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A Kinase-Independent Role for Unoccupied Insulin and IGF-1 Receptors in the Control of Apoptosis

Jeremie Boucher, Yazmin Macotella, Olivier Bezy, Marcelo A. Mori, Kristina Kriauciunas, and C. Ronald Kahn*

Joslin Diabetes Center and Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02215, USA

Abstract

Insulin and insulin-like growth factor 1 (IGF-1) act as anti-apoptotic hormones. We found that, unexpectedly, double knockout (DKO) cells that lacked both insulin and IGF-1 receptors (IR and IGF1R, respectively), were resistant to apoptosis induced through either the intrinsic or extrinsic pathway. This resistance to apoptosis was associated with decreased abundance of the pro-apoptotic protein Bax and increases in abundance of the anti-apoptotic proteins Bcl-2, Bcl-xL, XIAP, and Flip. These changes in protein abundance involved primarily post-transcriptional mechanisms. Restoration of the insulin or IGF-1 receptor to DKO cells also restored their sensitivity to apoptosis. Notably, expression of a catalytically inactive mutant form of the insulin receptor also restored susceptibility to apoptosis. Thus, the insulin and IGF-1 receptors have bidirectional roles in the control of cell survival and can be viewed as previously-unidentified dependence receptors. Insulin and IGF-1 binding stimulates receptor tyrosine kinase activity and blocks apoptosis, whereas unliganded insulin and IGF-1 receptors, acting through a mechanism independent of their catalytic activity, exert a permissive effect on cell death.

Keywords

Insulin receptor; IGF-1 receptor; Brown adipose tissue; Apoptosis; Bax; Bcl-2; Flip; Caspases; Dependence receptors

Introduction

Apoptosis, or programmed cell death, plays an important role in numerous physiological processes, including embryonic development, cell turnover, and removal of inflammatory cells (1;2). Conversely, defects in the regulation of apoptotic cell death contribute to many diseases, including cancer, ischemic damage, neurodegenerative diseases, and autoimmune disorders (3;4). Although the cellular and molecular mechanisms involved in apoptosis are not fully understood, two major pathways leading to induction of apoptosis have been

*Corresponding author: C. Ronald Kahn, MD, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215, Phone: (617)732-2635, Fax: (617)732-2487, c.ronald.kahn@joslin.harvard.edu.

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extensively characterized: the extrinsic pathway initiated by specific ligands, such as TNF α (tumor necrosis factor α) and Fas ligand (FasL), and the mitochondrially-mediated, intrinsic pathway initiated by growth factor withdrawal, DNA damage, oxidative stress, or toxic chemicals (5;6). Activation of the caspase cascade, which serves as the main effector of apoptosis, is a critical component of both pathways (7).

Insulin and insulin-like growth factor 1 (IGF-1) are major anti-apoptotic hormones, as well as metabolic and growth hormones. Their effects are mediated through activation of the insulin and IGF-1 receptors and the consequent activation of the PI3K-Akt (phosphatidylinositol 3-kinase to Akt) and the Ras-Raf-MEK-ERK (Ras to Raf to mitogen-activated or extracellular signal-regulated protein kinase kinase to extracellular signal-regulated protein kinase) pathways (8;9). Here, we explored the complementary roles of the insulin and IGF-1 receptors in apoptosis, using brown preadipocyte cell lines containing inactivated insulin receptors, IGF-1 receptors, or both. We found that wild-type (WT) cells and cells lacking either the insulin or the IGF-1 receptor (IRKO and IGFRKO cells, respectively) showed normal apoptotic responses to serum withdrawal and other stimuli, which could be largely rescued by either hormone. In contrast, cells deficient in both insulin and IGF-1 receptors (DKO) were resistant to apoptosis, induced either through the intrinsic or the extrinsic pathway. These DKO cells showed a marked reduction in caspase 3 activation and apoptosis-related events compared to WT cells. This resistance resulted from a coordinated series of post-transcriptional changes in the abundance of proteins involved in the apoptosis process, and could be reversed by re-introduction of either wild-type or catalytically inactive insulin receptors. Thus, in brown preadipocytes in the basal state, the presence of insulin or IGF-1 receptors plays a permissive role in apoptosis, which is blocked either by ligand binding or by deletion of both receptors. These findings reveal a specific role of unliganded receptors in control of apoptosis that is independent of their kinase activity.

Results

Creation of and signaling in IR- and IGF1R-deficient cells

To determine the contributions of IR and IGF1R in apoptosis, we used brown preadipocyte cell lines lacking the IR, the IGF1R, or both. At least three independent cell lines of each type were produced. IR and IGF1R mRNA or protein were not detectable in the corresponding single KO cell lines using primers specifically targeting the excised exons or the respective antibodies, and both were absent in the DKO cells [see Fig. 2B of (10)]. Increasing concentrations of insulin failed to activate Akt in DKO cells, indicating the complete absence of insulin and IGF-1 signaling (fig. S1A). Despite the absence of insulin and IGF-1 receptors, DKO and WT cells had similar growth rates and doubling times in culture (fig. S1B).

Apoptosis sensitivity in WT, IRKO, IGFRKO, and DKO cells

WT brown preadipocytes undergo apoptosis, as assessed by DNA fragmentation, in as little as 6 hour after serum withdrawal [(11); see also Fig. 1A]. Serum withdrawal also produced strong activation of the major executioner of apoptosis, caspase 3, as shown by its cleavage

and detection of a 17 kDa fragment (Fig. 1B). 6 hours after serum withdrawal, single receptor deletion mutant IRKO and IGFRKO cells displayed similar amounts of DNA fragmentation and similar degrees of caspase 3 cleavage (i.e., activation) as WT cells (Fig. 1A and 1B). Unexpectedly, however, DKO cells showed a >80% reduction in DNA fragmentation compared to WT, IRKO, or IGFRKO cells and little or no caspase 3 cleavage after serum deprivation, suggesting a greatly decreased sensitivity to serum withdrawal-induced apoptosis.

Apoptosis, as measured by DNA fragmentation, reached a maximum in confluent WT brown preadipocytes within 6 hours of serum withdrawal (Fig. 1C). DNA fragmentation was reduced by 70 to 90% in serum-deprived DKO cells at all time points studied. A similar difference was apparent when apoptosis was assessed by caspase 3 cleavage, with DKO cells showing no or very little caspase 3 activation (Fig. 1D). Consistent with this, the number of WT brown preadipocytes in culture decreased steadily following serum withdrawal, with a loss of >50% of the cells after 72 hours. In contrast, the number of DKO cells remained virtually constant even after three days of serum deprivation (Fig. 1E). Cytochrome c (cyt c) release from the mitochondria to the cytosol after serum deprivation was markedly reduced in DKO cells compared to WT cells, consistent with the decrease in apoptosis in these cells (fig. S2A). This was not due to a difference in mitochondrial mass between WT and DKO cells; both cell lines showed similar fluorescence after mitotracker staining (fig. S2B), no difference in abundance of the mitochondrial protein VDAC (voltage-dependent anion channel, fig. S2C), and no difference in the ratio of mitochondrial DNA to nuclear DNA (fig. S2D). Similar differences in apoptosis sensitivity were observed when chromatin condensation was visualized by Hoechst 33342 staining, with WT cells showing bright blue nuclear staining after serum deprivation, indicating chromatin condensation, whereas few DKO cells showed such staining (Fig. 1F). Finally, fluorescence-activated cell sorting (FACS) analysis revealed that annexin-positive cell number increased over time in serum-deprived WT cells and was consistently higher than in the apoptosis-resistant DKO cells at all time points (Fig. 1G). Quantitation of the percentage of cells in early [annexin-positive, but propidium iodide- (PI) negative cells] or late (both annexin- and PI-positive cells) phases of apoptosis showed that the percentage of early apoptotic cells increased from 5 to 22% during the first 12 hours of serum deprivation in WT cells, compared to an increase from 3 to 7% in DKO cells (fig. S3A). The total number of apoptotic and dead cells increased from 19 to 47% by 24 hours after serum withdrawal in WT cells, compared to an increase from 12 to 14% in DKO cells. Markedly reduced apoptosis was also observed when proliferating—rather than confluent—WT and DKO cells were used (fig. S3B).

Sensitivity to intrinsic and extrinsic pathway-mediated apoptosis in WT and DKO cells

To further explore the resistance of DKO cells to apoptosis, we subjected the cells to different inducers of apoptosis. Treatment for 24 hours with inducers of the intrinsic pathway such as etoposide (25 μ M), H₂O₂ (5 μ M), and paraquat (1 mM), or of the extrinsic pathway, FasL (100 ng/ml) and TNF α (50 ng/ml), induced DNA fragmentation in WT cells to the same extent as serum deprivation (Fig. 2A). DKO cells showed a 60 to 90% reduction in DNA fragmentation in response to all of these treatments (Fig. 2A). Similarly, etoposide,

H₂O₂, paraquat, FasL, and TNF α treatments all increased caspase 3 activation in WT cells, whereas almost no caspase 3 cleavage was detected in DKO cells (Fig. 2B). In WT cells, 100 nM IGF-1 or insulin strongly inhibited caspase 3 activation, indicating the potent anti-apoptotic effect of these hormones (Fig. 2C). Similarly, 100 nM IGF-1 inhibited caspase 3 activation induced by a TNF α (25 ng/ml) treatment in WT cells (Fig. 2D). Insulin and IGF-1 had no effect on DKO cells, consistent with the absence of their receptors on these cells. Thus, DKO cells are resistant to both the intrinsic and extrinsic pathways of apoptosis at some point upstream of caspase 3 cleavage, and neither insulin nor IGF-1 affects this process.

Apoptosis sensitivity in DKD cells

To further investigate the roles of IR and IGF1R in apoptosis, we repeated these experiments using brown preadipocytes in which relatively acute knock-down of the receptors was achieved by infection with an adenovirus encoding the Cre recombinase, and cells were studied 5 days after infection, without selection. In vitro Cre-recombination of IR and IGF1R floxed alleles was very effective; double knock-down (DKD) cells showed a 98% decrease in both *IR* and *IGF1R* mRNA abundance compared to control cells and a parallel decrease in IR and IGF1R protein (Fig. 3A). Serum deprivation increased apoptosis, as measured by FACS analysis of annexin binding, from 7.1 to 30.7% of control cells. Apoptosis in response to serum starvation was reduced by more than half in DKD cells, with an increase from 6.3% to 17.4% of cells (Fig. 3B). More strikingly, serum deprivation induced robust caspase 3 cleavage in control cells, which was barely detectable in DKD cells (Fig. 3C). Similar results were obtained in non-immortalized primary mouse embryonic fibroblasts (MEFs), in which acute deletion of IR and IGF1R was created by infecting double floxed cells with Cre adenovirus. Five days after infection, DKD MEFs showed a 90% reduction in both *IR* and *IGF1R* mRNA and protein levels (Fig. 3D). Serum deprivation was a weak inducer of apoptosis in this cellular model. However, the percentage of apoptotic cells increased from 8.4% to 65.8% in cells infected with control adenovirus after a 24 hours H₂O₂ treatment. DKD MEFs showed a marked resistance to apoptosis induced by H₂O₂ treatment as the percentage of apoptotic cells rose from 8.2% to only 18.4% (Fig. 3E). Together, these results indicate that the apoptosis resistance observed in cells lacking IR and IGF1R is apparent after short term knock-down in brown preadipocytes, as well as in primary mouse embryonic fibroblasts, and can occur independent of cell immortalization.

Abundance of anti-apoptotic and pro-apoptotic proteins in WT and DKO cells

The sensitivity of cells to any apoptotic stimulus can vary depending on the abundance of pro- and anti-apoptotic proteins. We therefore measured the abundance of proteins that have been implicated in the apoptotic process in WT and DKO cells under several conditions, including in proliferating cells, confluent cells in the presence of serum, and confluent cells after serum deprivation.

The Bcl-2 family of proteins, which regulate mitochondrial integrity, comprise a major family of proteins regulating apoptosis (12). There was a 50% decrease in the abundance of Bax, a pro-apoptotic member of the Bcl-2 family, at both the protein and mRNA levels, in

serum-fed or serum-deprived confluent DKO cells compared to WT cells (Fig. 4A and B). Protein abundance of other pro-apoptotic Bcl-2 family members, such as Bad (Fig. 4A and B), Bik, Bak, Bim, Bid, Bmf, and Puma, and of the anti-apoptotic protein Mcl-1 (fig. S4) did not differ between WT and DKO cells under either serum-fed or serum-deprived conditions. Similarly, mRNA levels of these proteins were identical between WT and DKO cells under either serum-fed or serum-deprived conditions, except for Bik and Bmf which showed an increase by a factor 5 after serum deprivation in WT cells but not in DKO cells (fig. S5). Bid mRNA was elevated by a factor 2 in both serum-fed and serum-deprived conditions in DKO cells compared to WT cells but this change was not reflected at the protein level (fig. S5).

The abundance of the anti-apoptotic protein Bcl-2, was increased by a factor 2.5 in proliferating or confluent DKO cells compared to WT cells, but showed no significant change in response to serum withdrawal. (Fig. 4A and C). The increase in Bcl-2 in DKO cells was post-transcriptional. No differences in *Bcl-2* mRNA levels were apparent between serum-fed WT and DKO cells (Fig. 4C), whereas serum deprivation induced a 650% increase in *Bcl-2* mRNA abundance in WT, but not DKO cells. The abundance of Bcl-xL, another anti-apoptotic protein of the Bcl-2 family, increased by a factor 2.5 in both serum-fed and serum-deprived DKO cells compared to the respective WT cells (Fig. 4A). This occurred with no change in *Bcl-xL* mRNA abundance (Fig. 4C).

Abundance of the anti-apoptotic protein Flip, a negative regulator of the extrinsic pathway (13-15), increased by a factor 4 in DKO compared to WT cells (Fig. 5A and B). The abundance of some members of the family of Inhibitor of Apoptosis Proteins (IAP), proteins that interact with and inhibit caspase-3, caspase-7, and caspase-9 (16-19), also increased. Thus, abundance of cIAP1 and 2 increased at least by a factor 4 in proliferating DKO cells compared to proliferating WT cells (Fig. 5A), and that of XIAP increased by a factor 2 in proliferating or confluent DKO cells compared to respective WT cells while survivin abundance was unchanged (Fig. 5A and B). In contrast to these changes in protein abundance, levels of the mRNAs encoding *Flip*, *cIAP1*, *cIAP2*, *XIAP*, and *survivin* were not different between WT and DKO cells (Fig. 5B and S5). In double knockdown MEFs, an increase in Bcl-xL, Flip, and XIAP abundance was observed similar to that in DKO cells, but there was no change in that of Bax or Bcl-2 (fig. S6).

Mitochondria release other pro-apoptotic proteins, such as Smac (second mitochondrial activator of caspases, also known as Diablo) and HtrA2 (high-temperature-regulated A2, also known as Omi), which act as IAP inhibitors (20-22), in addition to cytochrome c. The abundance of these two proteins in WT and DKO cells was similar (Fig. 5A and B). The abundance of apoptosis-inducing factor (AIF), a pro-apoptotic protein believed to play a central role in the regulation of caspase-independent cell death (23), was also unchanged in DKO compared to WT cells (fig. S4). Likewise, the expression of heat shock proteins 27, 60, 70, and 90 [all of which have been implicated in the apoptotic process (24)] was identical in WT and DKO cells (fig. S4).

Although we observed the entire repertoire of changes in the abundance of pro- and anti-apoptotic proteins, specifically including increased Bcl-2 abundance and decreased Bax abundance, only in DKO cells (fig. S7A and B, and Fig. 4), we did see some changes in the

abundance of these proteins in single knockout cells. For example, Flip was increased in both IRKO cells (by a factor 4) and IGF1RKO cells (by a factor 6), changes that were similar to the increase in DKO cells. As for the changes in protein abundance described above, this effect was post-transcriptional, with *Flip* mRNA levels being similar among the WT, IRKO, IGF1RKO, and DKO cell lines (fig. S7C). Serum deprivation induced an increase in *Bcl-2* mRNA levels in WT cells (650% increase) and to a lesser extent in IRKO and IGF1RKO cells (400% and 200%, respectively), but not in DKO cells. *Bax* mRNA levels were similar in WT, IRKO, and IGF1RKO cells in the presence or in the absence of serum but were decreased in DKO cells, similar to what we observed for Bax protein (fig. S7C).

Effects of re-expressing IR, IGF1R, or expressing a catalytically inactive IR in DKO cells

These data suggest that, in preadipocytes, the unliganded IR and IGF1R are pro-apoptotic. To test this hypothesis, we stably expressed functional human IRs (hIR), IGF1Rs (hIGFR), or both, in DKO cells. Thus, stimulation with 100 nM insulin induced Akt and ERK phosphorylation in DKO cells expressing hIR, hIGFR, or both, but not in DKO cells infected with empty vector controls, indicating that insulin signaling was restored in cells stably expressing the human receptors (Fig 6A). Notably, 6 hour serum deprivation induced caspase 3 cleavage in cells expressing hIR, hIGFR, or both, but not in DKO control cells, (Fig. 6B), indicating that introduction of IR and IGF1R increased the propensity for apoptosis.

The pro-apoptotic effect of IR and IGF1R was apparent in the absence of ligand, suggesting that it did not require receptor tyrosine kinase activity. To further assess the role of the tyrosine kinase activity of the IR in the pro-apoptotic effects of the unliganded receptor, we created an inactive mutant in which lysine residue 1030 in the ATP binding site was replaced with arginine (K1030R mutant) (25). As expected, insulin failed to stimulate Akt phosphorylation in DKO cells expressing the IR K1030R mutant (Fig. 6C). However, serum deprivation induced caspase 3 cleavage in these cells to an extent comparable to that in cells expressing the WT IR (Fig. 6D). As previously noted (Fig. 4), the relative abundance of key apoptotic proteins in DKO cells differed from that in WT cells; DKO cells re-expressing WT or mutant IR, IGF1R, or both WT IR and IGF1R, showed increased Bax abundance and decreased abundance of Bcl-2 and Bcl-xL, indicating partial rescue of the DKO phenotype (Fig. 6B and D and S8). Consistent with the lack of a role for “conventional” insulin signaling in the pro-apoptotic effect of the IR and IGF1R, apoptosis resistance (assessed by caspase 3 cleavage) could not be recapitulated in WT cells by treatment with PI3K inhibitors (LY294002 and wortmannin) or MAPK inhibitors (PD98059 and U0126) either alone or in combination (fig. S9A). Furthermore, 6 or 24 hours of insulin treatment of WT cells did not affect the abundance of Bax, Bcl-2 or Flip (fig. S9B), suggesting that the changes in abundance of these proteins induced by the lack of insulin and IGF-1 receptors did not depend on receptor activity. Together, these data indicate that the kinase activity of the IR is not essential for its permissive role in apoptosis, and that this role is independent of the PI 3-K and MAPK pathways.

Discussion

Insulin and IGF-1 promote cell growth and cell survival. IR and IGF1R signaling interfere with apoptosis through multiple pathways. Insulin receptor substrate (IRS)-1 is the predominant substrate for the anti-apoptotic effects of both insulin and IGF-1 (11); IRS-proteins are coupled to activation of the PI3-K-Akt pathway, which plays a pivotal role in inhibiting apoptosis in various cell types (26-29). The MEK-ERK pathway (27-29), a PI3-K-dependent but Akt-independent pathway (30), and other pathways independent of both MEK-ERK and PI3-K have also been implicated in the anti-apoptotic effects of insulin and IGF-1 (29;31;32).

Here, we identified a role for the IR and IGF-1R in promoting apoptosis. Using immortalized brown preadipocyte cell lines lacking the IR, the IGF-1R, or both, we found that, in contrast to WT cells or cells lacking either receptor alone, cells lacking both the IR, the IGF-1R were resistant to apoptosis. This was apparent through reductions in caspase 3 cleavage, annexin binding, chromatin condensation, and DNA fragmentation following serum deprivation, and when apoptosis was initiated through stimulation of either the intrinsic or the extrinsic pathway. We observed comparable results in at least three independently-derived WT and DKO cell lines, ruling out a clonal difference between cell lines as a possible explanation of this phenotype. Since the use of immortalized cell lines is a limitation in the present study, we repeated experiments in primary cultured MEFs which had been infected with a Cre adenovirus, and in brown preadipocytes which did not go under a clonal selection process. In both models, DKO cells were also resistant to apoptosis compared to control cells, substantiating a direct contribution of IR and IGF1R to apoptosis and indicating that these effects can occur independently of a selection process or SV40 immortalization. We infected MEFs with Cre adenovirus 2 to 3 passages after isolation and used them for apoptosis measurements the following passage; thus we consider it unlikely that a spontaneous transformation of the MEFs took place before CRE infection. Furthermore, proliferation of the cells slowed down considerably and eventually stopped shortly after a few passages. We observed decreased abundance of the pro-apoptotic protein Bax and increased abundance of the anti-apoptotic proteins Bcl-2, Bcl-xL, cIAP1 and 2, XIAP, and Flip in DKO compared to WT cells. This suggests that the decreased propensity of DKO cells to undergo apoptosis may depend on changes in the abundance of key proteins involved in the apoptotic process. Introduction of IR, IGF1R, or both, or of a catalytically-inactive mutant of IR restored sensitivity to apoptosis in DKO cells. Thus, the pro-apoptotic effect of unoccupied IR and IGF-1R appears to be independent of their catalytic activity. Thus, insulin and IGF-1 receptors can be considered members of the dependence receptor family, members of which can induce a 'positive' anti-apoptotic signal when their ligand is present, but also a 'negative' pro-apoptotic signal in the absence of ligand leading to apoptosis (33).

The resistance to apoptosis we observed in DKO cells--as well as the increased abundance of anti-apoptotic proteins and decreased abundance of pro-apoptotic proteins--was unanticipated. Bax and Bcl-2, two major proteins involved in apoptosis mediated through the intrinsic pathway, regulate mitochondrial integrity (12). Bax, which structure resembles various pore-forming proteins, has been suggested to form pores across the outer

mitochondrial membrane, leading to loss of membrane potential and efflux of cytochrome c, AIF, and the IAP inhibitors Smac and Htra2 (12). Conversely, Bcl-2 is thought to prevent such pore formation (12) while heterodimerizing with Bax (34). The abundance of Bax was decreased in DKO cells, whereas that of Bcl-2 was increased, providing a possible mechanism for the resistance of these cells to apoptosis mediated through the intrinsic pathway. In addition, the abundance of Bcl-xL, another anti-apoptotic protein in the Bcl-2 family, also increased in DKO cells (34).

We also observed coordinate changes in abundance of several other proteins implicated in apoptosis pathway in the DKO cells. Abundance of anti-apoptotic proteins of IAP protein family, including cIAP1, cIAP2 and XIAP, which directly inhibit caspase 3 and 9 activation (12), was increased in DKO cells, adding to the protection from apoptosis provided by the decrease in Bax and increase in Bcl-2 and Bcl-xL. The abundance of Flip, which inhibits caspase 8 activation, thereby acting as an inhibitor of the extrinsic pathway, was also increased in DKO cells, in agreement with the resistance of these cells to apoptosis mediated through the extrinsic pathway with TNF α or FasL treatment. The net result of the increases in anti-apoptotic proteins and decreases in pro-apoptotic proteins provides a potential mechanism for the highly decreased caspase 3 activation in response to stimulation of either the intrinsic or extrinsic apoptotic pathways in DKO cells.

These changes in susceptibility to apoptosis and in the abundance of pro- and anti-apoptotic proteins occur upon deletion of the IR and IGF1R, indicating that, in the unliganded state, these receptors send pro-apoptotic signals. These signals are distinct from the well-known anti-apoptotic signals mediated by the IR and IGF1R when they are activated by ligands. An association between unoccupied IR and IGF1R and apoptosis has been hinted at in studies by Nevado *et al.*, who found that re-expressing IR in immortalized hepatocytes in which the IR had been knocked out enhanced susceptibility to apoptosis (35), an observation consistent with the notion that basal expression of the insulin receptor creates a pro-apoptotic signal.

How these basal pro-apoptotic signals are mediated in the DKO cells remains unclear. Although the PI3K-Akt and MAPK-ERK pathways are important to the anti-apoptotic actions of insulin and IGF-1, they do not appear to mediate the pro-apoptotic effects of the IR and IGF1R, because resistance to apoptosis is not observed in WT cells treated with PI3K inhibitors or MAPK inhibitors either alone or in combination. Moreover, insulin treatment of WT cells did not alter the abundance of Bax, Bcl-2, or Flip. Furthermore, a catalytically-inactive form of the IR at least partially restored susceptibility to apoptosis in DKO cells indicating that IR kinase activity is not involved in its permissive effect on cell death. Whatever the signal, it produces a coordinated change in the abundance of pro- and anti-apoptotic proteins, which takes place primarily at the post-transcriptional level and leads to a pro-apoptotic state.

One potential mechanism would involve interaction of the unoccupied IR and IGF-1R with some other protein(s). In this scenario, removing both receptors would disrupt this interaction, leading to resistance to apoptosis. Although the nature of the possible interacting proteins remains to be defined, radiation inactivation experiments have shown that the IR, in its native membrane environment, behaves as if it is non-covalently associated with a high

molecular weight protein that modifies IR binding affinity (36). G α (i)-proteins (37), integrins (38), and β arrestin (39) all interact non-covalently with IRs and IGF-1Rs; the latter promotes IGF-1-dependent MAPK phosphorylation, as well as IGF1R endocytosis (40), ubiquitination, and down-regulation (41). B arrestin also serves as a molecular switch for G protein-coupled receptors, allowing ligands and the unoccupied receptor to elicit distinct effects from those mediated through the activated G protein (42). Another possible mechanism could be the release of pro-apoptotic fragments from the intracellular part of the receptors after caspase or other protease cleavage, as it has been shown for many other dependence receptors which includes several tyrosine kinase receptors like IR and IGF1R (33). Similar to what we observed for the IR, it has recently been shown that tyrosine kinase receptor A and C (TrkA and TrkC) induce apoptosis independent of their kinase activity since kinase inactive mutants of the receptors were also able to induce cell death (43). Whether or not IR and IGF1R can be cleaved by caspases or proteases and release intracellular pro-apoptotic fragments remains to be determined.

It remains unclear to what extent and when these pro-apoptotic effects occur in vivo. The simultaneous lack of both the IR and IGF1R during development results in marked intrauterine growth retardation and early fetal death (44). Likewise, simultaneous tissue-specific KO of both the IR and the IGF1R in vivo in β cells (45) or muscle (46) results in severe diabetes or early death from dilated cardiomyopathy, respectively. IR and IGF1R receptor expression change in many normal and pathological states (47), and it is conceivable that the pro-apoptotic effects of IR and IGF1R are directly proportional to receptor levels. This could be especially important during embryogenesis, and where varying degrees of IR and IGF1R abundance could confer differential susceptibility to apoptosis to specific groups of cells. Defects in apoptotic cell death have been linked to many diseases, including cancer, neurodegenerative diseases, and auto-immune disorders (3;4). The present data suggest that regulating IR and IGF1R abundance could provide a new approach to treating some diseases associated with defective apoptosis, or to promote resistance to apoptosis in cells or tissues being used for transplantation or stem cell therapy.

In conclusion, our data support a three state model for IR and IGF-1R signaling in control of apoptosis (Fig. 7A). In the occupied state, receptors act through their intrinsic tyrosine kinase to produce an anti-apoptotic effect. In the unoccupied state, receptors exert a permissive effect on apoptosis, which appears to be independent of PI3K-Akt and MAPK signaling, allowing apoptosis to occur under certain conditions. Finally, there is the "no receptor state", in which simultaneous deletion of the IR and IGF1R leads to resistance to apoptosis, as shown by a reduction in caspase 3 cleavage and DNA fragmentation. This resistance to apoptosis is apparent after stimulation of either the intrinsic or extrinsic pathway and may result, at least in part, from a change in the abundance of pro- and anti-apoptotic proteins. Our data indicate that the unliganded IR and IGF1R have a previously unrecognized role, exerting pro-apoptotic effects. The balance between the pro-apoptotic effects of the unliganded receptors and the anti-apoptotic effects of the occupied receptors may play a role in development and provide a new strategy for engineering cells for transplantation.

Materials and methods

Cell Isolation and Culture

Cells were created as previously described (10). Briefly, brown preadipocyte cells were isolated and immortalized from three strains of C57Bl/6 mice: 1) mice homozygous for a floxed allele of exon 4 of the insulin receptor (IRlox); 2) mice with a floxed allele of exon 3 of the IGF-I receptor (IGFRlox); and 3) mice with both floxed IR and IGF1R alleles (IRlox/IGFRlox). In vitro recombination of the insulin or IGF-I receptor was induced by infecting subconfluent cells with an adenovirus encoding *Cre* recombinase at a titer of 500 multiplicity of infection. After 1 h, the viral supernatant was replaced with culture medium. Individual colonies were selected, and IR or IGFR recombination or both was assessed by PCR of genomic DNA. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% v/v fetal bovine serum at 37 °C in a 5% CO₂ environment. Experiments were performed in the different KO cells lines using IRlox, IGFRlox, IRlox/IGFRlox, or wild type cell lines as a control. Because IRlox, IGFRlox, IRlox/IGFRlox, and wild type cell lines showed similar results, data obtained using the different control cell lines were pooled and referred to as WT in the text.

Insulin and IGF1R double knock-down (DKD) cells were generated from immortalized IRlox/IGFRlox brown preadipocyte cells (BAT) or non-immortalized mouse embryonic fibroblasts (MEF) cells infected overnight with an adenovirus encoding a GFP- tagged *Cre* and the corresponding control vector (Gene transfer vector core, University of Iowa). Cells were not selected, were kept in regular growth medium and split when reaching confluence, and experiments were performed 5 to 7 days after infection. MEF DKD cells were produced from IRlox/IGFRlox E.14.5 fetuses. Fetuses were decapitated and the liver was removed. Mesenchymal tissue was washed twice with PBS to remove blood clots, minced and digested with 0.5 g/L of trypsin and 0.2 g/L of EDTA in Hanks' Balanced Salt Solution without CaCl₂, MgCl₂, and MgSO₄ (Invitrogen) for 15 min at 37 °C with agitation. The cell suspension was homogenized vigorously with a pipette and plated on 165 cm² flasks (3 fetuses per flask) containing DMEM, 10% FBS, 10,000 units/mL penicillin, 10,000 µg/mL streptomycin, and 0.1 mg/mL Normocin (Invivogen). Cells were cultured for no more than 4 passages.

Retroviral infection

IR and IGF1R were stably introduced into DKO cells by retroviral infection with a pBABE retrovirus encoding hIR, hIGF1R, or control vectors. K1030R IR mutant was made starting with the pBABE retrovirus encoding hIR using Quikchange Lightning site-directed mutagenesis kit from Stratagene according to manufacturer's instructions. Plates (10 cm) of human embryonic kidney 293T cells were transiently transfected with 10 µg of retroviral expression vectors and the viral packaging vectors SV-E-MLV-env and SV-E-MLV using *TransIT-Express* transfection reagent (Mirus Bio Corp.). At 48 h after transfection, virus-containing medium was collected and passed through a 0.45-µm-pore-size syringe filter. Filter-sterilized Polybrene (hexadimethrine bromide; 12 µg/ml) was added to the virus-loaded medium. This medium was then applied to proliferating (40% confluent) DKO cells.

At 24 h after infection, cells were treated with trypsin and replated in medium supplemented with zeocin and hygromycin (Invitrogen) as a selection antibiotic.

Analysis of gene expression by quantitative PCR

Total RNA was extracted using an RNeasy mini kit (QIAGEN). 1 µg of RNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed starting with 12.5 ng cDNA and both sense and antisense oligonucleotides (300 nM each) in a final volume of 10 µl using the SYBR green PCR master mix (Applied Biosystems). Fluorescence was monitored and analyzed in an ABI Prism 7900 HT sequence detection system (Applied Biosystems). Analysis of Tata Binding Protein (TBP) expression was performed in parallel to normalize gene expression. Amplification of specific transcripts was confirmed by analyzing melting-curve profiles at the end of each PCR.

Cell lysates and immunoblotting

Cells were washed once with cold phosphate-buffered saline (PBS) and scraped in radioimmunoprecipitation assay lysis buffer containing 1% sodium dodecyl sulfate (SDS), 10 mM glycerophosphate, 10 mM NaF, 0.1 mM sodium orthovanadate, and 1% protease inhibitor cocktail (SIGMA). Protein concentrations were determined using the Bradford protein assay (Bio-Rad). Lysates (20 to 40 µg) were subjected to SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences) and immunoblotted with the appropriate antibodies. Secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) from donkey and HRP-conjugated anti-mouse IgG from sheep (Amersham). Proteins on the membranes were visualized using Supersignal West Pico substrate or Supersignal West Dura extended duration substrate (Pierce Biotechnologies).

Antibodies

Antibodies (with catalog number) to IR (sc-711), IGFR (sc-713), Bak (sc-832), cIAP2 (sc-7944), Smac (sc-22766), Mcl1 (sc-819), HSP27 (sc-1049), HSP60 (sc-1052) and HSP90 (sc-7938) were purchased from Santa Cruz Biotechnologies. Truncated Bid (PC645) was purchased from Calbiochem. Phospho-Ser473-Akt (#9271), caspase 3 (#9661), cleaved caspase 9 (#9509), Bcl-2 (#2876), Bcl-xL (#2762), Bax (#2772), Bik (#4592), Bad (#9292), Bim (#2819), Bid (#2003), cyt c (#4272), Flip (#3210), cIAP1 (#4592), survivin (#2808), Htra2 (#2176), Puma (#4976), AIF (#4642), HSP70 (#4872), and VDAC (#4866) were purchased from Cell Signaling.

Apoptosis assays

For induction of apoptosis, cells were washed once with PBS, and then incubated with medium containing 10% FBS and various apoptotic inducers, or in medium containing 0.1% BSA for serum deprivation. At the end of the incubation period, medium containing the floating cells was collected. Cells were rinsed with PBS, which was then added to the medium containing the floating cells. Attached cells were resuspended using trypsin and pooled with the floating cells. Cells were collected by centrifugation, washed once in PBS,

and then resuspended in PBS. For DNA fragmentation assays, 100,000 cells were resuspended in PBS and DNA fragmentation was assessed with a cell death detection enzyme-linked immunoabsorbent assay kit (Roche Molecular Biochemicals, Indianapolis, IN) according to manufacturer's instructions. For FACS analysis of annexin V binding, 100,000 cells were pelleted and resuspended in 100 μ l of binding buffer (0.1 M HEPES, pH 7.4, 1.4 M NaCl, 25 mM CaCl₂). Cells were then incubated with 5 μ l annexin V coupled to PE (BD Pharmingen) and 2.5 μ g/ml propidium iodide (SIGMA) for 15 minutes in the dark at room temperature. 400 μ l of binding buffer was added, and cells were subjected to FACS analysis. The remaining cells not used for DNA fragmentation or FACS analysis were pelleted and resuspended in lysis buffer for protein extracts and immunoblots. For DNA dye staining, cells were serum-deprived and Hoechst 33342 (Sigma) was added to the culture medium at a final concentration of 1 μ g/ml. Cells were then incubated for 15 minutes at 37°C in the dark.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Reduced apoptosis in DKO cells compared to WT cells

A) Confluent WT, IRKO, IGFRKO, and DKO cells were serum-deprived for 6 hours and DNA fragmentation performed. Data are mean $\pm$ S.E.M from 4 independent experiments. * indicates a significant difference compared to WT cells, p value <0.05 by Student's t test. AU: arbitrary unit B) Immunoblots to detect caspase 3 cleavage were performed on confluent WT, IRKO, IGFRKO, or DKO cells serum-deprived for 6 hours. One representative blot from 4 independent experiments is shown. C) Confluent WT or DKO cells were serum-deprived for 6 to 48 hours and DNA fragmentation was measured. Data are mean $\pm$ S.E.M from 4 independent experiments. D) Confluent WT or DKO cells were serum-deprived for 6 to 48 hours. One representative blot of caspase 3 cleavage from 3 independent experiments is shown. E) WT or DKO cells were grown in 6 well plates to about 90% confluence and then serum-deprived for 3 days. Cell number was determined after 24, 48, and 72 hours of serum deprivation. Data are mean $\pm$ S.E.M from 8 independent experiments. F) Confluent WT or DKO cells were serum-deprived for the indicated hours and incubated with Hoechst 33342 (1 μ g/ml). G) Confluent WT or DKO cells were serum-deprived for 6 to 48 hours. Cells were incubated with both annexin V PE and propidium iodide (PI) and FACS analysis performed. PI staining of the DNA indicates cell death, whereas PE staining shows binding of annexin V to the phosphatidyl serines of the membrane and thus apoptosis. One representative experiment of 3 is shown.

Fig. 2. DKO cells are resistant to apoptosis mediated through both the intrinsic and extrinsic pathways

Confluent WT or DKO cells were serum-deprived or exposed to etoposide (25 μ M), hydrogen peroxide (5 μ M), paraquat (1mM), FasL (100ng/ml), or TNF α (50 ng/ml) for 24 hours in the presence of serum. A) Floating cells were collected and pooled with attached cells recovered after trypsin treatment and DNA fragmentation was measured. Data are mean $\pm$ S.E.M from 4 independent experiments. * indicates a significant difference compared to WT cells, p value <0.05 by Student's t test. B) Protein extracts were subjected to SDS-PAGE and Western blot analysis for caspase 3. One representative of 4 independent experiments is shown. C) Confluent WT or DKO cells were incubated in medium without serum and treated or not with IGF-1 (100 nM) or insulin (100 nM) for 6 hours. Protein lysates from floating and attached cells were subjected to SDS-PAGE and Western blot analysis for caspase 3. One representative blot from 2 independent experiments is shown. D) Confluent WT or DKO cells were treated with TNF α (50 ng/ml) with or without IGF-1 (100 nM) for 6 hours. Protein lysates from floating and attached cells were subjected to SDS-PAGE and Western blot analysis for caspase 3. One representative blot from 2 independent experiments is shown.

Fig. 3. Apoptosis resistance in double knock down brown preadipocyte cells and MEFs

A) IR and IGF1R mRNA (graph) and protein (blot) abundance was measured in confluent IRlox/IGF1Rlox brown preadipocytes 5 to 7 days after infection with a GFP-tagged Cre adenovirus (BAT DKD) or with the GFP control adenovirus (BAT Cont). IR and IGF1R mRNA were measured by real time PCR and were normalized to Tata binding protein (*TBP*) mRNA. B) Confluent BAT Cont and BAT DKD cells were serum-deprived for 12 hours. Floating and attached cells were incubated with annexin V PE and propidium iodide (PI), and FACS analysis performed. Results represent the percentage of annexin-positive to total cells. Results are mean $\pm$ S.E.M from 5 independent experiments. * indicates a significant difference compared to control cells, p value <0.05 by Student's t test. C) Confluent BAT Cont and BAT DKD cells were serum-deprived for 12 hours. One representative blot of caspase 3 cleavage from 5 independent experiments is shown. D) IR and IGF1R mRNA (graph) and protein (blot) levels were measured in confluent IRlox/IGF1Rlox MEFs 5 to 7 days after infection with a Cre adenovirus (MEF DKD) or the control adenovirus (MEF Cont). IR and IGF1R mRNA were measured by real-time PCR and normalized to *TBP* mRNA. E) Confluent MEF Cont and MEF DKD cells were treated with 1mM H_2O_2 for 24 hours. Floating and attached cells were incubated with annexin V PE and propidium iodide (PI), and FACS analysis performed. Results represent the percentage of annexin-positive to total cells. Results are mean $\pm$ S.E.M from 5 independent experiments. * indicates a significant difference compared to control cells, p value <0.05 by Student's t test.

Fig. 4. Decreased Bax abundance and increased Bcl-xL and Bcl-2 abundance in DKO cells

A) Proliferating (50% confluent) or confluent WT or DKO cells were maintained in serum for 6 or 24 hours. Confluent cells were also serum-starved for 6 or 24 hours or treated with TNF α (50 ng/ml) for 24 hours. Immunoblots were performed on protein lysates from floating and attached cells. One representative Western blot from 3 independent experiments is shown. Bax (B) and Bcl-xL and Bcl-2 (C) protein and mRNA levels were quantified in WT or DKO confluent cells maintained in the presence or in the absence of serum for 24 hours. Results are normalized to β tubulin levels and are mean $\pm$ S.E.M from 5 independent experiments. mRNA levels were quantified by real-time PCR. The data were normalized to *TBP* mRNA. Results are mean $\pm$ S.E.M from 6 independent experiments. * indicates a significant difference compared to WT cells, p value <0.05 by Student's t test.

Fig. 5. Increased Flip and XIAP abundance in DKO cells

A) Proliferating (50% confluent) or confluent WT and DKO cells were maintained in the presence of serum for 6 or 24 hours. Confluent cells were also serum-starved for 6 or 24 hours or treated with TNF α (50 ng/ml) for 24 hours. Immunoblots were performed on protein lysates from floating and attached cells. One representative Western blot from 3 independent experiments is shown. B) Flip and XIAP protein and mRNA levels were quantified in WT and DKO confluent cells maintained in the presence or in the absence of serum for 24 hours. Results are normalized to β tubulin abundance and are mean $\pm$ S.E.M from 5 independent experiments. mRNA abundance was quantified by real-time PCR. The data were normalized to levels of *TBP* mRNA. Results are mean $\pm$ S.E.M from 6 independent experiments. * indicates a significant difference compared to WT cells, p value <0.05 by Student's t test.

Fig. 6. IR, IGF1R, or inactive IR mutant rescues susceptibility to apoptosis in DKO cells
 A) Confluent DKO cells expressing hIR, hIGFR, or both were serum-starved for 6 hours and then treated with 100 nM insulin for 5 minutes, after which cells were harvested and immunoblots were performed. One representative Western blot from 3 independent experiments is shown. B) Confluent DKO cells expressing hIR, hIGFR, or both were serum-starved for 6 hours. One representative Western blot from 4 independent experiments is shown. C) Confluent DKO cells expressing wild type human IR, or K1030R human IR were serum-starved for 6 hours and then treated with 100 nM insulin for 5 minutes, after which cells were harvested and immunoblots were performed. One representative Western blot from 3 independent experiments is shown. D) Confluent DKO cells expressing wild type human IR, or K1030R human IR mutant were serum-starved for 6 hours. One representative Western blot from 5 independent experiments is shown.

Fig. 7. Representation of the three state model for IR and IGF1R signaling in apoptosis
 IR and IGF1R are receptor tyrosine kinases. Ligand binding ("occupied state") stimulates their intrinsic kinase activity to produce anti-apoptotic signals. In the "unoccupied state", receptors are permissive for apoptosis, which takes place in response to various apoptotic inducers such as serum deprivation, etoposide, hydrogen peroxide, paraquat, FasL or TNF α treatments. In the "no receptor state", simultaneous deletion of the IR and IGF1R leads to marked resistance to apoptosis, associated with changes in abundance of apoptotic proteins.