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Akt Activation and Inhibition of Cytochrome C Release: Mechanistic Insights into Leptin-promoted Survival of Type II Alveolar Epithelial Cells

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ABSTRACT

Fetal growth restriction (FGR) increases the risk of perinatal death, partly due to defects in lung development. Leptin, a polypeptide hormone, is involved in fetal lung development. We previously demonstrated that treatment with exogenous leptin during gestation significantly promotes fetal lung maturity in the rat model of FGR. In this study, to delineate the molecular pathways through which leptin may enhance fetal lung development, we investigated the impact of leptin treatment on the survival of type II alveolar epithelial cells (AECs), essential leptin-responsive cells involved in lung development, in a rat model of FGR. The rat model of FGR was induced in pregnant Sprague-Dawley rats by partial uterine artery and vein ligation. In vivo and in vitro analyses of fetal lung tissues and freshly-isolated cultured AECs, respectively, showed that leptin protects type II AECs from hypoxia-induced apoptosis. Further molecular studies revealed the role of Akt activation in the leptin-mediated promotion of survival of type II AECs. The data also showed that the anti-apoptotic effects of leptin are dependent on phosphoinositol 3-kinase (PI3K) activation, and involve the down-regulation of caspases 3 and 9, upregulation of pro-survival proteins Bcl-2, and p-Bad, and inhibition of the release of cytochrome c from mitochondria. Taken together, our data suggested that leptin enhances the maturity of fetal lungs by mediating the regulation of caspase-3 and -9 during hypoxia-induced apoptosis of type II AECs and provide support for the potential of leptin as a therapeutic agent for promoting lung development in FGR. J. Cell. Biochem. 116: 2313–2324, 2015.

KEY WORDS: LEPTIN; FETAL GROWTH RESTRICTION; LUNG DEVELOPMENT; AKT

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H. Chen and Z-W. Liang contributed equally to this work.

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Fetal growth restriction (FGR) refers to a decrease in the growth rate of the fetus that prevents the infant from achieving an optimal growth potential as intrauterine growth restriction and is associated with an increased risk of perinatal morbidity 2.75–15.33% worldwide and about 6.39% in China [Tuuli et al., 2011]. FGR is caused by various unfavorable factors such as poor intrauterine nutrition, intrauterine infection [Prada and Tsang, 1998]. FGR is prominently associated with an increased risk of chronic lung disease [Cunningham et al., 2010]. Neonatal exposure to factors that restrict fetal growth can alter fetal lung development, inducing adverse structural changes in the fetal lung that limit, or impair proper lung function and respiratory health after birth [Lipsett J et al., 2006; Rehan et al., 2009]. Fetal hypoxemia is the major stimulus involved in the reduction in fetal growth, likely a natural adaptation to reduce the metabolic demands of the growing fetus, and may account for the significant contribution of FGR to perinatal morbidity and mortality and to the higher risks of developing diseases in adulthood [Aucott et al., 2004; Neerhof and Thaete, 2008].

Recent studies have shown that leptin may be involved in the development of respiratory diseases and that both fetal and adult lung tissues are leptin-responsive and -producing tissues [O′donnell et al., 1999; Malli et al., 2010]. Leptin ,the product of the *ob* gene, is synthesized and secreted mainly by white adipose tissue along with other tissues, including the placental trophoblasts [Zhang et al., 1994]. Leptin is also expressed in human peripheral lung tissue in bronchial epithelial cells, alveolar type II pneumocytes, and lung macrophages [Bruno et al., 2009; Vernooy et al., 2009]. Leptin-deficient *ob*/*ob* mice develop specific respiratory depression with alveolar hypoventilation and chronic hypercapnia [Groeben et al., 2004; O′Brien et al., 2007]. Studies of several models of pulmonary development suggest a modulatory role for leptin in fetal lung maturity [Henson et al., 2004; Huang et al., 2008; Malli et al., 2010; Chen et al., 2013]. Previous study showed that leptin enhances lung maturity in the fetal rat and the increase in fetal lung weight was accompanied by an increase in the number and maturation of type II alveolar epithelial cells (AECs) [Kirwin et al., 2006]. Meanwhile, isolated pulmonary type II AECs from the fetus exhibit a unique response to leptin stimulation [Harding et al., 2000]. Type II AECs are essential and critical components of the alveolar surface and can be converted to type I AECs to repair the damaged epithelium after lung injury or during fetal lung development [Uhal et al., 1997). Moreover, fetuses with FGR demonstrate predominantly smaller AECs [Orgeig et al., 2010; Rozance et al., 2011].

Therefore, abnormal survival of type II AECs may play a role in fetuses with FGR and the effect of leptin on the survival of these AECs may be one of the mechanisms of resistance to pulmonary hypoplasia in FGR. We previously demonstrated that leptin promotes fetal lung maturity and upregulates SP-A expression in pulmonary alveoli type II AECs in thyroid transcription factor-1 (TTF-1) dependent manner in FGR rat model which established by partial ligation of the uterine arteries and veins [Chen et al., 2013]. To further investigate the underlying mechanism in which leptin promotes fetal lung maturity, we hypothesized that the leptin might have a beneficial effect on survival of pulmonary alveoli type II AECs. To verify the hypothesis, in the present study, we aimed to investigate the impact of leptin on the survival of type II AECs to delineate the molecular mechanism of leptin-mediated lung development in fetuses with FGR.

MATERIALS AND METHODS

INDUCTION OF FGR IN RATS AND INTRAPERITONEAL INJECTION OF LEPTIN

The rat model of FGR was induced by partial uterine artery and vein ligation as described previously [Chen et al., 2013]. Briefly, pregnant Sprague-Dawley rats were randomly assigned into three groups with 10 animals per group on day 16 of gestation. Animals were anesthetized and the uterus was exposed under aseptic conditions. The uterine artery and vein and a 3/0-nylon thread were ligated together by 1-silk suture; then, the nylon thread was removed for partial ligation. The uterus was returned to the abdominal cavity and the incision was closed. On day 19 and 20 of gestation, maternal rats received an intraperitoneal injection of 1 mg/kg of body weight of leptin (Sigma–Aldrich, MO) or phosphate-buffered saline (PBS), as the negative control. The sham group underwent identical anesthetic and surgical procedures, except for the ligation. Caesarean section was performed on the 21st day. Newborn pups in the FGR and treatment groups were considered growth retarded when the initial body weight (iBW), measured within 24 h of birth, and was 2 standard deviations (−2 SDs) below of the mean iBW of the pups in the control group. The study was approved by the Animal Care and Ethics Committee of Sun Yat-sen University, and all animal studies were performed under an institutionally approved protocol according to the guidelines and the criteria from the committee (IACUC SYSU, No. 20061211015).

APOPTOSIS ANALYSIS IN VIVO

The terminal deoxynucleotidyl transferase end-labeling technique (TUNEL) was used for assaying apoptosis in vivo [Lipsett et al., 2006]. Briefly, after antigen retrieval, the slides were then incubated with biotinylated nucleotide mix and terminal deoxynucleotidyl transferase (TdT; Promega, Madison, WI) for 1 h at 37°C, followed by incubation with Alexa Fluor 647 streptavidin conjugate (Invitrogen, Carlsbad, CA) for 1 h at room temperature. Apoptotic type II AECs were detected by co-immunostaining with rabbit anti-prosurfactant protein C (Catalog No. AB3786; diluted 1:400; Millipore), followed by immunofluorescence-based detection (Cy3-labeled goat anti-rabbit IgG; Life Technologies). Tissues were stained with non-immune IgG in place of the primary antibody, serving as the native control for the TUNEL assay. Slides were analyzed using a Zeiss LSM 510 META microscope at 100× magnification, and images of five random fields from each slide were captured for quantification analysis.

ISOLATION OF FETAL LUNG TYPE II AECS AND TREATMENT WITH HYPOXIA AND LEPTIN

Type II AECs were isolated and cultured with a specific immunosorption procedure, as described previously [Wang et al., 2007]. Briefly, the fetal lungs were removed on the 20th day of the pregnancy and the lung tissue was sectioned into 1-mm^3 cubes and

digested with 0.05% trypsin for 20 min. After adding Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) for stopping the reaction, the cells were filtered through a 40-μm nylon mesh. The filtered cells were resuspended in 10% FBS and then plated onto culture dishes coated with rat IgG (1.5 mg of rat IgG/100 mm dish). After incubating the cells at 37°C for 1 h, the unattached cells were collected and resuspended in DMEM at a concentration of 1×10^7 cells/mL. The cell suspension was cultured overnight. Type II AECs were identified by surfactant protein C (SP-C) immunocytochemistry with diaminobenzidine (DAB) staining, along with electron micrography for assessing the lamellar body content. After culturing the unattached cells for 12 h, type II AECs were cultured in normal conditions or a hypoxic atmosphere (0.5% oxygen, 5% CO_2, and 94.5% nitrogen) at 37°C for 12 h, with or without leptin. For exposure to wortmannin, a potent, and specific inhibitor of PI3K, type II AECs were pretreated with wortmannin (200 nM) for 1 h and then incubated for another 12 h. Cells treated with 0.05% dimethylsulfoxide (DMSO) in normoxic conditions served as the vehicle control.

ASSESSMENT OF APOPTOSIS OF TYPE II AECS WITH FLOW CYTOMETRY

Early apoptosis was assayed by using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (BD Pharmingen, NJ), and analyzed with a Coulter Epic Elite apparatus (Hialeah, FL). Type II AECs were washed, trypsinized, and collected. Cells were centrifuged at 1300 rpm for 3 min at 4°C, rinsed with PBS, resuspended in 1× Binding Buffer, and then, 5 μL each of FITC-Annexin V and PI solutions were added. After vortexing gently, the cells were incubated for 15 min at room temperature (25°C) in the dark. Four hundred microliters of 1× binding buffer was added and the levels of apoptosis were then analyzed using flow cytometry.

MEASUREMENT OF CELL VIABILITY USING THE 3-[4,5-DIMETHYLTHIAZOL-2 -YL]-2,5-DEPHENYL TETRAZOLIUM BROMIDE (MTT) ASSAY

Type II AECs were seeded into 24-well plates in triplicate. The cells were allowed to adhere for 12 h and then subjected to hypoxia with without leptin treatment as describe above. The effect of leptin on the growth rate of type II AECs was determined after 1 d using the MTT (Sigma, MO) colorimetric assay. Briefly, 50 μL of an MTT (5 mg/mL) stock solution in PBS was added to each well. The microplate was incubated for 4 h, after which 550 μL of DMSO was added to each well. The optical density at 570 nm of the samples was measured by transferring the treated cell culture to disposable cuvettes. The cells were plated and treated in triplicate for each treatment/condition and the data represent the mean ± standard error of the mean (SEM). The values of the absorbance are calculated as percentages of the respective controls, and are shown at the relative cell viabilities.

WESTERN BLOT ANALYSIS

In brief, type II AECs cultured and treated as described above, and harvested and lysed with RIPA buffer (150 mM NaCl, 50 mM Tris with pH 7.4, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with phosphatase inhibitor cocktail from Sigma–Aldrich (code P8349) as described in our previous report [Gu et al., 2011; Li et al., 2014] . The protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad). The protein lysates (100 μg) were resolved using sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and subjected to western blot analysis with primary antibodies against phosphorylated (p)-Akt(Ser473), Akt, p-Bad(Ser136), Bad, procaspase-9, cleaved caspase-9, procaspase-3, and cleaved caspase-3 (Catalog Nos. 9271, 9272, 9295, 9292, 9506, 9507, 9662, and 9661, respectively; diluted 1:500; Cell Signaling Technology, Inc.). Antibodies for Bcl-2 were supplied by AbCam (ab7973, diluted 1:200) and antibodies for Bax were supplied by Santa Cruz Biotechnology (sc-7480, diluted 1:500). All incubations with primary antibodies were carried out at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and visualized using an enhanced chemiluminescence (ECL) detection kit (Pierce Biotechnology, IL) according to the manufacturer's recommendations. The same membrane was stripped and reprobed with an antibody specific to β-actin (Sigma–Aldrich; diluted 1:10000). The intensity of the protein-specific bands was quantified by analysis of scanned images of the western blot using the ImagePro Plus 6.0 software.

IN VITRO AKT KINASE ACTIVITY ASSAY

The Akt kinase activity assay was performed using a non-radioactive Akt kinase kit (Cell Signaling Technology, Inc.) according to the manufacturer's instructions. Briefly, following treatment, type II AECs were rinsed with ice-cold PBS, and lysed with lysis buffer (containing 1 mM phenylmethylsulfonyl fluoride [PMSF]) on ice for 10 min. The cells were scraped and centrifuged at 4°C for 10 min. Total cells lysates were prepared and 200 μL of cell lysate was immunoprecipitated with 20 μL of an anti-Akt antibody (Catalog No. 9272; diluted 1:500; Cell Signaling Technology, Inc.) by gentle rocking at 4°C overnight. After centrifugation, the immunoprecipitates were washed twice, consecutively in lysis buffer, and kinase buffer, and then resuspended in 40 μL kinase buffer supplemented with 200 μM ATP and 1 μg exogenous glycogen synthase kinase-3 (GSK-3), which served as the substrate for the p-Akt. After incubation for 30 min at 37°C, the reaction was stopped by adding SDS-PAGE sample buffer. Samples were analyzed by western blotting using an anti-p-GSK-3α/β Ser21/9 antibody (Catalog No. 9327; diluted 1:500; Cell Signaling Technology, Inc.). To confirm equal loading of protein samples, membranes were stripped and reprobed with an antibody specific to Akt.

ANALYSIS OF CYTOCHROME C RELEASE USING IMMUNOCYTOCHEMISTRY

Type II AECs grown on gelatin-coated coverslips were treated as above and rinsed twice with PBS. After fixation with 4% formaldehyde, the coverslips were incubated with blocking buffer (10% normal donkey serum, 0.3% Triton® X-100) for 45 min at room temperature. Then, the coverslips were incubated with an anti-cytochrome c antibody (Catalog No. sc-7159; diluted 1:500; Santa

Cruz Biotechnology) at 4°C overnight in a humidified chamber. After washing the coverslips with PBS, the secondary antibody (FITC-labeled goat anti-mouse antibody; diluted 1:10; R&D) was added and incubated for 1 h at room temperature in the dark. Coverslips were rinsed with PBS and then incubated with 4′,6-diamidino-2-phenylindole (DAPI) solution (Sigma) for 2 min. Finally, the coverslips were mounted with the cells facing towards the microscope slide using anti-fade mounting medium, and then analyzed using confocal immunofluorescence microscopy.

STATISTICAL METHODS

Experiments were routinely repeated three or more times and data are presented as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls posthoc test, was used for multiple group comparisons, performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL). A value of $P < 0.05$ was considered statistically significant.

RESULTS

LEPTIN INHIBITS APOPTOSIS OF TYPE II AECS IN THE RAT MODEL OF FGR

We previously demonstrated that treatment with exogenous leptin during gestation significantly promotes fetal lung maturity in the rat model of FGR. To address if leptin has a beneficial effect on survival of pulmonary alveoli type II AECs in vivo, the combined SP-C immunohistochemistry, and TUNEL assay was applied to determine the apoptosis of type II AECs. The immunohistochemistry data showed that FGR significantly induced apoptosis in type II AECs as compared with the control group and leptin treatment significantly protected type II AECs from apoptosis in the FGR pups (Fig. 1). The apoptotic index of type II AECs decreased from 26.65 ± 6.34% in the FGR group to 8.22 ± 3.36% in leptin-treated FGR group. These results demonstrate that leptin could promote fetal growth and lung maturity in FGR pups partially through the inhibition of the uteroplacental insufficiency-induced apoptosis of type II AECs.

LEPTIN PROTECTS TYPE II AECS AGAINST HYPOXIA-INDUCED APOPTOSIS

To further investigate the anti-apoptotic effect of leptin type II AECs, type II AECs were isolated,l and purified from fetal lung tissues for primary cell culture. The identity of the isolated type II AECs was confirmed by the presence of a characteristic cobblestone morphology (Fig. 2 A-1) and transmission electron micrographic analysis of lamellar bodies (Fig. 2 A-2), which are the universally-accepted gold standards for methods for identification of type II AECs. The homogeneity of the type II AEC culture was determined by immunostaining using an antibody specific to SP-C (Fig. 2 A-3).

Control type II AECs were cultured in normoxic conditions and cell growth under these conditions was assessed by observation of the cells under a light microscope (data not shown). Growth of the cells under hypoxic conditions induced morphologic changes, from a polygonal to a globular shape, in type II AECs along with an increase in the number of cells with an apoptotic phenotype (Fig. 2B). Then, the effect of leptin on hypoxia-induced apoptosis of type II AECs was investigated by qualitative and quantitative analysis using annexin V/PI staining and flow cytometry. Treatment with leptin significantly reduced apoptosis of type II AECs exposed to hypoxia, especially at higher leptin concentrations (400 and 800 ng/mL; Fig. 2B). Annexin V/PI staining and flow cytometry quantitative analysis also showed that leptin caused a strong dose-dependent

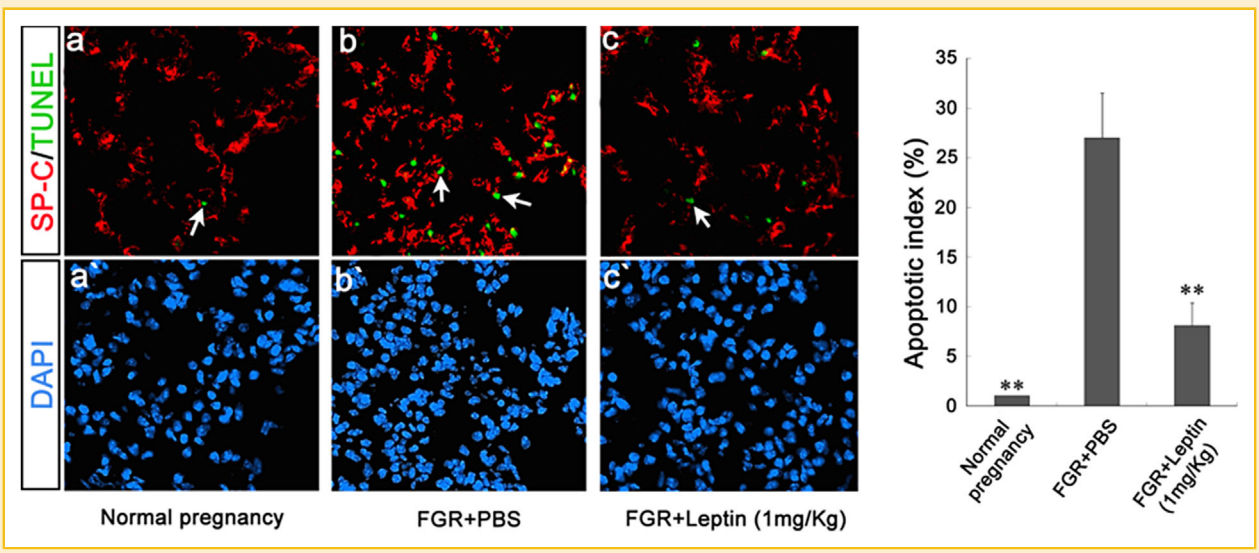

Fig. 1. Effect of leptin on survival of type II AECs in fetal lungs in the rat model of FGR. Apoptosis of type II AECs on day 21 as assessed by TUNEL staining (green fluorescence) and co-immunostaining for SP-C (red fluorescence; magnification, ×200). TUNEL-positive cells were quantified after nuclear staining with DAPI (blue fluorescence). Data are presented as a percentage after normalization to the value in the normal control ($^{**}P < 0.01$, vs. the FGR group). FGR, fetal growth restriction; PBS, phosphate-buffered saline.

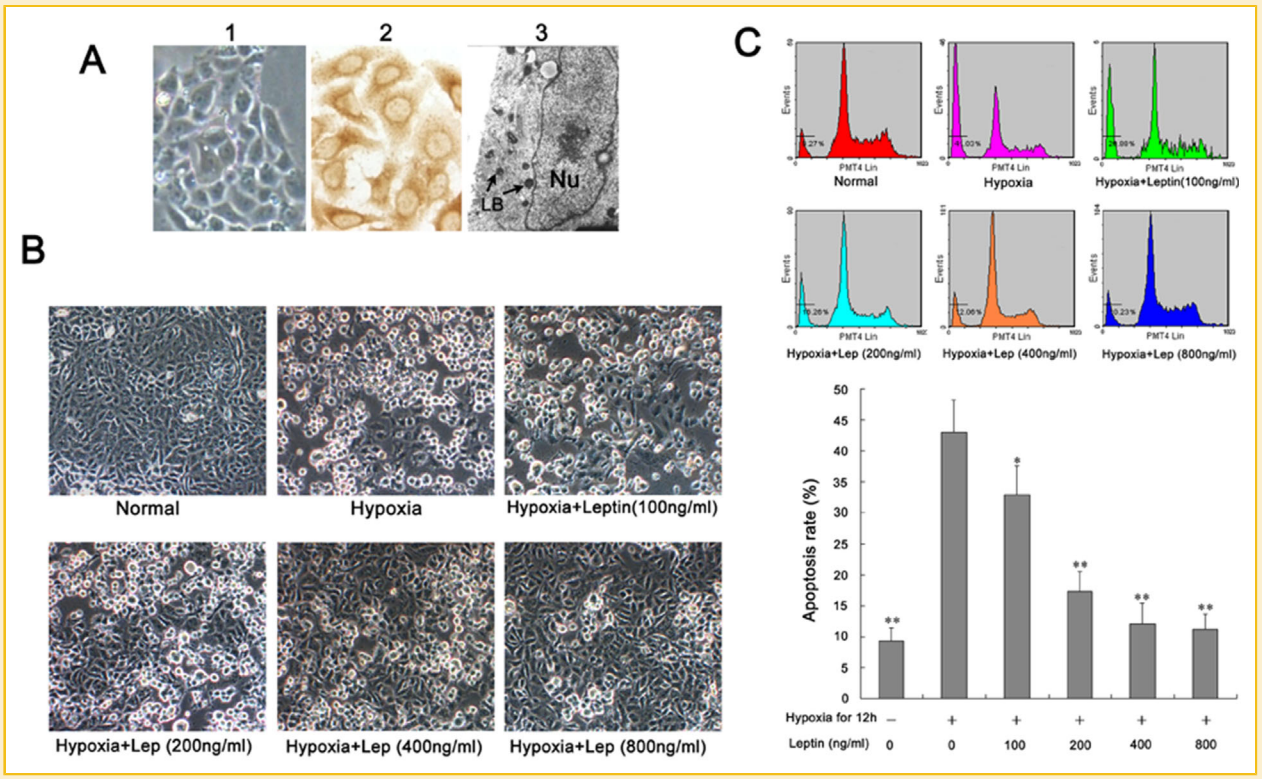

Fig. 2. Inhibition of hypoxia-induced apoptosis of type II AECs by leptin in vitro. A: Primary type II AECs were isolated from rat fetal lungs, and identified based on their appearance under the light microscope (1; magnification, ×400) and immunocytochemical staining of SP-C (2; magnification, ×400). Transmission electron micrographs displaying typical ultrastructural features, such as lamellar bodies (3; marked by arrows; magnification, ×1500) are shown. Nu, nucleus; Lb, lamellar body. B: Phenotypic aspects and representative images of cells exposed to hypoxia with or without leptin observed under a light microscope (magnification, ×400). C: Quantitative analysis of apoptosis of type II AECs by flow cytometry. Type II AECs were exposed to hypoxia, with or without treatment with different concentrations of leptin for 24 h, and stained with Annexin V and propidium iodide. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. the hypoxia-exposed cells. Lep, leptin.

inhibition on hypoxia-induced apoptotic death of type II AECs (Fig. 2C).

Taken together, these results indicate that leptin plays an important role in resistance to apoptosis in type II AECs in vitro and in vivo, supporting the hypothesis that leptin promotes fetal growth, and lung maturity in FGR by suppressing apoptosis.

INVOLVEMENT OF AKT ACTIVATION IN THE ANTI-APOPTOTIC EFFECT OF LEPTIN

To further elucidate the mechanisms of leptin-mediated cell survival, we examined the activation of Akt, a major signaling enzyme involved in mammalian cell survival. Akt activation was monitored by analyzing its phosphorylation at Ser473 using western blotting with antibodies specific to p-Akt (Ser473). Treatment with hypoxia significantly inhibited Akt phosphorylation at Ser473 in type II AECs (Fig. 3A; $P < 0.01$), while leptin treatment rescued the Akt phosphorylation in the presence of hypoxia, nearly to the levels observed in the control samples, in a dose-dependent manner (Fig. 3A). We also measured the specific Akt kinase activity in type II AECs using recombinant GSK-3 as the substrate. Hypoxia-treated type II AECs were treated with leptin for 12 h at increasing doses, and the Akt kinase activity in total cell lysates was analyzed in vitro. Type II AECs cultured in normoxia had a relatively high constitutive level of Akt kinase activity that was reduced significantly by hypoxia treatment (Fig. 3B). Furthermore, in the presence of hypoxia, leptin treatment restored the Akt kinase activity in an dose-dependent manner, with maximum stimulation of Akt activity to 3-times that above the hypoxia-induced baseline levels at a concentration of 800 ng/mL leptin (Fig. 3B, $P < 0.01$). These data suggest that the anti-apoptotic effects of leptin in type II AECs may be mediated through or involve the activation of the Akt signaling pathway.

LEPTIN-STIMULATED AKT PHOSPHORYLATION IS DEPENDENT ON PI3K IN TYPE II AECS

Akt is one of the major downstream effectors of the PI3K signaling pathway. To determine whether leptin-mediated anti-apoptotic activity in type II AECs is PI3K-dependent, the effect of the PI3K inhibitor wortmannin on cell viability and Akt phosphorylation in type II AECs was examined in the presence or absence of leptin. The cell viability of type II AECs clearly decreased under hypoxic conditions, compared with the control group (Fig. 4A). Leptin induced a significant increase in cell numbers under hypoxia (Fig. 4A). When cells were treated with a combination of leptin and wortmannin, the leptin-mediated rescue of type II AEC viability in hypoxic conditions was lost (Fig. 4A; $P < 0.01$), indicating that PI3K activity is necessary for mediating the anti-apoptotic effects of

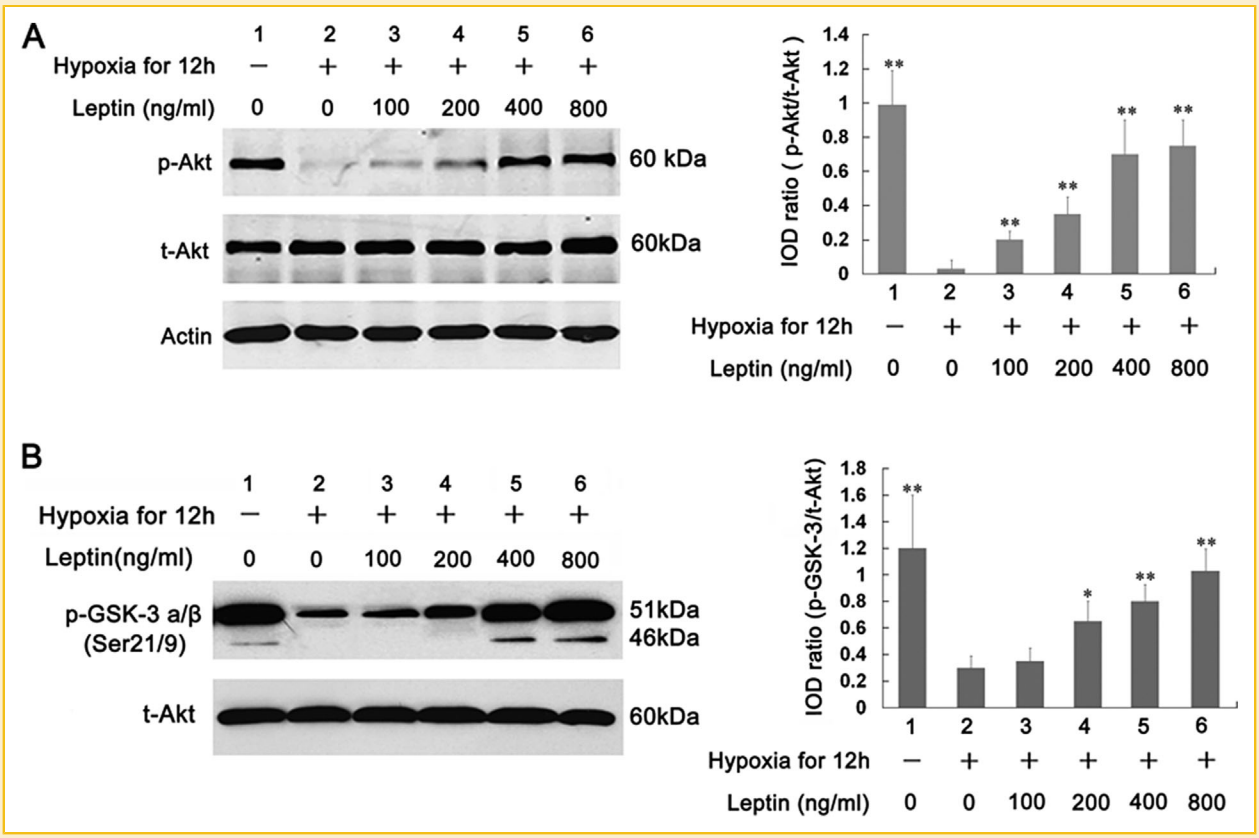

Fig. 3. Effects of leptin on Akt phosphorylation and Akt kinase activity in type II AECs treated with hypoxia. A: Type II AECs were exposed with hypoxia and leptin for 24 h. Cells were lysed and the expression of Akt and pAkt (Ser473) were assayed by western blotting. β-actin was used as the loading control, and the blots were quantified by densitometry. The ratio of the relative intensities of p-Akt (S473) to total Akt is shown ($^{**}P < 0.01$, vs. hypoxia-treated cells). B: Type II AECs were treated as above and lysates were collected for western blotting analysis and in vitro kinase assay. A GSK3 fusion protein was used as the Akt substrate after immunoprecipitation of Akt with an anti-Akt antibody. The relative abundance of GSK-3 α/β phosphorylated (Ser 21/9) by the immunoprecipitated Akt was normalized to the total Akt levels ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. hypoxia-treated cells). Integrated Optical Density (IOD), ; *t*-Akt, total Akt.

leptin. Similar changes also occurred in the leptin-mediated activation of Akt activation (Fig. 4A); leptin-mediated reversal of the hypoxia-induced decrease in Akt phosphorylation was completely suppressed by co-treatment with leptin and wortmannin (Fig. 4B, $P < 0.01$). These results indicate that the anti-apoptotic activity of leptin, and its effect on Akt activation, is mediated, at least in part, via the PI3K signaling pathway.

LEPTIN UPREGULATES SURVIVAL PROTEINS AND PREVENTS CYTOCHROME C RELEASE FROM MITOCHONDRIA IN TYPE II AECS

Next, we examined the changes in signaling molecules acting downstream of PI3K/Akt signaling that may modulate leptin-mediated type II AEC survival. Akt promotes cell survival by regulating the expression or activity of Bcl-2 family members, such as pro-apoptotic factors Bax and Bad and the anti-apoptotic factor Bcl-2 [Elmore, 2007; Manning and Cantley, 2007]. Akt-mediated phosphorylation of Bad leads to inactivation of Bad and to cell survival, whereas dephosphorylation of Bad results in apoptosis [Elmore, 2007; Manning and Cantley, 2007]. Treatment of type II AECs with hypoxia significantly inhibited the phosphorylation of Bad, comparing with that in the control cells (Fig. 5A). Leptin enhanced the level of phosphorylated Bad under hypoxia (Fig. 5A). When cells were treated with both leptin and wortmannin, leptin-induced phosphorylation of Bad was completely reversed by wortmannin (Fig. 5A). Similar changes in the p-Bad-to-Bad ratio was confirmed by semi-quantitative analysis of the western blots by densitometric analysis (Fig. 5A). Western blotting analysis revealed that the Bax levels were not altered in type II AECs treated with hypoxia, whereas Bcl-2 levels were significantly downregulated by hypoxia (Fig. 5B). Leptin treatment restored the levels of Bcl-2 protein in type II AECs under hypoxic conditions (Fig. 5 B). Furthermore, the leptin-mediated increase in Bcl-2 levels was prevented by co-treatment with wortmannin (Fig. 5B). Quantitative analysis of the Bax/Bcl-2 ratio also indicated that wortmannin blocked the leptin-mediated increase in Bax/Bcl-2 ratios in type II AECs (Fig. 5C). These results suggest that Bad and Bcl-2 are involved in the anti-apoptotic effects of leptin in type II AECs.

To determine the effect of leptin on cytochrome c release, a marker for apoptosis, type II AECs were treated with hypoxia, and leptin, and cytochrome c release was monitored by immunostaining with anti-cytochrome c antibodies and confocal microscopy. The cells treated with hypoxia for 12 h showed a diffuse cytosolic pattern of

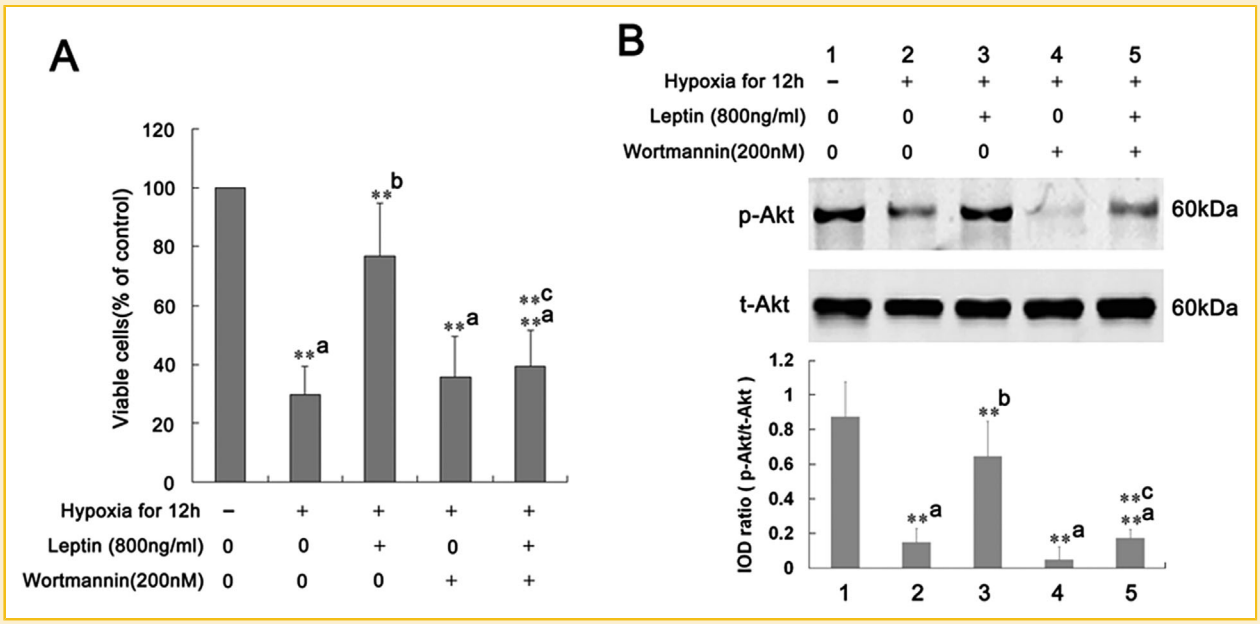

Fig. 4. The PI3K inhibitor wortmannin inhibits cell survival and blocks phosphorylation of Akt in leptin-treated Type II AECs. A: After exposure to hypoxia, followed by treatment with wortmannin and/or leptin, the cell viability of cultured type II AECs was assessed using the MTT assay. B: Type II AECs were exposed to hypoxia and treated with wortmannin or/and leptin for 12 h. Expression of p-Akt (Ser473) was determined by western blotting and analyzed using semiquantitative densitometry. The relative abundance of p-Akt was normalized to total Akt. ($^{**}P < 0.01$, a: vs. normoxia-treated cells (DMSO), b: vs. hypoxia-treated cells, c: vs. hypoxia- and leptin-treated cells).

cytochrome c staining, indicating that cytochrome c was released from the mitochondria (Fig. 5C, b), which was suppressed by treatment with leptin (Fig. 5C, c). After the addition of wortmannin, a diffuse cytosolic cytochrome c staining appeared in type II AECs (Fig. 5C, d), indicating that the protective effects of leptin require an intact PI3K signaling pathway.

Altogether, our data indicated that hypoxic treatment alters PI3K/Akt signaling, resulting in decreased Bcl-2 expression and Bad phosphorylation in vivo. Furthermore, our data show that leptin restores PI3K/Akt signaling-mediated regulation of survival proteins Bcl-2 and p-Bad, resulting in the prevention of hypoxia-induced apoptosis in type II AECs.

LEPTIN-MEDIATES DOWN-REGULATION OF HYPOXIA-INDUCED CASPASE-9 AND CASPASE-3 ACTIVITY IS PI3K-AKT-DEPENDENT

Caspase-9 and -3 are critical mediators of mitochondrial events in apoptosis [Manning and Cantley, 2007], and their cleavage generates key executioner proteins in the apoptotic process of cells. To investigate whether the anti-apoptotic effects of leptin involve regulation of caspase family proteins through the PI3K/Akt pathway, type II AECs were exposed to hypoxia, leptin, and wortmannin, alone or in combination, and the cleavage of caspases was analyzed by western blotting. Treatment of cells with hypoxia did not result in caspase-8 activation and cleavage (data not shown). However, hypoxia induced potent cleavage of caspase-9 and -3, along with significant upregulation of the protein levels of active forms of both caspases (data not shown). Consistent with our earlier findings, hypoxia-induced cleavage of caspase-9 and -3 was inhibited by exposure to leptin, in a dose-dependent manner (Fig. 6A and B). Treatment with wortmannin reversed the protective effect of leptin and significantly increased caspase-9 and -3 activities (Fig. 6C and D). These data show that the anti-apoptotic effect of leptin involves inhibition of caspase cleavage and activity during hypoxia-induced apoptosis (Fig. 7). The expression of cleaved caspase-9 and -3 was also increased by blocking PI3K activity with wortmannin. These data support the conclusion that leptin may promote the development of fetal lungs by preventing apoptosis of fetal lung cells, via down-regulation of caspase-9 and -3 activation.

DISCUSSION

Type II AECs have a multifunctional role in the lung, including participating in the development of alveoli. Therapeutic interventions interfering with the pathways that lead to the apoptosis of AECs should be of considerable benefit in the treatment of the pulmonary hypoplasia. The current disease paradigm for pulmonary hypoplasia is that ongoing or repetitive injurious stimuli in the presence of a genetic or acquired dysfunctional type II AEC phenotype results in increased AEC injury/apoptosis, deficiencies in regeneration of normal alveolar structure, and aberrant lung repair and fibroblast activation, leading to progressive fibrosis [Gharaee-Kermani et al., 2009; Zoz et al., 2011]. The principal findings of this study support the hypothesis that leptin enhances the maturity of fetal lungs by mediating the regulation of caspase-3 and -9 during hypoxia-induced apoptosis of type II AECs.

In this study, supraphysiological leptin concentrations were used both in vitro and in vivo experiments. The dose of leptin was chosen according to the previous studies. Previous in vitro studies showed that the biological effects of recombinant leptin were assessed in a

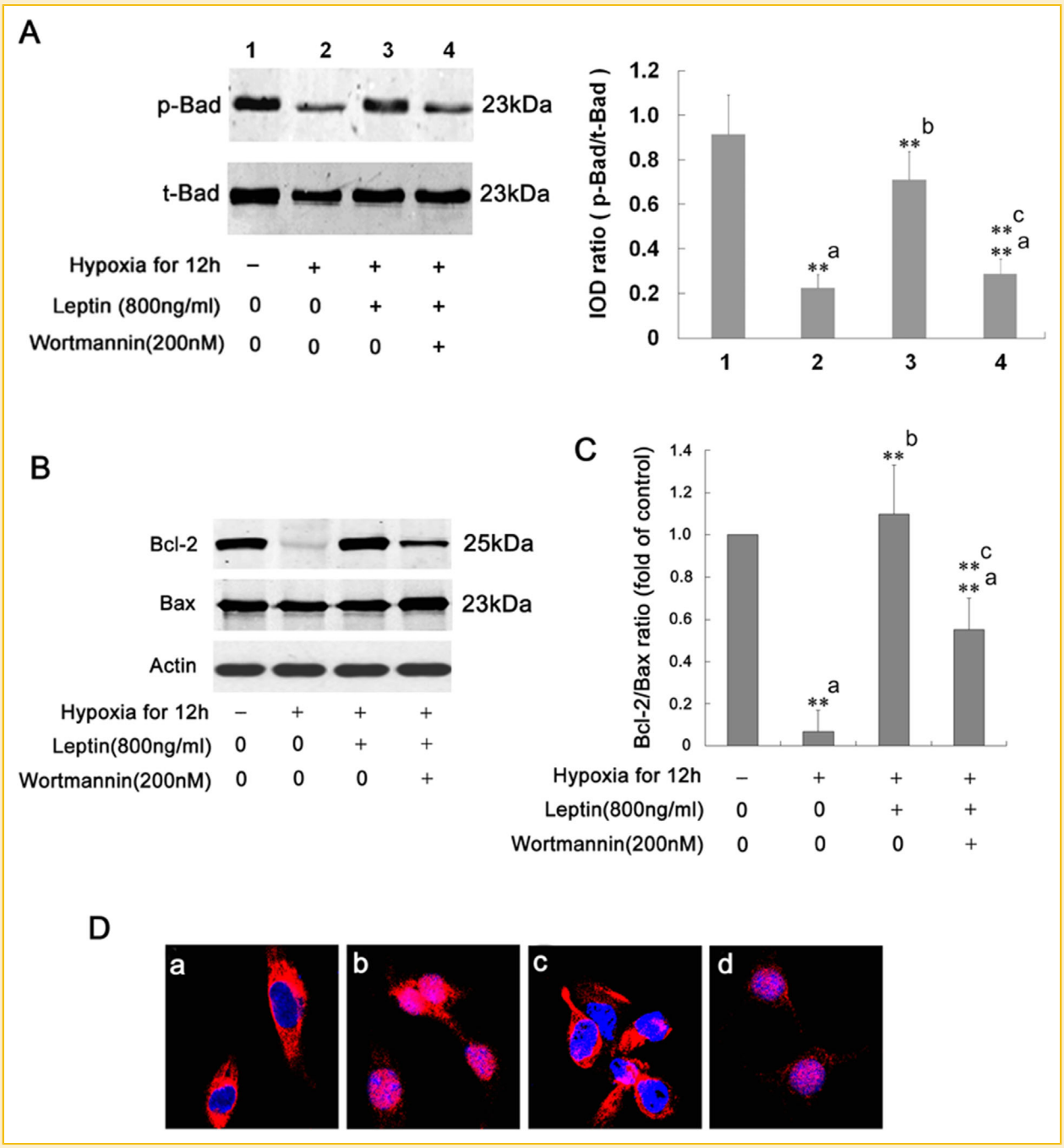

Fig. 5. Leptin modulates the expression of Bcl-2 family proteins and prevents cytochrome c release from mitochondria in Type II AECs. A: Type II AECs were incubated under hypoxic conditions, treated with wortmannin and/or leptin for 12 h, and cell extracts were subjected to western blotting analysis to determine the changes in p-Bad (Ser136). Total Bad was used as the loading control, and the protein bands were analyzed using semi-quantitative densitometry. B, C: Type II AECs were treated as above and protein lysates were analyzed using western blotting with anti-Bax and anti-Bcl-2 antibodies (B). The gel images were scanned and quantified by densitometry. Data are expressed as the ratio of Bcl-2 to Bax and represent the mean of 3 different samples in each group (C) ($^{**}P < 0.01$; a: vs. normoxia-treated cells, b: vs. hypoxia-treated cells, c: vs. hypoxia- and leptin-treated cells). D: Cytochrome c release was visualized by confocal immunofluorescence microscopy in Type II AECs (×1000). Immunolocalization of cytochrome c (red) and nuclear morphology (blue) is shown in normal cells (a), hypoxia-treated cells (b), hypoxia- and leptin-treated cells (c), and in cells treated with hypoxia, leptin, and wortmannin (d). Cells were fixed 4 h after treatment and immunostained with an anti-cytochrome c monoclonal antibody and DAPI. *t*-Bad, total Bad.

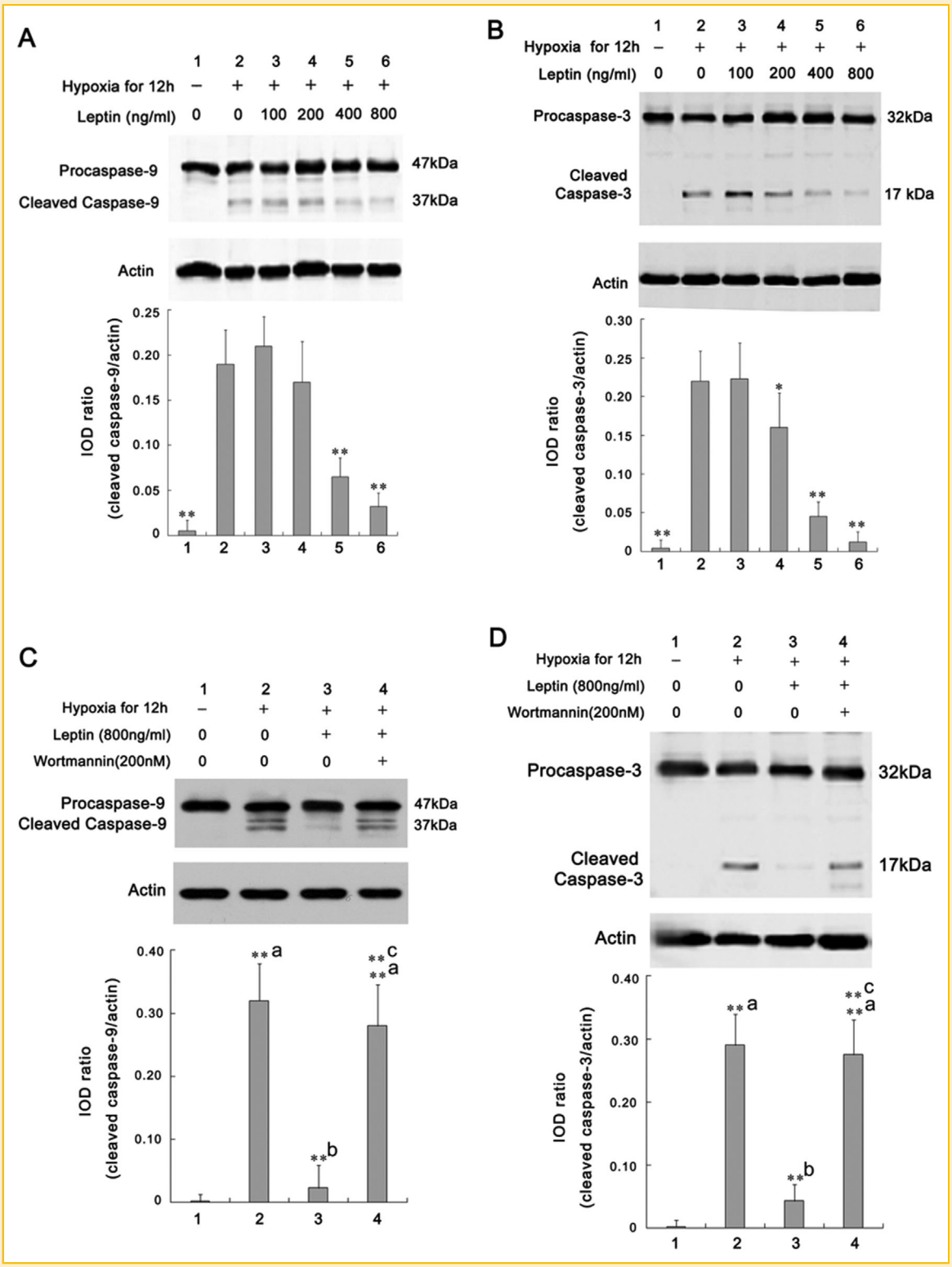

Fig. 6. Inhibitive effects of leptin on caspase-9 and -3 cleavage in Type II AECs. A, B: Type II AECs were exposed to hypoxia and treated with leptin (0–800 ng/mL) for 12 h, and western blotting analysis was performed for cleaved caspase-9 (A) and caspase-3 (B) ($^{**}P < 0.01$, $^{*}P < 0.05$ vs. hypoxia-treated cells. C, D: Type II AECs were incubated under hypoxic conditions, and then treated with wortmannin and/or leptin for 12 h. After the treatments, cell extracts were analyzed using western blotting to determine the protein levels of caspase-9 and cleaved caspase-9 (C), as well as caspase-3 and cleaved caspase-3 (D), respectively. The intensity of band was determined by a densitometry using β-actin for normalization. ($^{**}P < 0.01$; a: vs. normoxia-treated cells, b: vs. hypoxia-treated cells, c: vs. hypoxia- and leptin-treated cells).

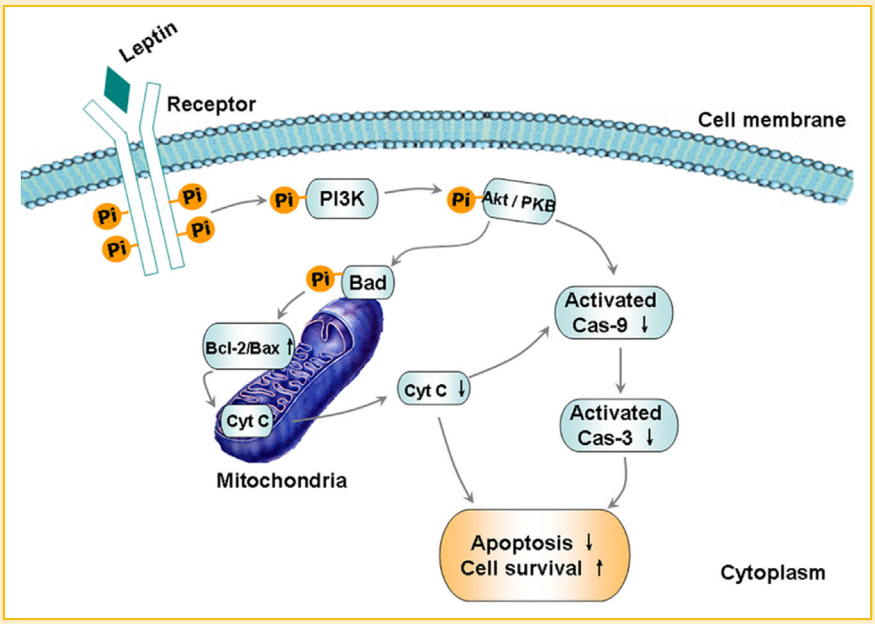

Fig. 7. A schematic overview of signaling events involved in leptin-mediated inhibition of apoptosis in Type-II AECs. Leptin may prevent hypoxia-induced apoptosis of type II AECs, in part, through activation of PI3K/Akt signaling, resulting in increased p-Bad/Bad and Bcl-2/Bax levels, thus preventing cytochrome c release and caspase-9 and caspase-3 cleavage. Cyt c, cytochrome c; Pi, inorganic phosphate; PKB, protein kinase B; PI3K, phosphoinositol 3-kinase; cas, caspase.

concentration ranging from 10 to 1,000 ng /mL (Ramsay,2003; Cui et al., 2006; Lam et al., 2007; Greco et al., 2008; Lin et al., 2009), which is far beyond the physiological range for swine, 2–10 ng/mL [Cameron et al., 2000]. Previous in vivo experiments also used the dose of 1 mg/kg leptin [Cleary et al., 2001; Ramsay , 2003; Kirwin et al., 2006; Hsu et al., 2007; Sato et al., 2011], and this dose of exogenous leptin could restore phenotypes of ob/ob mice [Cleary et al., 2001]. The supraphysiological leptin concentrations in these studies may be attribute to the recombinant leptin [Cleary et al., 2001; Ramsay , 2003; Kirwin et al., 2006; Hsu et al., 2007; Sato et al., 2011]. The dose of recombinant leptin is totally different from the physiological concentration of leptin [Ramsay, 2003].

Recent studies demonstrated that leptin is closely involved with appropriate progress/development of gestation, lactation, fetal development, and fetal lung maturity [Tsuchiya et al., 1999; Dal Farra et al., 2000; Torday et al., 2002; Torday JS and Rehan, 2002; Vlahakis and Hubmayr, 2003; Israel and Chua, 2010]. Moreover, leptin is critical to postnatal lung remodeling, particularly related to increased lung volume, and an enlarged alveolar surface area [Malli et al., 2010]. Studies have also shown that leptin stimulates surfactant protein synthesis, when added to fetal rat lung explant cultures or fetal alveolar type II cell cultures, thus supporting the hypothesis that leptin plays an important role in lung development by regulating pulmonary surfactant production [Tsuchiya et al., 1999; Torday et al., 2002]. Our previous study found that exogenous leptin improves lung structure and function in growth-restricted rats, when leptin was administered to maternal rats at the later stages of gestation [Chen et al., 2013]. In the current study, we demonstrated the protective effect of leptin on type II AECs that were isolated the fetal lungs of the newborn rats in this FGR model. Leptin inhibited hypoxia-induced apoptosis and promoted survival of the isolated type II AECs. Analysis of the expression/activation of key molecular factors involved in AEC apoptosis/survival indicated that the leptin-mediated protective effects depend on the PI3K/Akt signaling pathway and involve the stimulation of Akt phosphorylation, prevention of mitochondrial cytochrome c release, as well as suppression of caspase-3 and -9 cleavage and activation. These data suggest that leptin may be an important factor regulating pulmonary epithelial differentiation and FGR fetal lung maturity.

Our data provide insights into the mechanism(s) through which leptin may mediate the survival and inhibit apoptosis of type II AECs. Although leptin was originally described as a hormone that regulates food intake and energy expenditure, recent studies have highlighted its role as a pleiotropic hormone that exerts anti-apoptotic, and cell growth-promoting effects, especially in some cancers [Caldefie-Chezet et al., 2005; Terzidis et al., 2009; Geng et al., 2012; Chen et al., 2013]. A previous study showed that leptin promotes maturation of fetal lung tissue by promoting adequate production of the SP-A surfactant protein, mediated by transcriptional regulation via thyroid transcription factor-1 (TTF-1) [Chen et al., 2013]. In this study, our data revealed another pathway through which leptin modulates the function of type AECs during lung maturation. The data showed that the anti-apoptotic effects of leptin on type II AECs were PI3K-dependent, in that co-treatment of cells with a PI3K inhibitor and leptin abolished the leptin-mediated protection from hypoxia-induced apoptosis, including the molecular changes associated with leptin treatment, such as Akt phosphorylation at Ser 473 and Bad phosphorylation. Leptin also increased the expression Bcl-2, which prevents the opening of mitochondrial pores during the apoptotic process. Recent reports showed that the

leptin-mediated activation of some typical signaling pathways such as PI3K-Akt [Wang et al., 2012;Zhang et al., 2013] and MAPK [Pérez-Pérez et al., 2008] in tumor cells, neurons, and other cells. Therefore, we then investigated the effects of leptin on the MAPK signal pathway in type II AECs under the same experimental conditions. As shown in the supplementary Fig. 1, hypoxia treatment has no effect on the level of p-ERK1/2 in type II AECs, and the specific MEK inhibitor PD98059 fully inhibited the ERK1/2 pathway. However, there is no significant effect of leptin on phosphorylation of ERK1/2 in Type II AECs exposed to hypoxia. Therefore, it is plausible to propose that the protective action of leptin in type II AECs is mediated via the PI3K/Akt but not the ERK1/2 pathway.

There are still some limitations in this study. Although the protective effect of leptin on type II AECs via PI3K/Akt signaling pathway was confirmed in vitro in this study, this data should be further verified in a rat model. In addition, if other leptin-activated signaling pathways such as JAK2/STAT are associated with the protective effect of leptin on type II AECs also remain to be investigated. Furthermore, we did not analyze the status of cleaved PARP fragment as a predictor for cell death. These issues should be addressed in the following studies.

The results suggest that the uteroplacental insufficiency in mid-pregnancy induces a high rate of FGR and changes in lung structure and lung function in newborns, proving the establishment of a rat model for FGR. Antenatal treatment with leptin has a positive effect on fetal growth and lung development. Leptin-mediated promotion of lung maturity involves prevention of hypoxia-induced apoptosis of type II AECs, through regulation of the PI3K/Akt signaling pathway, leading to the inhibition of cleavage, and activation of caspase-3 and -9. Our data provide the basis for further investigation and development of leptin as a protective therapeutic agent for promoting lung development in FGR.

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