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Insulin reduces apoptosis and increases DNA synthesis and cell size via distinct signalling pathways in *Drosophila* Kc cells

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Abstract. During development of *Drosophila*, cell proliferation and size are known to be regulated by insulin. Here we use *Drosophila* Kc cells to examine the molecular basis for the control of cell growth by insulin. Growing cells in the presence of insulin increased cell number above control levels at 16, 24, 48 and 72 h. We have demonstrated a novel anti-apoptotic effect of insulin (~50%) in these cells, measured by caspase 3-like activity, which contributed to the increase in cell number. The anti-apoptotic effect was observed both in control cells and those in which apoptosis was induced by ultraviolet irradiation. An approximately 2-fold stimulation of bromodeoxyuridine incorporation demonstrated that insulin also increased Kc cell proliferation by stimulating new DNA synthesis. The ability of insulin to increase cell number, stimulate bromodeoxyuridine incorporation and reduce caspase 3-like activity was prevented by PD98059, which inhibits activation of the *Drosophila* extracellular signal regulated kinase (DERK) pathway, and was unaffected by wortmannin, an inhibitor of *Drosophila* phosphatidylinositol 3-kinase (DPI3K). Insulin also increased cell size approximately 2-fold and this was prevented by wortmannin and rapamycin, an inhibitor of Drosphilia target of rapamycin (DTOR). In summary, we show that DERK plays an important role in mediating the effect of insulin to reduce apoptosis and increase DNA synthesis whereas the DPI3K/DTOR/Dp70S6 kinase pathway mediates effects of insulin on cell size in *Drosophila* Kc cells.

INTRODUCTION

Cell growth, division and survival are temporally co-ordinated to regulate development in *Drosophila melanogaster* (Bangs & White, 2000; Johnston & Gallant 2002). Recent work has begun to characterize the mechanisms that control growth and organ size. The insulin-signalling pathway has emerged as the principal regulator of cell growth and development in *Drosophila* (Brogiolo *et al.* 2001; Leevers 2001; Oldham *et al.* 2002; Oldham & Hafen 2003). For example, expression of an insulin-like peptide leads to an increase in body mass (Brogiolo *et al.* 2001) and studies in the developing eye show that over-expression of the insulin receptor increases

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growth (Brogiolo *et al.* 2001). Furthermore, when insulin signalling is inhibited, imaginal disc development and overall fly size are reduced (Edgar 1999). The association of mutations in the insulin receptor or defects in insulin signalling with leprechaunism in humans and with small flies in *Drosophila* suggests that an evolutionarily conserved function of insulin signalling is the control of cell growth and total body size (Brogiolo *et al.* 2001).

The insulin-signalling system is very well conserved from *Drosophila* to humans (Garofalo 2002). This has led to *Drosophila* being exploited as an excellent system in which to investigate insulin signalling. There is one insulin receptor (DInR) (Fernandez *et al.* 1995) and seven insulin-like peptides in *Drosophila* (Brogiolo *et al.* 2001). Downstream signalling pathways involve the homologue of the mammalian insulin receptor substrates (Oldham *et al.* 2000; Poltilove *et al.* 2000), phosphatidylinositol 3-kinase (DPI3K) (Leevers *et al.* 1996), phosphatase and tensin homologues on chromosome 10 (DPTEN) (Goberdhan *et al.* 1999), protein kinase B/Akt (DAkt) (Verdu *et al.* 1999), p70 S6 kinase (DS6K) (Watson *et al.* 1996), extracellular signal-regulated kinase (DERK) (Lim *et al.* 1999) and p38 mitogen-activated kinase (Dp38) (Han *et al.* 1998) amongst others. Many studies have demonstrated a role for components of these insulin-signalling pathways in the control of cell division or cell size.

It has also been shown that insulin can rescue many types of cell from apoptotic death (Bertrand *et al.* 1998; Lee-Kwon *et al.* 1998; Yenush *et al.* 1998). Programmed cell death by apoptosis is a common means of destroying unwanted and superfluous cells (Vaux & Korsmeyer 1999). Apoptosis is mediated by a group of cysteine proteases, termed caspases (Thornberry & Lazebnik 1998). These enzymes, which cleave their substrates after an aspartate residue, are normally present as inactive precursors in cells. Upon receiving an apoptotic signal, the pro-forms of caspases (pro-caspases) undergo proteolytic processing to generate active enzymes (Kumar & Colussi 1999; Nicholson 1999; Kumar & Doumanis 2000; Richardson & Kumar 2002). There is a total of seven caspases in *Drosophila*; DCP-1, DREDD, DRICE, DRONC, DECAY, DAMM and STRICA (Cakouros *et al.* 2002; Richardson & Kumar 2002).

The use of whole flies to study insulin signalling and growth control by insulin/insulin-like growth factor I (IGF-I) has been and will continue to be extremely useful. In addition, the availability of *Drosophila*-derived cell lines which exhibit characteristics of the parent organism are ideal for this purpose. In this study, our aim was to examine the mitogenic and anti-apoptotic effects of insulin on *Drosophila* Kc cells and to elucidate the underlying signalling mechanisms responsible for these effects. The results of the present investigation demonstrate that insulin increases Kc cell proliferation and suppresses apoptosis via DERK-dependent and DPI3K-independent signalling pathways.

MATERIALS AND METHODS

Determination of cell number

Kc cells (a kind gift from Dr Lucy Cherbas, University of Indiana, Indianapolis) were propagated at 26 °C in Schneider's *Drosophila* media (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% foetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells (0.5×10^6 in 2 ml; at a concentration of 0.25×10^6/ml) were grown in a six-well culture dish for 16, 24, 48 and 72 h in the presence or absence of 1 μM insulin. Where indicated, cells were also pre-treated for 30 min with PD98059 [Bioshop, Burlington, ON (5 mM)] or wortmannin (Calbiochem, La Jolla, CA; 100 nM). At each time point an aliquot of 50 μl was taken from each well and counted using a haemocytometer. Trypan blue dye exclusion was used to confirm viability of cells.

Bromodeoxyuridine (BrdUrd) incorporation assay

The effect of insulin on DNA synthesis was assessed by BrdUrd incorporation using a colorimetric enzyme-linked immunosorbent-based assay kit from Oncogene Research Products (La Jolla, CA). In brief, cells grown in 96-well plates were stimulated with insulin 1 μM for 12 h; then BrdUrd label (1 : 2000 dilution) was added with subsequent incubation for an additional 12 h. Where indicated, cells were also treated with PD98059 (5 mM) or wortmannin (100 nM) for 30 min prior to the addition of insulin. Thereafter, DNA was denatured and cells were incubated with anti-BrdUrd antibody followed by quantification of antibody binding by measuring absorbance at dual wavelengths of 450–540 nm.

Determination of apoptosis in Kc cells

Apoptosis was measured by determining caspase 3-like activity using a kit from Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan). This assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labelled substrate DEVD-*p*NA. Where indicated, 2×10^6 cells were then treated with UV light in the UV-B range (302 nm) for 60 min to induce apoptosis and were subsequently grown for 24 h in the presence or absence of insulin 1 μM. Cells were also pre-treated with PD98059 (5 mM) or wortmannin (100 nM) for 30 min prior to the addition of insulin where indicated. Caspase 3-like activity was then determined according to the manufacturer's instructions.

Microscopy and image analysis to determine cell size

Cells (1×10^6) were grown in six-well plates on sterile coverslips coated with poly D-lysine. They were then incubated (where indicated with inhibitors: wortmannin: 100 nM, rapamycin: 20 ng/ml) for 1 h, followed by insulin (1 μM) for 24 h. Cells were identified using an Olympus FV300 laser scanning microscope (at 60× magnification). Images of cells were digitally aquired and the surface area of individual cells was measured using FLUOVIEW software Version 3.0 (Carsen Group, Markham, ON) as described previously (Lnenicka & Keshishian 2000; Cashion *et al*. 2003).

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test. The accepted level of significance was set at $P < 0.05$.

RESULTS

Insulin increases cell number via a DERK-dependent signalling pathway

Figure 1 shows that at all time-points measured over a 72-h period (16, 24, 48 and 72 h) insulin significantly increased cell number, as assessed by cell counting using a haemocytometer. We also demonstrated that the specific inhibitor of DERK activation, PD98059, prevented the insulin-induced increase in cell number at 16, 24, 48 and 72 h in a statistically significant manner (Fig. 1a). Importantly, PD98059 had no effect on the growth of cells cultured in the absence of insulin (Fig. 1a). We also used wortmannin to test the involvement of the DPI3K pathway in insulin-induced increases in cell number. We found that wortmannin had no effect on cell number under basal or insulin-treated conditions from 0 to 72 h (Fig. 1b).

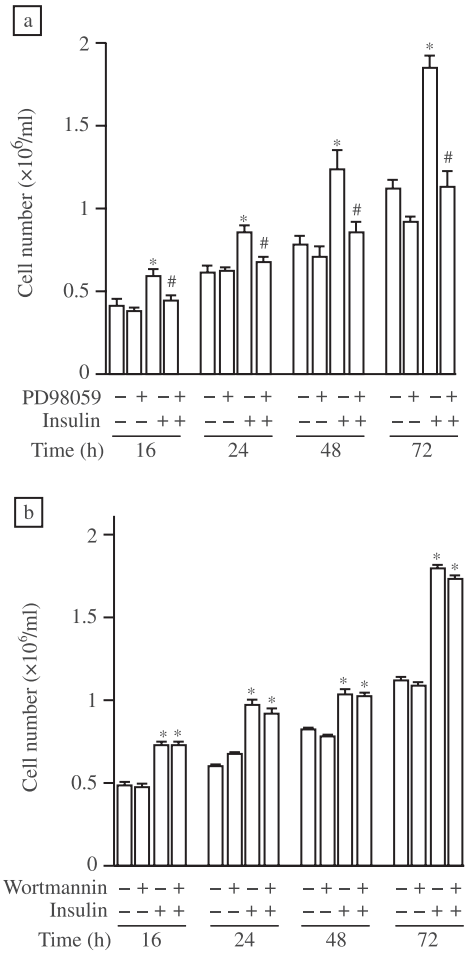

Figure 1. Insulin increases cell number via a DERK-dependent signalling pathway. Cells (at a starting concentration of 0.25×10^6/ml) were grown in a six-well culture dish for 16, 24, 48 and 72 h in the presence or absence of 1 μM insulin. Where indicated, cells were also pre-treated for 30 min with (a) PD98059 (5 mM) or (b) wortmannin (100 nM). At each time-point an aliquot of cells was removed and cell number was determined. Data are expressed as the mean ± SEM of three individual experiments. Statistical analysis was performed by one-way analysis of variance and $P < 0.05$ indicated with respect to control (*) and insulin (#) at each time-point.

Insulin increases BrdUrd incorporation in Kc cells in a DERK-dependent manner

Having shown that insulin increased cell number (Fig. 1) we next determined whether an increase in cell proliferation was responsible for this effect. To examine DNA synthesis we utilized the BrdUrd incorporation assay. Figure 2 shows that insulin increased BrdUrd incorporation in Kc cells (2.06 ± 0.03 fold). The ability of insulin to increase BrdUrd incorporation was reduced in cells which were pre-incubated with PD98059 (Fig. 2). Basal levels of BrdUrd incorporation were unaffected by pre-treatment with PD98059 (Fig. 2). We also tested the effect of wortmannin and found that it had no effect on basal or insulin-stimulated rates of DNA synthesis (Fig. 2).

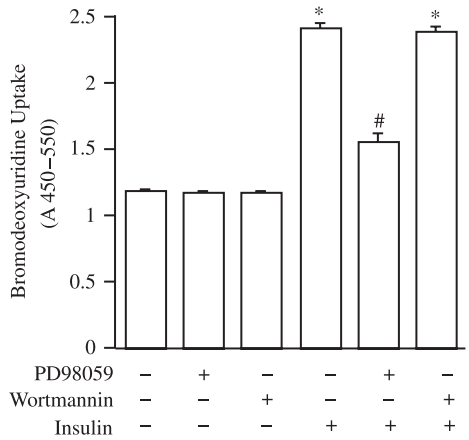

Figure 2. Insulin increases BrdUrd incorporation in Kc cells in a DERK-dependent manner. Cells were stimulated with insulin 1 μM for 12 h, BrdUrd was added for a further 12 h then incorporation of BrdUrd was determined. Where indicated, cells were also treated with PD98059 (5 mM) or wortmannin (100 nM) for 30 min prior to the addition of insulin. Data are expressed as the mean ± SEM of four individual experiments. Statistical analysis was performed by one-way analysis of variance and $P < 0.05$ indicated with respect to control (*) and insulin (#).

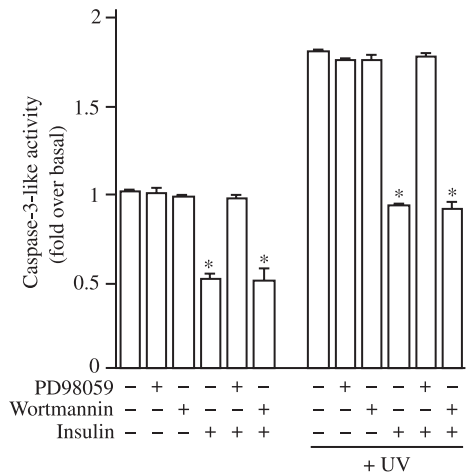

Figure 3. Insulin exerts anti-apoptotic effects in Kc cells in a DERK-dependent manner. Cells were treated with UV light (302 nm) for 60 min and subsequently grown for 24 h in the presence or absence of insulin 1 μM. Cells were also pre-treated with PD98059 (5 mM) or wortmannin (100 nM) for 30 min prior to the addition of insulin where indicated. Caspase 3-like activity was then determined. Data are expressed as the mean ± SEM of four individual experiments. Statistical analysis was performed by one-way analysis of variance and $P < 0.05$ is indicated with respect to control (*).

Insulin exerts anti-apoptotic effects in Kc cells in a DERK-dependent manner

The ability to protect cells from apoptosis may also contribute to the ability of insulin to increase cell numbers (Fig. 1). Therefore, we examined apoptosis using an assay kit to measure caspase 3-like activity. Insulin was shown to decrease the levels of caspase 3-like activity in Kc cells under both control and UV-treated conditions by 50 and 49%, respectively (Fig. 3). The ability of insulin to reduce basal levels or to protect cells from UV-induced apoptosis was prevented by prior incubation of cells with PD98059 but not wortmannin (Fig. 3). Control or UV-induced levels of caspase 3-like activity were unaffected by PD98059 or wortmannin (Fig. 3).

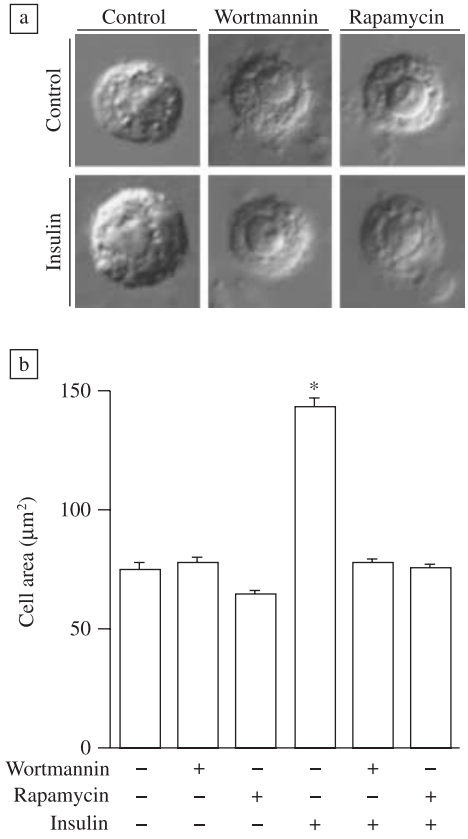

Figure 4. Insulin increases cell size via DPI3K- and Dp70S6K-dependent signalling pathways. Cells (1×10^6) grown in six-well plates were incubated where indicated with wortmannin (100 nm) or rapamycin (20 ng/ml) for 1 h followed by insulin 1 μM for 24 h. Representative images of cells are shown (a) and the surface area of 10 randomly chosen individual cells was measured, then the mean ± SEM was calculated (b). Statistical analysis was performed by one-way analysis of variance and $P < 0.05$ is indicated with respect to control (*).

Insulin increases cell size via DPI3K- and DTOR/Dp70S6K-dependent signalling pathways

Cell size was determined by digitally acquiring images of cells grown under the indicated conditions and measuring surface area by image analysis using FLUOVIEW software. Treatment of cells with insulin for 24 h caused a statistically significant increase in cell size of 1.93-fold (Fig. 4; control, 75.2 mm^2 and insulin, 145.3 mm^2). This effect was totally abolished when cells were pre-treated with wortmannin (78.6 mm^2) or rapamycin (75.8 mm^2) whereas control cell size was not significantly altered by the presence of these inhibitors (Fig. 4).

DISCUSSION

Accurate control of cell number and cell size plays a vital role in determining the development of multicellular organisms. *Drosophila* has often been used as a model system to study the

molecular mechanisms controlling cell growth because the signalling pathways responsible are well conserved from *Drosophila* to man (Garofalo 2002). It is clear from studies to date that the insulin-signalling pathway plays a fundamental role in the control of *Drosophila* cell growth. Over-expression of one of the seven insulin-like peptides expressed in *Drosophila* or the insulin receptor leads to increased growth in *Drosophila* (Brogiolo *et al.* 2001). Conversely, attenuation of insulin receptor function or insulin-signalling pathways causes severe growth retardation (Edgar 1999). Thus, studies in whole flies have conclusively shown an important role for insulin in controlling cell growth. It is now necessary to develop a more detailed molecular understanding of the mechanisms whereby insulin controls cell growth and division.

Here we show that growing *Drosophila* Kc cells in the presence of insulin caused an increase in cell number. Furthermore, we have shown that insulin achieved this effect via a DERK-dependent signalling pathway, in agreement with the fact that the role of ERK in regulating proliferation has been extensively characterized in various species (Fisher *et al.* 2001; Kavurma & Khachigian 2003; Masaki *et al.* 2003). Our results also support a recent report identifying a role of DERK in insulin-stimulated cell growth in *Drosophila* Schneider's S2 cells (Kwon *et al.* 2002). It was also demonstrated recently that over-expression of DAkt caused an increase in cell size but no alteration in the rate of cell proliferation (Verdu *et al.* 1999). One conclusion from this study was that *Drosophila* cell proliferation was independent of Akt. The inability of the PI3K inhibitor wortmannin to prevent insulin-stimulated increases in cell number in our study supports the notion that the DPI3K/DAkt pathway does not play a role in insulin-stimulated Kc cell proliferation.

The increase in cell number detected in cells grown in the presence of insulin could conceivably be the result of an increase in new DNA synthesis and thus cell proliferation, or of an ability of insulin to decrease rates of cell death. Therefore, we first examined the effect of insulin on new DNA synthesis by measuring BrdUrd incorporation in Kc cells. BrdUrd is a thymidine analogue which is incorporated into DNA, and this can be quantified using antibodies to BrdUrd (Dolbeare 1996). We found that insulin increased BrdUrd incorporation, via a DERK-dependent signalling pathway, suggesting that DNA synthesis and cell division was increased by the hormone. Wortmannin did not prevent the insulin-stimulated increase in cell number. To date, surprisingly little is known regarding control of DNA synthesis by insulin in *Drosophila*. A previous study using 32D cells transfected with a chimeric insulin receptor (human insulin binding domain and *Drosophila* intracellular domain) showed that although DPI3K could be activated by the chimaeric receptor, co-transfection of insulin receptor substrate 1 (IRS-1) to allow activation of ERK was necessary for insulin-stimulated DNA synthesis (Yenush *et al.* 1996). Our studies use *Drosophila* Kc cells expressing only endogenous *Drosophila* insulin receptor and produce a similar conclusion; namely DERK, but not DPI3K, activation is necessary for insulin-stimulated mitogenesis.

Accurate co-ordination of cell growth and apoptosis is necessary for development of complex multicellular organisms (Vaux & Korsmeyer 1999; Tapon, Moberg & Hariharan 2001). Thus, it is conceivable that insulin increased cell number by regulating the rate of Kc cell apoptosis. Indeed, it has been reported previously that insulin can rescue several types of cells from apoptotic death (Bertrand *et al.* 1998, Lee-Kwon *et al.* 1998; Yenush *et al.* 1998). However, here we tested, for the first time, the ability of insulin to control apoptosis in Kc cells and found that the basic rate of apoptosis or the increased rate induced by exposure to UV light were both reduced by insulin. Several studies have previously implicated components of the insulin-signalling pathway in controlling apoptosis in *Drosophila* (Bohni *et al.* 1999; Huang *et al.* 1999; Verdu *et al.* 1999). Here we demonstrate using *Drosophila* Kc cells that insulin exerts a protective effect against apoptosis via a DERK-signalling pathway. Our results also suggest that PI3K activation is not necessary for this effect of insulin.

In mice, deletion of the *p70S6K* gene generated an animal that was reduced in size (Shima *et al.* 1998). Many recent studies have established the important role of DTOR (Oldham & Hafen 2003) and Dp70S6K (Thomas 2002) in control of *Drosophila* growth and development. Importantly, in *Drosophila* mutation of *p70S6K* affects cell size but not cell number (Radimerski *et al.* 2002). Activation of TOR and p70S6K signalling is conventionally viewed as being PI3K-dependent. However, a recent study suggested that DTOR-mediated p70S6K activation is DPI3K-independent (Radimerski *et al.* 2002). Indeed, the phosphorylation site required for murine TOR activation by Akt is absent in the *Drosophila* homologue of TOR (Oldham *et al.* 2000). Thus, it appears that DPI3K signalling diverges to regulate cell growth in *Drosophila* via both DAkt and Dp70S6K. Activation of p70S6K leads to translation of mRNAs which mostly encode ribosomal proteins and translation initiation factors. Thus, activation of this kinase is commonly associated with increased protein synthesis and cell growth. Increased cell growth in response to Akt activation is thought to result via phosphorylation and inactivation of the translation initiation factor eIF-4E and subsequently increased cap-dependent translation initiation (Toker 2000). Alternatively, Akt may act to stimulate progression of the cell cycle (Rossig *et al.* 2001). Here we demonstrate that inhibition of either DPI3K or DTOR prevents insulin-induced increases in Kc cell size. This fits with the model proposed above. We also find that inhibition of either DPI3K or DTOR is sufficient to attenuate totally the increase in cell size caused by insulin. This suggests that a linear signalling pathway involving DPI3K and subsequently DTOR may mediate the ability of insulin to cause *Drosophila* Kc cell hypertrophy. It has been shown previously that DERK does not play a role in insulin-stimulated increases in *Drosophila* cell size (Kwon *et al.* 2002).

In summary, we show that insulin exerts a mitogenic effect on *Drosophila* Kc cells. Importantly, the increased number of cells observed when cultures were grown in the presence of insulin ensues as a result of both increased DNA synthesis and reduced apoptosis. DERK plays an important role in mediating the effect of insulin to increase DNA synthesis and reduce apoptosis whereas the DPI3K/DTOR/Dp70S6 kinase pathway mediates the effect of insulin on cell size in *Drosophila* Kc cells.

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