Title

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Journal

Journal of Molecular Endocrinology, 53(3)

ISSN

0952-5041

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Publication Date

2014-12-01

DOI

10.1530/jme-14-0056

Peer reviewed

HHS Public Access

Author manuscript

J Mol Endocrinol. Author manuscript; available in PMC 2015 December 01.

Published in final edited form as:

J Mol Endocrinol. 2014 December ; 53(3): 417–427. doi:10.1530/JME-14-0056.

CRFR1 activation protects against cytokine-induced beta cell death

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Abstract

During diabetes development beta cells are exposed to elevated concentrations of proinflammatory cytokines, TNFα and IL-1β which *in vitro*, induce beta cell death. The class B G-protein-coupled receptors (GPCRs): Corticotropin releasing factor receptor 1 (CRFR1) and CRFR2 are expressed in pancreatic islets. As downstream signalling by other class B GPCRs can protect against cytokine-induced beta cell apoptosis we evaluated the protective potential of CRFR activation in beta cells in a pro-inflammatory setting.

CRFR1/CRFR2 ligands activated AKT and CRFR1 signalling reduced apoptosis in human islets. In rat and mouse insulin secreting cell lines (INS-1 and MIN6) CRFR1 agonists upregulated insulin receptor substrate 2 (IRS2) expression, increased AKT activation, counteracted cytokine-mediated decrease in BAD phosphorylation, and inhibited apoptosis. The anti-apoptotic signalling was dependent on prolonged exposure to CRF family peptides and following PKA activation mediating IRS2 upregulation. This suggests that CRFR signalling counteracts proinflammatory cytokine-mediated apoptotic pathways by upregulation of survival signalling in beta-cells. Interestingly, CRFR signalling also counteracts basal apoptosis in both cultured INS-1 cells and intact human islets.

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Declaration of interest

L.B., G.L.C., M.M., T.v.d.M., M.O.H and N.B. have nothing to declare. W.W.V. was a co-founder, 356 member of the Board of Directors, and a shareholder of Neurocrine Biosciences, a company that is 357 developing small molecule antagonists of corticotropin releasing factor.

Author contribution statement

L.B. designed the study, performed experiments, evaluated data and wrote the manuscript. G.L.C. performed experiments, evaluated data and wrote the manuscript, M.M., T.v.d.M., M.O.H, and N.B evaluated data and participated in writing the manuscript, and W.W.V. designed the study and evaluated data.

This article is dedicated to Wylie W. Vale, Ph.D., who passed away unexpectedly on January 3rd, 2012 while this work was in progress. Dr. Vale was an extraordinary mentor and contributed greatly to the understanding of CRF/urocortins effects in beta cells and islet.

Keywords

Beta cells; Apoptosis; Survival; Urocortins; GPCR; CRFR; Cytokines

3. Introduction

Pancreatic beta cell death occurs in type 1 as well as type 2 diabetes mellitus leading to a progressive decline in beta cell function and beta cell mass. Although the initiating mechanism differs, some signalling pathways are relevant for both diabetes types as they converge on common effectors. This is true for IL-1β and TNFα signalling as both these pathways lead to activation of two key pro-apoptotic signalling pathways in the beta cell, i.e. the nuclear factor κB (NFκB) and mitogen-activated protein kinases (MAPKs) (Donath et al., 2003). Prior to cytokine exposure, NFκB is sequestered in the cytoplasm bound to inhibitor protein kappa Bα (IκBα). Following IL-1β or TNFα exposure, IκBα is phosphorylated, ubiquitinated and degraded by the proteasomal complex, liberating NFκB to translocate to the nucleus and induce expression of several inflammatory genes (Bladwin et al., 1996, Flodstrom et al., 1996, Heimberg et al., 2001, Patel et al., 2009). Inhibition of the NFκB pathway protects pancreatic beta cells from cytokine-induced apoptosis *in vitro* and from multiple low-dose streptozotocin (STZ)-induced diabetes *in vivo* (Patel et al., 2009).

The c-Jun N-terminal kinase (JNK) is a member of the MAPKs and an important mediator of cytokine-induced beta cell death. Blocking JNK signaling protects against IL-1β-induced apoptosis in insulin-secreting cells (Ammendrup et al., 2000, Bonny et al., 2000, Bonny et al., 2001, Nikulina et al., 2003) and prevents cytokine-induced suppression of viability in human islets (Aikin et al., 2004). Two other MAPKs, the extracellular signal-regulated kinase 1/2 (ERK1/2) and p38, have also been shown to be involved in mediating deleterious cytokine effects in beta cells (Pavlovic et al., 2000, Saldeen et al., 2001), although ERK1/2 may be more known for its proliferative capacity (Blandino-Rosano et al., 2008). The cross-talk between JNK and the Ser/Thr kinase AKT, has great impact on survival prospects of human islets (Aikin et al., 2004). AKT is widely involved in cell growth and survival and its importance in beta cell survival has been substantiated in various mouse models with transgenic modification of components in the AKT pathway (Elghazi et al., 2009). AKT is commonly activated in a phosphatidylinositol 3-kinase (PI3K)-dependent manner (Elghazi et al., 2009). The insulin receptor substrate 2 (IRS2), a substrate of the insulin/insulin-like growth factor signaling cascade responsible for compensatory beta cell growth, function and survival throughout life (Jhala et al., 2003, White 2003) mediates anti-apoptotic signaling through activation of AKT. Correlation between increase in cAMP levels and the IRS2/AKT signaling pathway in beta cells has been demonstrated (Jhala et al., 2003, Van de Velde et al., 2011). AKT signaling inhibits several pro-apoptotic components including the JNK pathway (Aikin et al., 2004) and the pro-apoptotic Bcl-2 family member BAD (Zha et al., 1996). AKT-induced BAD phosphorylation is antagonized by JNK-stimulated BAD dephosphorylation. The latter leads to BAD-mediated functional blockage of anti-apoptotic Bcl-Xl and Bcl-2, initiation of the caspase cascade and induction of beta cell death (Sunayama et al., 2005, Zha et al., 1996).

The corticotropin releasing factor (CRF) family of peptides include CRF, urocortin (Ucn) 1, 2 and 3 (Lewis et al., 2011, Perrin and Vale 1999, Reyes et al., 2001, Vaughan et al., 1995). These peptides bind to two subtypes of CRF receptors, CRFR1 and CRFR2, with varying affinity. CRF is a preferred CRFR1 agonist (Perrin and Vale 1999) in contrast to Ucn2 and Ucn3, which are selective CRFR2 agonists (Hsu and Hsueh 2001, Lewis et al., 2001, Reyes et al., 2001). Ucn 1 binds to both receptors with high affinity (Perrin and Vale 1999). Members of this family were initially recognised as coordinators of the mammalian stress response. Since then, these peptides as well as their receptors have been identified in many tissues throughout the periphery suggesting a potential involvement in other physiological responses (Fekete and Zorilla 2007, Florio et al., 2004, Kimura et al., 2002, Lee et al., 2011, Kuperman and Chen 2008). In fact, the pancreatic beta cell is one of the most abundant sites of Ucn 3 expression (Li et al., Endo 2003).

Pancreatic islets were recently found to express both CRFR1 and CRFR2 in equal abundance (Huising et al., 2011). In clonal beta cells such as MIN6 and INS-1 cells the expression levels of CRFR1 is much higher than CRFR2, a balance that can be overturned by exposure to glucocorticoids (Huising et al., 2010, Huising et al., 2011). These receptors belong to the class B GPCRs, as do receptors for incretins such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (Brubaker and Drucker 2002). This subclass of receptors couples with Gαs and activates adenylate cyclase to stimulate cAMP production. We have previously shown that CRFR1 activation increases insulin secretion from pancreatic islets (Huising et al., 2010). Signalling induced by GIP and GLP-1 is known to prevent cytokine-induced apoptosis in beta cells (Ferdaoussi et al., 2008, Li et al., 2005, Natalicchio et al., 2010), prompting us to investigate the potential of CRFR signalling to promote beta cells survival and protect against cytokine-induced beta cell death.

4. Materials and methods

4.1 Reagents

Recombinant murine IL-1β was from BD Pharmingen and mouse TNFα from R&D. Antalarmin was a kind gift from Dr. Chrousos at NIH. All peptides used in this study were synthesized using BOC chemistry and provided by Dr. Jean Rivier (Salk Institute, La Jolla, CA). LY294002 and H89 were from Calbiochem (Billerica, MA).

4.2 Cells

INS-1 cells and MIN6 insulinoma cells (obtained at passage 18 from Ulupi Jhala (UCSD, La Jolla, CA) were maintained in DMEM (Invitrogen, Carlsbad, CA) containing Glutamax and 11 mM of glucose and supplemented with 10 % FBS (Sigma, St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 10 μmol/l β-mercaptoethanol (Sigma). Cells were cultured under standard cell culture conditions at 37°C in a humidified atmosphere containing 5% CO_2.

4.3 Human islets

Human islets were obtained through the Integrated Islet Distribution Program (IIDP islet program) funded by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and with support from the Juvenile Diabetes Research Foundation International (JDRFI). Upon arrival islets were washed twice in CMRL with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Islets were re-picked and cultured for 1–2 days before experimental set-up.

4.4 Immunoblotting

One hundred human islets were seeded in 24-well plates (Nunc, Rochester, NY) or 400,000 INS-1 cells were seeded in 12-well plates. Receptor antagonists were added 30 min prior to peptides and cells/islets incubated for various time points (30 min to 24 h). Cytokines were added 16 h following peptide treatment and cultured continued for another 20 min to 24 h. Cells were lysed in PLC-lysis buffer (50mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM Naf, 10 mM NaPPi, 50 μM $NaVO_4$, supplemented with protease inhibitors (Roche Diagnostics, Indianapols, IN) and 1mM DTT). Protein content was measured by the Bradford method prior to separation by gel electrophoresis using 3–7% or 10% BisTris gels (Invitrogen) and transferred by electroblotting to nitrocellulose membranes (Invitrogen). Membranes were blocked in TBST buffer (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 containing 5% nonfat dry milk), and incubated in primary antibody overnight at 4°C. After washing in TBST, membranes were incubated with secondary antibody at room temperature. Antibodies: Active motif; IκBα (#40903). Cell Signaling (Beverly, MA); phospho-ERK1/2 (#4377), total ERK1/2 (#9102), phospho-p38 (#4511), phospho-JNK (#4668), phospho-AKT (#9271), total AKT, cleaved caspase-3 (#9661) and phospho-BAD (#4366). BD Pharmingen (San Diego, CA); iNOS (#610332). Santa Cruz Biotechnology (Santa Cruz, CA); total JNK1 (#Sc-571) and IRS2 (#Sc-1555). Millipore (Bedford, MA); IRS2 (# MABS15). Abcam (Cambridge, MA); β-actin (ab6276). Secondary antibodies: horseradish peroxidase conjugated donkey anti rabbit or sheep anti mouse (GE Healthcare, Bucks, UK) and rabbit anti goat (Thermo Scientific Pierce, Rockford, IL). Immune-complexes were detected by chemiluminescent substrate (Thermo Scientific Pierce), and light emission captured on film (Kodak, Rochester, NY).

4.5 Gene reporter assay

Two hundred thousand INS-1 cells were seeded in triplicates in 24-well dishes and preincubated for 2 days. At the day of transfection, media was discarded and cells transiently co-transfected with a total of 0.7 μg plasmid DNA using Lipofectamin 2000 (Invitrogen): NFkB dependent expression plasmid (Strategene, Santa Clara, CA) together with a β-galactosidase expression plasmid for normalization. Following 6 h of transfection media was replaced and cells were cultured o.n. before addition of cytokines for 4 h. Cells were subsequently washed in HDB and lysed in Gly-Gly buffer (25 mM Gly-Gly pH 7.5, 15 mM $MgSO_4$, 4 mM EGTA) added 10% Triton X-100 and 1mM DTT. At the time of measurements samples were diluted in Gly-Gly buffer including 10 mM potassium phosphate, 1.25 mM DTT and 1.25 mM ATP. Luciferase activity was detected by addition

of Gly-Gly buffer containing 0.1 mM luciferase and read at a Modulus plate reader. Consecutively, β-galactosidase buffer (0.875 mg/ml ONPG in Sodium phosphate, 100 mM Sodium phosphate pH 7.5, 0.3% β-mercaptoethanol, 1 mM $MgCl_2$) was added and cells were incubated until color change was observed and absorbance could be measured at 450 nM.

4.6 Cell death detection ELISA

Sixty human islets or 200.000 INS-1 cells were seeded in 24-well plates. Following pre-treatment with peptides, cells or islets were cultured in the presence or absence of cytokines for 6 days or 24h, respectively. The degree of apoptosis was measured by cell death detection ELISA plus (Roche) according to manufacturer's description, detecting the amount of DNA-histone complexes present in the cytoplasmic lysates.

4.7 Statistical analyses

All data are presented as mean ± SEM of *n* independent experiments. Statistical analysis was done using a paired Student's *t* test or a one-way Anova followed by posthoc Dunnett multiple comparison analysis where appropriate. A *p* value less than 0.05 was considered significant.

5. Results

5.1 CRF family members differentially activate AKT in beta cells

AKT has previously been shown be involved in beta cell survival signalling mediated by class B GPCRs and we investigated if members of the CRF family could affect AKT activity in beta cells. INS-1 cells were treated with the CRFR1 selective agonist, ovine CRF (oCRF), for various time points ranging from 0–24 h. A time-dependent increase in phoshorylation of AKT, peaking at around 16 h of oCRF exposure was demonstrated (fig. 1a). The experiment was repeated using either mouse Ucn3 (mUcn3) or rat Ucn1 (rUcn1) to activate either CRFR2 alone or both CRFR1 and CRFR2, simultaneously. Exposing cells to rUcn1 for 16 h robustly upregulated the levels of phosphorylated AKT compared to untreated cells, as seen for oCRF (fig. 1b and 1c for rat INS-1 or mouse MIN6 cells, respectively). In contrast no effect was seen by mUcn3.

5.2 CRFR1-mediated upregulation of IRS2 and PI3K-dependent activation of AKT in beta cells

Beta cells, as opposed to intact islets, mainly express CRFR1 and pre-incubating the cells with 10 μM of the CRFR1-selective antagonist Antalarmin fully blocked both basal and oCRF- and rUcn1-mediated phosphorylation of AKT, suggesting that this is a CRFR1-mediated effect (fig. 2a).

As PI3K is a common upstream activator of AKT, we used LY2940002, a pharmacological inhibitor of PI3K signalling, to investigate if PI3K was involved in AKT activation in our cell systems. As evident from fig. 2b, pre-incubation with 10 μM of LY294002 fully inhibited oCRF- and rUcn1-mediated phosphorylation of AKT. Additionally, we found that oCRF dose-dependently affected transcription from a Glucose-6-phosphatase promoter

construct in a luciferase assay, a promoter previously reported responsive to activation of the AKT signalling pathway (Schmoll et al., 2000) (fig. 2c), further confirming activation of the Akt pathway.

CRFR1 activation did not lead to acute activation of AKT in beta cells (fig. 1), indicating that the observed effects are dependent on another factor. IRS2 is thought to promote islet cell survival in response to insulin and IGFI signalling (Withers et al., 1999, Fernández et al., 2003) and may act upstream of PI3K (Jhala et al., 2003). To investigate if CRFR1 agonists could increase IRS2 expression in beta cells, INS-1 cells were treated with oCRF or rUcn1 for various time points ranging from 30 min to 24 h. From this experiment we conclude that CRFR1 activation leads to increased protein levels of IRS2, peaking at around 8 h of treatment (fig. 2d). Pre-treating the cells with 10 μM of H89, a pharmacological inhibitor of the cAMP-dependent protein kinase A (PKA), prior to exposure to the CRFR1 ligands revealed partial inhibition of oCRF- or rUcn1-induced upregulation of IRS2 protein expression and AKT phosphorylation (fig. 2e). Together these data suggest CRFR1-mediated PKA-dependent upregulation of IRS2 precedes AKT activation.

5.3 CRFR1 activation increases survival signalling in cytokine-exposed beta cells

The pro-inflammatory cytokines IL-1β and TNFα have been shown to induce apoptosis by inhibiting the activity of AKT. As seen in fig. 3a, exposure to IL-1β (160 pg/ml) or TNFα (20 ng/ml) for 24 h reduced the levels of phosphorylated AKT compared to untreated INS-1 cells. To examine if CRFR1 activation preserves AKT activity in the presence of cytokines, INS-1 cells were pre-treated with oCRF, rUcn1 or mUcn3 for 16 h, followed by cytokine-exposure to IL-1β or TNFα. Cytokine-induced reduction of phosphorylated AKT was counteracted by pre-treatment with either oCRF or rUcn1. In line with previous observations, we did not observe any protection against cytokine-mediated decrease in AKT phosphorylation by mUcn3 treatment.

One mechanism by which AKT promotes survival is by phosphorylation-induced inactivation of the pro-apoptotic BAD. Indeed, exposure of INS-1 cells to TNFα partly reduced levels of phosphorylated BAD compared to untreated INS-1 cells (fig. 3b) and as for AKT, cytokine-mediated reduction in phosphorylated BAD was counteracted by pre-culturing the cells in the presence of oCRF or rUcn1 but not by mUcn3. A similar effect was seen exposing INS-1 cells to IL-1β or MIN6 cells to a combination of IL-1β and TNFα (data not shown). We thus conclude that CRFR1 agonists can increase survival signalling and inhibit the cytokine-mediated apoptosis.

5.4 CRFR1 activation counteracts pro-apoptotic signalling in cytokine-exposed beta cells

The MAPKs are activated by cytokines, such as IL-1β and TNFα. To explore any potential effects of CRFR activation on MAPK activity, INS-1 cells were pre-treated with CRF family members as described above and subsequently exposed to either IL-1β or TNFα for additional 20 min. Western blotting results represented in fig. 4a, show that, as expected, cytokines activated all three MAPKs. Neither peptide inhibited MAPK phosphorylation, but interestingly cytokine-induced ERK1/2 phosphorylation was potentiated in cells pre-treated with oCRF or rUcn1. No effect was seen by mUcn3.

Taking advantage of an NFκB-reporter construct we looked at NFκB-mediated gene regulation. As expected, both IL-1β and TNFα significantly increased NFκB promoter activity in INS-1 cells (fig. 4b). Cells pre-treated with oCRF exhibited less cytokine-mediated increase in promoter activity, indicating that CRFR1 activation can affect cytokine-induced NFκB-mediated gene regulation. TNFα and IL-1β increase NFκB transcriptional activity by promoting IκBα degradation. However, neither peptide was able to inhibit this cytokine-induced degradation of IκBα, suggesting that CRF family members do not exert their modulation of NFκB signalling by affecting IκBα directly (fig. 4c). Similar results were obtained using MIN6 cells (data not shown).

5.5 CRFR1 activation prevents cytokine-induced apoptosis

Since CRFR1 activation affects key survival and apoptotic pathways, we next explored the effect of cytokines and the CRF family members on cleavage of the executor caspase 3. Stimulation by the combination of IL-1β and TNFα resulted in the cleavage of caspase 3 (fig. 5a). We found that pre-treatment with either oCRF or rUcn1 but not mUcn3 reduced this cytokine-mediated cleavage of caspase 3, supporting the concept that activation of the CRFR1 protects against cytokine-induced beta cell death.

To confirm this we evaluated beta cell apoptosis in form of DNA doublestrand breaks by use of the Cell death detection ELISA assay. This assay revealed an increase in apoptosis in INS-1 cells following TNFα exposure for 24 h. Pre-treating the cells with either oCRF or rUcn1 completely blocked TNFα-induced apoptosis (fig. 5b). Similarly, oCRF or rUcn1 but not mUcn3 significantly inhibited IL-1 β- or TNFα-induced apoptosis in MIN6 cell (fig. 5c). Taking a closer look at basal apoptosis, oCRF and rUCN1 also inhibited basal apoptosis in INS1-cells (fig 5d). This effect was counteracted by 30 min pretreatment with the CRFR1 selective antagonist antalarmin (figure 5d).

5.6 CRFR1 and CRFR2 activation promote basal survival of human islets

The effect of CRFR1 and CRFR2 activation was investigated in human islets from three individual donors. Intact human islets were cultured in the presence of oCRF, rUcn1 or hUcn3 and cell lysates analysed for phosphorylation of AKT. As evident from fig. 6a, AKT phosphorylation was apparent in response to both CRFR1 and CRFR2 ligands following 8–16 h of treatment (fig. 6a).

In human islets from one donor oCRF, rUcn1 or hUcn3 reduced apoptosis induced by a combination of IL-1β, TNFα and IFNγ with 49%, 17% and 42%, respectively (data not shown). In experiments using islets from two other donors, cytokines induced substantially more apoptosis which could not be inhibited by the CRF family members tested (data not shown). However, in islets from all three donors, basal apoptosis levels in freshly isolated human islets were significantly reduced when cultured in the presence of oCRF or rUcn1 (fig. 6b). Human Ucn3 did not significantly reduce apoptosis (p =0.06). These data suggest that the CRFR1 selective agonists can activate AKT and inhibit apoptosis in cultured human islets.

6. Discussion

In this study we characterized CRFR signaling in beta cells and examined the role of CRF family members on cytokine-induced beta cell apoptosis. Signalling through CRFR1 and CRFR2 protects cultured hippocampal and neocortical neurons from a range of neurotoxic mediators (Facci et al., 2003) and positively affects survival of cardiac myocytes in models of heart failure (Brar et al., 2000, Brar et al., 2002). Recently, CRF was shown to inhibit basal apoptosis in INS-1 cells following 72 h of culturing but the underlying mechanisms were not investigated (Schmid et al., 2011). In this study we confirm this observation using both cell lines and human islets and demonstrate for the first time the ability of the CRF family members to protect beta cells against apoptotic stimuli associated with the development of diabetes.

An inhibition of pro-apoptotic NFκB signalling was apparent as CRFR1 activation reduced NFκB-mediated gene transcription induced by either TNFα or IL-1β. No effect was seen on IkBa degradation. The ability of NFκB to recruit the transcriptional apparatus and stimulate target gene expression in the nucleus is ensured by post-translational modification (i.e. phosphorylation, acetylation etc.) of NFκB itself and its surrounding chromatin environment. CRFR1 agonists may inhibit NFκB-mediated gene transcription by preventing crucial post-translational modifications, although this has not been investigated further.

Additionally, CRFR1 signalling prevented cytokine-mediated decrease in AKT phosphorylation as well as dephosphorylation of the AKT target BAD, all favouring survival.

A time-course experiment revealed that a prolonged exposure (16 h) to CRFR1 ligands was required for a robust activation of AKT in beta cells. This is in line with what is reported for GLP-1 receptor-mediated AKT activation in MIN6 cells and human islets. The proposed mechanism involved is a cAMP/PKA and CREB-dependent upregulation of IRS2 protein levels working upstream of PI3K (Velmurugan et al., 2012, Altejeros and Montminy 2011, Jhala et al., 2003, Li et al., 2005, Park et al., 2006, Van de Velde et al., 2011). We have previously demonstrated robust CRFR1-mediated increase in CREB phosphorylation (Huising et al., 2010) and in the current study reveal a time- and PKA-dependent increase in IRS2 protein expression following CRFR1 activation in INS-1 cells peaking before maximum AKT activation.

CRFR1 and CRFR2 belong to the same receptor class as receptors for incretins, such as GLP-1. GLP-1 has been shown to inhibit beta cell apoptosis and protection against cytokine-induced apoptosis was correlated with inhibition of JNK activity (Ferdaoussi et al., 2008, Natalicchio et al., 2010) but no effect was observed on p38 activity.

As for GLP-1 we did not observe any effect of CRFR1 activation on p38 activity, however we did not detect any inhibitory effect of any of the peptides on JNK phosphorylation in INS-1 cells. In MIN6 cells TNFα-induced JNK activation was inhibited by CRFR1 agonists (data not shown) suggesting some discrepancies between the signalling in these two cell lines. In both cell types, CRFR1 ligands protected against apoptosis induced by exposure to either cytokine individually or in combination implying that inhibition of JNK activity is not

decisive for beta cell survival under these conditions. Surprisingly, cytokine-induced ERK1/2 phosphorylation was potentiated by CRFR1 agonists in INS-1 cells. Cytokine-induced ERK1/2 phosphorylation has previously been linked to cytokine-induced nitric oxide formation and beta-cell apoptosis. However, ERK1/2 is generally involved in regulation of proliferation (Blandino-Rosano et al., 2008) and our previous results showed oCRF-induced ERK1/2 activation associated with increased beta cell proliferation in neonatal rat islets (Huising et al., 2010). Whether increased ERK1/2 activation following short-term exposure to cytokines acts as a survival/proliferative mechanism which is potentiated by the presence of CRFR1 agonists remains to be investigated.

Throughout the study, we observed no protective effect of the CRFR2-selective agonist Ucn3 on clonal beta cells, INS-1 and MIN6. However, in intact human islets Ucn3 robustly activated AKT but did not significantly reduce apoptosis ($p = 0.06$). This is in agreement with our published observations suggesting relative low expression of CRFR2 on clonal beta cell lines as opposed to primary rodent and human islets (Huising et al., 2011). Ucn3 itself is abundantly expressed in the beta cell and regulates glucose-stimulated insulin secretion and energy homeostasis (Li et al., 2007). These observations strongly suggest that Ucn3 plays important roles in regulating beta cell function *in vivo*. Furthermore, CRFR2 expression is robustly upregulated in both primary islets and clonal beta cells in response to glucocorticoids increasing the sensitivity to Ucn3 (Huising et al., 2010). This study also demonstrated that basal expression of CRFR2 is insufficient to mediate ligand-induced response in INS-1 and MIN6 cells but it is likely that conditions with increased CRFR2 levels would enable Ucn3 to promote beta cell survival in a manner similar to agonists of CRFR1.

Testing three different batches of human islets we consistently observed CRFR1-mediated inhibition of basal apoptosis, in line with our data on INS-1 cells. Additionally, CRFR1 as well as CRFR2-signalling decreased cytokine-induced apoptosis in islets from one of the donors. Human islets from the two other donors exhibited a more robust induction of apoptosis in response to the combination of TNFα, IL-1β and IFNγ for six days, and CRFR activation was not capable of protecting against cytokine-induced apoptosis in these islets (data not shown). The considerable variation in islet function and quality from preparation to preparation is a well-known concern when using human islets for experiments suggesting additional batches of human islets and perhaps lower concentrations of or shorter exposure to cytokines are needed to explore the potential for CRF family members to protect against cytokine-induced apoptosis in human islets.

6.2 Conclusion

Results from the present study suggest that CRFR1 signalling promotes beta cell survival and protects beta cells from the negative consequences of pro-inflammatory cytokines. The outcome of CRFR1-mediated signalling pathways is a shift in the balance between pro- versus anti-apoptotic signalling pathways towards increased beta cell survival. Activation of intracellular stress signaling pathways during human islet preparation has a negative impact on the prospects of the graft survival following islet transplantation (Abdelli et al. 2004, Aikin et al. 2004). The capacity of members of the CRF family to significantly reduce

apoptosis in human islets following isolation suggests a therapeutic potential of these peptides in protection of islet survival. Our data prompt further analysis exploring the potential use of CRFR agonists as a therapeutic approach aimed at enhancing beta cell survival, thereby reducing or delaying cytokine-mediated beta cell destruction in the development of type 1 and type 2 diabetes.

Acknowledgments

Human islets were obtained courtesy of the Islet cell Resource Basic Science Islets Distribution Program. We thank Jean Rivier and Judit Erchegyi (The Salk Institute, La Jolla, USA) for providing the peptides used in this study and Helle Fjordvang for excellent technical assistance.

Funding

This work was supported in part by The Alfred Benzon Foundation, the Clayton Medical Research Foundation, Inc., the Juvenile Diabetes Research Foundation, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) grant PO1 DK026741-30, The Danish Council for Independent Research, Medical Sciences and The Novo Nordisk Foundation.

References

Abdelli S, Ansite J, Roduit R, Borsello T, Matsumoto I, Sawada T, Allaman-Pillet N, Henry H, Beckmann JS, Hering BJ, Bonny C. Intracellular stress signaling pathways activated during human islet preparation and following acute cytokine exposure. Diabetes. 2004; 53(11):2815–23. [PubMed: 15504961]

Aikin R, Maysinger D, Rosenberg L. Cross-talk between phosphatidylinositol 3-kinase/AKT and c-Jun NH2-terminal kinase mediates survival of isolated human islets. Endocrinology. 2004; 145:4522–4531. [PubMed: 15242986]

Altarejos JY, Montminy M. CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. Nature Reviews Molecular Cell Biology. 2011; 12:141–51. Review. [PubMed: 21346730]

Ammendrup A, Maillard A, Nielsen K, et al. The c-Jun amino-terminal kinase pathway is preferentially activated by interleukin-1 and controls apoptosis in differentiating pancreatic beta-cells. Diabetes. 2000; 49:1468–1476. [PubMed: 10969830]

Baldwin AS. The nf-kappa-b and i-kappa-b proteins—new discoveries and insights. Annual Review of Immunology. 1996; 14:649–683.

Blandino-Rosano M, Perez-Arana G, Mellado-Gil JM, Segundo C, Aguilar-Diosdado M. Anti-proliferative effect of pro-inflammatory cytokines in cultured beta cells is associated with extracellular signal-regulated kinase 1/2 pathway inhibition: protective role of glucagon-like peptide -1. Journal of Molecular Endocrinology. 2008; 41:35–44. [PubMed: 18487229]

Bonny C, Oberson A, Steinmann M, Schorderet DF, Nicod P, Waeber G. IB1 reduces cytokine-induced apoptosis of insulin-secreting cells. Journal of Biological Chemistry. 2000; 275:16466–16472. [PubMed: 10748095]

Bonny C, Oberson A, Negri S, Sauser C, Schorderet DF. Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. Diabetes. 2001; 50:77–82. [PubMed: 11147798]

Brar BK, Jonassen AK, Stephanou A, Santilli G, Railson J, Knight RA, Yellon DM, Latchman DS. Urocortin protects against ischemic and reperfusion injury via a MAPK-dependent pathway. Journal of Biological Chemistry. 2000; 275:8508–8514. [PubMed: 10722688]

Brar BK, Stephanou A, Knight R, Latchman DS. Activation of protein kinase B/Akt by urocortin is essential for its ability to protect cardiac cells against hypoxia/reoxygenation-induced cell death. Journal of Molecular Cellular Cardiology. 2002; 34:483–92. [PubMed: 11991736]

Brubaker PL, Drucker DJ. Structure-function of the glucagon receptor family of G protein-coupled receptors: the glucagon, GIP, GLP-1, and GLP-2 receptors. Receptors Channels. 2002; 8:179–88. [PubMed: 12529935]

Donath MY, Størling J, Maedler K, Mandrup-Poulsen T. Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes. Jounal of Molecular Medicine. 2003; 81:455–70.

Elghazi L, Bernal-Mizrachi E. Akt and PTEN: beta-cell mass and pancreas plasticity. Trends in Endocrinology and Metabolism. 2009; 20:243–51. [PubMed: 19541499]

Facci L, Stevens DA, Pangallo M, Franceschini D, Skaper SD, Strijbos PJ. Corticotropin-releasing factor (CRF) and related peptides confer neuroprotection via type 1 CRF receptors. Neuropharmacology. 2003; 45:623–36. [PubMed: 12941376]

Fekete EM, Zorrilla EP. Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: ancient CRF paralogs. Frontiers in Neuroendocrinology. 2007; 28:1–27. [PubMed: 17083971]

Ferdaoussi M, Abdelli S, Yang JY, Cornu M, Niederhauser G, Favre D, Widmann C, Regazzi R, Thorens B, Waeber G, et al. Exendin-4 protects beta-cells from interleukin-1 beta-induced apoptosis by interfering with the c-Jun NH2-terminal kinase pathway. Diabetes. 2008; 57:1205–15. [PubMed: 18252896]

Fernández E, Martín MA, Fajardo S, Escrivá F, Alvarez C. Increased IRS-2 content and activation of IGF-I pathway contribute to enhance beta-cell mass in fetuses from undernourished pregnant rats. American Journal of Physiology Endocrinology and Metabolism. 2007; 292:E187–95. [PubMed: 16912057]

Flodstrom M, Welsh N, Eizirik DL. Cytokines activate the nuclear factor kappa B (NF-kappa B) and induce nitric oxide production in human pancreatic islets. FEBS Letters. 1996; 385:4–6. [PubMed: 8641463]

Florio P, Vale W, Petraglia F. Urocortins in human reproduction. Peptides. 2004; 25:1751–7. [PubMed: 15476942]

Heimberg H, Heremans Y, Jobin C, Leemans R, Cardozo AK, Darville M, Eizirik DL. Inhibition of cytokine-induced NF-kappaB activation by adenovirus-mediated expression of a NF-kappaB super-repressor prevents beta-cell apoptosis. Diabetes. 2001; 50:2219–24. [PubMed: 11574401]

Huising MO, Pilbrow AP, Matsumoto M, van der Meulen T, Park H, Vaughan JM, Lee S, Vale WW. Glucocorticoids differentially regulate the expression of CRFR1 and CRFR2α in MIN6 insulinoma cells and rodent islets. Endocrinology. 2011; 52:138–50. [PubMed: 21106875]

Huising MO, van der Meulen T, Vaughan JM, Matsumoto M, Donaldson CJ, Park H, Billestrup N, Vale WW. CRFR1 is expressed on pancreatic beta cells, promotes beta cell proliferation, and potentiates insulin secretion in a glucose-dependent manner. Proceedings of the National Academy of Sciences U S A. 2010; 07:912–7.

Hsu SY, Hsueh AJ. Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. Nature Medicine. 2001; 7:605–611.

Jhala US, Canettieri G, Screaton RA, Kulkarni RN, Krajewski S, Reed J, Walker J, Lin X, White M, Montminy M. cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. Genes and Development. 2003; 17:1575–80. [PubMed: 12842910]

Kimura Y, Takahashi K, Totsune K, Muramatsu Y, Kaneko C, Darnel AD, Suzuki T, Ebina M, Nukiwa T, Sasano H. Expression of UCN and corticotropin-releasing factor receptor subtypes in the human heart. Journal of Clinical Endocrinology and Metabolism. 2002; 87:40–346.

Kuperman Y, Chen A. Urocortins: emerging metabolic and energy homeostasis perspectives. Trends in Endocrinol and Metabolism. 2008; 19:122–9.

Lee S, Braden B, Kang SS, Rivier C. Urocortins are present in the rat testis. Neuropeptides. 2011; 45:131–7. [PubMed: 21256589]

Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, Vaughan J, Reyes TM, Gulyas J, Fischer W, et al. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. Proceedings of the National Academy of Sciences USA. 2001; 98:7570–7575.

Li C, Chen P, Vaughan J, Blount A, Chen A, Jamieson PM, Rivier J, Smith MS, Vale W. Urocortin III is expressed in pancreatic beta-cells and stimulates insulin and glucagon secretion. Endocrinology. 2003; 144:3216–24. [PubMed: 12810578]

Li C, Chen P, Vaughan J, Lee KF, Vale W. Urocortin 3 regulates glucose-stimulated insulin secretion and energy homeostasis. Proceedings of the National Academy of Sciences U S A. 2007; 104:4206–11.

Li L, El-Kholy W, Rhodes CJ, Brubaker PL. Glucagon-like peptide-1 protects beta cells from cytokine-induced apoptosis and necrosis: role of protein kinase B. Diabetologia. 2005; 48:1339–49. [PubMed: 15902400]

Natalicchio A, De Stefano F, Orlando MR, Melchiorre M, Leonardini A, Cignarelli A, Labarbuta R, Marchetti P, Perrini S, Laviola L, et al. Exendin-4 prevents c-Jun N-terminal protein kinase activation by tumor necrosis factor-alpha (TNFalpha) and inhibits TNFalpha-induced apoptosis in insulin-secreting cells. Endocrinology. 2010; 151:2019–29. [PubMed: 20219981]

Nikulina MA, Sandhu N, Shamim Z, Andersen NA, Oberson A, Dupraz P, Thorens B, Karlsen AE, Bonny C, Mandrup-Poulsen T. The JNK binding domain of islet-brain 1 inhibits IL-1 induced JNK activity and apoptosis but not the transcription of key proapoptotic or protective genes in insulin-secreting cell lines. Cytokine. 2003; 24:13–24. [PubMed: 14561487]

Park S, Dong X, Fisher TL, Dunn S, Omer AK, Weir G, White MF. Exendin-4 uses Irs2 signaling to mediate pancreatic beta cell growth and function. Journal of Biological Chemistry. 2006; 281:1159–68. [PubMed: 16272563]

Patel S, Santani D. Role of NF-kappa B in the pathogenesis of diabetes and its associated complications. Pharmacological Reports. 2009; 61:595–603. Review. [PubMed: 19815941]

Pavlovic D, Andersen NA, Mandrup-Poulsen T, Eizirik DL. Activation of extracellular signal-regulated kinase (ERK)1/2 contributes to cytokine-induced apoptosis in purified rat pancreatic beta-cells. European Cytokine Network. 2000; 11:267–274. [PubMed: 10903806]

Perrin MH, Vale WW. Corticotropin releasing factor receptors and their ligand family. Ann NY Acad Sci. 1999; 885:312–328. [PubMed: 10816663]

Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, Hogenesch JB, Gulyas J, Rivier J, Vale WW, et al. Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. Proceedings of the National Academy of Sciences U S A. 2001; 98:2843–8.

Saldeen J, Lee JC, Welsh N. Role of p38 mitogenactivated protein kinase (p38 MAPK) in cytokine-induced rat islet cell apoptosis. Biochemical Pharmacology. 2001; 61:1561–1569. [PubMed: 11377386]

Schmid J, Ludwig B, Schally AV, Steffen A, Ziegler CG, Block NL, Koutmani Y, Brendel MD, Karalis KP, Simeonovic CJ, et al. Modulation of pancreatic islets-stress axis by hypothalamic releasing hormones and 11β.hydroxysteroid dehydrogenase. Proceedings of the National Academy of Sciences. 2011; 108:13722–13727.

Schmoll D, Walker KS, Alessi DR, Grempler R, Burchell A, Guo S, Walther R, Unterman TG. Regulation of glucose-6-phosphatase gene expression by protein kinase Balpha and the forkhead transcription factor FKHR. Evidence for insulin response unit-dependent and -independent effects of insulin on promoter activity. Journal of Biological Chemistry. 2000; 275:36324–33. [PubMed: 10960473]

Sunayama J, Tsuruta F, Masuyama N, Gotoh Y. JNK antagonizes Akt-mediated survival signals by phosphorylating 14-3-3. Journal of Cell Biology. 2005; 170:295–304. [PubMed: 16009721]

Van de Velde S, Hogan MF, Montminy M. mTOR links incretin signaling to HIF induction in pancreatic beta cells. Proceedings of the National Academy of Sciences U S A. 2011; 108:16876–82.

Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C, et al. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. Nature. 1995; 378:287–292. [PubMed: 7477349]

Velmurugan K, Balamurugan AN, Loganathan G, Ahmad A, Hering BJ, Pugazhenthi S. Antiapoptotic actions of Exendin-4 against Hypoxia and cytokines are augmented by CREB. Endocrinology. 2012; 153:1116–28. [PubMed: 22253425]

White MF. Insulin signaling in health and disease. Science. 2003; 302:1710–1711. [PubMed: 14657487]

Withers DJ, Burks DJ, Towery HH, Altamuro SL, Flint CL, White MF. Irs-2 coordinates Igf-1 receptor-mediated beta-cell development and peripheral insulin signaling. Nature Genetics. 1999; 23:32–40. [PubMed: 10471495]

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell. 1996; 87:619–28. [PubMed: 8929531]

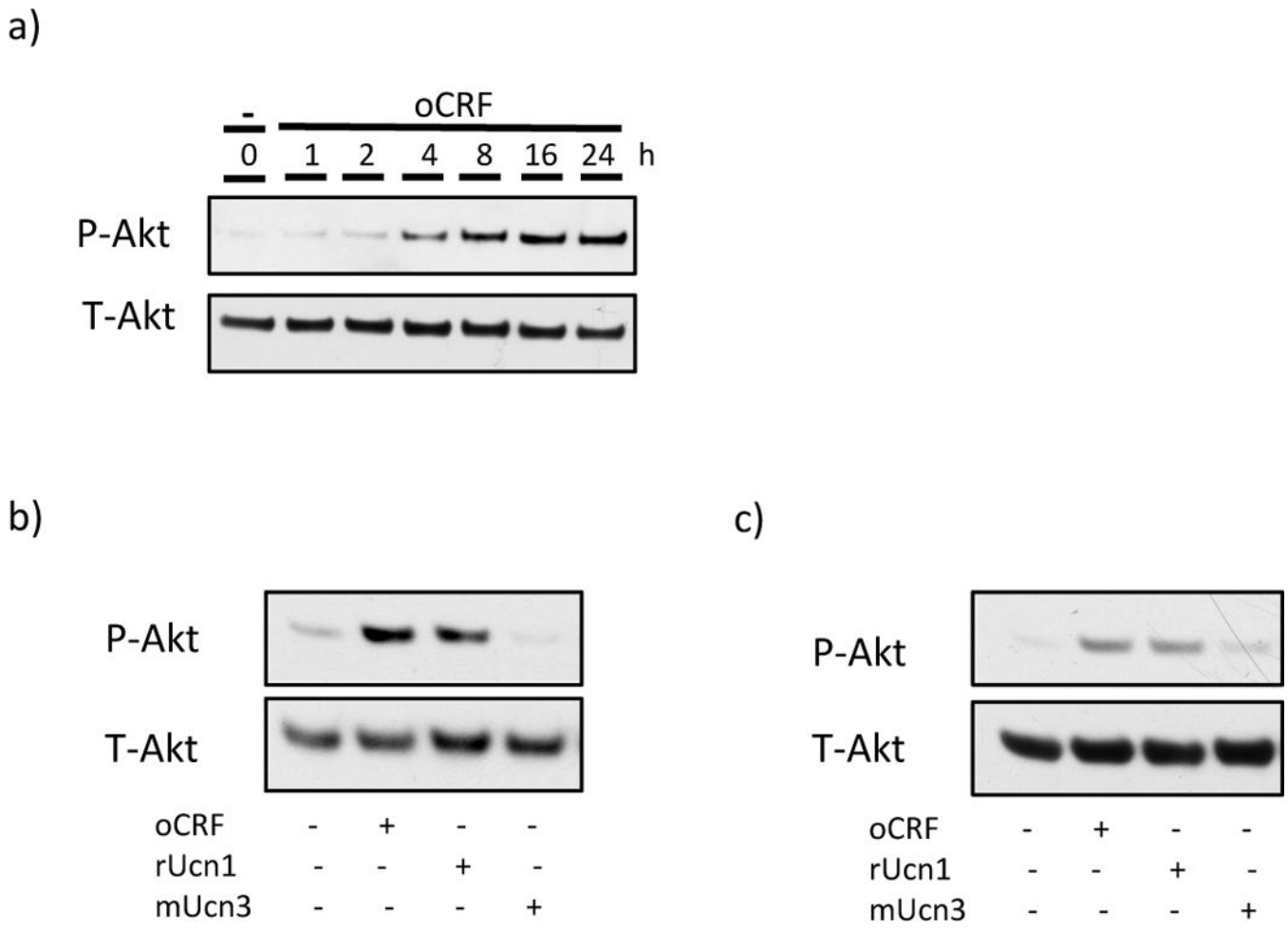

Fig. 1. Effect of CRFR signalling on AKT activation

INS-1 cells (a) were treated with 50 nM of oCRF for various time points ranging from 1 h to 24 h. Lysates were subjected to immunoblotting using antibodies against phosphorylated (P-AKT) or total AKT (T-AKT). A representative blot is shown (n=3). INS-1 cells b) or MIN6 cells c) were treated with 50 nM of either oCRF, rUcn1 or mUcn3 for 16 h and lysates were treated as above (n = 4).

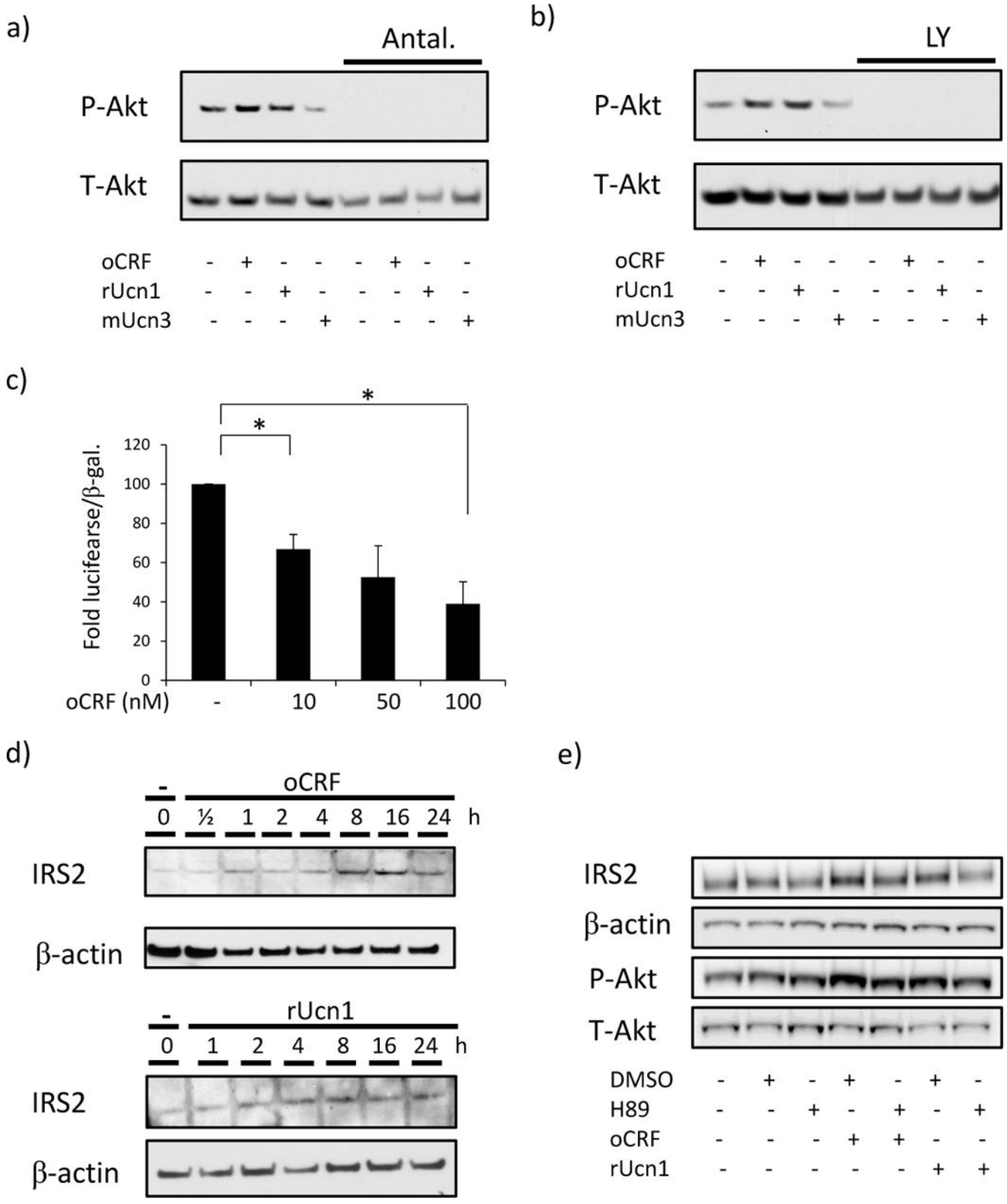

Fig. 2. Determination of receptor type and signalling components

a) INS-1 cells were treated with 5 μM of the CRFR1 antagonist, antalarmin 30 min prior to addition of 50 nM of oCRF, rUcn1 or mUcn3 and cultured for 16 h. Lysates were analysed by immunoblotting using antibodies against phosphorylated (P-AKT) or total AKT (T-AKT). A representative blot is shown (n=4). b) INS-1 cultured with 10 μM of the PI3K inhibitor LY294002 30 min prior to treatment with oCRF, rUcn1 or mUcn3 and handled as above. c) INS-1 cells were transiently transfected with a glucose-6-phosphatase promotor construct leading to luciferase expression and a constitutive active β-galactosidase construct. Cells were cultured with increasing concentration of oCRF. Data are presented as mean luciferase activity normalised to β-galactosidase ± SEM from three independent experiments each performed in triplicates.*$p<0.05$, ***$p<0.001$ vs. untreated cells (*t* test). d) INS-1 cells were treated with 50 nM of oCRF or rUcn1 for various time points ranging from 30 min to 24 hours. Lysates were subjected to immunoblotting using antibodies against IRS2 and the housekeeping protein β-actin. A representative blot from three independent experiments is shown (n=3). e) INS-1 cells were pre-treated with 10 μM of H89 for 30 min prior to addition of 50 nM of oCRF or rUcn1 and culturing for additional 16 h. Lysates were analysed for IRS2 expression, β-actin, phosphorylated AKT (P-AKT) and total AKT (T-AKT). The blot shown is a representative of three independent experiments (n=3).

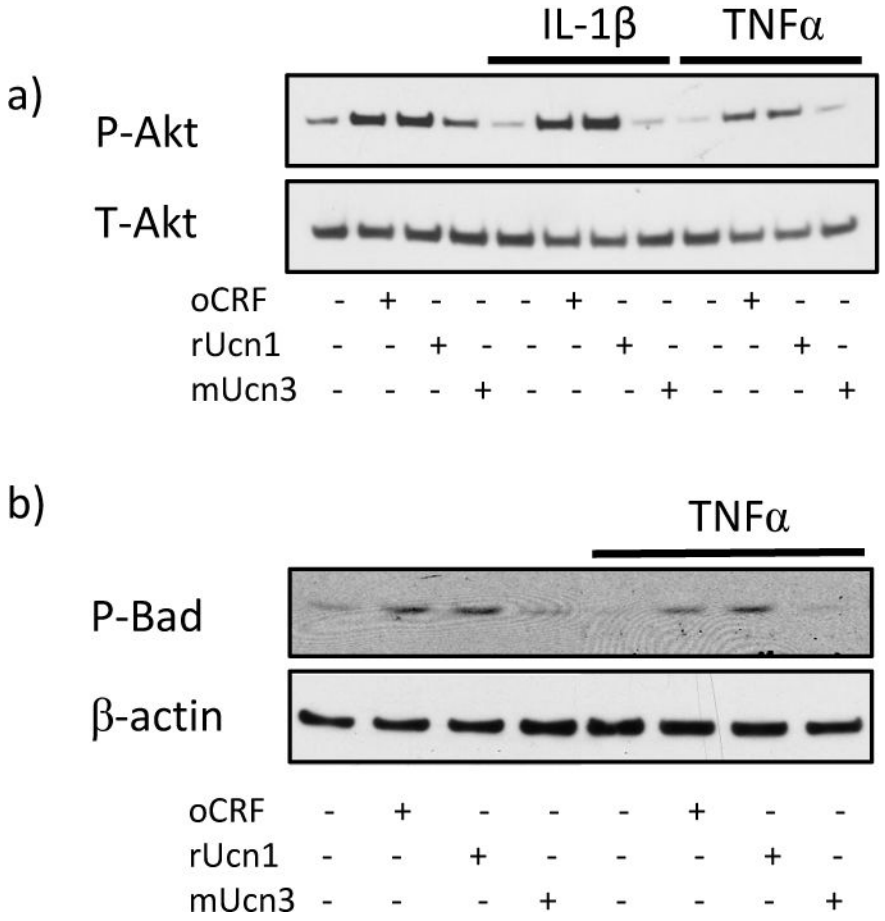

Fig. 3. Effect of CRFR signalling on survival pathways

a) INS-1 cells were pre-cultured with oCRF, rUcn1 or mUcn3 for 16 h. Subsequently, IL-1β (160 pg/ml) or TNFα (20ng/ml) was added to the cells and culture continued for another 24 hours. Cell extract was analysed for phosphorylated (P-AKT) or total (T-AKT) AKT by western blot analysis. A representative blot is shown (n=5). b) INS-1 cells were pre-treated as above and exposed to TNFα for 24 h. Levels of phosphorylated BAD and β-actin were analysed by western blot analysis. A representative blot of 3 independent experiments is shown (n=3).

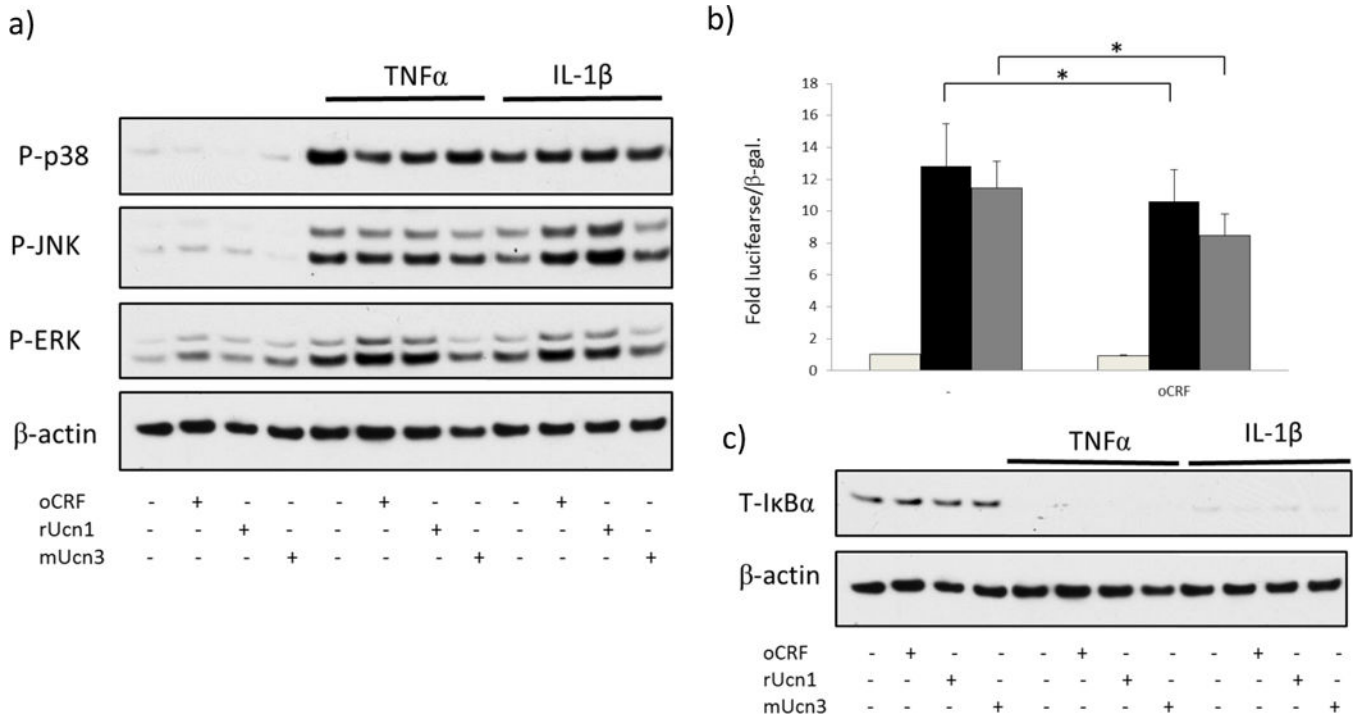

Fig. 4. Effect of CRFR signalling on cytokine-induced MAPK and NFκB activation

a) INS-1 cells were pre-cultured with oCRF, rUcn1 or mUcn3 for 16 h followed by IL-1β (160 pg/ml) or TNFα (20 ng/ml) exposure for additional 20 min. Cell extracts were analysed for phosporylated (P) JNK, p38, ERK1/2. A representative blot from three independent experiments is shown (n=3). b) INS-1 cells were transiently co-transfected with NFκB-responsive reporter construct together with a constitutively active β-galactosidase construct, incubated with oCRF for 16 h and exposed to IL-1β or TNFα for another 6 hours. Data are presented as mean luciferase activity normalised to β-galactosidase ± SEM from four independent experiments each performed in triplicates (n=4).*p<0.05 vs. cytokine-treated cells (t test). White bars represent controls, black bars IL-1β-treated and grey bars TNFα-treated cells. c) INS-1 cells were treated as above and degradation of IκBα analysed by western blot analysis using whole cell lysates and antibodies against IκBα and the housekeeping protein β-actin. A representative blot from three independent experiments is shown (n=3).

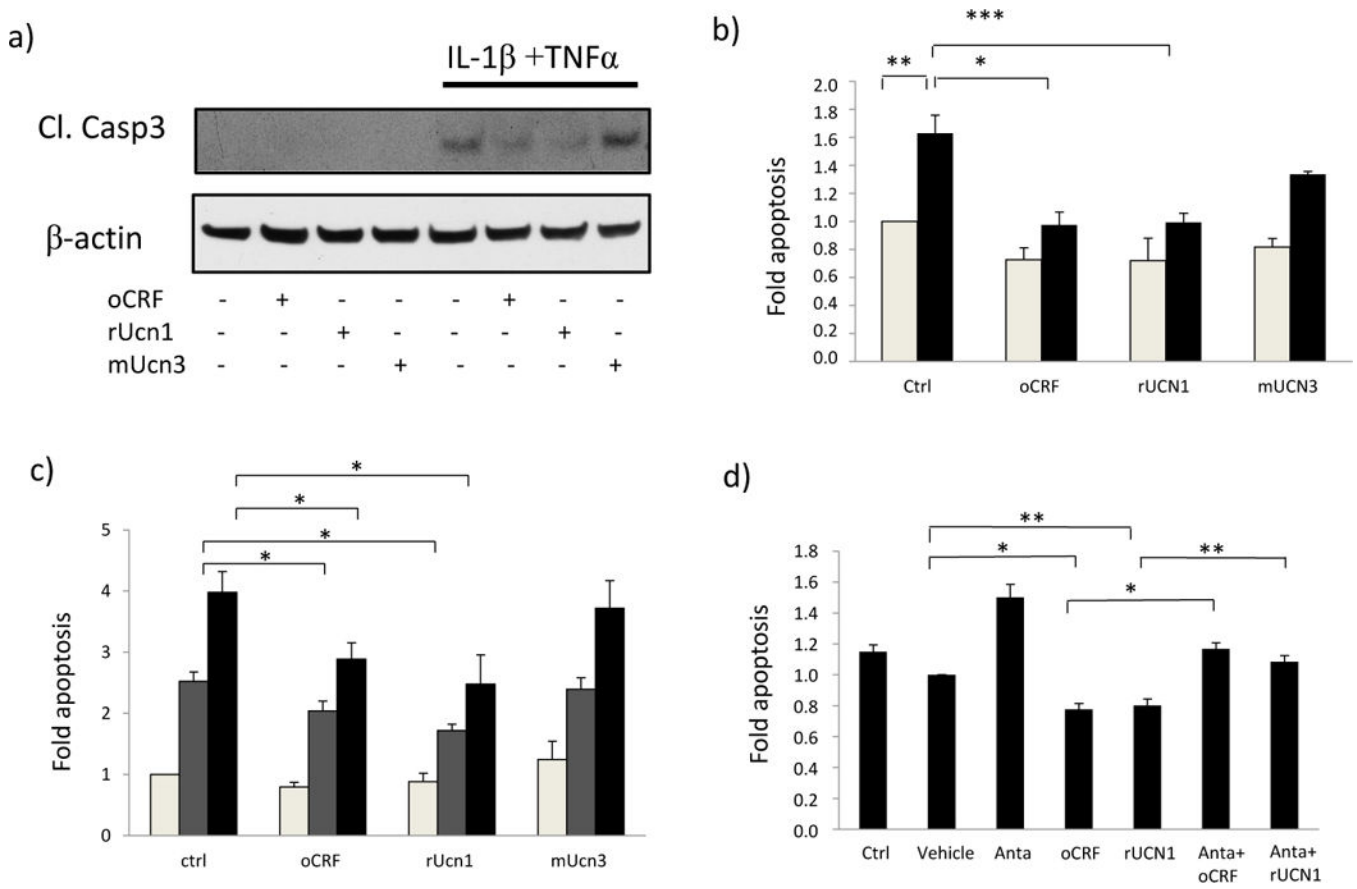

Fig. 5. Effects of CRFR1 signalling on cytokine-induced apoptosis

a) MIN6 cells were pre-treated with 50 nM of oCRF, rUcn1 or mUcn3 for 16 h. Subsequently cells were exposed to IL-1β (160 pg/ml) in combination with TNFα for 24 h. Following lysis, western blotting was performed and apoptosis detected by antibodies recognising the cleaved form of caspase 3 as well as β-actin. A representative blot from three independent experiments is shown (n=3).

b) INS-1 cells were pre-treated as for a) Subsequently TNFα (20 ng/ml) or was added and cells were cultured for another 24 h. c) MIN6 cells were treated as above and exposed to IL-1β (160 pg/ml) or TNFα (20 ng/ml) for 24 h. d) INS-1 cells were pretreated with vehicle (EtOH) or 5μM antalarmin for 30 min before stimulation with 50nM oCRF or rUCN1 for 60 hours. Apoptosis was detected by cell death detection ELISA and data are presented as mean fold induction of apoptosis ± SEM from three to four independent experiments (n=3–4).*p<0.05, ** p<0.01, *** p<0.001 vs. cytokine-stimulated cells (t test). White bars represent controls, black bars TNFα-treated cells and grey bars IL-1β-treated cells.

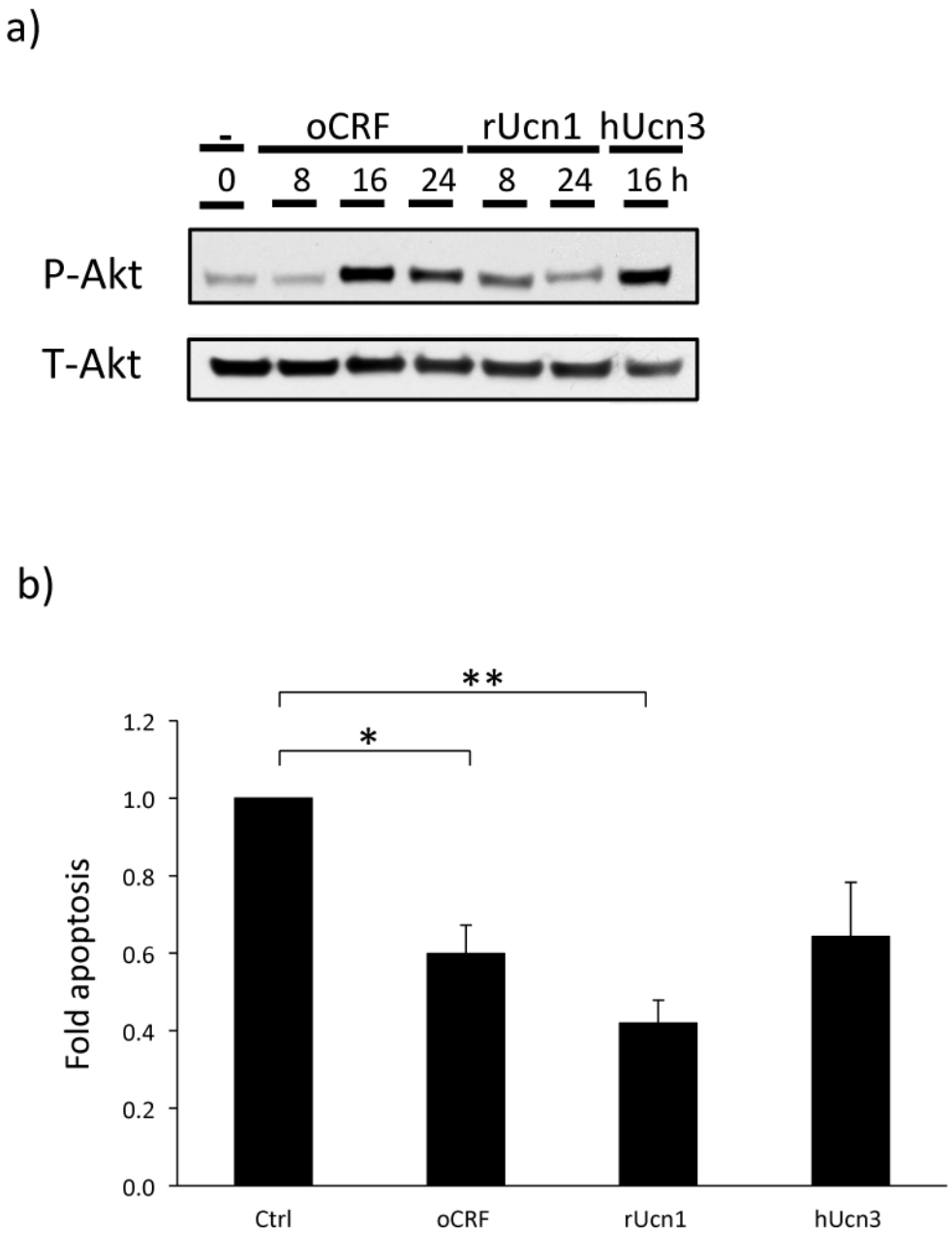

Fig. 6. Effect of CRFR signalling on human islets

a) Human islets were pre-treated with 50 nM of oCRF, rUcn1 or mUcn3 as indicated and analysed for levels of phosphorylated AKT (P-AKT) or total AKT (T-AKT) by western blot analysis. A representative of three independent experiments is shown (n=3). b) Human islets were pre-treated as described above and cultured for 6 days. Apoptosis was determined by the cell death detection ELISA. Experiment was performed independently on islets from three different donors and data presented as mean apoptosis ± SEM.*p<0.05, **p<0.01 vs. untreated islets (t test) (n=3).