Insulin-Dependent Phosphatidylinositol 3-Kinase/Akt and ERK Signaling Pathways Inhibit TLR3-Mediated Human Bronchial Epithelial Cell Apoptosis

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Takanori Numata,* Jun Araya,* Satoko Fujii,* Hiromichi Hara,* Naoki Takasaka,* Jun Kojima,* Shunsuke Minagawa,† Yoko Yumino,* Makoto Kawaishi,* Jun Hirano,† Makoto Odaka,‡ Toshiaki Morikawa,‡ Stephen L. Nishimura,† Katsutoshi Nakayama,* and Kazuyoshi Kuwano*

TLR3, one of the TLRs involved in the recognition of infectious pathogens for innate and adaptive immunity, primarily recognizes viral-associated dsRNA. Recognition of dsRNA byproducts released from apoptotic and necrotic cells is a recently proposed mechanism for the amplification of toxicity, suggesting a pivotal participation of TLR3 in viral infection, as well as in lung diseases where apoptosis plays a critical role, such as asthma and chronic obstructive pulmonary disease. In addition to metabolic control, insulin signaling was postulated to be protective by inhibiting apoptosis. Therefore, we explored the role of insulin signaling in protecting against TLR3-mediated apoptosis of human bronchial epithelial cells. Significant TLR3-mediated apoptosis was induced by polyinosinic-polycytidylic acid, a dsRNA analog, via caspase-8–dependent mechanisms. However, insulin efficiently inhibited TLR3/ polyinosinic-polycytidylic acid-induced human bronchial epithelial cell apoptosis via PI3K/Akt and ERK pathways, at least in part, via upregulation of cellular FLIPs and through protein synthesis-independent mechanisms. These results indicate the significance of TLR3-mediated dsRNA-induced apoptosis in the pathogenesis of apoptosis-driven lung disease and provide evidence for a novel protective role of insulin. The Journal of Immunology, 2011, 187: 000–000.

Insulin, an essential hormone for glucose homeostasis, was also proposed to have anti-inflammatory and cytoprotective properties (1). Insulin-mediated PI3K/Akt, MAPK, and primitive insulin-induced signaling pathways are known to play important regulatory and protective roles in apoptosis in several cell types (2–4). In the clinical setting, a growing body of evidence demonstrates the beneficial pulmonary effects of tight blood glucose control with intensive insulin therapy in critically ill patients (5, 6).

Apoptosis, a type of programmed cell death, is a physiologic mechanism for cell deletion that is necessary for the maintenance of homeostatic plasticity in the lung (7). Apoptosis is tightly regulated by activation of effector caspases. However, excessive apoptosis in the airway from noxious environmental stimuli may overwhelm apoptotic cellular clearance mechanisms and contrib-

*Division of Respiratory Diseases, Department of Internal Medicine, Jikei University School of Medicine, Tokyo 105-8461, Japan; †Department of Pathology, University of California, San Francisco, San Francisco, CA 94110; and ‡Division of Chest Diseases, Department of Surgery, Jikei University School of Medicine, Tokyo 105-8461, Japan

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Address correspondence and reprint requests to Dr. Jun Araya, Division of Respiratory Diseases, Department of Internal Medicine, Jikei University School of Medicine, 3-25-8 Nishi-Shinbashii, Minato-ku, Tokyo 105-8461, Japan. E-mail address: araya@ jikei.ac.jp

Abbreviations used in this article: BEGM, bronchial epithelial growth medium; BFA, bafilomycin A; cFLIP, cellular FLIP; CHX, cycloheximide; HBEC, human bronchial epithelial cell; IAP, inhibitor of apoptosis protein; PI, propidium iodide; poly-IC, polyinosinic-polycytidylic acid; siRNA, small interfering RNA.

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Materials and Methods

Cell culture, Abs, and reagents

Normal airways were collected from first- through fourth-order bronchi from pneumonectomy and lobectomy specimens from resections performed for primary lung cancer. Informed consent was obtained from all surgical participants as part of an approved ongoing research protocol by the ethical committee of Jikei University School of Medicine. HBECS were isolated with protease treatment and characterized as previously described (15). Freshly isolated HBECS were plated onto rat tail collagen type I-coated (10 mg/ml) dishes and incubated overnight; thereafter the medium was changed to bronchial epithelial growth medium (BEGM; Lonza, Tokyo, Japan) containing bovine pituitary extract, retinoic acid, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, triiodothyronine, gentamicin, and 5 μg/ml insulin. HBECS showed >95% positive staining with anti-cytokeratin Ab (Lu-5; BioCare Medical, Concord, CA) and <5% positive staining with anti-vimentin Ab (Sigma-Aldrich, Tokyo, Japan) Ab (data not shown) (16). HBECS were serially passaged and used for experiments until passage 4. For experiments of apoptosis induction by poly-IC, HBECS were treated in BEGM without insulin supplement. Bronchial fragments were obtained as previously described (15). Briefly, treated cells were harvested and immediately immobilized by 70% ice-cold ethanol overnight. Then the cells were incubated with 100 μg/ml RNase and 50 μg/ml PI in PBS-Triton X-100 (0.05%) for 40 min at 37°C. The quantity of cells with hypodiploid DNA was measured on a FACScan at the FL-2 channel (Becton Dickinson).

DNA fragmentation analysis was performed as previously described (17). After treatment, collected cells were lysed in 100 μl cell lysis buffer (10 mM Tris·HCl [pH 7.4], 10 mM EDTA [pH 8], and 0.5% Triton X-100). After centrifugation, the supernatant was treated with 2 μl proteinase K (20 mg/ml) for 30 min at 37°C and then incubated with 2 μl proteinase K (20 mg/ml) for 30 min at 37°C. DNA in the supernatant was precipitated overnight by the addition of 20 μl 3 M NaCl and 120 μl isopropanol. After centrifugation, the DNA pellet was dissolved in Tris-EDTA buffer, followed by electrophoresis on a 2% agarose gel. The agarose gel was stained with ethidium bromide, and the resulting DNA-fragmentation pattern was revealed by UV illumination.

Fluorescence microscopic detection of apoptotic cells was performed, as previously described, with minor modifications (17). Harvested cells were stained with 2-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole (Hoechst 33258: Sigma Aldrich), seeded on a glass slide,
and photographed with a fluorescent microscope. The percentage of apoptotic cells was assessed by manually counting 300 cells/condition.

TUNEL was performed using the In Situ Apoptosis Detection Kit (Takara Bio, Shiga, Japan), according to the manufacturer’s instructions. The TUNEL+ bronchial epithelial cells in each fragment were detected using fluorescence microscopy (Nikon, Tokyo, Japan). The percentage of apoptotic cells was assessed by manual counting of all epithelial cells in each fragment.

Immunofluorescence staining was performed as previously described (15). The primary Abs were applied according to the manufacturer’s instructions.

Western blotting

After 24 h of starvation with BEBM, HBECs grown on six-well culture plates were treated with insulin in the presence or absence of inhibitors and lysed in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, IL) with protease inhibitor mixture (Roche Diagnostics, Tokyo, Japan) and 1 mM sodium orthovanadate (Sigma Aldrich). Western blotting was performed, as previously described, with minor modification. After transfer to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, MA), blotting with specific primary Abs was performed overnight at 4°C. Proteins were detected by HRP-conjugated secondary Ab (Bethyl Laboratories, TX), followed by ECL (GE Healthcare, Tokyo, Japan) with the LAS-4000 UVmini system (Fujifilm, Tokyo, Japan).

Small interfering RNA transfection

The TLR3, FLIP, and negative-control small interfering RNAs (siRNAs) were purchased (Applied Biosystems, Carlsbad, CA), and transfections of HBECs were performed using the AmaxaNucleofector (AmaxaBiosystems), using matched optimized-transfection kits for airway epithelial cells.

RNA isolation and PCR

RNA isolation, reverse transcription, and PCR were performed as previously described (16). The primers used were TLR3 sense primer, 5'-AAATTGGCCAAGAACTCACAGG-3', TLR3 antisense primer, 5'-GTGGTCCAGAGCCGTGCTA-3', β-actin sense primer 5'-TGACGGG-GTACCACACTGTTGCC-3', and β-actin antisense primer 5'-CTAGAAGCATTGCGTGACGATGGAAGG-3'. These primer sets yielded PCR products of 320 and 662 bp for TLR3 (14) and β-actin, respectively. Aliquots of the PCR products were subjected to agarose gel electrophoresis in Tris–acetate–EDTA buffer and visualized by ethidium bromide staining.

Statistics

The Student t test was used for comparison of two data sets; ANOVA was used for multiple data sets. The Tukey or Dunn test was used for parametric and nonparametric data, respectively, to find where the difference lay. Significance was defined as p < 0.05. Statistical software used was Prism v.5 (GraphPad Software, San Diego, CA).

Results

Poly-IC induces apoptosis in primary HBECs in the absence of insulin

To elucidate the regulatory role of insulin signaling in TLR3-mediated cytotoxicity in HBECs, we treated them with poly-IC, a dsRNA analog, in the absence or presence of insulin (5 μg/ml) in BEGM with other supplements. Trypan blue dye exclusion demonstrated that, in the absence of insulin, poly-IC treatment for 8 h increased the number of dead cells in a dose-dependent manner, which was significant at a poly-IC concentration of 10 μg/ml (Fig. 1A). Hence, a poly-IC concentration of 10 μg/ml was chosen for further analysis. To further characterize the cell death induced by poly-IC treatment as apoptosis, we performed nuclear staining with Hoechst 33258, DNA-fragmentation assay, and flow cytometric analysis with PI staining. Fluoro-

![FIGURE 2](http://www.jimmunol.org/)

**A** Western blotting using anti–caspase-9, anti–caspase-8, and anti–β-actin in control-treated HBECs (lane 1), HBECs treated with poly-IC in the absence of insulin (lane 2), HBECs treated with poly-IC in the presence of insulin (lane 3), HBECs treated with poly-IC and PD098059 (20 μM) in the presence of insulin (lane 4), and HBECs treated with poly-IC and wortmannin (100 nM) in the presence of insulin (lane 5). Protein samples were collected after 3 h of treatment with poly-IC (10 μg/ml). Shown is a representative experiment of three showing similar results. **B** Western blotting using anti–p-ERK, anti-ERK, anti–p-Akt, and anti-Akt of HBEC lysates at the indicated time points following insulin treatment. After 48 h of starvation with BEBM, HBECs were stimulated with BEBM + insulin (5 μg/ml). **C** Western blotting using anti–p-ERK, anti-ERK, anti–p-Akt, and anti-Akt. After 48 h of starvation with BEBM, HBECs were stimulated with BEBM + insulin (5 μg/ml) for 30 min in the presence or absence of wortmannin (100 nM) or PD098059 (20 μM). Shown is a representative experiment of three showing similar results. **D** Measurement of DNA contents by flow cytometric analysis with PI staining. Shown is the percentage (± SE) of hypodiploid apoptotic cells from six independent experiments. Open bars represent no treatment, and filled bars represent poly-IC treatment (10 μg/ml). HBECs were treated with indicated inhibitors of wortmannin (100 nM), rapamycin (100 μM), or PD098059 (20 μM) or a combination of wortmannin and PD098059 (last two bars). *p < 0.05, **p < 0.001.
rescence microscopic detection of nuclear staining demonstrated the characteristic features of apoptosis by poly-IC treatment at 6 h, which was represented by surface blebbing and nuclear condensation in the absence of insulin (mean 25.3%) (Fig. 1B). DNA-fragmentation assays showed clear DNA ladder formation at a poly-IC concentration of 10 μg/ml at 6 h in the absence of insulin, but ladder formation was diminished in the presence of insulin (Fig. 1C). Measurement of DNA content demonstrated an increased percentage of cells with hypodiploid DNA (mean, 24.6%) after poly-IC treatment in the absence of insulin at 6 h (Fig. 2D). Insulin significantly inhibited poly-IC–induced HBEC apoptosis at a concentration of 10 ng/ml at 6 h in the absence of insulin, but ladder formation was diminished in the presence of insulin (Fig. 1D). Measurement of DNA content demonstrated an increased percentage of cells with hypodiploid DNA (mean, 24.6%) after poly-IC treatment in the absence of insulin at 6 h (Fig. 2D). Insulin significantly inhibited poly-IC–induced HBEC apoptosis at a concentration of 10 ng/ml at 6 h in the absence of insulin, but ladder formation was diminished in the presence of insulin (Fig. 1D), which is ∼10-fold higher than physiological blood levels of insulin during a nonfasting state. These findings indicated that poly-IC induces apoptotic cell death in HBECs, which is efficiently inhibited by insulin treatment.

To further confirm poly-IC–induced apoptosis in HBECs and insulin’s antiapoptotic effect in more physiologically relevant models, we used intact human bronchial tissue fragments, which maintain the structure of the bronchial wall and are composed of bronchial epithelial cells, basement membrane, and subepithelial stromal components, as previously described (Fig. 3A) (16). Consistent with in vitro experiments, poly-IC induced apoptosis in HBECs, as determined by TUNEL+ nuclear staining (30.7%), which was completely inhibited by insulin (5 μg/ml) treatment (Fig. 3B).

TLR3 recognition is crucial for poly-IC–induced apoptosis in primary HBECs
dsRNA is recognized by at least four intracellular sensing molecules, including retinoic acid-inducible gene-I, MDA5, dsRNA-dependent protein kinase R, and TLR3. Among them, a growing body of evidence supports the involvement of TLR3 in apoptosis induction via association with the TLR-adapter protein, TRIF (18). In this context, we examined the involvement of TLR3 in poly-IC–induced apoptosis in HBECs. HBECs were transfected with siRNA targeting TLR3; RT-PCR and FACS analysis for both cell surface and intracellular protein 48 h posttransfection showed that TLR3 siRNA efficiently reduced the amount of both mRNA and protein (Fig. 4A, 4B). Regardless of treatment with insulin, TLR3 siRNA-transfected HBECs demonstrated resistance to poly-IC–induced apoptosis (Fig. 4C), which correlated with levels of knock down of TLR3, indicating an important regulatory role for TLR3 in dsRNA-induced apoptosis. TLR3 expression has been known to be localized both on the cell surface and the endosomal compartment. To further elucidate the involvement of endosome-localized TLR3 in our system, we used BFA, an endosomal acidification inhibitor, which is known to be a functional inhibitor of endosomal TLR3 signaling (19). Interestingly, BFA nearly completely abrogated poly-IC–induced apoptosis of HBECs in the absence of insulin, suggesting the involvement of endosome-localized TLR3 in poly-IC–induced apoptosis of HBECs (Fig. 4D).

Poly-IC–induced NF-κB activation is not involved in apoptosis regulation in HBECs
Poly-IC was demonstrated to activate the antiapoptotic transcription factor NF-κB (14). To elucidate the involvement of NF-κB activation in the regulation of poly-IC–induced apoptosis in HBECs, we performed immunofluorescence staining to detect nuclear translocation of the NF-κB subunit, p65. Poly-IC treatment clearly induced NF-κB activation (Fig. 5A). Therefore, we examined the effect of dexamethasone, a known inhibitor of NF-κB activation. However, no change in apoptosis in the presence of

![FIGURE 3. TUNEL staining in human bronchial fragments. A, After 7 d of culture without poly-IC treatment, bronchial fragments were fixed with 4% paraformaldehyde. Low-magnification (left panel) and high-magnification (right panel) view of bronchial fragment (H&E). B, TUNEL+ bronchial epithelial cells (arrowheads) in each fragment were detected using fluorescence microscopy. Nuclear staining with Hoechst 33258 (left panels) and TUNEL staining (right panels). Shown is a representative experiment of three showing similar results.](http://www.jimmunol.org/)
dexamethasone was observed (Fig. 5B), indicating NF-κB activation was not involved in the regulation of apoptosis by poly-IC via TLR3 recognition in HBEC.

**Poly-IC induces apoptosis in primary HBECs via the extrinsic-apoptotic pathway of caspase-8**

TLR3-associated TRIF-induced apoptosis requires caspase activity initiated by the FADD–caspase-8 axis (18). Therefore, we speculated that poly-IC–induced apoptosis is mediated via the extrinsic apoptotic pathway of caspase-8. After a 3-h treatment, poly-IC activated only caspase-8, but not caspase-9, as shown by the detection of its cleavage form in Western blotting (Fig. 2A); this activation was efficiently inhibited with insulin treatment, indicating the possible involvement of the extrinsic-apoptotic pathway in poly-IC–induced HBEC apoptosis.

**Insulin-mediated activation of both PI3K-Akt and ERK signaling is responsible for the inhibition of poly-IC–induced apoptosis in HBECs**

Insulin demonstrated an inhibitory effect on poly-IC–induced apoptosis in HBECs, and it was shown to activate several intracellular signaling pathways, which potentially inhibit the apoptotic process (1). To elucidate the intracellular signaling pathway involved in insulin-dependent apoptosis inhibition, we treated HBECs with poly-IC in the presence or absence of wortmannin (PI3K inhibitor) and PD098059 (ERK inhibitor), alone and in combination. Initially, the activation of both PI3K and ERK signaling pathways by insulin were confirmed by the detection of phosphorylation of Akt and ERK by Western blotting (Fig. 2B). Activation was efficiently inhibited by wortmannin or PD098059, respectively (Fig. 2C). Wortmannin, in the presence of insulin, restored poly-IC–induced apoptosis to 14.5% (mean value), just slightly lower than that seen with poly-IC without insulin (Fig. 2D). Wortmannin also resulted in detection of cleaved caspase-8 (Fig. 2A). However rapamycin, a mammalian target of rapamycin inhibitor, a downstream signaling molecule of PI3K/Akt, which is implicated in playing an inhibitory role in apoptosis and caspase activation (20), showed no obvious effect on apoptosis induction in the presence of insulin (Fig. 2D). MAPKs are composed of three main signaling pathways, including ERK, p38 MAPK, and JNK. Although the involvement in both pro- and antiapoptotic processes has been widely demonstrated in all MAPK signaling pathways, ERK is likely the most potent signaling pathway for cell survival and proliferation (3). PD098059 demonstrated a more efficient inhibition of the antiapoptotic effect of insulin, because PD098059 increased poly-IC–induced apoptosis to 26.9% (mean value), levels equal to that of poly-IC without insulin (Fig. 2D). Furthermore, wortmannin and PD098059 in combination showed an additive increase in apoptosis (mean, 49.7%), suggesting that PI3K/Akt and ERK signaling pathway antiapoptotic effects are not identical. The combined effects seen with these inhibitors were not due to a cytotoxic effect, because no significant increase in apoptotic cell number was observed without poly-IC treatment (Fig. 2D). Taken together, PI3K/Akt and ERK

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**FIGURE 4.** The role of TLR3 in poly-IC–induced apoptosis in HBECs. A, RT-PCR using primers to TLR3 (upper row) and GAPDH (lower row) was performed from total RNA harvested from control siRNA (lane 1)- or TLR3 siRNA (lane 2)-transfected HBECs after 48 h of incubation. B, Graphs of flow cytometric analysis using anti-TLR3 on permeabilized cells from control siRNA- or TLR3 siRNA-transfected HBECs 48 h posttransfection (left panel). Shown is the relative TLR3 mean fluorescence intensity levels in TLR3 siRNA-treated cells compared with that of control siRNA (right panel). C, Measurement of DNA contents by flow cytometric analysis with PI staining. Shown is the percentage (± SE) of hypodiploid apoptotic cells from three independent experiments. Open bars represent no treatment and filled bars represent poly-IC treatment (10 μg/ml) for 6 h in control-transfected and TLR3 siRNA-transfected cells, respectively, in the absence of insulin. Poly-IC treatment was started 48 h post-siRNA transfection. D, Measurement of DNA contents by flow cytometric analysis with PI staining. Shown is the percentage (± SE) of hypodiploid apoptotic cells from three independent experiments. Open bars represent no treatment and filled bars represent poly-IC treatment (10 μg/ml) in the absence of insulin and with or without BFA (100 nM) for 6 h, respectively. **p < 0.001.
signaling pathways play a major role in mediating insulin-dependent and insulin-independent inhibition of poly-IC–induced apoptosis.

Insulin-induced cFLIP expression partly mediates the antiapoptotic effect of insulin

PI3K/Akt and ERK signaling pathways were reported to upregulate the expression of the antiapoptosis protein, cFLIP (20, 21). To examine a possible role for cFLIP in insulin-mediated protection from apoptosis, we examined the insulin-mediated changes in cFLIP expression levels by Western blotting. cFLIP was detectable at 0 min and clearly increased after a 10-min incubation with insulin (Fig. 6A). Both PD098059 and wortmannin tended to inhibit insulin-induced expression of cFLIP; however, only PD098059 showed significant inhibition, indicating a larger contribution of ERK signaling to the regulation of cFLIP (Fig. 6B). cFLIP expression was necessary for the inhibition of poly-IC–induced apoptosis by insulin, because FLIP knockdown with siRNA efficiently restored poly-IC–induced apoptosis in HBECs in the presence of insulin (Fig. 6C, 6D). Interestingly, cFLIP knockdown seemed to slightly enhance poly-IC–induced apoptosis in the absence of insulin compared with control cells, indicating a possible antiapoptotic role for cFLIP. To further clarify whether insulin-mediated protein synthesis is indispensable for apoptosis inhibition, we examined the effect of cycloheximide (CHX), a protein synthesis inhibitor. CHX-treated HBECs, in the presence of poly-IC and insulin, showed apoptosis in 42.2% (mean value) of the cells, and CHX further enhanced poly-IC–induced apoptosis to 63.0% (mean value) in the absence of insulin, indicating that insulin was also able to inhibit poly-IC–induced apoptosis even without protein synthesis (Fig. 7A). In contrast, protein synthesis was necessary for the insulin-independent inhibition of CH11-induced apoptosis (Fig. 7B). CH11 is an agonistic Fas Ab, and Fas is a representative inducer of the extrinsic pathway of apoptosis, indicating a specific inhibitory role for insulin-dependent protein synthesis on the TLR3-mediated extrinsic pathway of apoptosis. Interestingly, insulin partially inhibited CH11-induced apoptosis and completely inhibited H2O2-induced apoptosis in the presence of CHX. These data suggested that insulin signaling-mediated apoptosis inhibition, in the absence of protein synthesis, might be responsible for both the intrinsic and extrinsic pathway of apoptosis, which is mainly regulated via the PI3K/Akt signaling pathway (Fig. 7B).

Collectively, insulin-elicited PI3K/Akt and ERK signaling pathways suppressed poly-IC–induced apoptosis, in part by synthesis of the antiapoptotic protein cFLIP, as well as by mechanisms that do not require new protein synthesis (Fig. 8).

Discussion

Recent studies revealed that insulin-elicited PI3K/Akt- and concomitant MAPKs signaling pathways are involved in the regulation of blood glucose levels, as well as in a diverse array of cellular processes, including proliferation, survival, and senescence (1, 22). Indeed, nonmetabolic effects, including anti-inflammation, prevention of endothelial dysfunction, and antiapoptosis, were also postulated to be part of the cell-protective mechanisms responsible for clinical benefits seen with intensive insulin treatment (1). Because of the tissue-specific differences of insulin action in glucose-uptake and mitogenic activity, it is reasonable to clarify possible insulin-dependent, cell type-specific protective mechanisms in lung epithelial cells in terms of the prevention of lung injury. Although it was reported that the insulin family of soluble growth factors are crucial determinants for HBEC and BEAS-2B bronchial epithelial cell line survival in vitro culture conditions...
Our findings clearly demonstrate the proapoptotic mechanisms of poly-IC through TLR3 signaling in an in vitro culture model of HBECs in the absence of insulin, further supporting a possible participation of dsRNA in inflammation, as well as in apoptosis-related pulmonary diseases.

Insulin’s antiapoptotic effect was demonstrated in a model of ischemic reperfusion injury of the heart (26) and was also implicated in the regulation of renal tubular epithelial cell apoptosis in the pathogenesis of acute kidney injury (27). Interestingly, development of acute renal failure in critically ill patients in intensive care units was reduced by intensive insulin treatment, which was mediated mainly through a nonmetabolic effect of insulin (28), which may be due to the involvement of a direct antiapoptotic mechanism. The widespread induction of apoptosis by multiple mechanisms, such as viral infections and oxidative stress, which might overwhelm the capacity of epithelial cell repopulation and apoptotic cellular clearance in the airway and lung, plays an integral part in the underlying mechanisms for progression of idiopathic pulmonary fibrosis, bronchial asthma, and chronic obstructive pulmonary disease, including the setting of exacerbations (8–10, 29). Therefore, manipulating excessive apoptosis in epithelial cells is required for the prevention of disease deteriorations. The antiapoptotic mechanisms orchestrated by PI3K/Akt and ERK signaling are involved in multiple steps of apoptotic processes in different cell types, including caspase-3 inhibition through upregulation of IAP1 on activation of the TRIF–mediated main pathways, inactivation of caspase-9 and proapoptotic bcl-2 family protein Bad via their phosphorylation, and increased expression of antiapoptotic cFLIP (30–33). This suggests two major apoptotic signaling pathways, mitochondria-dependent intrinsic and mitochondria-independent extrinsic, as potential targets of antiapoptotic activity by insulin. We demonstrated activation of the extrinsic apoptotic pathway by poly-IC treatment in HBECs, as shown by cleavage of caspase-8, and suppression of its activation by insulin treatment, which is regulated via both PI3K/Akt and ERK signaling pathways. Modestly induced expression of cFLIP was involved in the antiapoptotic effect of insulin; however, cFLIP expression was mainly regulated by ERK, rather than PI3K/Akt-signaling pathway, suggesting the possibility that cFLIP, as well as de novo protein synthesis of other antiapoptotic proteins, might be required. Although a recent paper showed the inhibitory effect of IAP1 on activation of the TRIF–
caspase-8 axis (34), we did not observe any change in IAP1 protein levels after insulin treatment (data not shown). However, direct interference in apoptotic-signaling pathways by predominantly PI3K/Akt signaling may explain the antiapoptotic mechanism of insulin (Figs. 7, 8). Indeed, the antiapoptotic effect seen with insulin treatment was, at least in part, protein synthesis independent. Insulin signaling may be inhibiting both the intrinsic- and extrinsic-apoptotic pathway, because apoptosis-induced by both CH11 and H2O2, in the presence of CHX, was partially and completely inhibited by insulin. Indeed, H2O2 potentially induces both the extrinsic and intrinsic pathways of apoptosis (35).

In the current study, we demonstrated the involvement of TLR3, which is responsible for ~60% of poly-IC–induced apoptosis, according to knockdown experiments using siRNA (Fig. 4). We speculate that incomplete knockdown of TLR3 expression by our siRNA experiment might contribute to this modest effect, because BFA, a functional inhibitor of endosomal TLR3 signaling, showed complete inhibition of poly-IC–induced apoptosis in HBECs (Fig. 4). However, different modalities of approach are also warranted to determine the specific role of endosomal TLR3 signaling due to the diverse array of cellular effects of BFA. TLR3-induced intracellular signaling has been implicated in the regulation of poly-IC–induced pancreatic β cell apoptosis, predominantly via IFN regulatory factor-mediated transcription (36). However, in that report, pancreatic β cells were treated for 5 d with poly-IC before apoptosis was detected, suggesting involvement of different mechanisms than those observed in our study, in which apoptosis started within 3 h after poly-IC treatment (data not shown). TLRs recruit several intracellular TIR domain-containing adaptor proteins to elicit distinct signaling pathways, including apoptosis induction. Among the TIR domain-containing adaptor proteins, TRIF mediates apoptosis through activation of extrinsic caspase-8 (18, 34). TRIF is responsible for both TLR3 and TLR4 signaling, and the TLR4–TRIF–TRAF6 cascade was recently implicated in the regulation of severity of acute lung injury in severe acute respiratory syndrome, anthrax, and H5N1 influenza infection (37). Although TLR4 signals via two different intracellular adaptor proteins, TRIF and MyD88, TLR3 signals via TRIF alone. Furthermore, both severe acute respiratory syndrome and influenza virus are ssRNA viruses, and dsRNA synthesis in infected cells is an inevitable process during virus replication, which is recognized by TLR3. Indeed, a dsRNA intermediate has been known to be a critical determinant for the outcome of viral infections (38), and both influenza A virus and poly-IC induce the expression of TLR3 in lung epithelial cells (14), suggesting the further implication of TLR3 in the recognition of dsRNA byproducts released from apoptotic and necrotic cells, as well as the dsRNA intermediate produced during viral infection (Fig. 8). Most interestingly, our results indicated that the protein synthesis-dependent, antiapoptotic effect of insulin is relatively specific to the TLR3-mediated extrinsic pathway of apoptosis (Fig. 7). This suggests the possible application of intensive insulin therapy for reduction of the development of apoptosis-driven disease, including airway disease, as well as acute respiratory distress syndrome, especially...
that caused by TLR3 signaling. However, in contrast to a previous randomized study (28), recent large, international, randomized trials demonstrated an increased mortality by intensive glucose control with insulin accompanied by more episodes of severe hypoglycemia (39). Although the methodological differences might explain the different outcomes between these studies (40), optimal glucose target levels by insulin treatment in variable ICU settings remain controversial. Furthermore, the development of a novel delivery system to increase local concentrations in the lung epithelial cell milieu without hypoglycemia is a prerequisite for clinical application of insulin to lung diseases, because it was shown that circulating insulin levels with intensive insulin therapy are only transiently higher than in conventional treatment (5), and the antiapoptotic effect of insulin occurred in our in vitro model at concentrations 10-fold higher than physiologically levels during a nonfasting state. However, it is not unexpected that more than physiological levels of insulin might be indispensable to demonstrate the antiapoptotic effect, especially in in vitro experimental conditions as used in recent studies (27, 41).

In summary, our study demonstrated the involvement of dsRNA in lung epithelial cell apoptosis through activation of the extrinsic TLR3–caspase-8 cascade. Insulin-dependent PI3K/Akt and ERK signaling pathways are responsible for the inhibition of poly-IC–induced apoptosis via both protein synthesis of cFLIP and protein synthesis-independent mechanisms. Although the clinical implication of both TLR3-mediated apoptosis in lung disease pathogenesis and insulin-mediated antiapoptotic effect in lung disease prevention remains to be elucidated and needs more study using physiologically relevant animal models, we speculate that our findings indicate the novel protective potential of insulin administration for apoptosis-related pulmonary illnesses.

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Disclosures
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