oxygenase-2)	5'-CCTACTACAAGTGTCTTTTTTGCATT-3'	5'-TCACACCATAGTTTCAGAGAGGTAATCA-3'
<i>Cpt1a</i>	5'-CAATGCAATTTTTACTCCTTCCA-3'	5'-TTACCTCCTCCTTTGAACACTTACAG-3'
<i>Ccnd2</i> (cyclin D2)	5'-TGACTGAACATTTTGGATGTAAGAA-3'	5'-CCCGGACGCTCAGTCTTG-3'
<i>Gpx1</i>	5'-GCGGCCCTGGCATTG-3'	5'-GGACCAGCGCCCATCTG-3'
<i>Ikkb</i> (IKK β)	5'-ACAGCCAGGAGATGGTACG-3'	5'-CGGACTTTGCTACAGGCGAT-3'
<i>Nos2</i> (inducible nitric oxide synthase (iNOS))	5'-CCTCCTTTGCCCTCACTCT-3'	5'-CTTCAGTCAGGAGGTTGAGTTTTTC-3'
<i>Isl1</i>	5'-GGAGATGACGGGCTCAGT-3'	5'-CTGCGTTTCTTGCTCCTTGCA-3'
<i>Jun</i> (c-jun)	5'-CCAGCAATGGGCACATCAC-3'	5'-TGCTCGTCCGGTCACGTTCT-3'
<i>Mapk1</i>	5'-TGCTGTGGAGTTGATGGTGTAT-3'	5'-TTGAACAAGGCATATTTCTCATCAG-3'
<i>Pdx1</i>	5'-TTTGAAGTCAGTCAGTTGCTCCTT-3'	5'-CCTTCAACCCCTCTCTTGCTATT-3'
<i>Sod2</i> (MnSOD)	5'-CACATTAACGCGCAGATCATG-3'	5'-CCAGAGCCTCGTGGTACTTCTC-3'
<i>Sod3</i>	5'-AGGTGGATGCTGCCGAGAT-3'	5'-TCCAGACTGAAATAGGCCCTCAAG-3'
<i>Tnfaip3</i> (A20)	5'-TGGTTCCAATTTTGCTCCTT-3'	5'-CGTTGATCAGGTGAGTCGTG-3'
<i>Ucp2</i>	5'-CATCGCCTCCCCTGTTGAT-3'	5'-TGGCCCAAGGCAGAGTTC-3'

Immunohistochemistry

Pancreas was harvested from mice just prior to islet isolation by taking a small piece of tissue from adjacent to the spleen and fixing in buffered formalin for 24 h. The tissues were dehydrated and embedded in paraffin using standard procedures and 5 μ m sections cut and affixed to glass slides. Following dewaxing, sections were blocked using 5% normal serum complementary to the respective secondary antibody. Unmasking was done by immersing the slides in heated PBS (pH 7.2) for 30 min. The sections were then incubated with primary antibody overnight at 4 °C. Following stringent washing in PBS, appropriate secondary antibodies (conjugated to AlexaFluor dyes of 488 or 546 nm excitation wavelength) were applied for 1 h at room temperature. This process was repeated for blocking primary and secondary antibodies for dual-labeling experiments. Slides were coverslipped in SlowFade Gold containing DAPI (Invitrogen) in order to visualize the nuclei. Digital photomicrographs were obtained using a Zeiss fluorescence microscope and Axiovision 4.6 software.

Statistical analysis

All results are expressed as mean \pm S.E.M. Data were analyzed by using one or two-way ANOVA. Statistical analyses were done using Prism software (Graphpad, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

NO production is enhanced by UCP2 null expression

Increased NO and superoxide production has been reported in immune tissues of *Ucp2*^{-/-} mice (Bai *et al.* 2005; Fig. 1). In pancreatic islets, basal *Nos2* mRNA (Fig. 1A) and iNOS protein expression (Fig. 1B) were barely detectable relative to β -actin in both *Ucp2*^{+/+} and *Ucp2*^{-/-} islets, but NO production, measured by Griess assay, was increased by 2-fold in *Ucp2*^{-/-} versus *Ucp2*^{+/+} islets ($P < 0.05$, Fig. 1C). Interestingly, the iNOS inhibitor 1400W at a concentration of 10 μ mol/l more potently reduced basal NO production in *Ucp2*^{-/-} than *Ucp2*^{+/+} islets (Fig. 1C), although a concentration of 50 μ mol/l reduced NO to not-detectable levels in both islets (data not shown). Higher doses of 1400W non-specifically inhibit other NOS isoforms. Cytokine exposure of intact islets for 24 h increased iNOS mRNA (Fig. 1A) and protein (Fig. 1B). NO was increased significantly in *Ucp2*^{+/+} but not in *Ucp2*^{-/-} islets when cytokines were present ($P < 0.05$, Fig. 1C). In both cases, NO production was suppressed by 1400W but not by CAPE.

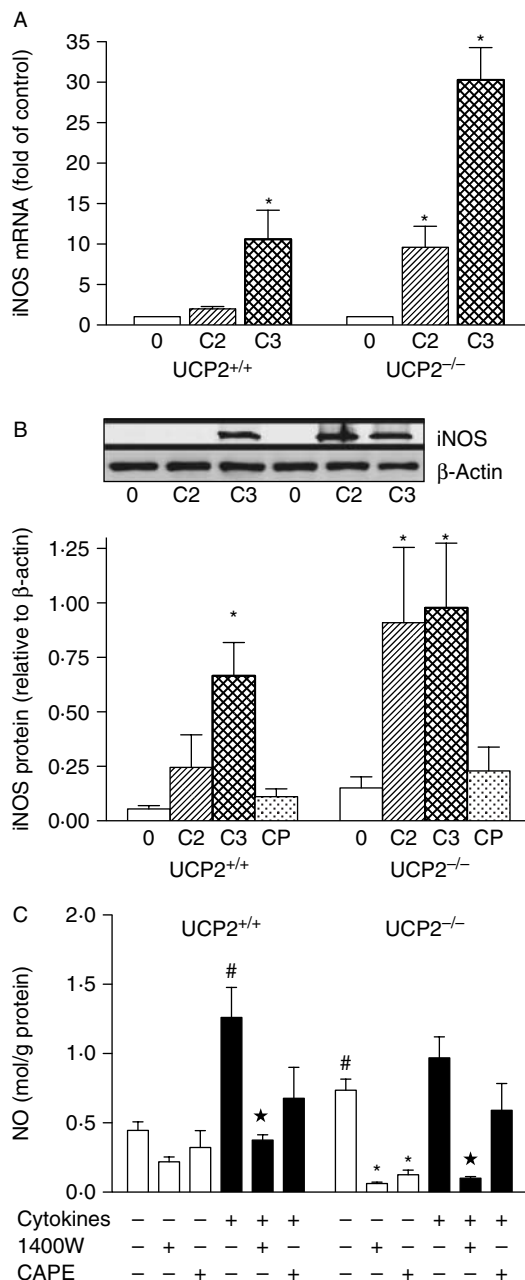


Figure 1 Basal and cytokine-stimulated production of nitric oxide in islets. Expression of (A) iNOS (*Nos2*) mRNA and (B) iNOS protein in UCP2^{+/+} and UCP2^{-/-} islets. 0=untreated control islets, C2=IL1 β +IFN γ and C3=IL1 β +IFN γ +TNF- α . Data were normalized to β -actin expression, as shown in the representative blot (inset). The effect of CAPE (CP) in the presence of three cytokines is also shown. $N=3$ or greater. (C) Nitric oxide (as nitrite normalized to total protein) was measured in supernatant from cultures of islets after 24 h in the absence or presence of iNOS inhibitor 1400W (10 μ mol/l) or CAPE (10 μ mol/l). $N=4-8$. * $P < 0.05$ compared with respective basal group; star indicates $P < 0.05$ compared with UCP2^{+/+} basal; # $P < 0.05$ compared with respective cytokine-treated group.

No genotype-related differences in ROS formation were found under any experimental conditions (data not shown). Mn-superoxide dismutase, which may be induced by ROS, was quantified by western blotting and was similar between *Ucp2*^{-/-} and *Ucp2*^{+/+} islets basally and after exposure to cytokines for 24 h. mRNA expression of *Sod2*, *Sod3* and glutathione peroxidase-1 (*Gpx1*) was also not significantly different between genotypes (data not shown).

Effect of UCP2 null expression and cytokines on expression of selected genes

The adaptive response to cytokine exposure was compared between *Ucp2*^{-/-} and *Ucp2*^{+/+} islets (Figs 2 and 3A). A significant increase in basal cyclin D2 (*Ccnd2*) and *Tnfaip3* mRNA was detected in *Ucp2*^{-/-} islets, whereas the mRNA expression of *Myc* and *Jun* was reduced (Fig. 2A). Basal protein expression of TNFAIP3 was also increased in *Ucp2*^{-/-} islets (Fig. 2B). Immunohistochemistry also revealed strong staining of JUN in *Ucp2*^{+/+} relative to *Ucp2*^{-/-} islets, and the majority positive cells also stained positively for insulin (Fig. 3A).

Expression of these genes was assessed after cytokine treatment of the islets (Fig. 2C). The mRNA for *Tnfaip3*, an important anti-apoptosis gene in β -cells, was induced ~6-fold in *Ucp2*^{-/-} islets ($P < 0.01$) compared with 3-fold in *Ucp2*^{+/+} islets. The redox-sensitive transcription factor gene *Jun* was significantly increased in *Ucp2*^{-/-} but not in *Ucp2*^{+/+} islets by 24 h cytokine treatment, whereas *Ddit3* (chop), expressed in endoplasmic reticulum stress, was 3-fold higher in *Ucp2*^{+/+} islets after cytokine treatment (Fig. 2C). The expression of *Myc* was not altered by either cytokines in either genotype, while cytokines reduced expression of the rate-limiting gene of fat metabolism, *Cpt1*, in both (not shown). Lastly, expression of genes involved in cell growth and differentiation was measured. Overall, similar inhibition of expression of *Ccnd2*, *Isl1*, *Mapk* and *Pdx1* was observed in both *Ucp2*^{+/+} and *Ucp2*^{-/-} islets exposed to cytokines (not shown).

TNFAIP3 and NF- κ B immunostaining: effects of STZ

TNFAIP3 is characterized as a stress-response gene and may have a critical role in protecting β -cells (Liuwantara *et al.* 2006; Figs 3B and C and 4). TNFAIP3 immunostaining was detected in both *Ucp2*^{+/+} and *Ucp2*^{-/-} islets using H-100 polyclonal antibody. In *Ucp2*^{+/+} islets, TNFAIP3 was localized in the perinuclear region of cells on the periphery of islets and did not co-localize with insulin. However, in *Ucp2*^{-/-} islets, perinuclear localization was detected in non- β -cells, while diffuse cytoplasmic staining was detected in β -cells (Fig. 3B).

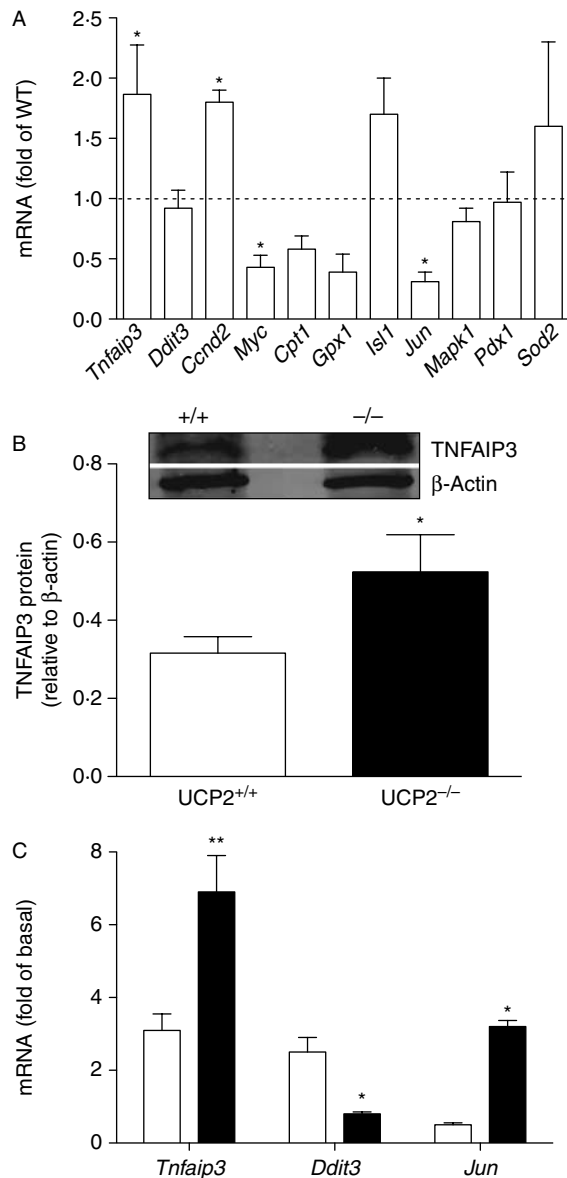


Figure 2 Expression of selected genes regulated by NF- κ B or important for β -cell differentiation and function. (A) Basal expression of selected genes. Data show mRNA levels in *UCP2*^{-/-} islets relative to *UCP2*^{+/+} islets (dashed line). Carnitine palmitoyl transferase-1, *Cpt1*; chop, *Ddit3*; cyclin D2, *Ccnd2*; glutathione peroxidase-1, *Gpx1*; mitogen-activated protein kinase-1, *Mapk1*; pancreas duodenum homeobox-1, *Pdx1*; superoxide dismutase-2, *Sod2*. (B) Protein expression of TNFAIP3 in *UCP2*^{+/+} and *UCP2*^{-/-} islets detected by immunoblotting. (C) Effects of cytokine treatment (IL1 β + IFN γ + TNF- α) for 24 h on mRNA expression of TNFAIP3, *Ddit3* (chop) and *Jun* expressed as fold of untreated controls in *UCP2*^{+/+} and *UCP2*^{-/-} islets. * $P < 0.05$ and ** $P < 0.01$ for $n = 3-4$ separate preparations.

Whether TNFAIP3 co-localized with glucagon was investigated and found to differ by genotype. As shown in the micrographs at the far right of Fig. 3B, TNFAIP3 and glucagon were co-localized in *Ucp2*^{-/-}

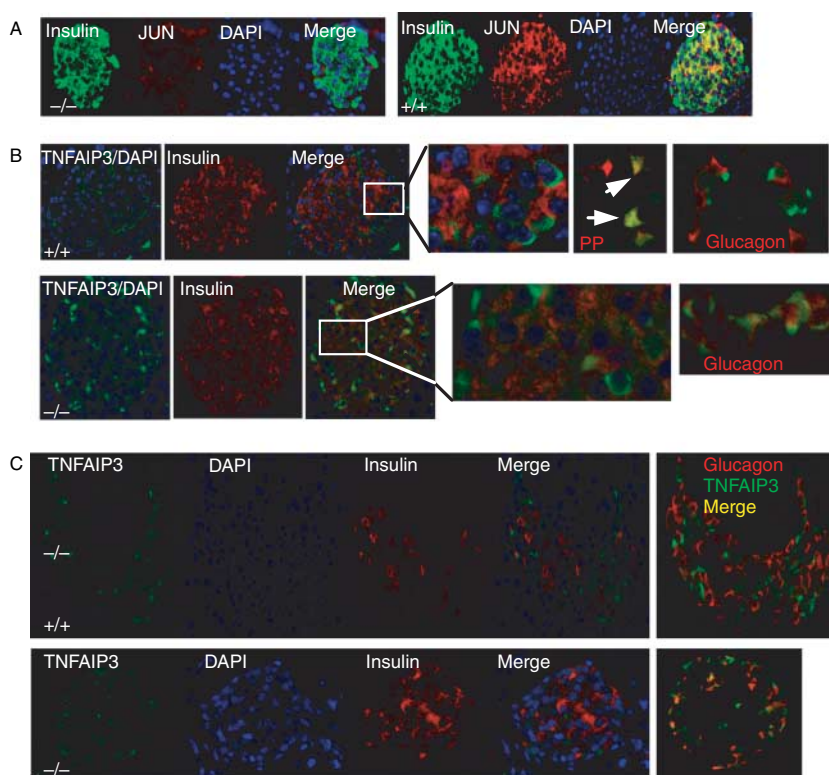


Figure 3 (A) Protein expression of JUN in $UCP2^{+/+}$ and $UCP2^{-/-}$ islets detected by immunohistochemistry. When images were merged, co-localization of insulin (green) and JUN (red) was detected as yellow in $UCP2^{+/+}$ islets. The intensity of JUN staining also appeared more intense in the $UCP2^{+/+}$ sections. (A) Representative photomicrographs showing localization of TNFAIP3 in islets by immunohistochemistry. TNFAIP3 (green) and insulin (red) were independently detected in islets from $UCP2^{+/+}$ (top) and $UCP2^{-/-}$ (bottom) mice. Nuclei were visualized with DAPI. Merged images indicated that in $UCP2^{+/+}$ islets, TNFAIP3 was detected mainly in the perinuclear region of non- β -cells (top, expanded from inset). There was co-labeling of TNFAIP3 with some pancreatic polypeptide-expressing cells (arrows) but not glucagon-labeled cells (far right). In $UCP2^{-/-}$ islets, TNFAIP3 was detected not only in the perinuclear regions of non- β -cells but also in the cytoplasm of cells co-staining for insulin (bottom, expanded from inset). In $UCP2^{-/-}$ islets, co-staining of TNFAIP3 and glucagon was detected (far right). (C) Effects of STZ treatment on TNFAIP3 immunostaining in islets. Tissues were obtained 14 days after STZ administration. In $UCP2^{+/+}$ islets (top), TNFAIP3 staining was confined to non- β - and non- α -cells. Islets from $UCP2^{-/-}$ mice (bottom) expressed TNFAIP3 in most α -cells.

but not in $Ucp2^{+/+}$ islets. In $Ucp2^{+/+}$ islets, the TNFAIP3 staining overlapped with a majority of pancreatic polypeptide-expressing cells. No TNFAIP3 staining was detected in somatostatin-expressing cells (not shown). A similar staining pattern of TNFAIP3 was found when a monoclonal antibody (A-12) was used.

STZ induces oxidative/nitrosative stress and kills β -cells. TNFAIP3 localization in pancreatic tissue from STZ-treated $Ucp2^{-/-}$ and $Ucp2^{+/+}$ mice was compared 14 days after induction of diabetes. TNFAIP3 staining in $Ucp2^{+/+}$ islets was not associated with either β - or α -cells (Fig. 3C, top panel), whereas in $Ucp2^{-/-}$ islets, some α -cells staining for glucagon also stained for TNFAIP3 (Fig. 3C, bottom panel).

NF- κ B regulates *Tnfaip3* transcription and TNFAIP3 acts to negatively regulate NF- κ B transactivation in islets (Grey *et al.* 2003). We therefore investigated whether altered NF- κ B expression or cellular localization could be detected in pancreatic sections. NF- κ B immunostaining of pancreatic islets was more intense than of acinar cells and was similar in intensity in $Ucp2^{-/-}$ and $Ucp2^{+/+}$ islets in fixed pancreatic tissue. NF- κ B staining co-localized with α -cells in both $Ucp2^{+/+}$ and $Ucp2^{-/-}$ islets (Fig. 4A and B). In islets from $Ucp2^{-/-}$ mice, NF- κ B staining distinctly above background was also detected in non- α -cells. Because these cells were central to the islet, they appeared to be β -cells, although it was difficult to detect co-localization with insulin.

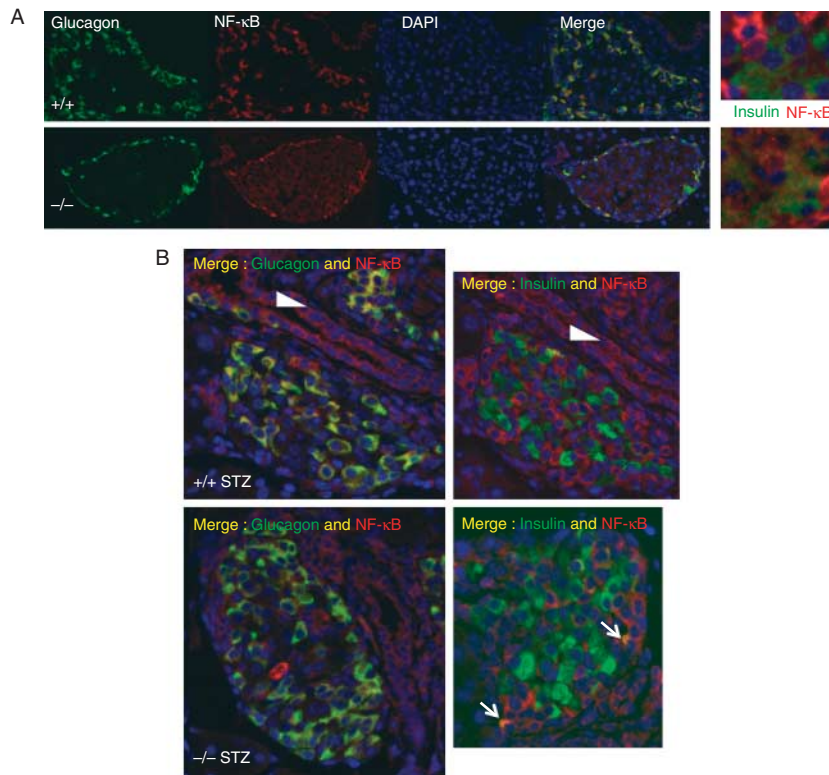


Figure 4 Localization of NF- κ B by immunohistochemistry. (A) Glucagon (green) and NF- κ B (red) were independently detected in islets from UCP2^{+/+} (top) and UCP2^{-/-} (bottom) mice. Nuclei were visualized with DAPI. Merged images indicated that in UCP2^{+/+} islets, NF- κ B was detected in non- β -cells of both phenotypes. Less-intense staining was also detected in the islet core of UCP2^{-/-} islets. Co-localization of insulin with NF- κ B was detected in UCP2^{-/-} but not in UCP2^{+/+} islets (far right panels). For these images, insulin staining was blunted equally in both photomicrographs using Image J (intensity range 43–446) and the maximum intensity of NF- κ B increased equally (intensity range 0–140). (B) Effects of STZ treatment on NF- κ B immunostaining in islets. Tissues were obtained 14 days after STZ administration. In UCP2^{+/+} islets (top), NF- κ B staining was confined to non- β -cells that also stained for glucagon (left panel), whereas co-localization with insulin was not detected (right panel). Islets from UCP2^{-/-} mice (bottom) expressed NF- κ B in α -cells. A minority of β -cells also appeared to express NF- κ B (arrows). In both genotypes, NF- κ B was detected in duct cells (arrowheads).

Following STZ treatment, there appeared to be increased NF- κ B staining intensity in non- β -cells and also in duct cells in islets from both genotypes (Fig. 4C and D). In all cases, the majority of staining was non-nuclear. A minority of β -cells also appeared to co-localize NF- κ B in the Ucp2^{-/-} islets.

Lack of UCP2 modifies basal NF- κ B activation and apoptosis in isolated islets

Because of the potential induction of NF- κ B in islets from Ucp2^{-/-} mice, further characterization was performed in isolated islets (Fig. 5). Basal NF- κ B activity was significantly increased ($P < 0.05$) by 2-fold in Ucp2^{-/-} versus Ucp2^{+/+} islets after overnight culture (Fig. 5A). The compound BAY, which blocks

phosphorylation of I κ B α to prevent NF- κ B nuclear translocation, inhibited NF- κ B activation by >50% in Ucp2^{+/+} islets but had no effect in Ucp2^{-/-} islets when applied for 2 h. However, blocking NO production with the iNOS inhibitor 1400W significantly inhibited basal NF- κ B translocation in both genotypes. Likewise, CAPE, which interferes with the binding of the RelA subunit to DNA, reduced NF- κ B activation in islets from both genotypes. The increase in basal NF- κ B activity in Ucp2^{-/-} islets could be attributed to 2-fold greater basal I κ B α phosphorylation (Fig. 5B), downstream from increased basal IKK β expression, as suggested from measurement of mRNA (Fig. 6C). I κ B α phosphorylation was significantly inhibited by BAY within 2 h of application in Ucp2^{-/-} and Ucp2^{+/+} islets, suggesting that, in a relatively acute timeframe, lack of

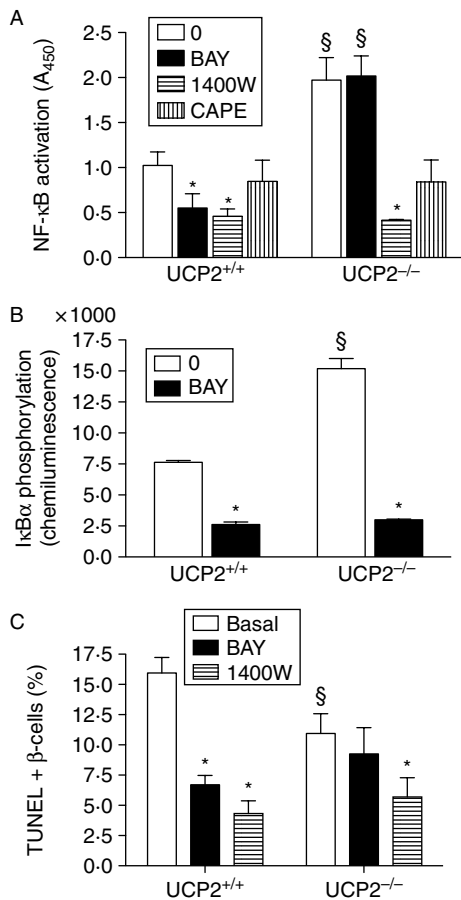


Figure 5 Characterization of basal NF- κ B pathway and apoptosis in islets from UCP2^{+/+} and UCP2^{-/-} mice. (A) Basal NF- κ B (RelA subunit) activation ($n=4$) in control cells and those incubated with IKK β inhibitor BAY 11-7082 (10 μ M) or iNOS inhibitor 1400W (10 μ M) for 2 h. Data were normalized to protein concentration. (B) Basal I κ B α phosphorylation in the presence or absence of BAY ($n=4$). Effects were quantified by chemiluminescence and normalized to protein concentration. (C) Basal apoptosis, as a percentage of total β -cells, in UCP2^{+/+} and UCP2^{-/-} islets, comparing the effects of BAY or 1400W with untreated controls. Cell apoptosis was assessed by TUNEL staining. Two thousand cells were counted on average at 200 \times magnification ($n=4$). * $P<0.05$, effect of inhibitor; § $P<0.05$, effect of genotype).

effect of BAY on NF- κ B activation is due to its sequestration in the nucleus.

To determine the effects of increased basal NF- κ B activation on β -cell viability, we assessed apoptosis frequency by TUNEL, which was significantly higher in dispersed cells from Ucp2^{+/+} than Ucp2^{-/-} islets (Fig. 5C). Ucp2^{+/+} islets had a significantly ($P<0.05$) reduced percentage of apoptotic cells when treated with BAY for 24 h but there was no effect in Ucp2^{-/-} islets. Blocking NO production with 1400W reduced apoptosis by $\sim 75\%$ in Ucp2^{+/+} islets. There was a 36% decrease in apoptosis in Ucp2^{-/-} islets, from 11.0 to $\sim 7.0\%$ ($P<0.05$).

Cytokine-mediated cell death has altered dependence upon NF- κ B in Ucp2^{-/-} islets

Culture for 24 h with a combination of three cytokines (50 ng/ml IL1 β +50 ng/ml IFN γ +5 ng/ml TNF- α) increased apoptosis assessed by the TUNEL method in Ucp2^{+/+} but not in Ucp2^{-/-} β -cells (Fig. 6A). In β -cells exposed to cytokines plus iNOS inhibitor 1400W, apoptosis was strongly inhibited in both genotypes (Ucp2^{+/+}: 34.1 \pm 9.5% vs 8.4 \pm 0.7% and Ucp2^{-/-}: 13.1 \pm 2.2 vs 5.8 \pm 0.9% in the absence and presence of 1400W, respectively, $P<0.01$). A significant reduction ($P<0.01$) in apoptotic cell number was observed in cytokine-exposed Ucp2^{+/+} but not in Ucp2^{-/-} β -cells treated concurrently with BAY for 24 h (Fig. 6A). Necrosis (PI fluorescence) in both genotypes was increased equally by cytokines (data not shown).

NF- κ B was stimulated by 2.5-fold by cytokines in Ucp2^{+/+} islets ($P<0.05$) and this was inhibited by $>90\%$ with 2 h BAY treatment ($P<0.001$). Conversely, neither cytokines nor BAY affected NF- κ B activity in islets from Ucp2^{-/-} mice (Fig. 6B). However, NF- κ B activation was strongly inhibited by 1400W and CAPE in both genotypes. I κ B α phosphorylation triggers translocation of RelA subunit of NF- κ B to the nucleus. In Ucp2^{+/+} mice, cytokines increased I κ B α phosphorylation, as expected, and this was significantly reduced by BAY. Ucp2^{-/-} islets did not demonstrate any increase in I κ B α phosphorylation in the presence of cytokines but BAY did reduce phosphorylation by 85% (Fig. 6C). By western blotting, there was no difference in total expression of either NF- κ B or I κ B α in Ucp2^{+/+} versus Ucp2^{-/-} islets (data not shown). However, the mRNA expression of IKK β , which phosphorylates I κ B α , was elevated 2–3-fold in Ucp2^{-/-} compared with Ucp2^{+/+} islets, both basally and after 24 h cytokine treatment (Fig. 6D).

Discussion

Null expression of UCP2 enhances β -cell adaptation to metabolic stress as shown by high-fat feeding studies in which glucose-stimulated insulin secretion remained robust and β -cell mass was expanded more than that in Ucp2^{+/+} mice (Joseph *et al.* 2002, 2004). These results are considered paradoxical by some authors in light of the hypothesis that UCP2 induction protects cells from oxidative stress (Pi *et al.* 2009). Indeed, this is demonstrated in stroke survival (Mehta & Li 2009). The purpose of the current study was to examine potential mechanisms contributing to β -cell protection in the absence of UCP2. We show that isolated Ucp2^{-/-} islets have increased basal NO production and expression of *Tnfaip3* (also called A20) and *Ccnd2*, two genes associated with cell proliferation, and

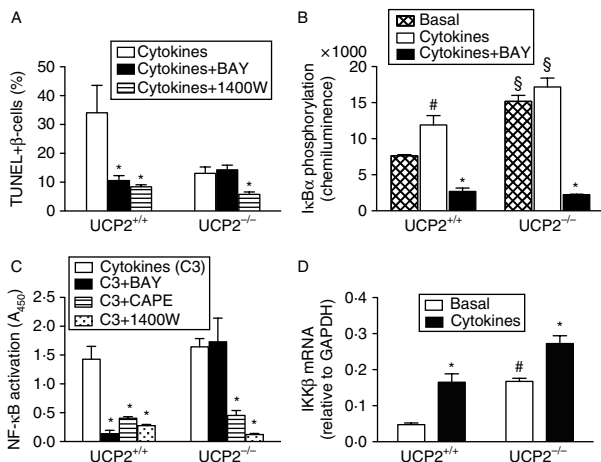


Figure 6 Effects of 24 h exposure to cytokines on β -cell death and NF- κ B pathway in islets from UCP2^{+/+} and UCP2^{-/-} mice. In all cases, $n=4$. Basal = untreated control islets, cytokines = IL1 β + IFN γ + TNF- α . (A) Cytokine-induced apoptosis, as percentage of total β -cells from UCP2^{+/+} and UCP2^{-/-} islets after 24 h exposure. Effects of concurrent BAY 11-7082 (10 μ M) are shown. The iNOS inhibitor 1400W (10 μ M, 24 h) inhibited apoptosis induced by cytokines to 8.4 \pm 0.7% in UCP2^{+/+} and 5.8 \pm 0.9% in UCP2^{-/-} islets ($P < 0.05$ for both). (B) Effects of 24 h cytokines on NF- κ B activation in UCP2^{+/+} and UCP2^{-/-} islets in cytokine-treated cells and those incubated concurrently with inhibitors. The IKK β inhibitor BAY strongly inhibited NF- κ B activation in UCP2^{+/+} but not in UCP2^{-/-} islets. The iNOS inhibitor 1400W inhibited NF- κ B transactivation in both UCP2^{+/+} and UCP2^{-/-} islets ($P < 0.05$ for both). CAPE also inhibited NF- κ B transactivation in both UCP2^{+/+} and UCP2^{-/-} islets ($P < 0.05$ for both). (C) Phosphorylation of I κ B α basally or stimulated by cytokines in the presence and absence of BAY in UCP2^{+/+} and UCP2^{-/-} islets. (D) Expression of IKK β mRNA in control and cytokine-treated islets. * $P < 0.05$, effect of cytokines; \$ $P < 0.05$ for genotype effect; # $P < 0.05$, effect of cytokines compared with basal).

reduced expression of *Myc* and *Jun*, two genes associated with apoptosis. The link between NO and the altered expression of these genes appears to be the transcription factor NF- κ B. The resultant phenotype in isolated islets includes activation of NF- κ B and induction of and/or altered cellular localization of TNFAIP3. These traits are linked to protection of β -cells from isolation- and cytokine-mediated apoptosis.

Basal production of NO was 2-fold higher in islets from Ucp2^{-/-} than from Ucp2^{+/+} mice. Because basal NO synthesis of Ucp2^{-/-} islets was inhibited by 1400W, enhanced activity of iNOS is suggested, whereas in Ucp2^{+/+} islets, basal production of NO was not inhibited by 1400W. Islets express neuronal NOS and endothelial NOS, both constitutively active isoforms (Spinas 1999) not sensitive to 1400W (Garvey *et al.* 1999), that are possibly the source of basal NO in Ucp2^{+/+} islets. These isoforms may also contribute to the fraction of basal NO not inhibited by 1400W in Ucp2^{-/-} islets. Exposure to cytokines, which are known to induce iNOS, elicited similar increase in iNOS mRNA and protein in Ucp2^{+/+} and Ucp2^{-/-} islets.

The NO produced under cytokine stimulation was inhibited by 75 and 90% by 1400W in Ucp2^{+/+} and Ucp2^{-/-} islets, respectively. These data suggest higher basal activity of iNOS in Ucp2^{-/-} islets and is consistent with other studies showing elevated circulating NO, increased NO production by macrophages and increased iNOS expression in spleen (Bai *et al.* 2005). The physiological relevance of this relatively small increase in NO production remains unclear, but low concentrations of NO can act in signaling pathways, having been shown to promote insulin secretion via modulation of ATP-dependent K channels (Sunouchi *et al.* 2008).

NO is implicated in the regulation of gene transcription, working via NF- κ B and the cAMP-response element binding protein, MYC (Contestabile 2008). A collection of pro-proliferative, pro-apoptotic, islet-specific and metabolic genes was screened for basal and cytokine-stimulated mRNA expression. NF- κ B is known to respond to cytokines and is differentially regulated in immune tissues in Ucp2^{-/-} mice (Bai *et al.* 2005); thus, some genes selected are regulated by NF- κ B. Basally, four genes from this panel were differentially expressed in Ucp2^{-/-} compared with Ucp2^{+/+} islets. Reduced expression of *Myc* and *Jun* is predicted to promote cell survival in Ucp2^{-/-} islets. MYC is a transcription factor implicated in starvation-induced, caspase-mediated apoptosis in β -cells (Van de Castele *et al.* 2003), and JUN, also a transcription factor, is involved in cytokine-mediated apoptosis (Ammendrup *et al.* 2000). In islets, *Myc* is regulated by NF- κ B but *Jun* is induced by cytokines independent of NF- κ B (Cardozo *et al.* 2001). Conversely, we observed induction of *Ccnd2*, which is essential for postnatal β -cell replication (Kushner *et al.* 2005) and could be associated with the enhanced proliferation of β -cells observed in islets from high-fat diet-fed mice (Joseph *et al.* 2002). Each of these observations is consistent with reduced frequency of apoptosis in Ucp2^{-/-} β -cells observed after islet isolation.

The fourth gene of interest was *Tnfaip3*, which has been identified as a cardinal gene regulating anti-apoptosis in β -cells (Liuwantara *et al.* 2006) and the strong mRNA and protein induction observed is consistent with lower basal apoptosis in UCP2^{-/-} islet cells. Changes in TNFAIP3 might occur as a result of islet isolation, which is known to induce apoptosis in a fraction of the β -cells (Thomas *et al.* 2009). We used immunohistochemistry to study *in situ* TNFAIP3 expression patterns in a less-stressed state, which revealed an altered pattern of expression in islets. In Ucp2^{+/+} islets, TNFAIP3 was most abundantly expressed in non- β -cells that stained for pancreatic polypeptide and was predominantly perinuclear in distribution. Conversely, TNFAIP3 was detected in both β - and non- β -cells in Ucp2^{-/-} islets and was

predominantly dispersed throughout the cytoplasm of β -cells while maintaining perinuclear localization in non- β -cells. Liuwantara *et al.* (2006) demonstrated localization of TNFAIP3 predominantly in the cytoplasm of β -cells but used cytokine-stimulated, dispersed FACS-purified cells for immunostaining, whereas the current results are from intact islets without exogenous cytokine stimulation. Predominantly perinuclear localization of TNFAIP3 was noted in a COS7 cell model (Li *et al.* 2008). Dispersal of TNFAIP3 throughout the cytoplasm in *Ucp2*^{-/-} β -cells may have important functional consequences. Interestingly, islets from pancreatectomized rats exhibit strong, nearly 5-fold induction of *Tnfaip3* mRNA and these rats are protected from cell death after STZ treatment (Laybutt *et al.* 2002), as are mice overexpressing TNFAIP3 in pancreas (Yu *et al.* 2004). *Ucp2*^{-/-} mice are also partially resistant to STZ, maintaining higher circulating insulin concomitant with lower plasma glucose, although β -cell mass was not significantly protected (Lee *et al.* 2009). After STZ treatment, TNFAIP3 localization in *Ucp2*^{+/+} islets was maintained in both phenotypes and did not appear elevated relative to untreated islets. We speculate that co-localization of TNFAIP3 in α -cells of *Ucp2*^{-/-} islets has important adaptive effects because *Ucp2*^{-/-} α -cells are highly sensitive to cell death stimuli (Diao *et al.* 2008). Examination of islets in the early aftermath of STZ treatment (e.g. 3–7 days instead of 14 days) might reveal greater adaptive response and genotype differences.

Both iNOS and TNFAIP3 in islets are regulated by the transcription factor NF- κ B (Cardozo *et al.* 2001, Laybutt *et al.* 2002). Although total NF- κ B expression was similar to *Ucp2*^{+/+} islets (data not shown), translocation to the nucleus of RelA was significantly greater in isolated *Ucp2*^{-/-} mouse islets. The mechanism appeared to be at the level of IKK β expression, which was 3-fold higher in *Ucp2*^{-/-} islets, and correlated with 2-fold higher I κ B α phosphorylation. Normally, NF- κ B RelA is retained in the cytoplasm by association with I κ B α . IKK β phosphorylation of I κ B α allows it to enter the ubiquitin pathway for degradation, thereby permitting translocation of NF- κ B RelA subunit to the nucleus (Magnani *et al.* 2000). Laybutt *et al.* (2002) also found increased NF- κ B activity in their pancreatectomy model with induced TNFAIP3 expression and resistance to STZ. The current findings were also consistent with those in *Ucp2*^{-/-} immune tissues, where IKK β activity was enhanced and persistent NF- κ B activation was noted (Bai *et al.* 2005). Curiously, while the IKK β inhibitor BAY strongly reduced I κ B α phosphorylation in *Ucp2*^{-/-} islets, it had no effect on NF- κ B nuclear translocation or apoptosis. One explanation is that once NF- κ B is sequestered in the nucleus, it is immune to regulatory events occurring in the cytoplasm. This hypothesis is supported by the fact that CAPE, which

inhibits NF- κ B by blocking its binding to DNA, reduced basal and cytokine-stimulated NF- κ B activation in *Ucp2*^{-/-} islets as well as iNOS expression. However, further experiments to test this hypothesis more directly are required. *In situ* examination of NF- κ B expression patterns by immunohistochemistry did not confirm increased translocation to the nucleus. Thus, like the changes seen in TNFAIP3, islet isolation may be the initial precipitating factor leading to increased RelA detected in the nucleus. Likewise, the lack of effect of STZ on the cellular distribution of NF- κ B is possibly due to the extended sampling period of 14 days post-treatment.

In most studies of islets, NF- κ B is associated with an increase in apoptosis but a growing amount of data (Laybutt *et al.* 2002, Hagerkvist *et al.* 2007, Kim *et al.* 2007), including the current report, suggest that longer-term or constitutive activation of NF- κ B confers a pro-proliferative, anti-apoptotic phenotype in β -cells. The failure to detect a significant elevation in apoptosis in isolated *Ucp2*^{-/-} islets may be due to strong induction of TNFAIP3 during islet isolation and suppression of *Myc* and *Jun*. Over-expression of TNFAIP3 in islets protected from NF- κ B activation and cytokine-mediated apoptosis (Grey *et al.* 1999) and increased islet survival after transplantation (Grey *et al.* 2003). Cytokine-treated human islets also had increased expression of TNFAIP3 and other anti-apoptotic genes (Sarkar *et al.* 2009). NF- κ B activation is also required for induction of other anti-apoptotic genes in mouse islets, including XIAP and c-FLIP, two inhibitors of caspases (Kim *et al.* 2007), which merits further investigation in our model. While concomitant up-regulation of TNFAIP3 and activation of NF- κ B appear paradoxical, we concur with the suggestion of others (Laybutt *et al.* 2002) now generally accepted in multiple cell types (Hymowitz & Wertz 2010) that TNFAIP3 is working as a brake under these conditions and, possibly combined with increased expression of other anti-apoptotic genes, is protective. Of interest is the finding that TNFAIP3 is a substrate of IKK β (Hutti *et al.* 2007). With an increase in IKK β expression, as documented here, TNFAIP3 phosphorylation at serine 381 is predicted to be increased, which would increase its ability to inhibit the NF- κ B pathway (Hutti *et al.* 2007). TNFAIP3 acts upstream of IKK β to deubiquitinate substrates involved in NF- κ B signaling, such as RIPK1 and TRAF6 (Hymowitz & Wertz 2010).

In conclusion, we find that isolated islets from *Ucp2*^{-/-} mice display increased transactivation of NF- κ B, which may contribute to enhanced expression of anti-apoptotic genes such as *Tnfaip3* and pro-proliferative genes such as *Ccnd2*. This phenotype appears to be revealed by stressful events such as islet isolation because nuclear localization of NF- κ B was not observed in intact pancreatic tissue. One possible

mechanism is up-regulation of NO-dependent IKK β activity. TNFAIP3 is of particular interest because of its ability to exert negative feedback on NF- κ B signaling pathways to modulate apoptosis and other downstream events. Interestingly, TNFAIP3 had altered cellular and sub-cellular distribution in the islets from *Ucp2*^{-/-} mice. This phenotype may contribute to the so-called paradoxical improved outcomes of *Ucp2*^{-/-} mice to stressors such as cytokines, as shown here, or high-fat diet challenge (Joseph *et al.* 2002, 2004). Furthermore, the localization of TNFAIP3 in non- β -cells *in situ* has not previously been reported but may be of importance to islet function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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