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Transforming growth factor-β1 stimulates heme oxygenase-1 expression via the PI3K/Akt and NF-κB pathways in human lung epithelial cells

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Abstract

A previous report showed that transforming growth factor-β1 (TGF-β1) can induce heme oxygenase-1 (HO-1) expression, attenuate cellular injury, and maintain tissue homeostasis. In this study, we investigated the involvement of phosphoinositide-3-OH-kinase (PI3K)/Akt and the nuclear factor-κB (NF-κB) signaling pathway in TGF-β1-induced HO-1 expression in human lung epithelial cells (A549). Treatment of A549 cells with TGF-β1 caused HO-1 to be expressed in a concentration- and time-dependent manner. Treatment of A549 cells with LY 294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one, a PI3K inhibitor), an Akt inhibitor, and the dominant negative mutant of Akt (Akt DN) inhibited TGF-β1-induced HO-1 expression and HO-1-luciferase activity. Stimulation of cells with TGF-β1 caused an increase in Akt phosphorylation in a time-dependent manner, which was inhibited by wortmannin and LY 294002 (PI3K inhibitors). In addition, treatment of A549 cells with Bay 117082 ((E)-3-[4-methylphenylsulfonyl]-2-propenenitrile, an IκB phosphorylation inhibitor), pyrrolidine dithiocarbamate (PDTC, an NF-κB inhibitor), and the dominant negative mutant of IκBα (IκBαM) inhibited TGF-β1-induced HO-1 expression and HO-1-luciferase activity. Treatment of A549 cells with TGF-β1-induced IκB kinase α/β (IKKα/β) phosphorylation, IκBα phosphorylation, IκBα degradation, p65 Ser536 phosphorylation, and κB-luciferase activity. The TGF-β1-mediated increases in IKKα/β phosphorylation, p65 Ser536 phosphorylation, and κB-luciferase activity were inhibited by LY 294002, an Akt inhibitor, and Akt DN. Taken together, these results suggest that the PI3K/Akt dependent IKKα/β/NF-κB signaling pathway plays an important role in TGF-β1-induced HO-1 expression in A549 cells.

Keywords: Akt; HO-1 [Heme oxygenase-1]; Human lung epithelial cell; NF-κB [Nuclear factor-κB]; PI3K [Phosphoinositide-3-OH-kinase]; TGF-β1 [Transforming growth factor-β1]

1. Introduction

The transforming growth factor-β (TGF-β) family consists of three closely related isoforms (TGF-β1, -β2, and -β3) that are prototypes of the larger TGF-β superfamily (Massague, 1998; Robert, 1999). TGF-β family members elicit a diverse range of cellular responses including cell proliferation, migration, fibrosis, inflammation, and wound repair (Massague, 1998; Robert, 1999). The biological functions of TGF-β1 are of widespread importance in embryogenesis and in fibroproliferative disorders in adults, for example, liver cirrhosis, chronic glomerulonephritis, and atherosclerosis (Border and Noble, 1994). TGF-β1 knockout mice develop diffuse mononuclear cell infiltrates that prove lethal within a few weeks of birth (Shull et al., 1992). Recent studies have suggested a fundamental role for TGF-β1 as a critical mediator of the tissue response to lung injury (Pittet et al., 2001). In addition, TGF-β1 plays a protective role in attenuating cellular damage and maintaining tissue homeostasis through induction of cytoprotective proteins such as heme oxygenase-1 (HO-1) (Hill-Kapturczak et al., 2000; Kutty et al., 1994; Ning et al., 2002).

HO plays critical roles in physiological iron homeostasis, antioxidant defense, anti-inflammation, and antiapoptotic effects (Morse and Choi, 2002). HO catalyzes the conversion of heme to

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biliverdin, releasing equimolar amounts of carbon monoxide and iron. Subsequently, biliverdin is converted to bilirubin by biliverdin reductase (Montellano, 2000). Three isoforms of HO (HO-1, -2, and -3) have been identified (Maines, 1997). HO-1 is an inducible enzyme that is most-highly concentrated in tissues that are heavily involved in the catabolism of heme proteins (Shibahara et al., 1985). HO-2 is a non-inducible isoform that is present in highest concentrations in the brain and testes and is thought to be particularly involved in signaling pathways (Maines et al., 1986). HO-3 is an isoform with low catalytic activity and its physiological role is uncertain (McCoubrey et al., 1997). HO-1 is induced by heme products and a wide variety of non-heme stimuli, which include oxidant stress and TGF-β1 (Ning et al., 2002; Stocker, 1990). Several consensus sequences, including those for nuclear factor-κB (NF-κB), activating protein-2 (AP-2), and heat shock-responsive element (HSE) in the 5′ region of the *HO-1* gene, have been identified as regulatory sequences that can induce HO-1 expression in response to various stimuli (Lavrovsky et al., 1994; Ryter et al., 2002). NF-κB, an important transcription factor for regulating HO-1 expression, is a dimer of the transcription factors p50 and p65 (Baldwin, 1996). In resting cells, NF-κB is thought to be retained in the cytoplasm by a series of inhibitory proteins referred to as inhibitors of κB (IκBs) (Baldwin, 1996). Binding of NF-κB to IκB molecules masks the nuclear localization signal in NF-κB dimers, thereby preventing NF-κB nuclear translocation and transcription activity (Baeuerle and Baltimore, 1988). IKK, which is activated through stimulation by cytokines and growth factors, phosphorylates IκBs to produce ubiquitination of IκBs at lysine residues and degradation by the 26S proteasome (Woronicz et al., 1997). This process releases active NF-κB, which is then translocated from the cytosol to the nucleus, to bind specific DNA enhancer sequences and to induce gene expression (Baldwin, 1996). However, little is known about the signal transduction event; in particular, the phosphoinoside-3-OH-kinase (PI3K)/Akt and IKKα/β/NF-κB pathways, which lead to the expression of HO-1 by TGF-β1 stimulation, are unclear.

The TGF-β1 signaling pathway acts through a system of transmembrane serine/threonine kinase receptors composed of type I and II receptors (TGF-β receptor I and TGF-β receptor II) (Attisano and Wrana, 2002). Ligand binding to TGF-βII recruits and activates the TGF-βI receptor, which phosphorylates Smad2 and Smad3 at their respective SSXS motifs. The phosphorylated Smad2 and Smad3 form stable complexes with Smad4, which are then translocated into the nucleus where they mediate TGF-β1-responsive genes (Zawel et al., 1998). However, accumulating data suggest that Smad-independent pathways can also be activated by TGF-β1, including p38 mitogen-activated protein kinase (MAPK), PI3K, and Akt (Krymskaya et al., 1997; Ning et al., 2002; Zhu et al., 2004). These signaling pathways can potentially contribute to TGF-β1 responses, but little is known about how TGF-β1 regulates the induction of HO-1 protein expression.

PI3K and its downstream serine/threonine kinase, Akt, are important signal transduction pathways involved in many cellular processes, including cell cycle progression, proliferation, and survival (Downard, 1998; Songyang et al., 1997). PI3K/Akt can be activated by a variety of growth factors, such as insulin, nerve growth factors, and TGF-β1 (Downard, 1998; Horowitz et al., 2004). Activation of the PI3K/Akt pathway mediates TGF-β1-induced matrix metalloproteinase-13 expression in hepatic stellate cells (Lexhuga et al., 2004). Moreover, PI3K/Akt-dependent NF-κB activation is involved in TGF-β1-induced neuroprotection (Zhu et al., 2004). There is limited information, however, on the role and regulation of this pathway in TGF-β1-induced HO-1 expression in lung epithelial cells.

The roles of PI3K/Akt and NF-κB in TGF-β1-induced HO-1 expression remain unclear. Therefore, in the present study, we attempted to elucidate the roles of PI3K/Akt and NF-κB in TGF-β1-mediated HO-1 expression in human lung epithelial cells (A549). Our findings revealed that TGF-β1 triggering of the PI3K/Akt signaling pathway leading to activation of IKKα/β/NF-κB plays an important role in TGF-β1-induced HO-1 expression in lung epithelial cells.

2. Materials and methods

2.1. Materials

TGF-β1 was obtained from PeproTech (London, UK). LY 294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one) and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma (Saint Louis, MO, U.S.A.). Wortmannin was purchased from Calbiochem–Novabiochem (San Diego, CA, U.S.A.). The Akt inhibitor (1L-6-hydroxymethyl-chiroinositol2[(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate]) and Bay 117082 ((E)-3-[4-methylphenylsulfonyl]-2-propenenitrile) were purchased from Alexis (Laufelfingen, Switzerland). A dominant negative mutant of IκBα (IκBαM) was purchased from Clontech (Mountain View, CA, U.S.A.). pGL2-ELAM-Luc (which is under the control of one NF-κB binding site) and pBK-CMV-Lac Z were kindly provided by Dr. Wan-Wan Lin (National Taiwan University, Taipei, Taiwan). A dominant negative mutant of Akt (Akt DN) was kindly provided by Dr. Che-Ming Teng (National Taiwan University, Taipei, Taiwan). A human HO-1 promoter-luciferase construct, PGL2/hHO3.2-Luc (−3106/+186) was kindly provided by Dr. Yu-Chih Liang (Taipei Medical University, Taipei, Taiwan). Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/Ham's F-12), fetal calf serum, penicillin/streptomycin, and Lipofectamine Plus™ reagent were purchased from Life Technologies (Gaithersburg, MD, U.S.A.). Antibodies specific for IκBα, IκBα phosphorylated at Ser32, IKKα/β, HO-1, Akt1/2, p65, and anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidases were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Akt phosphorylated at Ser473, IKKα/β phosphorylated at Ser180 (IKKα)/Ser181 (IKKβ), and p65 phosphorylated at Ser536 were purchased from New England Biolabs (Beverly, MA, U.S.A.). All materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Hercules, CA, U.S.A.). All other chemicals were obtained from Sigma (San Diego, CA, U.S.A.).

2.2. Cell culture

A549 lung epithelial cells were obtained from the American Type Culture Collection (Livingstone, MT, U.S.A.), and cells were maintained in DMEM/Ham's F-12 nutrient mixture containing 10% fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin in a humidified 37 °C incubator. After reaching confluence, cells were seeded onto 6-cm dishes for Western blotting and onto 12-well plates for cell transfection and the κB-luciferase activity assay. Before the addition of TGF-β1, the growth medium was removed and replaced with DMEM/Ham's F-12 in the absence of fetal calf serum.

2.3. Western blot analysis

To determine the expressions of HO-1, IKKα/β phosphorylation at Ser180 (IKKα) or Ser181 (IKKβ), IκBα phosphorylation at Ser32, Akt phosphorylation at Ser473, p65 phosphorylation at Ser536, IKKα/β, IκBα, Akt1/2, and p65 in A549 cells, proteins were extracted, and Western blot analysis was performed as described previously (Chen et al., 2004). Briefly, A549 cells were cultured in 6-cm dishes. After reaching confluence, the growth medium was removed and replaced with 2 ml of DMEM/Ham's F-12 in the absence of fetal calf serum for 24 h. Cells were treated with vehicle and TGF-β1, or pretreated with specific inhibitors as indicated followed by TGF-β1. After incubation, cells were washed twice in ice-cold phosphate-buffered saline (PBS) and solubilized in lysis buffer containing 10 mM Tris (pH 7.0), 140 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 0.5% NP-40, 0.05 mM pepstatin A, and 0.2 mM leupeptin. Samples of equal amounts of protein (80 μg) were subjected to SDS-PAGE, then transferred onto a polyvinylidene fluoride membrane which was then incubated in Tris-buffered saline with 0.1% Tween-20 (TBST) buffer containing 5% bovine serum albumin. Proteins were visualized by specific primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies. The immunoreactivity was detected using enhanced chemiluminescence following the manufacturer's instructions. Quantitative data were obtained using a computing densitometer with scientific imaging systems (Kodak, Rochester, NY, U.S.A.).

2.4. Transfection and HO-1- or κB-luciferase assay

A549 cells (2×10^5) were seeded onto 12-well plates, and cells were transfected the following day using Lipofectamine Plus™ reagent containing 0.5 μg of PGL2/hHO3.2-Luc or 0.5 μg of pGL2-ELAM-Luc, and 0.5 μg of pBK-CMV-Lac Z. After 24 h, the medium was aspirated and replaced with fresh DMEM/Ham's F-12 devoid of fetal calf serum, and then stimulated with TGF-β1 (10 ng/ml) for another 24 h before being harvested. To assess the effects of the indicated inhibitors, drugs were added to cells 20 min before the addition of TGF-β1. To assess the effects of the Akt DN and IκBαM, cells were cotransfected with PGL2/hHO3.2-Luc and pBK-CMV-Lac Z or pGL2-ELAM-Luc and pBK-CMV-Lac Z. Luciferase activity was determined with a luciferase assay system (Promega), and was normalized on the basis of Lac Z expression. The level of induction of luciferase activity was compared as a ratio of cells with and without stimulation.

2.5. Statistical analysis

Results are presented as the means±S.E.M. from at least three independent experiments. One-way analysis of variance (ANOVA) followed by, when appropriate, Bonferroni's multiple range test was used to determine the statistical significance of the difference between means. A P value of <0.05 was considered statistically significant.

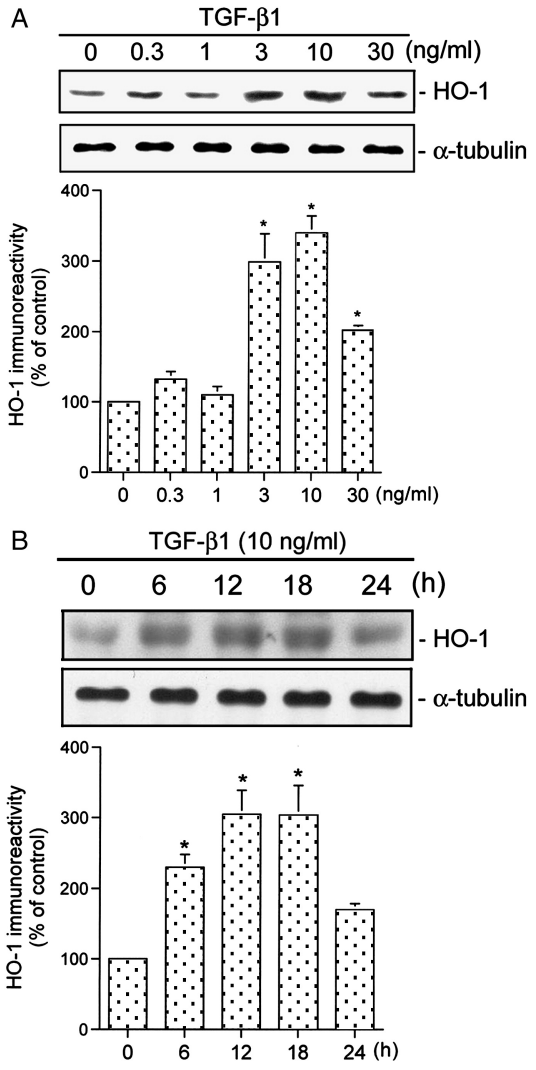

Fig. 1. TGF-β1 induces HO-1 expression. A, A549 cells were incubated with various concentrations of TGF-β1 with 18 h, and then HO-1 or α-tubulin protein levels were determined. Immunoblots are representative of three experiments, which are presented as the mean±S.E.M. *$P<0.05$ compared with the control group. B, Cells were incubated for various time intervals with TGF-β1 (10 ng/ml), and then HO-1 and α-tubulin protein levels were determined. Immunoblots are representative of three experiments, which are presented as the mean±S.E.M. *$P<0.05$ as compared with the control group.

3. Results

3.1. PI3K/Akt is involved in TGF-β1-induced HO-1 expression in A549 cells

Human lung epithelial cells (A549) were chosen to investigate the signal pathways of TGF-β1 in HO-1 expression. Treatment with TGF-β1 (0.3–30 ng/ml) for 18 h induced HO-1 protein expression in a concentration-related manner (Fig. 1A); this induction also occurred in a time-dependent manner, beginning at 6 h and reaching a maximum at 12–18 h (Fig. 1B). After 18 h of treatment with 10 ng/ml TGF-β1, the HO-1 protein had increased by 304±42% (Fig. 1B). To understand the connection between HO-1 expression of TGF-β1 and its PI3K/Akt signaling pathway, the PI3K inhibitor, LY294002 (Chen et al., 2002), and the Akt inhibitor, 1L-6-hydroxymethyl-chiroinositol2-[(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate] (Hu et al., 2000), were used. As a result, the TGF-β1-induced elevation of HO-1 expression was inhibited by 10 μM LY 294002 and 100 nM of the Akt inhibitor by 76±8% and 83±3%, respectively (Fig. 2A). In addition, treatment of cells with LY 294002 (10 μM) and an Akt inhibitor (100 nM) did not affect cell viability, which was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (data not shown). Moreover, transfection of A549 cells with 0.5 μg of Aktc induced an increase in HO-1 expression by 424±31% (Fig. 2B). To further confirm whether TGF-β1 can induce HO-1-luciferase activity and PI3K/Akt signaling pathway mediates this effect, A549 cells treated with 10 ng/ml TGF-β1 for 24 h showed an increase in HO-1-luciferase activity of 365±69%, and this effect was inhibited by LY 294002 (10 μM) and Akt DN (0.5 μg) by 77±13% and 75±12%, respectively (Fig. 2C). These results suggest that the PI3K/Akt signaling pathway is necessary for TGF-β1-induced HO-1 expression. Ser473 residue phosphorylation of Akt by a PI3K-dependent signaling pathway causes enzymatic activation (Alessi et al., 1996). To directly confirm the crucial role of PI3K/Akt in HO-1 expression, we determined Akt Ser473 phosphorylation in response to TGF-β1. As shown in Fig. 3A, treatment of A549 cells with 10 ng/ml TGF-β1 resulted in time-dependent phosphorylation of Akt Ser473. Akt Ser473 phosphorylation began at 3 min, peaked at 30 min, and then declined to 60 min after TGF-β1 treatment (Fig. 3A, upper panel). The protein levels of Akt1/2 were not affected by TGF-β1 treatment (Fig. 3A, bottom panel). In addition, TGF-β1-induced Akt Ser473 phosphorylation was also inhibited by treatment with 100 nM wortmannin and 10 μM LY 294002 (Fig. 3B).

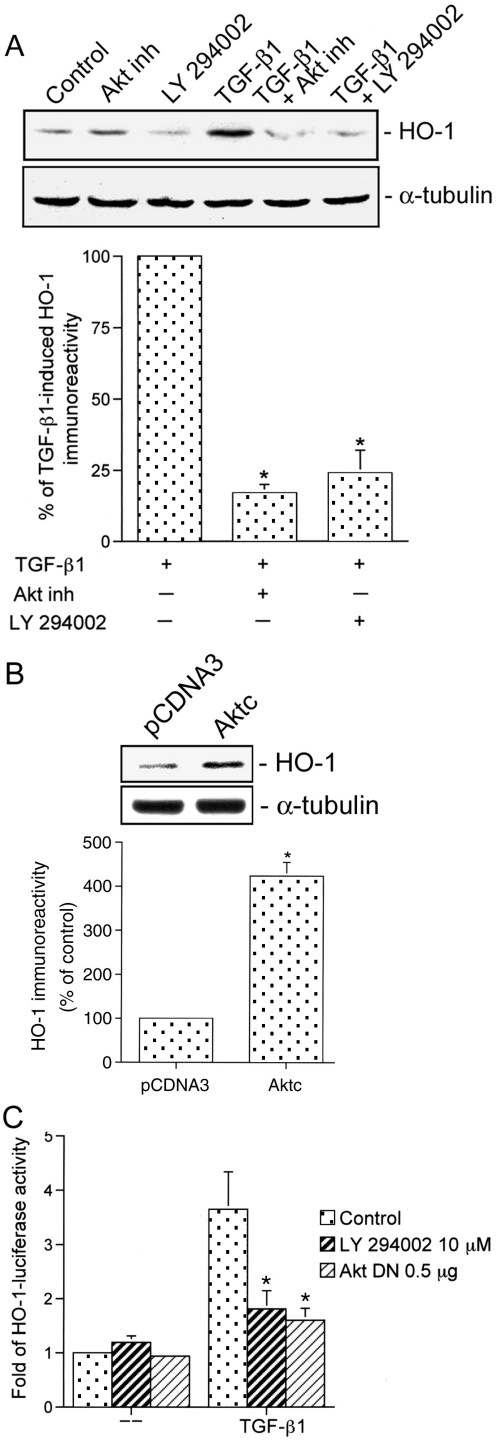

Fig. 2. PI3K/Akt is involved in TGF-β1-induced HO-1 expression in A549 cells. A, Cells were pretreated for 30 min with 10 μM LY 294002 and 100 nM of the Akt inhibitor (Akt inh) and then stimulated with 10 ng/ml TGF-β1. After an 18 h incubation, HO-1 and α-tubulin protein levels were determined. Immunoblots are representative of three experiments, which are presented as the mean±S.E.M. **P*<0.05 as compared with the TGF-β1 treatment. B, A549 cells were transiently transfected with 0.5 μg of pCDNA3 or 0.5 μg of Aktc for 24 h. The medium was aspirated and replaced with fresh DMEM/Ham's F12 for another 18 h. Cell lysates were prepared, and then HO-1 or α-tubulin protein levels were determined. Immunoblots are representative of three experiments, which are presented as the mean±S.E.M. **P*<0.05 as compared with the pCDNA3 group. C, A549 cells transiently transfected with 0.5 μg of pGL2/hHO3.2-Luc and 0.5 μg of pBK-CMV-Lac Z for 24 h were either cotransfected with 0.5 μg Akt DN or pretreated with 10 μM LY 294002 for 30 min, before incubation with 10 ng/ml TGF-β1 for 24 h. Cells were then harvested for the HO-1-luciferase assay as described in "Material and methods". Data represent the mean±S.E.M. of three experiments performed in duplicate. **P*<0.05 compared with TGF-β1 treatment.

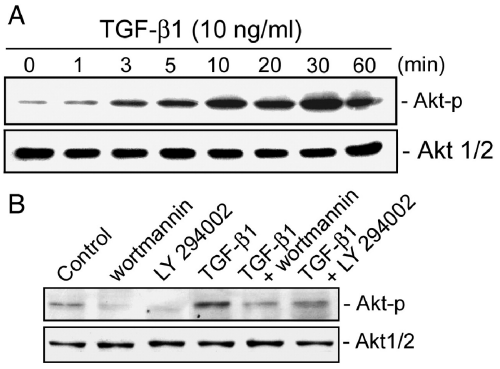

Fig. 3. TGF-β1 induces Akt Ser473 phosphorylation in A549 cells. A, A549 cells were incubated with 10 ng/ml TGF-β1 for 0–60 min. Cell lysates were prepared, and Akt Ser473 phosphorylation was determined by immunoblotting using the phospho-Akt Ser473 antibody. Immunoblots are representative of three experiments with similar results. B, Cells were pretreated for 30 min with 100 nM wortmannin and 10 μM LY 294002 and then stimulated with 10 ng/ml TGF-β1. After 30 min of stimulation, Akt Ser473 phosphorylation was determined. Immunoblots are representative of two experiments with similar results.

3.2. NF-κB is involved in TGF-β1-induced HO-1 expression

As previously mentioned, NF-κB activation is necessary for HO-1 expression (Brouard et al., 2002). To examine whether NF-κB activation is involved in TGF-β1-induced HO-1 expression, an NF-κB inhibitor, PDTC (Chen et al., 1998), was used. Fig. 4A shows that A549 cells pretreated with 30 μM PDTC and inhibited TGF-β1-induced HO-1 expression by 86±3% (n=3) (Fig. 4A). Furthermore, A549 cells pretreated with 10 μM Bay 117082, an IκBα phosphorylation inhibitor (Pierce et al., 1997), reduced TGF-β1-induced HO-1 expression by 45±4% (n=3) (Fig. 4A). In addition, treatment of cells with neither PDTC (30 μM) nor Bay 117082 (10 μM) affected cell viability (data not shown). Moreover, transfection of A549 cells with 0.5 μg of IκBαM inhibited the TGF-β1-induced HO-1-luciferase activity by 71±4% (Fig. 4B). These results indicated that NF-κB activation is important for TGF-β1-induced HO-1 expression in A549 cells.

3.3. TGF-β1 causes an increases in IKKα/β phosphorylation, IκBá phosphorylation, IκBá degradation, p65 Ser536 phosphorylation, and κB-luciferase activity

To further determine the upstream molecules involved in thrombin-induced NF-κB activation, stimulation of cells with 10 ng/ml TGF-β1 induced an increase in IKKá/β phosphorylation in a time-dependent manner, beginning at 5 min, and the effect was sustained to 60 min (Fig. 5A). In parallel with IKKá/β phosphorylation, 10 ng/ml TGF-β1-induced IκBá phosphorylation increased beginning at 5 min and was sustained to 60 min (Fig. 5B). IκBá degradation was apparent after 20–60 min of treatment with 10 ng/ml TGF-β1 (Fig. 5C). Previous studies showed that p65 Ser536 phosphorylation increases NF-κB transactivation (Madrid et al., 2001; Viatour et al., 2005), and the antibody specific against phosphorylated p65 Ser536 was used to examine p65 phosphorylation. Treatment of A549 cells with TGF-β1 (10 ng/ml) for various time intervals resulted in p65 Ser536 phosphorylation which began at 10 min and was sustained to 60 min (Fig. 6A, upper panel). The protein levels of p65 were not affected by TGF-β1 treatment (Fig. 6A, bottom panel). To directly determine NF-κB activation after TGF-β1 treatment, A549 cells were transiently transfected with pGL2-ELAM-κB-luciferase as an indicator of NF-κB activation. As shown in Fig. 6B, TGF-β1 (0.3–30 ng/ml) treatment of A549 cells for 24 h caused a concentration-dependent increase in κB-luciferase activity. Cells treated with 10 ng/ml TGF-β1 showed an increase in κB-luciferase activity of 247±22% (n=3).

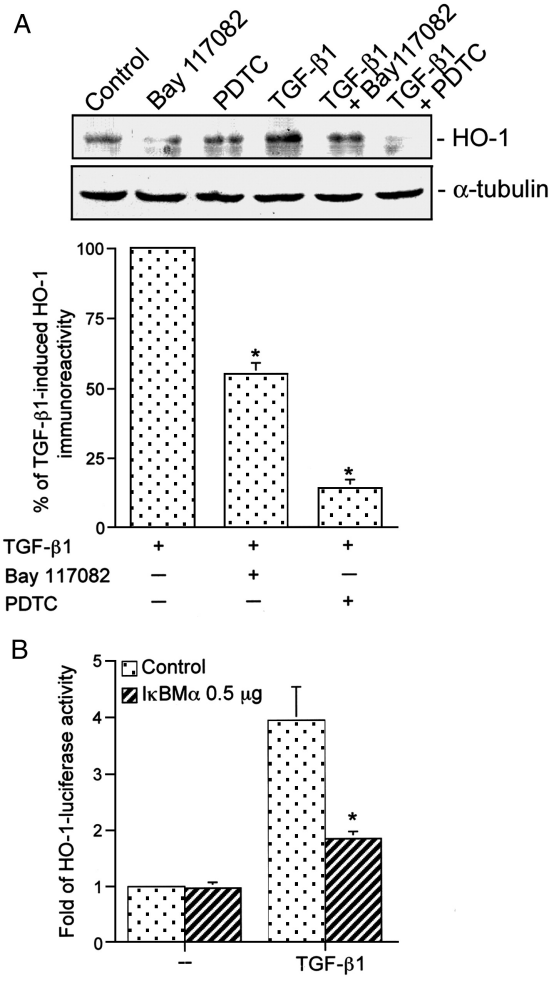

Fig. 4. NF-κB is involved in TGF-β1-induced HO-1 expression in A549 cells. A, Cells were pretreated for 30 min with 10 μM Bay 117082 and 30 μM PDTC and then stimulated with 10 ng/ml TGF-β1. After an 18-h incubation, HO-1 and α-tubulin protein levels were determined. Typical traces are representative of three experiments, which are presented as the mean±S.E.M. *P<0.05 as compared with the TGF-β1 treatment. B, A549 cells transiently transfected with 0.5 μg of pGL2/hHO3.2-Luc and 0.5 μg of pBK-CMV-Lac Z or cotransfected with 0.5 μg IκBM for 24 h, and then stimulated with 10 ng/ml TGF-β1 for another 24 h. Cells were then harvested for the HO-1-luciferase assay as described in Fig. 2C. Data are presented as the mean±S.E.M. of three experiments performed in duplicate. *P<0.05 compared with TGF-β1 treatment.

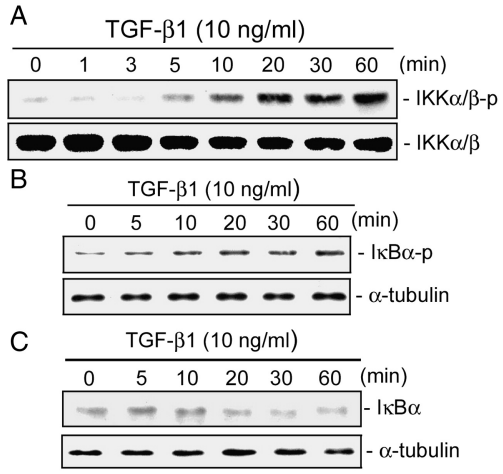

Fig. 5. TGF-β1-induced IKKα/β phosphorylation, IκBα phosphorylation, and IκBα degradation in A549 cells. A549 cells were incubated with 10 ng/ml TGF-β1 for different time intervals. Cell lysates were prepared, and then immunoblotted with antibodies for (A) phospho-IKKα/β (top panel) or IKKα/β (bottom panel), (B) phospho-IκBα (top panel) or α-tubulin (bottom panel), and (C) IκBα (top panel) or α-tubulin (bottom panel). Immunoblots are representative of two experiments with similar results.

Similarly, 10 ng/ml TNF-α, a potent lung epithelium NF-κB stimulator (Rahman et al., 2003), increased κB-luciferase activity by 280±21% (n=3).

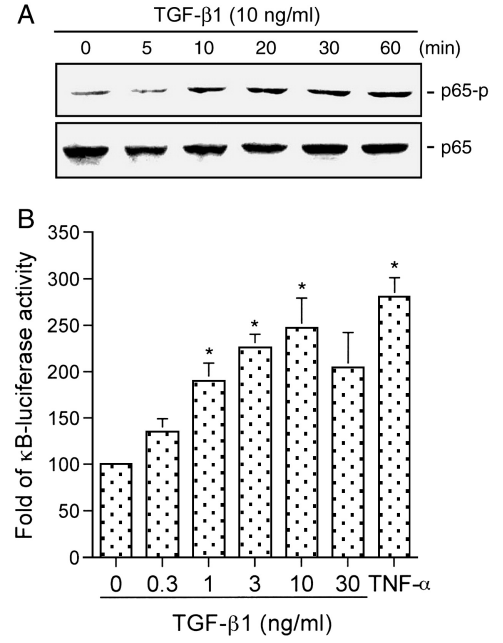

Fig. 6. TGF-β1-induced p65 Ser536 phosphorylation and κB-luciferase activity in A549 cells. A, A549 cells were incubated with 10 ng/ml TGF-β1 for 0–60 min. Cell lysates were prepared, and then immunoblotted with antibodies for phospho-p65 Ser536 (top panel) or p65 (bottom panel), respectively. B, A549 cells were transiently transfected with 0.5 μg of pGL2-ELAM-Luc and 0.5 μg of pBK-CMV-Lac Z for 24 h. Cells were then stimulated with TGF-β1 (0.3–30 ng/ml) or TNF-α (10 ng/ml) for another 24 h. Luciferase activities were determined as described in "Materials and methods". The level of induction of luciferase activity was compared to that of cells without agonist treatment. Data are presented as the mean±S.E.M. of three experiments performed in duplicate. *P<0.05 compared to the control without agonist treatment.

3.4. PI3K/Akt mediates TGF-β1-induced IKKα/β phosphorylation, p65 phosphorylation, and κB-luciferase activity

To further investigate whether TGF-β1-induced IKKα/β phosphorylation, p65 Ser536 phosphorylation, and NF-κB

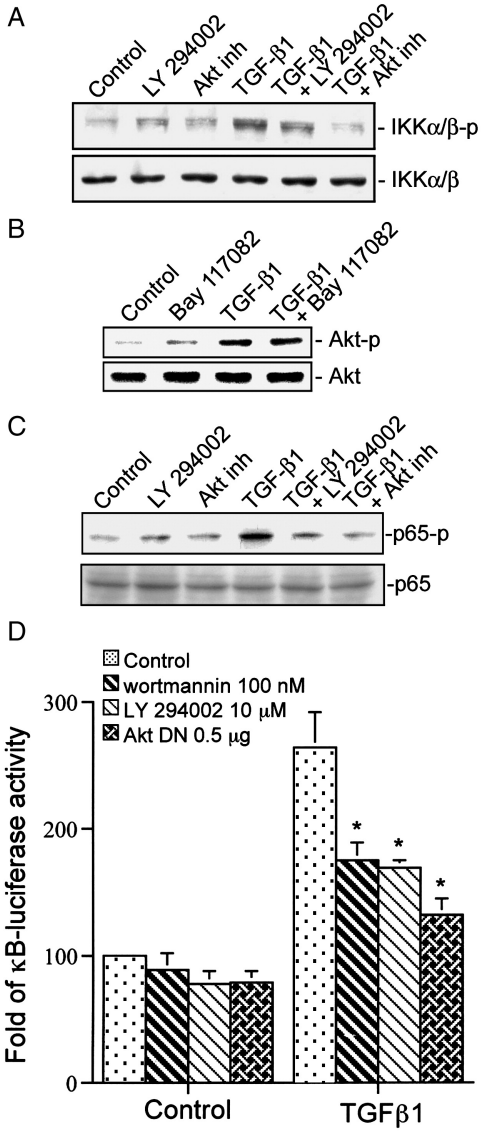

Fig. 7. PI3K and Akt inhibitors affected the TGF-β1-induced increases in IKKα/β phosphorylation, p65 phosphorylation, and κB-luciferase activity in A549 cells. A549 cells were pretreated with 10 μM LY 294002, 100 nM of the Akt inhibitor (Akt inh), or 10 μM Bay 117082 for 30 min before treatment with 10 ng/ml TGF-β1 for another 20 min, after which (A) IKKα/β phosphorylation, (B) Akt phosphorylation, and (C) p65 Ser536 phosphorylation were determined by immunoblotting with antibodies specific for phospho-IKKα/β, phospho-Akt, and phospho-p65, respectively. Equal loading in each lane is shown by the similar intensities of IKKα/β, Akt1/2, and p65, respectively. Immunoblots represent two experiments with similar results. D, A549 cells transiently transfected with 0.5 μg of pGL2-ELAM-Luc and 0.5 μg of pBK-CMV-Lac Z for 24 h were either cotransfected with 0.5 μg Akt DN or pretreated with 100 nM wortmannin or 10 μM LY 294002 for 30 min, before incubation with 10 ng/ml TGF-β1 for 24 h. Cells were then harvested for the κB-luciferase assay as described in Fig. 6B. Data are presented as the mean±S.E.M. of three experiments performed in duplicate.*P<0.05 compared with TGF-β1 treatment.

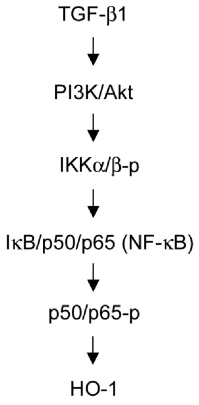

Fig. 8. Schematic summary indicating how signal transduction by TGF-β1 induces HO-1 expression in human airway epithelial cells (A549). TGF-β1 activates the PI3K/Akt pathway, which in turn induces IKKα/β phosphorylation, p65 Ser536 phosphorylation, and NF-κB activation, which leads to HO-1 expression in human lung epithelial cells.

activation occur through the PI3K/Akt pathway, A549 cells were pretreated for 30 min with LY 294002 (10 μM) and the Akt inhibitor (100 nM), which inhibited the TGF-β1-induced increase in IKKα/β phosphorylation as shown in Fig. 7A. Neither inhibitor affected basal IKKα/β phosphorylation. In contrast, Bay 117082 did not affect TGF-β1-induced Akt phosphorylation (Fig. 7B). Moreover, the TGF-β1-induced increase in p65 Ser536 phosphorylation was also attenuated by 10 μM LY 294002 and 100 nM of the Akt inhibitor (Fig. 7C). In addition, the TGF-β1-induced increase in κB-luciferase activity was inhibited by treatment with 100 nM wortmannin, 10 μM LY 294002, and 0.5 μg Akt DN by 49±9%, 47±4%, and 68±8%, respectively ($n=3$) (Fig. 7D). Taken together, these data suggest that activation of PI3K/Akt is required for TGF-β1-induced IKKα/β phosphorylation, p65 Ser536 phosphorylation, and NF-κB activation in lung epithelial cells.

4. Discussion

In this study, we investigated the effects of TGF-β1-induced HO-1 expression in human lung epithelial cells (A549). Our data for the first time demonstrate that TGF-β1 induces HO-1 protein expression via activation of PI3K/Akt-dependent IKKα/β phosphorylation, p65 ser536 phosphorylation, and NF-κB activation in A549 cells. The induction of HO-1 in response to cytokines and growth factors has been demonstrated to be a consequence of *de novo* transcription (Brouard et al., 2002; Kutty et al., 1994). In humans, the *HO-1* gene promoter contains multiple potential regulatory transcription factor binding sites, including HSE, NF-κB, AP-2, and interleukin-6-responsive elements (Choi and Alam, 1996; Lavrovsky et al., 1994), suggesting a potential role for these transcription factors in modulating HO-1 expression. Smad7 has been implicated in the regulation of HO-1 expression by TGF-β1 in human renal epithelial cells (Hill-Kapturczak et al., 2000); however, the NF-κB that controls TGF-β1-induced HO-1 expression has yet to be identified. The results of this study showed that NF-κB activation is essential for HO-1 expression stimulated by TGF-β1. This is based on the fact that PDTC and Bay 117082 inhibited TGF-β1-induced HO-1 expression. Furthermore, TGF-β1 induced increases in IKKα/β phosphorylation, IκBα phosphorylation, IκBα degradation, p65 Ser536 phosphorylation, and κB-luciferase activity. Consistent with the results of our study, NF-κB plays a critical role in the regulation of age-dependent increases in *HO-1* gene expression (Lavrovsky et al., 2000). Therefore, these results suggest that NF-κB activation is required for HO-1 expression by TGF-β1 in human pulmonary epithelial cells.

The cytoprotective activity of TGF-β1 associated with the induction of HO-1 has been documented for different cell types (Hill-Kapturczak et al., 2000; Kutty et al., 1994; Ning et al., 2002). Ning et al. reported that in human pulmonary epithelial cells, p38 MAPK plays a crucial role in TGF-β1-induced HO-1 expression. Hill-Kapturczak et al. showed that in TGF-β1-treated human renal epithelial cells, Smad7 inhibited TGF-β1-mediated HO-1 expression. In this study, we present data to support the role of the PI3K/Akt pathway in TGF-β1-induced HO-1 expression in human lung epithelial cells. We found that both the blockade of PI3K by LY 294002 and the inhibition of Akt by the Akt inhibitor significantly inhibited TGF-β1-induced HO-1 expression. Moreover, we also found that TGF-β1 activated Akt Ser473 phosphorylation, while wortmannin and LY 294002 inhibited TGF-β1-mediated Akt Ser473 phosphorylation. In addition, we focused our attention on the PI3K/Akt pathway, a major cascade mediating activation of the NF-κB signaling pathway in human lung epithelial cells (Chang et al., 2004; Pan et al., 1999). Akt-mediated induction of NF-κB transcriptional activity has been shown to be necessary and sufficient for cyclooxygenase-2 expression (Chang et al., 2004). Furthermore, several studies have shown direct associations of Akt and IKKα/β with increases in IKKα/β and NF-κB activities in many cell types (Lu and Wahl, 2005; Romashkova and Makarov, 1999). In this study, we found that the TGF-β1-induced increase in κB-luciferase activity was abolished by wortmannin, LY 294002, and the dominant negative mutant of Akt, indicating that the PI3K/Akt pathway is involved in the underlying mechanism of NF-κB activation. Interestingly, further investigations revealed that the TGF-β1-induced increase in Akt phosphorylation occurred at 3 min, whereas IKKα/β phosphorylation occurred at 5 min. In addition, IKKα/β phosphorylation caused by TGF-β1 was inhibited by both LY 294002 and the Akt inhibitor. However, Bay117082 did not affect the TGF-β1-induced increase in Akt phosphorylation. Therefore, PI3K/Akt is involved in TGF-β1-induced NF-κB activation through phosphorylation of IKKα/β in A549 cells.

Many NF-κB activation pathways have been reported, and all of them rely on sequentially activated kinase cascades (Viatour et al., 2005). The classical pathway is triggered by various proinflammatory cytokines such as IL-1β and TNF-α (Viatour et al., 2005). These extracellular signals activate the IKK complex which phosphorylates IκBα at Ser32 and Ser36 and signals for ubiquitin-related degradation. The released NF-κB is then translocated into the nucleus where it promotes NF-κB-dependent transcription (Viatour et al., 2005). Besides the phosphorylation and degradation of the IκB signal pathway, an IκB-independent

pathway such as p65 phosphorylation for optimal NF-κB activation has been defined (Buss et al., 2004; Viatour et al., 2005). p65 Ser276 is phosphorylated by the protein kinase A catalytic subunit and mitogen- and stress-activated protein kinase-1, and this phosphorylation increases p65 transcriptional activity (Vermeulen et al., 2003; Zhong et al., 1997). In addition, p65 is phosphorylated at Ser536 by a variety of kinases through various signaling pathways, and this enhances the p65 transactivation potential. TNF-α induces rapid p65 phosphorylation at Ser536 through IKKs, resulting in increased transcriptional activity of p65 (Sakurai et al., 1999). PI3K/Akt also mediates phosphorylation of p65 Ser536 via IKKs or p38 MAPK pathways (Madrid et al., 2001; Sizemore et al., 2002). The results of this study showed that the PI3K/Akt pathway contributed to TGF-β1-induced p65 Ser536 phosphorylation in A549 cells. TGF-β1-induced IKKα/β phosphorylation as well as an increase in p65 phosphorylation at Ser536 which began at 5 and 10 min, respectively, and both LY 294002 and the Akt inhibitor inhibited TGF-β1-induced p65 phosphorylation at Ser536. These results indicate that PI3K/Akt may act through IKKα/β to increase p65 phosphorylation at Ser536 and enhance NF-κB transactivation.

In conclusion, our study for the first time provides fundamental information on the regulatory molecular mechanisms of TGF-β1-induced HO-1 expression through the PI3K/Akt signaling pathway in increasing IKKα/β phosphorylation, p65 Ser536 phosphorylation, NF-κB activation, and HO-1 protein expression in human lung epithelial cells (A549). Fig. 8 is a schematic representation of the signaling pathway involved in the enhancement of HO-1 expression in response to TGF-β1 in human lung epithelial cells. Our results provide a mechanism linking TGF-β1 and HO-1, and provide additional support for the notion that TGF-β1 plays a protective role in lung disease.

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