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Putative Role of the Phosphatidylinositol 3-Kinase–Akt Signaling Pathway in the Survival of Granulosa Cells

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Insulin-like growth factor-I (IGF-I) is an important differentiation and survival factor for granulosa cells. The purpose of this study was to test the hypothesis that IGF-I promotes survival of porcine granulosa cells by signaling through the phosphatidylinositol (PI) 3-kinase/Akt signal transduction pathway. Treatment with IGF-I (100 ng/mL) for 10 min stimulated PI 3-kinase and Akt protein kinase activity. IGF-I stimulated the phosphorylation and activation of Akt in a time- and concentration-dependent manner. The PI 3-kinase inhibitors wortmannin and LY294002 blocked IGF-I induced increases in PI 3-kinase activity and phosphorylation of Akt. Additionally, IGF-I treatment prevented apoptosis. The survival response to IGF-I was blocked by treatment with either wortmannin or LY294002. These data suggest that IGF-I-induced phosphorylation of Akt is mediated through PI 3-kinase and that inactivation of this pathway results in granulosa cell apoptosis. We conclude that the PI 3-kinase/Akt signaling serves as a functional survival pathway in the ovary.

Key Words: Granulosa cell; insulin-like growth factor-I; apoptosis; phosphatidylinositol 3-kinase; Akt; signal transduction; ovary.

Introduction

Insulin-like growth factor I (IGF-I) is an important intraovarian regulator of follicular development and function (reviewed in refs. *1* and *2*). The granulosa cells of the follicle produce IGF-I, and it is well established that IGF-I stimulates the proliferation as well as the differentiation of granulosa cells of many species. Moreover, IGF-I has been acknowledged to serve as a critical intrafollicular survival factor *(3–5)*. In this regard, IGF-I has been shown to protect cells in intact rat follicles from the commitment to apoptosis *(4)* and to attenuate apoptosis in cultured porcine granulosa cells *(5)*. Despite the important role of IGF-I in the follicle, little is known about the intracellular mechanism of action of IGF-I.

Recent reports have provided compelling evidence implicating the serine/threonine protein kinase Akt as a central mediator of IGF-I-mediated cell survival *(6,7)*. Akt, the cellular homolog of v-akt, was initially identified by its similarity to protein kinase A and C, and therefore has been referred to as protein kinase B or as related to A and C kinase (RAC-PK). Interest in this protein kinase stems from its position as a downstream target of phosphatidylinositol (PI) 3-kinase activity. PI 3-kinase-generated phosphatidlyinositol-3,4-bisphosphate (PtdIns-3,4-P_2) and PtdIns-3,4,5-P_3 are essential for the activation of Akt (reviewed in refs. *8–12*). These unique phospholipids interact with the pleckstrin homology (PH) domain of Akt to initiate its colocalization at the plasma membrane with the protein kinases that phosphorylate and activate Akt. Stimulation of PI 3-kinase activity results in the phosphorylation of Akt on two residues, Thr308 and Ser473, both of which are required for maximal activation. The recently discovered protein kinase 3-phosphoinositide-dependent kinase 1 (PDK1) is responsible for phosphorylating Akt at Thr308 *(11)*. Moreover, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 alter the conformation of Akt, allowing Thr308 to be accessible to PDK1 *(8–12)*.

Relevant to this study is the ability of Akt to suppress apoptosis in a variety of cell systems. Akt has been shown to play a critical role in IGF-I-mediated cell survival in fibroblasts *(6)* and cerebellar neurons *(7,13)*, in Interleukin-3 dependent survival of hematopoietic cells *(14)*, and in nerve growth factor support of neuron survival *(15)*. In each of these studies it was found that Akt-mediated cell survival could be blocked by utilizing the PI 3-kinase-specific inhibitors wortmannin *(16,17)* and LY294002 *(18,19)*. The object of the present study was to determine whether or not the PI 3-kinase/Akt kinase signaling pathway might be involved in IGF-I-mediated cell survival in granulosa cells.

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Results

Western blot analysis employing antibodies to Akt and the phosphorylated form of Akt established the presence of Akt in primary cultures of porcine granulosa cells (Fig. 1). We observed that treatment with IGF-I (100 ng/mL) for 10 min increased the phosphorylation of Akt (Fig. 1A). To verify that the increase in Akt phosphorylation was associated with an increase in the activity of the enzyme, we employed Akt immunocomplex protein kinase assays. Akt protein kinase activity, assayed by the extent of substrate peptide phosphorylation, was increased following IGF-I treatment for 10 min (Fig. 1B). Time course studies revealed that Akt phosphorylation increased within 2 min following IGF-I treatment and that Akt phosphorylation was sustained for at least 30 min (Fig. 2A). Other studies (data not shown) indicated that IGF-I-induced Akt phosphorylation was sustained in 4-h incubations. IGF-I-induced Akt phosphorylation was concentration dependent, with maximal phosphorylation occurring at 10–30 ng/mL (Fig. 2B).

Pretreatment for 30 min with the PI 3-kinase inhibitor wortmannin (200 n*M*) completely blocked IGF-I-stimulated phosphorylation of Akt in cultured granulosa cells (Fig. 3A). Similar results were observed following a 30-min pretreatment with 10 μ*M* LY294002 (not shown). The stimulatory effects of IGF-I on Akt phosphorylation were similar in cultured (Fig. 3A) and freshly isolated (Fig. 3B) granulosa cells. Likewise, pretreatment with the PI 3-kinase inhibitor wortmannin (200 n*M*) for 30 min prevented IGF-I-stimulated Akt phosphorylation in freshly isolated granulosa cells (Fig. 3B).

To verify that the PI 3-kinase inhibitors were working as predicted, we measured PI 3-kinase activity in granulosa cells. Treatment with IGF-I for 10 min increased PI 3-kinase activity approximately fivefold (Fig. 4). The PI 3-kinase inhibitors wortmannin and LY294002 abrogated the stimulatory effect of IGF-I on PI 3-kinase activity.

Isolated granulosa cells incubated in the absence of serum or growth factor support spontaneously undergo apoptosis *(3–5)*. Experiments were performed to determine the involvement of the PI 3-kinase signaling pathway in the survival response to IGF-I. Compared to nonincubated controls (time, 0 h), freshly harvested granulosa cells incubated for 18 h in serum free medium exhibited low molecular weight oligonucleosomal DNA fragmentation, a hallmark of cells undergoing apoptotic cell death *(3–5)* (Fig. 5). Treatment with IGF-I (100 ng/mL) prevented granulosa cells from undergoing commitment to apoptosis. In fact, the level of DNA laddering in IGF-I-treated cells was comparable to non-incubated controls. The DNA laddering pattern in cells treated with the PI 3-kinase inhibitor LY294002 was similar to that of control cells incubated for 18 h without IGF-I. More important, pretreatment for 30 min with the PI 3-kinase inhibitor LY294002 (10 μ*M*) reduced the protective effects of IGF-I (Fig. 5). This result

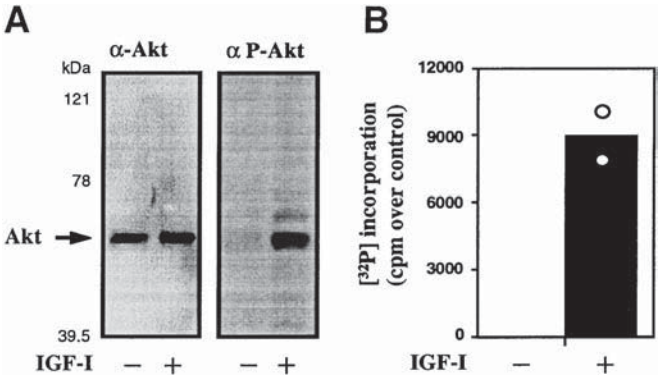

Fig. 1. IGF-I mediated phosphorylation and activation of Akt in granulosa cells. Granulosa cells were treated with IGF-I (100 ng/mL) for 10 min and total cell lysates were prepared. **(A)** Western blot analysis was performed using Akt (αAkt) or phospho-Akt (αP-Akt) antibodies. **(B)** Akt protein kinase activity was determined in αAkt immunocomplex protein kinase assays. The average increases (bar) in two experiments (s) are shown.

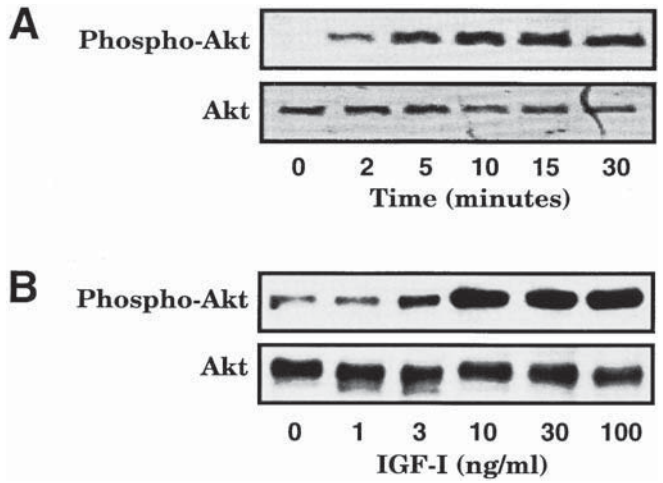

Fig. 2. IGF-I stimulates Akt phosphorylation in a time- and concentration-dependent manner. **(A)** Granulosa cells were treated with IGF-I (100 ng/mL) for 0 to 30 min. **(B)** Granulosa cells were treated with 0–100 ng/mL of IGF-I for 10 min. Western blot analysis was performed using Akt or phospho-Akt antibodies. Membranes were sequentially probed for phospho-Akt and Akt.

suggests that IGF-I-mediated cell survival is sensitive to inhibitors of the PI 3-kinase/Akt signaling pathway.

In addition to using freshly isolated granulosa cells, we also examined whether IGF-I exerted effects on cell survival in primary cultures of porcine granulosa cells. May et al. *(20)* have demonstrated using serum-free culture conditions that IGF-I treatment for 18 h stimulated an increase in DNA synthesis in porcine granulosa cell cultures. However, under these cell culture conditions, IGF-I did not promote cellular proliferation even after 3 or 6 d in culture *(20)*. Figure 6 shows that treatment for 18 h with IGF-I stimulated a concentration-dependent increase in DNA synthesis, as

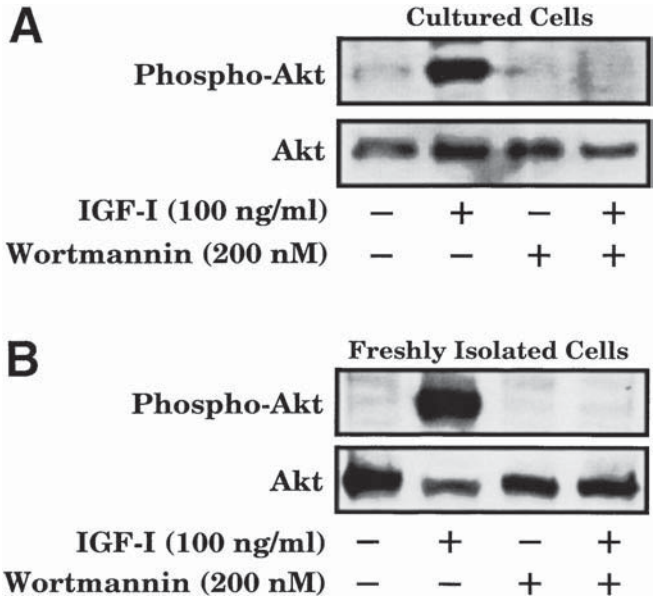

Fig. 3. The PI 3-kinase inhibitor wortmannin blocks IGF-I-induced phosphorylation of Akt. Cultured granulosa cells **(A)** or freshly isolated granulosa cells **(B)** were pretreated for 30 min with wortmannin (200 n*M*) prior to treatment with IGF-I (100 ng/mL) for 10 min. Western blot analysis of total cell lysates was performed using Akt or phospho-Akt antibodies.

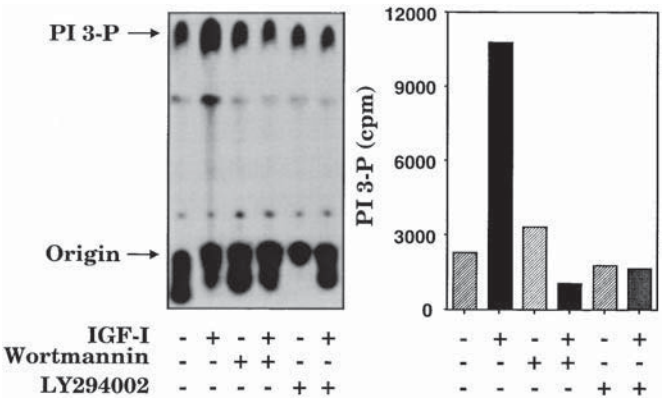

Fig. 4. IGF-I stimulates PI 3-kinase activity. Granulosa cells were pretreated for 30 min with wortmannin (200 n*M*) or LY294002 (10 μ*M*) prior to the addition of IGF-I (100 ng/mL) for 10 min. **(Left)** Autoradiograph of a thin-layer chromatography plate demonstrating the formation of phosphatidylinositol 3-phosphate (PI 3-P); **(Right)** radioactivity associated with PI 3-P.

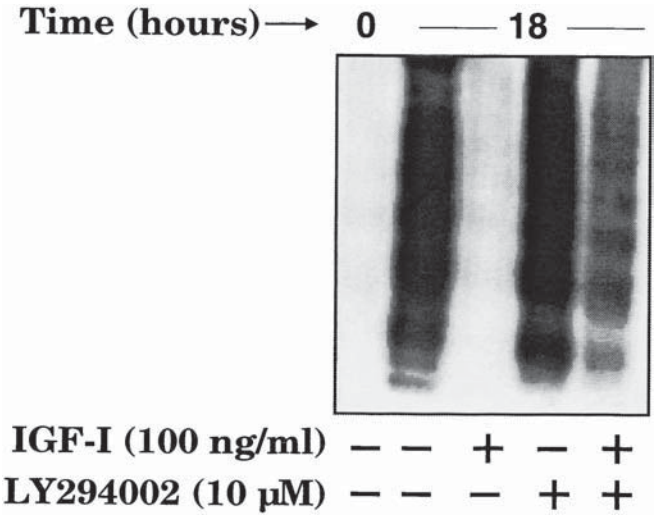

Fig. 5. PI 3-kinase inhibitors attenuate IGF-I-stimulated granulosa cell survival. Aliquots of freshly isolated granulosa cells were stopped prior to incubations (time 0) or after 18 h of incubation in basal medium or medium containing IGF-I (100 ng/mL). Some cells were pretreated for 30 min with LY294002 (10 μ*M*). Cells were collected for analysis of DNA fragmentation prior to (time 0) or following 18 h of treatment.

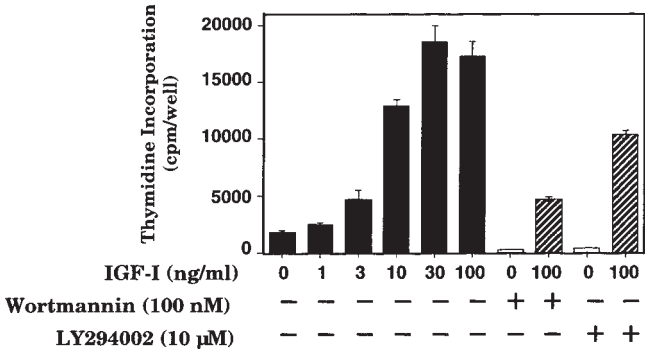

Fig. 6. IGF-I-stimulated DNA synthesis in granulosa cells is reduced by treatment with PI 3-kinase inhibitors. Serum-free cultures of granulosa cells were incubated in the presence or absence or IGF-I and/or the PI 3-kinase inhibitors wortmannin (200 n*M*) or LY294002 (10 μ*M*) for 18 h. Cells were pulse labeled with [^{3}H]thymidine for 4 h.

measured by [^{3}H]thymidine incorporation. The stimulatory effect of IGF-I on DNA synthesis was similar to that reported by May et al. *(20)*. Treatment with either PI 3-kinase inhibitor, wortmannin (200 n*M*), or LY294002 (10 μ*M*) attenuated the stimulatory effect of IGF-I on thymidine incorporation (Fig. 6). A similar approach was adapted for our studies on cell survival; that is, following attachment, granulosa cell cultures were incubated in serum-free Ham's F-12: Dulbecco's modified Eagle's Medium (F:D) medium for 18 h. In the absence of cell proliferation under serum-free culture conditions *(20)*, alterations in cell number presumptively reflect changes in IGF-I-mediated survival. Figure 7 illustrates the morphology of granulosa cell cultures following 18 h of treatment. Compared with cultures treated with IGF-I (Fig. 7C), cultures treated with control medium (Fig. 7A) had fewer attached cells. Levels of cells following treatment with LY294002 were less than those observed in control and IGF-I-treated cultures, respectively (Fig. 7B, D). The cells that had detached from the plates had morpho-

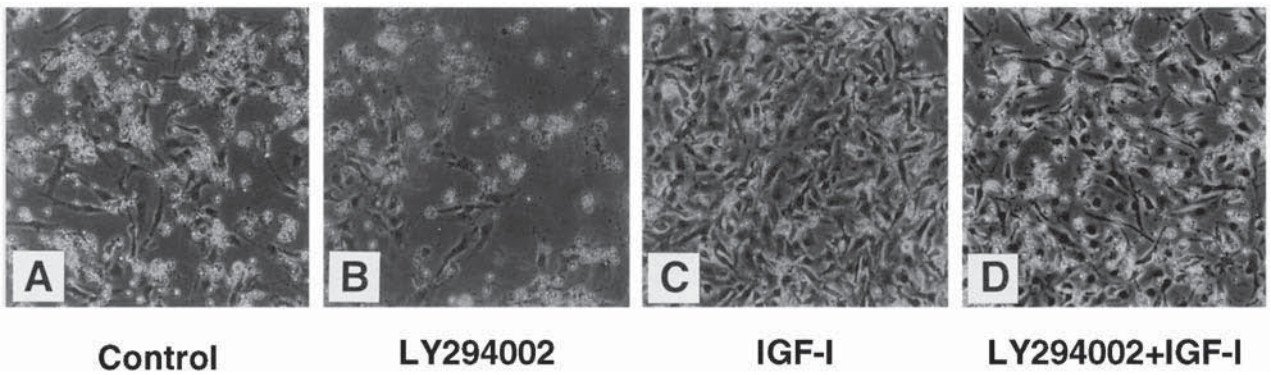

Fig. 7. The PI 3-kinase inhibitor LY294002 attenuates IGF-I-stimulated cell survival in primary cultures of porcine granulosa cells. Cultures of granulosa cells were treated for 18 h with or without IGF-I (100 ng/mL), in the presence or absence of LY294002 (10 μ*M*). Morphology of cells was examined and photomicrographs were prepared. **(A)** Control; **(B)** LY294002 (10 μ*M*); **(C)** IGF-I (100 ng/mL); **(D)** LY294002 + IGF-I.

logical characteristics of apoptotic cells *(3)*; for example, the cells were shrunken with condensed and fragmented nuclei. Figure 8 represents a quantitative assessment of cell numbers in primary cultures treated with or without IGF-I in the presence or absence of the PI 3-kinase inhibitor LY294002.

Discussion

Apoptotic cell death is essential to normal ovarian function *(3,4)*. However, the intracellular mechanisms that control follicular cell fate have not been established. The requirement for IGF-I in follicular development *(21)* and the increases in IGF-I during the follicular phase *(1,2)* strongly suggest that IGF-I is involved in the growth of ovulatory follicles. As important as IGF-I is to follicular development, little is understood about the signaling mechanisms inherent to its actions. In the present study we have shown that IGF-I signaling through a PI 3-kinase-dependent pathway leads to the activation of the protein kinase Akt, which may mediate granulosa cell survival.

PI 3-kinase is a major downstream effector of IGF-I receptor signaling *(6–7,22)*. Activation of the 110-kDa PI 3-kinase catalytic subunit results from the binding of the src-homology 2 domains of its 85-kDa PI 3-kinase regulatory subunit to phosphorylated tyrosine residues on insulin receptor substrate-1, (IRS-1) *(23)*. Earlier studies have shown that treatment with IGF-I increases IGF-I receptor tyrosine kinase activity *(22,24)*, IRS-1 phosphorylation *(24)*, and PI 3-kinase activity *(22)* in rat and bovine luteal cells. We now demonstrate that IGF-I increases PI 3-kinase activity in porcine granulosa cells. Propagation of the IGF-I signal downstream of PI 3-kinase is thought to be mediated by such effectors as PDK1, serum- and glucocorticoid-inducible kinase (SGK), p70 ribosomal S 6-kinase, protein kinase Cζ, p21ras, and Akt *(8–12,23)*. The cellular events that utilize PI 3-kinase as a signaling component include growth, transformation, differentiation, cytoskeletal

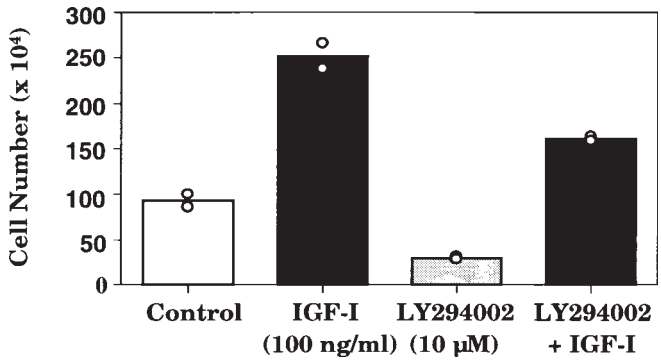

Fig. 8. The PI 3-kinase inhibitor LY294002 attenuates IGF-I-stimulated cell survival in primary cultures of porcine granulosa cells. Cultures of granulosa cells were treated for 18 h with or without IGF-I (100 ng/mL), in the presence or absence of LY294002 (10 μ*M*). Representative fields of granulosa cells were counted as described in the Materials and Methods.

organization, and apoptosis. Because these cellular events are integral parts of follicular development *(25,26)*, it seems likely that many of them may be mediated by IGF-I-stimulated PI 3-kinase signaling.

The present study confirms the results of Guthrie et al. *(5)*, who demonstrated that IGF-I treatment prevented apoptosis in suspensions of freshly isolated granulosa cells; that is, both studies show that IGF-I inhibits low molecular weight oligonucleosomal DNA fragmentation. To examine the involvement of PI 3-kinase in IGF-I-mediated granulosa cell survival, we employed two specific inhibitors of PI 3-kinase: wortmannin *(16,17)* and LY294002 *(18,19)*. We found that both compounds effectively blocked IGF-I-stimulated PI 3-kinase and reduced IGF-I-mediated granulosa cell survival. The present studies also demonstrated that IGF-I treatment promotes cell survival in primary cultures of porcine granulosa cells. Thus, although having no mitogenic effect in serum-free medium *(20)*, IGF-I serves to maintain granulosa cells in culture. Consistent with results obtained with

freshly isolated granulosa cells, the PI 3-kinase inhibitor reduced levels of cells and DNA synthesis in control and IGF-I-stimulated cultures.

The results of the present show, for the first time, that Akt is present in granulosa cells and is rapidly activated in response to IGF-I. Furthermore, Akt is positioned downstream of PI 3-kinase because the stimulatory effects of IGF-I on Akt were blocked by neutralizing PI 3-kinase activity with wortmannin or LY294002. In addition, the IGF-I-mediated increases in Akt phosphorylation and activity were associated with IGF-I-mediated cell survival responses.

Apoptosis in the ovary is thought to be regulated by the balance of antiapoptotic (e.g., Bcl-2, Bcl-X_{long}, Mcl-1) to proapoptotic (e.g., Bad, Bax, Bok, Bak) Bcl-2 cell death protein family members *(3,4)*. Of these cell death protein family members, Bad is a likely signaling mediator for the survival effect of Akt *(7–10)*. Bad is expressed in the rat ovary and its overexpression leads to increased cell death *(27)*. Research using neural or hematopoietic cells has led to the idea that Bad induces cell death by forming heterodimers with Bcl-X_{long}, an antiapoptotic Bcl-2 family member *(7–10)*. The binding of Bad to Bcl-X_{long} results in an increase in Bax homodimers that destabilizes mitochondrial membranes *(28)*. This causes the release of cytochrome-*c* from mitochondria, a critical step in the activation of the caspase protease cascade, an early event leading to cell death *(29)*. Following treatment with IGF-I, Akt phosphorylates Bad, resulting in the dissociation of Bad from Bcl-X_{long}. The phosphoserine binding protein 14-3-3 binds to and sequesters phosphorylated Bad, allowing Bcl-X_{long} to exert its antiapoptotic effect. We have observed that Bad mRNA is expressed in porcine granulosa cells (unpublished data), and future studies will evaluate whether Bad is involved in IGF-I-mediated granulosa cell survival. IGF-I signaling via PI 3-kinase/Akt may also regulate other cellular components that affect cell fate. In addition to Bad, Akt has been shown to phosphorylate and inactivate caspase 9 *(30)*, resulting in the inhibition of the apoptotic caspase protease cascade and repression of cell death. PI 3-kinase signaling may also regulate the expression of members of the Bcl-2 family of proteins *(31)*, providing a more favorable ratio of antiapoptotic to proapoptotic proteins.

Activated Akt may enter the nucleus and play a role in the regulation of gene expression *(8–12)*. Akt has recently been shown to phosphorylate members of the Forkhead family of transcription factors—FKHRL1 *(32)* and AFX *(33)*—resulting in an inhibition of their transcriptional activity. The phosphorylation of the forkhead transcription factor FKHRL1 has been implicated in the inhibition of apoptosis in response to IGF-I in CCL39 fibroblasts *(32)*. Similar to the actions of Akt on the phosphorylation of the Bad protein, the phosphorylation of FKHRL1 allows it to associate with the 14-3-3 protein and be retained in the cytoplasm. In the absence of survival factors, the transcription factor is dephosphorylated, enters the nucleus, and induces genes that are critical for cell death, such as the Fas ligand gene *(32)*. Peng et al. *(34)* recently demonstrated that apoptosis in porcine granulosa cells was associated with increased expression of Fas antigen and Fas ligand. It seems possible, therefore, that the induction of apoptosis in porcine granulosa cells may involve a reduction in PI 3-kinase/Akt signaling that allows Forkhead transcription factors to increase the expression of Fas ligand.

In addition to its role as a survival factor, IGF-I is well known for its ability to enhance follicle-stimulating hormone (FSH)-stimulated granulosa cell differentiation *(1,2)*. Recent reports demonstrate that the expression of SGK is well correlated with FSH-induced granulosa cell differentiation in the rat ovary *(35)*. Because IGF-1 is known to activate SGK via a PI 3-kinase/PDK1 dependent pathway in 293 cells *(36,37),* it would be of great interest to know whether IGF-I-stimulated PI 3-kinase activity plays a role in the activation of SGK in granulosa cells. Such experiments would point to the involvement of PI 3-kinase signaling in follicular survival and differentiation.

Our present work and that of others *(4,5)* demonstrate that IGF-I supports follicular development by enhancing granulosa cell survival. Our study provides the initial evidence that IGF-I activates the PI 3-kinase/Akt signaling pathway in granulosa cells and that inhibition of this pathway leads to granulosa cell death. However, our observations that the PI 3-kinase inhibitors completely blocked the activity of PI 3-kinase and Akt phosphorylation but only partially blocked the survival responses to IGF-I, suggest that additional signaling events may also participate in IGF-I-mediated cell survival. Further studies are needed to explore the role of this signaling pathway in the mechanisms controlling growth and differentiation of granulosa cells.

Materials and Methods

Preparation of Porcine Granulosa Cells

Granulosa cells were harvested from small follicles (2 to 3 mm) of ovaries obtained from pigs at slaughter *(38)*. Cells were plated in F:D medium (1:1) (Life Technologies, Grand Island, NY) containing 5% fetal bovine serum (Life Technologies). Granulosa cells were cultured for 48 h after which the medium was removed and replaced with serum-free F:D medium. Following equilibrium for 2 to 3 h, cells were treated with or without IGF-I (0–100 ng/mL) (Upstate Biotechnology, Lake Placid, NY). When used, the PI 3-kinase inhibitors, wortmannin or LY294002 (Sigma, St. Louis, MO) were added to cultures 30 min before addition of IGF-I. In some experiments, freshly isolated cells were suspended in serum-free F:D, equilibrated for 2 h, and treated as described above.

Western Blot Analysis

Following treatment, cells were lysed with nondenaturing buffer A (20 m*M* Tris, pH 7.4; 140 m*M* NaCl, 10 m*M* EDTA; 1 m*M* phenylmethylsulfonyl fluoride, 100 m*M* NaF; 2 m*M*

Na_3VO_4, 10% glycerol; 1% Igepal; 10 μg/mL of aprotinin; 10 mM $Na_4P_2O_7$; and 2 n*M* oakadaic acid). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% gels and transferred to polyvinylidene difluoride nylon membranes (Millipore, Bedford, MA). Buffer containing 1% milk and 1% bovine serum albumin (BSA) was used to block nonspecific binding, and membranes were probed with either phospho-Akt (Ser 473) (1:1000) or Akt antibodies (1:1000) (ca. no. 9271 and 9272, respectively; New England Biolabs, Beverly MA).

PI 3-Kinase Assays

Lysates were prepared in buffer A, and PI 3-kinase assays were performed as previously described *(22)*.

Akt Kinase Assays

Total cell lysates prepared with buffer A were incubated with 4 μg of Akt antibody overnight at 4°C. Protein G-agarose beads (Upstate Biotechnology) were added for 4–6 h to immunoprecipitate Akt. Lysates treated with protein G-agarose beads without Akt antibody served as internal assay controls. The immunoprecipitates were washed in buffer (25 m*M* HEPES, 1% BSA, 10% glycerol, 1 m*M* dithiothreitol [DTT], 1% Triton X-100, 1 M NaCl) and incubated for 20 min at 30°C in a reaction mixture containing 20 m*M* $MgCl_2$, 20 μ*M* adenosine triphosphate (ATP), 2 μCi of [γ-^{32}P]ATP, 100 m*M* Tris (pH 7.4), 2 m*M* DTT, and 100 μ*M* Akt specific substrate peptide 12-340 (Upstate Biotechnology). Reaction tubes were mixed at 5-min intervals. To terminate the reaction, samples were rapidly centrifuged and supernatants were removed and placed into a tube with an equal volume of 40% trichloroacetic acid (TCA). After 10 min, the samples were centrifuged and aliquots of the supernatant were transferred onto P81 cellulose paper squares (Whatman, Maidstone, England). Squares were washed three times with 0.75% phosphoric acid and transferred to scintillation vials for counting.

Cell Counts

Following attachment of granulosa cells, serum-containing medium was removed from the cultures, and the cells were allowed to reequilibrate in serum-free F:D medium for 1 to 2 h. Cells were then treated with IGF-I (100 ng/mL) in the presence or absence of LY294002 (10 μ*M*) or wortmannin (100 n*M*) for 18 h. The medium was removed and cells were fixed with ice-cold methanol. Fields of cells were counted using light microscopy as previously reported *(39)*.

Thymidine Incorporation

Following serum removal, granulosa cell cultures were incubated in the presence or absence of IGF-I, with or without the PI 3-kinase inhibitors wortmannin or LY294002. After 18 h treatments were removed and fresh F:D medium containing [^{3}H]thymidine (4 μCi) (ICN, Costa Mesa, CA) was added for 4 h. The medium was removed and cultures were stopped by the addition of ice-cold TCA (10%). Cultures were washed three times with 5% TCA. The acid-insoluble fraction was solubilized in 0.2 *N* NaOH. [^{3}H]Thymidine incorporation was determined by liquid scintillation counting.

DNA Isolation and Analysis

Isolation and analysis of DNA fragmentation was performed as previously described *(40)*.

Acknowledgment

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