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AKT Controls Human First Trimester Trophoblast Cell Sensitivity to FAS-Mediated Apoptosis by Regulating XIAP Expression[1]

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ABSTRACT

The PIK3/AKT pathway plays an important role in both the inhibition of the apoptotic cascade and the promotion of cell growth and proliferation. Multiple apoptosis-related targets of phosphatidylinositide 3-kinase (PIK3) and protein kinase B (AKT) have been identified, including the antiapoptotic protein XIAP. By phosphorylating XIAP, AKT was previously shown to prevent the ubiquitinization and degradation of XIAP. First-trimester trophoblast cells express high levels of XIAP, which protects them from certain apoptotic stimuli. In this study, we determine that the inhibition of the PIK3/AKT pathway induces XIAP inactivation and the activation of caspase 3 in first-trimester trophoblast cells. Using a specific AKT inhibitor and a XIAP mutant construct, which mimics the AKT phosphorylated form of XIAP, we also demonstrate that these effects are dependent on the phosphorylation of XIAP by AKT. Finally, we show that the selective inhibition of AKT renders normally resistant first-trimester trophoblast cells sensitive to FAS-mediated apoptosis by regulating XIAP expression. Our findings may provide a link between AKT, XIAP, and the regulation of the FAS apoptotic cascade in first-trimester trophoblast cells and contribute to our current knowledge of the molecular mechanisms mediating normal trophoblast physiology during pregnancy.

AKT, apoptosis, FAS, PIK3, placenta, pregnancy, trophoblast, XIAP

INTRODUCTION

Despite expressing both FAS (CD95) and FAS LIGAND (FASLG) [1–8], trophoblast cells from first-trimester placentas do not undergo FAS-induced apoptosis under normal conditions [9–12]. Our laboratory and others have demonstrated previously that first-trimester trophoblast cells express high levels of the antiapoptotic protein, XIAP, which protects them from FAS-mediated apoptosis [3, 11–13]. Once XIAP is inactivated, however, reactivation of the caspase cascade occurs, and first-trimester trophoblast cells become sensitive to FAS stimulation [11, 12]. How XIAP expression and inactivation are regulated and whether other regulators outside of XIAP function to control first-trimester trophoblast cell sensitivity to FAS-induced apoptosis are unknown.

Phosphatidylinositide 3-kinase (PIK3) and protein kinase B (AKT), one of the downstream targets of PIK3, are known to play an important role in both the inhibition of apoptosis and the promotion of cell growth and proliferation. Indeed, the inhibition of PIK3 and/or AKT induces apoptosis in several cell types [14, 15] and has been shown to sensitize first-trimester trophoblast cells to oxygen-induced cell death [16]. In addition, the activation of the PIK3/AKT pathway represents the mechanism by which both epidermal growth factor [17, 18] and hepatocyte growth factor [19] inhibit trophoblast apoptosis. Moreover, PIK3 and AKT have been shown to protect other cells from FAS-induced apoptosis [20]. A role for the PIK3/AKT pathway in the regulation of first-trimester trophoblast cell resistance to FAS-mediated apoptosis, however, has not been demonstrated.

AKT, also referred to as RAC-PK, is a serine/threonine kinase that is known to have at least three isoforms, all of which are activated by PIK3. As a serine/threonine kinase, AKT has been shown to have both prosurvival and antiapoptotic functions via phosphorylation of its downstream targets. One of the first AKT substrates identified to have antiapoptotic effects was BAD [21], the proapoptotic BCL2 family member that initiates apoptosis by binding antiapoptotic BCL2 family members and causing the release of cytochrome *c* from mitochondria. Since then, AKT has been reported to directly phosphorylate and inactivate caspase 9 [22] as well as inhibit the activation of BAX through an unknown mechanism [23, 24]. More recently, AKT was shown to prevent the ubiquitination and degradation of XIAP via phosphorylation both in vitro and in vivo [25].

Because AKT can stabilize XIAP expression [25], and the PIK3/AKT pathway has been demonstrated to inhibit FAS-induced apoptosis in other cells [20], we hypothesized that AKT may control trophoblast cell sensitivity to FAS stimulation by regulating XIAP expression and function in first-trimester trophoblast cells. Therefore, the aim of this study was to determine whether the PIK3/AKT pathway plays a role in the regulation of XIAP expression and first-trimester trophoblast cell resistance to FAS-mediated apoptosis.

MATERIALS AND METHODS

Tissue

First-trimester placentas were obtained from the fetal side of a normal pregnancy that was voluntarily terminated for reasons unrelated to the present study. A signed, written consent form was obtained from the patient. The use of

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placental tissue specimens and consent forms was approved by the Yale University Human Investigation Committee. The tissue specimen was collected in cold, sterile PBS and immediately transported to the laboratory for cell culture preparation.

Reagents and Antibodies

The agonistic anti-human FAS monoclonal antibody (mAb; clone E0S9.1) was obtained from BD PharMingen (San Diego, CA). Although the PIK3/AKT inhibitor, LY-294,002, was purchased from Sigma-Aldrich (St. Louis, MO), the selective AKT inhibitor, API-2, which is a small molecule that has been described previously [14], was a kind gift from Dr. Jin Q. Cheng (University of South Florida College of Medicine, Tampa, FL). The pan-caspase inhibitor, Z-VAD-FMK, was obtained from R&D Systems Inc. (Minneapolis, MN). Both the rabbit anti-total AKT (no. 9272; 1:2000) and the rabbit anti-phosphorylated AKT (no. 9271; 1:1000) were purchased from Cell Signaling Technology Inc. (Beverly, MA). The mouse anti-XIAP mAb (clone 28; 1:1000) was obtained from BD Transduction Labs (San Diego, CA), whereas the rabbit polyclonal antibody for BETA ACTIN (ACTB; A2066; 1:10 000) was purchased from Sigma-Aldrich. Primary antibody signals were detected using either a horseradish peroxidase (HRP)-conjugated horse anti-mouse or an HRP-conjugated goat anti-rabbit secondary antibody (1:10 000) from Vector Laboratories (Burlingame, CA).

Cell Lines

The first-trimester human cytotrophoblast cell line, 3A, which was transformed by SV40 tsA255 [26], was purchased from American Type Culture Collection (Manassas, VA), whereas the SVneo-transformed first-trimester human extravillous trophoblast cell line, HTR-8/SVneo [27], was a gift from Dr. Charles Graham (Queen's University, Kingston, ON, Canada). Both trophoblast cell lines were cultured in RPMI 1640 (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 10 mM Hepes, 0.1 mM minimum essential medium (MEM) nonessential amino acids, 1 mM sodium pyruvate, and 100 units/ml penicillin/streptomycin (Gibco) and maintained at 37°C/5% CO_2.

First-Trimester Primary Trophoblast Cell Isolation and Culture

Primary trophoblast cells were isolated from first-trimester placentas according to Loke et al. [28], with a few modifications [11]. In brief, first-trimester placental tissue was washed with cold Hanks Balanced Salt Solution (HBSS) without calcium and magnesium (Gibco) to remove excess blood. Cells were removed from the membranes by scraping and were transferred to trypsin-ethylenediaminetetraacetic acid (Gibco) digestion buffer and incubated at 37°C for 10 min with shaking at 200 rpm. An equal volume of Dulbecco modified eagle medium (DMEM) media containing 10% fetal bovine serum (FBS) was added to inactivate the trypsin. This mixture was vortexed for 20 sec and allowed to sediment, and the supernatant was collected. The two previous steps were repeated twice, and the collected supernatant was centrifuged at 1500 rpm for 10 min. The pellet was resuspended in DMEM with 10% FBS, filtered through a 70-μm cell strainer (BD Falcon, San Diego, CA) to remove tissue pieces, and centrifuged at 1500 rpm for 10 min. Contaminating red blood cells were removed from the filtrate by resuspending the cellular pellet in HBSS, layering this suspension over Lymphocyte Separation Media (ICN Biomedicals Inc., Aurora, OH), and centrifuging the gradient at 2000 rpm for 25 min. The interface, containing the trophoblast cells, was removed and incubated with an anti-CD45 mAb conjugated to magnetic beads (Dynabeads 450; Dynal, Oslo, Norway) at 4°C with rotation for 30 min [29]. After this incubation, the immune cells were magnetically separated from the negative cell fraction, and the unbound cells were collected, washed, and cultured at 37°C/5% in DMEM media supplemented with 10% Human Serum (Gemini Bio-Products, Woodland, CA).

Plasmid Constructs and Transient Transfection

The phosphomimic XIAP construct, XIAP-S87D, which was generated by subcloning FLAG-tagged XIAP into the p3XFLAG-CMV-10 vector (Sigma-Aldrich) and mutagenized as described previously [25], was a generous gift from Dr. Jin Q. Cheng. First-trimester trophoblast cells were transfected using FuGENE 6 (Roche, Basel, Switzerland) at a transfection reagent:plasmid DNA ratio of 3:1. The transfection reagent was prepared in reduced serum phenol-depleted Opti-MEM (Gibco) and allowed to incubate at room temperature for 5 min. A total of 2 μg of plasmid DNA was added, and the transfection reagent/DNA complex was incubated at room temperature for 15–20 min before being added to the cells. Cells were transiently transfected overnight (16–24 h) at 37°C/5% CO_2 and allowed to recover in media with 10% FBS for 0–48 h. 3XFLAG-tagged proteins were confirmed by Western blot analysis using antibodies against the FLAG epitope.

Western Blot Analysis

A total of 5×10^5 first-trimester trophoblast cells were plated in 35-mm^2 Petri dishes (BD Biosciences) and grown to 70% confluence for treatment. After treatment, cells were lysed in nondenaturing lysis buffer purchased from Cell Signaling Technology Inc. (Beverly, MA) in the presence of 0.2 μg/ml PMSF and a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Protein concentrations were calculated by BCA assay (Pierce Biotechnology, Rockford, IL). A total of 20 μg of total cellular protein was loaded per lane, separated by SDS-PAGE using 12% polyacrylamide gels, and transferred to polyvinylidene fluoride membranes (NEN Life Sciences, Boston, MA) as described previously [30]. The membranes were stained with Ponceau Red to ensure efficient transfer and equal loading of proteins. To inhibit nonspecific binding, membranes were blocked with 5% powdered milk in PBS/0.05% Tween-20 (PBS-T) prior to immunoblotting. The membranes were then incubated with primary antibody overnight at 4°C, followed by the appropriate secondary antibody for 1 h at room temperature in PBS-T/1% powdered milk. After each step, the membranes were washed three times with PBS-T for 10 min. Finally, the blots were developed using the enhanced chemiluminescence system (NEN Life Sciences). As a negative control, membranes were incubated with secondary antibody alone to validate the specificity of the signal.

Cell Viability Assay

Cell viability was evaluated using the CellTiter 96 assay according to the manufacturer's instructions (Promega, Madison, WI). Briefly, 5×10^3 first-trimester trophoblast cells were plated in triplicate wells in a 100-μl volume per well in a 96-well microtiter plate (BD Biosciences). The cells were grown to 70% confluence, at which stage the medium was replaced with reduced serum phenol-depleted Opti-MEM (Gibco), and the cells were cultured for an additional 6 h prior to treatment. After treatment, 20 μl of the CellTiter 96 Aqueous One Solution was added to each well, and the plate was incubated at 37°C for 1–4 h. Optical densities of the samples were measured at 490 nm using an automatic microplate reader (Model 550; Bio-Rad). The values of the treated cells were compared with the values generated from the untreated control and were reported as percent viability.

Caspase 3 Activity Assay

Caspase 3 activity was measured using the Caspase 3 Glo assay according to the manufacturer's instructions (Promega). Briefly, 10 μg of total cellular protein from cell lysates was incubated at room temperature for 1 h in the dark with the proluminescent caspase 3 substrate. After incubation, luminescence was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). All samples were assayed in triplicate. Luminescence was expressed as Relative Light Units (RLUs) and is proportional to the amount of caspase 3 activity present in the sample.

Statistical Analysis

The data are represented as the average ± SD and analyzed for statistical significance ($P < 0.05$) or less using one-way ANOVA with the Bonferonni correction. All experiments were repeated three times with similar results.

RESULTS

The Inhibition of the PIK3/AKT Pathway Induces XIAP Inactivation, the Activation of Caspase 3, and First-Trimester Trophoblast Cell Apoptosis

Because the inhibition of the PIK3/AKT pathway has been shown to induce apoptosis in other cell types [14, 15], our initial objective was to determine whether the inhibition of the PIK3/AKT pathway had any effects on trophoblast cell viability. Therefore, first-trimester trophoblast cells (3A) were treated with 1, 10, and 25 μM PIK3/AKT inhibitor, LY-294,002, for 24 h, and cell viability was evaluated using the CellTiter 96 assay. As Figure 1A indicates, cell viability decreased in a dose-dependent manner after treatment with LY-

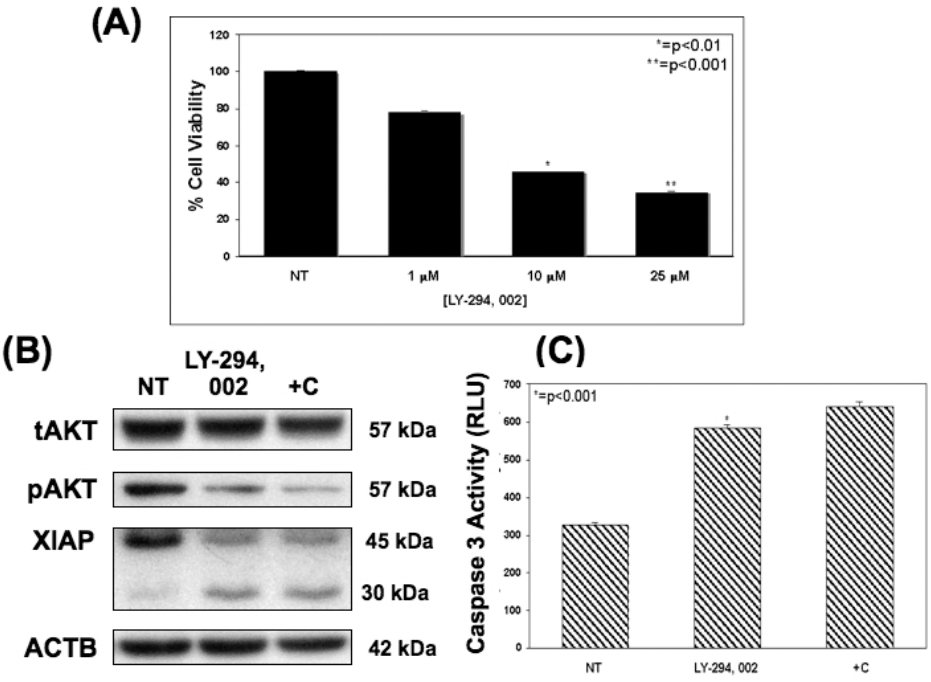

FIG. 1. LY-294,002 treatment decreases first-trimester trophoblast cell viability and induces XIAP inactivation and the activation of caspase 3. First-trimester trophoblast cells (3A) were treated with 1, 10, or 25 μM PIK3AKT inhibitor LY-294,002 for 24 h. **A**) Cell viability was evaluated using a colorimetric assay. Bar graph shows percent cell viability relative to the untreated control (NT). After treatment with 25 μM LY-294,002, cell lysates were analyzed for (**B**) the expression of total AKT (tAKT) and phosphorylated AKT (pAKT) and the activation status of XIAP by Western blot analysis, and for (**C**) caspase 3 activity using a luminescent assay. Bar graph shows caspase activity expressed as RLUs. LY-294,002-treated HTR-8/SVNEO first-trimester trophoblast cells were used as a positive control (+C). ACTB was used as a loading control.

294,002, with the optimal dose of LY-294,002 required to significantly reduce trophoblast cell viability between 10 μM (45.5% ± 1.02%; $P < 0.01$) and 25 μM (34.8% ± 1.01%; $P < 0.001$).

Once we had determined that the inhibition of the PIK3/AKT pathway induced trophoblast cell death, our next aim was to determine the effects of LY-294,002 treatment on the intracellular apoptotic cascade in first-trimester trophoblast cells. To accomplish this, first-trimester trophoblast cells (3A) were treated with 25 μM LY-294,002 for 24 h, and the expression and activation status of total AKT, phosphorylated AKT, and XIAP was evaluated by Western blot analysis. Although LY-294,002 treatment had no effect on total AKT expression (57 kDa), the expression of phosphorylated AKT (57 kDa) decreased after treatment with LY-294,002 (Fig. 1B). This decrease in phosphorylated AKT expression correlated with the activation status of XIAP, as evidenced by the decrease in the active form of XIAP (45 kDa) and the increase in the inactive fragment of XIAP (30 kDa). Moreover, a significant increase ($P < 0.001$) in caspase 3 activity was also observed after LY-294,002 treatment (Fig. 1C). Similar results were obtained with HTR-8/SVNEO first-trimester trophoblast cells (+C in Fig. 1, B and C) and 7-wk primary trophoblast cells (Fig. 2), which suggests a potential link between the PIK3/AKT pathway and the regulation of XIAP and the caspase cascade in first-trimester trophoblast cells.

The Downregulation of the Active Form of XIAP Is Caspase Independent, Whereas LY-294,002-Induced XIAP Cleavage Is Caspase Dependent

After determining that the inhibition of the PIK3/AKT pathway resulted in XIAP inactivation and the activation of caspase 3, we next sought to determine whether LY-294,002-induced XIAP inactivation was caspase dependent. Therefore, 7-wk primary trophoblast cells were treated with 25 μM LY-294,002 for 24 h in the absence and presence of the pan-caspase inhibitor, Z-VAD-FMK, using LY-294,002-treated HTR-8/SVNEO first-trimester trophoblast cells as a positive control. Although caspase 3 activity was significantly abrogated ($P < 0.001$) in the presence of Z-VAD-FMK (Fig. 2A), the downregulation of both the phosphorylated form of AKT (57 kDa) and the active form of XIAP (45 kDa) was still observed after treatment with LY-294,002 (Fig. 2B). In contrast, the cleavage product of XIAP (30 kDa) could not be detected in LY-294,002-treated primary trophoblast cells with the addition of the pan-caspase inhibitor (Fig. 2B). This suggests that the two forms of XIAP are differentially regulated in first-trimester trophoblast cells. Although the expression of the active form of XIAP appears to be regulated by the PIK3/AKT pathway and is caspase independent, the expression of the inactive fragment of XIAP is dependent on caspase activation.

The Selective Inhibition of AKT Similarly Induces XIAP Inactivation and the Activation of Caspase 3

Because the PIK3 inhibitor, LY-294,002, inhibits the phosphorylation and subsequent activation of AKT indirectly through PIK3, our next objective was to determine whether the selective inhibition of AKT had similar effects on the activation status of XIAP and the caspase cascade in first-trimester trophoblast cells using the specific AKT inhibitor, API-2 [14], and API-2-treated HTR-8/SVNEO trophoblast cells as a positive control. To determine the concentration at which API-2 effectively inhibited AKT, first-trimester trophoblast cells (3A) were treated with increasing doses of API-2 for 18 h, and the expression of total AKT and phosphorylated AKT was evaluated by Western blot analysis. Analogous to the findings above, treatment with API-2 had no effect on total AKT expression (57 kDa) but did decrease the expression of phosphorylated AKT (57 kDa) in a dose-dependent manner (Fig. 3A). Moreover, the concentration of API-2 that appeared to be most effective in inhibiting the phosphorylation of AKT was 10 μM. To ensure that this decrease in phosphorylated AKT expression was not due to the dimethyl sulfoxide (DMSO) in which API-2 was prepared, first-trimester trophoblast cells were also incubated with a similar dose of DMSO for 18 h. As Figure 3A indicates, no decrease in the expression of phosphorylated AKT could be detected in the presence of

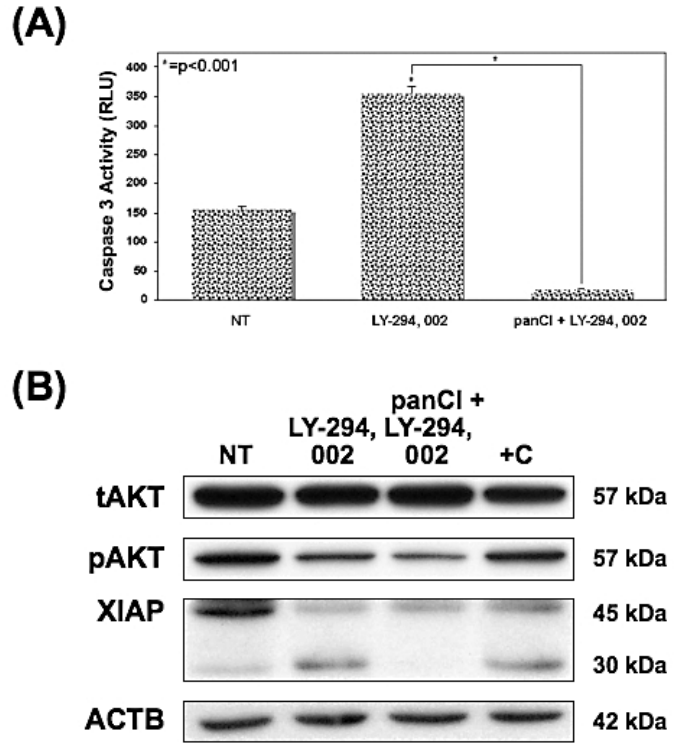

FIG. 2. The LY-294,002-induced downregulation of the active form of XIAP is caspase independent, whereas XIAP cleavage is caspase dependent. Primary first-trimester trophoblast cells (7 wk) were treated with 25 μM PIK3/AKT inhibitor LY-294,002 in the absence and presence of a pan-caspase inhibitor (panCI). **A**) Caspase 3 activity was evaluated using a luminescent assay. Bar graph shows caspase activity expressed as RLUs. **B**) The expression of total AKT (tAKT) and phosphorylated AKT (pAKT) and the activation status of XIAP were assessed by Western blot analysis using ACTB as a loading control. LY-294,002-treated HTR-8/SVNEO first-trimester trophoblast cells were used as a positive control (+C). NT, no treatment.

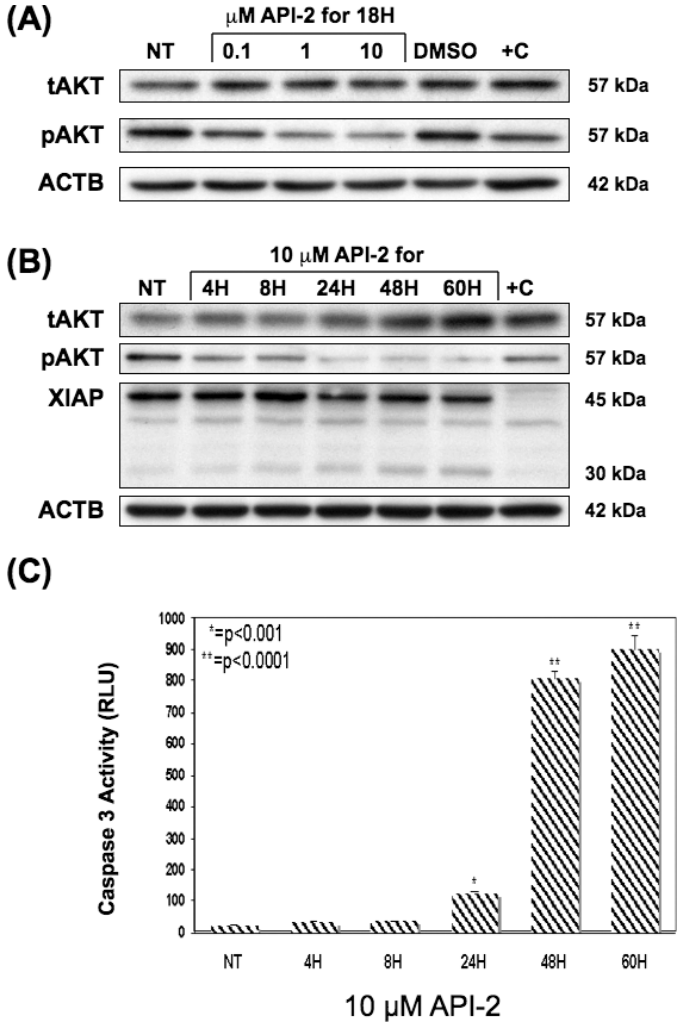

FIG. 3. API-2 treatment induces XIAP inactivation and the activation of caspase 3. **A**) First-trimester trophoblast cells (3A) were treated with 0.1, 1, or 10 μM specific AKT inhibitor, API-2, for 18 h. The expression of total AKT (tAKT) and phosphorylated AKT (pAKT) was evaluated by Western blot analysis. Treatment with a similar concentration of DMSO and API-2-treated HTR-8/SVNEO first-trimester trophoblast cells (+C) served as controls. After treatment with 10 μM API-2 for 4–60 h, 3A cell lysates were analyzed by (**B**) Western blot analysis for the expression of tAKT and pAKT and the activation status of XIAP, and for (**C**) caspase 3 activity using a luminescent assay. Bar graph shows caspase activity expressed as RLUs. All blots were reprobed for ACTB as a loading control. NT, no treatment for 60 h.

DMSO alone, confirming that the results obtained from API-2 treatment were specific.

Once we had determined the concentration at which to treat first-trimester trophoblast cells with API-2, we next sought to determine the effects of API-2 treatment on the activation status of XIAP and caspase 3. Therefore, first-trimester trophoblast cells (3A) were treated with 10 μM API-2 for 4–60 h, and the expression and activation status of XIAP were evaluated by Western blot analysis. Consistent with previous findings, maximal inhibition of phosphorylated AKT expression (57 kDa) was achieved between 8 and 24 h and maintained for 48 and 60 h of API-2 treatment (Fig. 3B). This decrease in the expression of phosphorylated AKT correlated with the activation status of XIAP, as demonstrated by the time-dependent decrease in the active form of XIAP (45 kDa) and the increase in the cleavage fragment of XIAP (30 kDa) in first-trimester trophoblast cells. Moreover, caspase 3 activity also significantly increased over time (Fig. 3C), with a 6-fold increase at 24 h ($P < 0.001$) and between a 40-fold and a 45-fold increase in caspase 3 activity observed at 48 h ($P < 0.0001$) and 60 h ($P < 0.0001$) of API-2 treatment, respectively. Interestingly, an increase in total AKT expression (57 kDa) was detected after 48–60 h of incubation with API-2 (Fig. 3B), suggesting that prolonged API-2 treatment has effects on total AKT expression to compensate for the loss of phosphoryated AKT. Similar results were obtained with primary first-trimester trophoblast cells (data not shown). Based on these results, we concluded that it was the selective inhibition of AKT rather than the inhibition of PIK3 itself that accounted for the change in XIAP expression and function in first-trimester trophoblast cells.

API-2-Induced XIAP Inactivation and Activation of Caspase 3 Are Dependent on the Phosphorylation of XIAP by AKT

It has been demonstrated previously both in vitro and in vivo that AKT inhibits XIAP ubiquitination and degradation by phosphorylating XIAP at residue serine 87 [25]. To determine the effect of XIAP phosphorylation on API-2-induced XIAP inactivation and the activation of caspase 3, first-trimester trophoblast cells (3A) were transiently transfected with phosphomimic XIAP (XIAP-S87D), which mimics the AKT

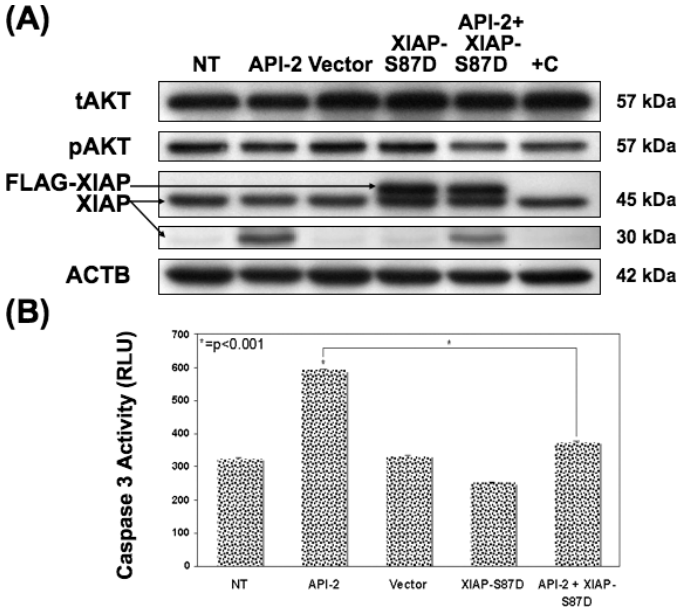

FIG. 4. API-2-induced caspase 3 activation depends on XIAP phosphorylation by AKT. First-trimester trophoblast cells (3A) were transiently transfected with phosphomimic XIAP (XIAP-S87D), which mimics the AKT phosphorylated form of XIAP, in the absence and presence of the specific AKT inhibitor API-2 (10 μM). The empty p3XFLAG-CMV-10 vector (Vector) was used as a control. **A**) The expression of total AKT (tAKT) and phosphorylated AKT (pAKT) and the activation status of XIAP (top: 1-sec exposure; bottom: 1-min exposure) were assessed by Western blot analysis using untreated HTR-8/SVNEO first-trimester trophoblast cells (+C) and ACTB as a positive and loading control, respectively. **B**) Caspase 3 activity was measured using a luminescent assay. Bar graph shows caspase activity expressed as RLUs. NT, no treatment for 72 h.

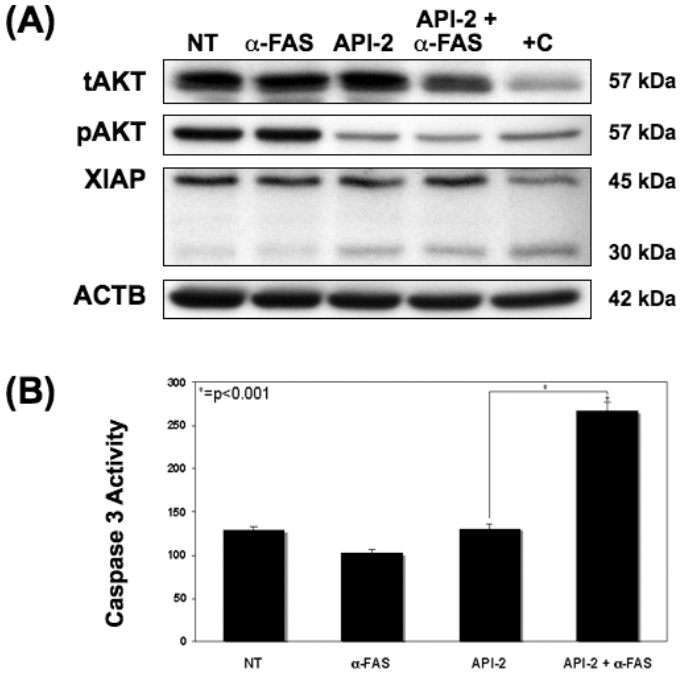

FIG. 5. Treatment with API-2 sensitizes first-trimester trophoblast cells to FAS-mediated apoptosis. First-trimester trophoblast cells (3A) were pretreated with or without 10 μM specific AKT inhibitor API-2 and then were treated with or without 500 ng/ml agonistic anti-FAS (α-FAS) mAb for an additional 24 h. **A**) Cell lysates were evaluated for the expression of total AKT (tAKT) and phosphorylated AKT (pAKT) and the activation status of XIAP by Western blot analysis using anti-FAS-treated Jurkat T cells as a positive control (+C). **B**) Caspase 3 activity was assessed using a luminescent assay. Bar graph shows caspase activity expressed as RLUs. ACTB was used as a loading control. NT, no treatment.

phosphorylated form of XIAP, and treated for an additional 24 h with 25 μM API-2 using untreated HTR-8/SVNEO trophoblast cells as a positive control. As shown above, treatment with API-2 induced XIAP inactivation (Fig. 4A) and an increase in caspase 3 activity ($P < 0.001$; Fig. 4B) in first-trimester trophoblast cells. However, when first-trimester trophoblast cells (3A) were treated with API-2 after XIAP-S87D transfection, this API-2-induced increase in capase 3 activity was attenuated with a concomitant decrease in the inactive form of XIAP ($P < 0.001$; Fig. 4B). In addition, the overexpression of S87D-XIAP by itself had no effect on the expression of the inactive XIAP fragment (Fig. 4A), confirming that two forms of XIAP are differentially regulated in first-trimester trophoblast cells. This suggests that AKT protects first-trimester trophoblast cells from apoptosis by preventing the degradation of the active form of XIAP via phosphorylation and the subsequent activation of caspase 3.

The Selective Inhibition of AKT Sensitizes First-Trimester Trophoblast Cells to FAS-Mediated Apoptosis

Because treatment with the AKT inhibitor, API-2, resulted in XIAP inactivation and the activation of caspase 3, we next sought to determine whether the inhibition of AKT also sensitized first-trimester trophoblast cells to FAS-mediated apoptosis. To accomplish this, first-trimester trophoblast cells (3A) were treated with 25 μM API-2 for 24 h and then incubated with or without 500 ng/ml of an agonistic anti-FAS mAb for an additional 24 h. After treatment, the expression and activation status of AKT and XIAP were evaluated by Western blot analysis using FAS-sensitive Jurkat T cells as a positive control. Moreover, the activity of caspase 3 was also assessed. As Figure 5A indicates, no difference in the expression or activation status of total AKT (57 kDa), phosphorylated AKT (57 kDa), or the active (45 kDa) and inactive (30 kDa) forms of XIAP could be observed between the untreated and anti-FAS-treated trophoblast cells. However, in the presence of API-2, phosphorylated AKT expression (57 kDa) decreased, and the cleavage product of XIAP (30 kDa) was detected with and without FAS stimulation (Fig. 5A). More importantly, a significant increase in caspase 3 activity ($P < 0.001$; Fig. 5B) was also observed in the anti-FAS-treated first-trimester trophoblast cells (3A) after API-2 treatment in comparison with incubation with API-2 alone. Interestingly, total AKT expression (57 kDa) decreased only in the presence of both API-2 and anti-FAS (Fig. 5A), suggesting that the activation of the FAS apoptotic cascade has effects on AKT degradation. Similar results were obtained with primary first-trimester trophoblast cells (data not shown). This confirmed that the inhibition of AKT renders normally resistant first-trimester trophoblast cells sensitive to FAS stimulation. It also suggests that AKT can control first-trimester trophoblast cell sensitivity to FAS-mediated apoptosis by regulating XIAP expression.

DISCUSSION

The PIK3/AKT pathway has been shown to play an important role in both the inhibition of the apoptotic cascade and the promotion of cell growth and proliferation. In trophoblast cells, PIK3 and AKT are also involved in trophoblast differentiation [31] as well as extravillous trophoblast invasion and migration [32–35]. By inhibiting proapop-

totic factors and activating antiapoptotic factors, AKT is able to simultaneously regulate multiple downstream targets via phosphorylation. More recently, XIAP was added to the growing list of apoptosis-related AKT substrates [25]. In this study, we characterize the relationship between AKT and XIAP expression in first-trimester trophoblast cells and show that AKT protects first-trimester trophoblast cells from FAS-mediated apoptosis.

Although the inhibition of the PIK3/AKT pathway has been shown to induce apoptosis in other cell types [14, 15], we initially determined whether treatment with the PIK3 inhibitor LY-294,002 had similar effects on trophoblast cell viability. Indeed, LY-294,002 treatment significantly decreased cell viability in first-trimester trophoblast cells at concentrations similar to those observed in previous studies [15, 36]. Interestingly, LY-294,002 also inhibits casein kinase 2 [37], a protein kinase that has been shown to protect other cells from apoptosis [38, 39]. Because casein kinase 2 was demonstrated previously to phosphorylate and upregulate AKT [40], the results obtained from LY-294,002 treatment confirm a role for the PIK3/AKT pathway in the regulation of trophoblast apoptosis.

Not only did LY-294,002 treatment decrease first-trimester trophoblast cell viability, but it also induced XIAP inactivation and the activation of caspase 3. In the presence of a pan-caspase inhibitor, however, the cleavage product of XIAP was no longer detected, suggesting that the expression of this form of XIAP is dependent on caspase activation. A decrease in the expression of the active form of XIAP, on the other hand, was still observed after addition of the pan-caspase inhibitor. This suggests that the active form of XIAP is regulated by PIK3/AKT pathway, which is in accordance with previous studies from our laboratory demonstrating that phenoxodiol treatment simultaneously downregulates active XIAP and AKT expression [29]. Therefore, the two forms of XIAP appear to be differentially regulated in first-trimester trophoblast cells.

Because LY-294,002 inhibits AKT indirectly through PIK3 or casein kinase 2 [37], we determined whether the use of the specific AKT inhibitor API-2 had similar effects on the activation status of XIAP and caspase 3. Indeed, XIAP inactivation and the activation of caspase 3 increased over time, although the effect of the selective inhibition of AKT on the activation status of XIAP was not as pronounced as that observed after PIK3 inhibition. This may be due, however, to differences in the concentration of inhibitor used (10 μM API-2 vs. 25 μM LY-294,002). The effect of API-2 treatment on first-trimester trophoblast cells was confirmed with the phosphomimic XIAP, S87D-XIAP, which attenuated the API-2-induced caspase 3 activation and expression of the inactive form of XIAP. In addition, the overexpression of S87D-XIAP alone had no effect on the expression of the inactive XIAP fragment, confirming that the two forms of XIAP are differentially regulated in first-trimester trophoblast cells.

Finally, the effect of AKT inhibition on first-trimester trophoblast cell sensitivity to FAS-mediated apoptosis was evaluated. After the addition of anti-FAS, a further increase in caspase 3 activity was observed in first-trimester trophoblast cells, and this was statistically significant compared with API-2 treatment alone. Interestingly, a decrease in the active form of XIAP was not detected after treatment with either API-2 or API-2 and anti-FAS. This may be due to the design of the experiment, because API-2 treatment was removed upon the addition of the agonistic anti-FAS mAb, and first-trimester trophoblast cells were incubated with anti-FAS alone for an additional 24 h, giving the cells ample time to recover from the API-2-induced downregulation of the active form of XIAP. If first-trimester trophoblast cells were incubated with anti-FAS in the presence of API-2, a decrease in the active form of XIAP might have been observed. However, the effects of adding API-2 and anti-FAS treatment simultaneously were not tested in order to determine whether the intracellular apoptotic cascade could be activated only with anti-FAS by first sensitizing the cells with API-2. Given that API-2 is a small molecule and must therefore pass through the cell membrane to mediate its effects, and the anti-FAS antibody is agonistic to FAS receptors present on the cell surface, we also sought to eliminate the possibility that API-2 and anti-FAS treatment might interfere with one another. Nevertheless, the results obtained by caspase activity assay suggest that AKT can control the sensitivity of first-trimester trophoblast cells to FAS-induced apoptosis.

The activation of the PIK3/AKT pathway is important for the inhibition of apoptosis and the promotion of trophoblast survival. In this study, we demonstrate a potential link between AKT, XIAP, and the regulation of the FAS apoptotic in first-trimester trophoblast cells. Although it was known that the activation of the PIK3/AKT pathway protects certain cells from FAS-mediated apoptosis [20] and that AKT can prevent the degradation of XIAP [25], the finding that AKT can control FAS sensitivity by regulating XIAP expression was unknown. Because AKT and/or XIAP are thought to be involved in trophoblast differentiation, invasion, and migration [3, 31–35], our findings may strengthen our understanding of the molecular mechanisms mediating these processes during normal pregnancy and in cases of aberrant trophoblast physiology.

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