Role of glycogen synthase kinase 3 in squamous differentiation of pig airway epithelial cells: A primary study

CHEN Wen-Shu, WU Ren-Liang*, TIAN Dan, WANG Xi
Department of Pathology, Tongji Medical College, Huazhong University of Science and Technology; Pulmonary Disease Laboratory, the Ministry of Health of China, Wuhan 430030, China

Abstract: To investigate if glycogen synthase kinase 3 (GSK3) is involved in squamous differentiation of airway (tracheobronchial) epithelial cells, primary pig airway epithelial cells were treated with lithium chloride, a highly selective inhibitor of GSK3. Change in morphology of cells was monitored under microscopy, and expression of β-catenin, phosphorylated GSK3 and involucrin, a squamous differentiation marker, were detected by Western blotting, while expression of mRNA of another squamous differentiation marker, small proline-rich protein, was detected by RT-PCR. Further, luciferase reporter assay was used to assess the activation of β-catenin/Tcf signaling. The results demonstrated that lithium was able to induce a squamous morphology of the cells, and to enhance the expression of involucrin and small proline-rich protein mRNA. Moreover, lithium increased inhibitory phosphorylation of GSK3, augmented nuclear translocation of β-catenin in a dose- and time-dependent manner. Activation of β-catenin/Tcf signaling was observed after the elevation of squamous differentiation markers. Taken together, these data suggest that GSK3 is possibly involved in squamous differentiation of pig airway epithelial cells.

Key words: glycogen synthase kinase 3; β-catenin; squamous differentiation; airway epithelial cell; pig

Squamous cell metaplasia or squamous cell differentiation of conductive airway epithelium is the process that stratified squamous cells replace the pseudostratified mucociliary columnar cells. This switch is accompanied with an increased expression of transglutaminase, involucrin, small proline-rich protein (cornifin) and other protein precursors of cornified cell envelope characteristic of terminally differentiating stratified squamous epithelia. Squamous cell differentiation can be induced by diverse stimuli such as vitamin A deficiency, tobacco smoke and carcinogens. Commonly seen in chronic obstructive pulmonary diseases (COPD), this change of cell phenotype has been thought

Received 2004-12-06   Accepted 2005-03-10
This work was supported by the National Natural Science Foundation of China (No.30200115).
*Corresponding author. Tel: +86-27-83692619; E-mail: renliangwu@hotmail.com
to be an adaptive response to chronic injury as well as a precancerous lesion of lung squamous carcinoma [1]. The molecular mechanisms of squamous metaplasia are not fully elucidated to date [2], though through the studies of classical squamous metaplasia inducers, vitamin A deficiency and phorbol ester, AP-1 (activator protein-1) signaling has been shown to be involved in this process [3-5].

Glycogen synthase kinase 3 (GSK3), a serine/threonine kinase that has a variety of putative substrates, plays important roles in metabolism, cell proliferation, differentiation and survival [6]. Phosphorylation of proteins by GSK3 is most often inhibitory, therefore GSK3 serves as a negative regulator of various signal pathways, including AP-1, and Wnt/β-catenin pathway [6,7], a cascade involved in morphogenesis and carcinogenesis, which has recently been associated with squamous metaplasia of mammary, prostatic and other secretory epithelia [8-11]. Given that GSK3 is constitutively active in resting cells [6] and rich in lung tissue [12], it is likely that GSK3 may be implicated in the maintenance of mucociliary phenotype of airway epithelial cells.

Lithium is a highly selective inhibitor of GSK3 in vitro and in vivo, and has been widely used to test the putative function of GSK3 [13,14]. In this primary study, we took advantage of lithium to examine if GSK3 was involved in squamous differentiation of cultured pig airway epithelial cells. This would help to understand an aspect of molecular events that underlie the cell phenotype change as well as COPD.

1 MATERIALS AND METHODS

1.1 Cell culture

Pig airway epithelial cells (PAEC) were prepared as previously described [15]. Briefly, the tracheae and bronchi were resected from freshly slaughtered pigs, rinsed with cold D-Hanks solution containing antibiotics (500 IU/ml penicillin, 500 µg/ml streptomycin and 5 µg/ml amphotericin B), and protease solution (0.1% protease XIV (Sigma, USA), 100 IU/ml penicillin, 100 µg/ml streptomycin in D-Hanks solution) followed by incubation at 37°C for about 1 h with gentle rocking. The protease solution was then removed, and tracheae and bronchi were intensively washed with DMEM/F-12 (Hyclone, USA) containing antibiotics and 10% new calf serum (Zhejiang Sanli Biotechnology Company, China). The washing solution was mixed with protease solution and centrifugated to collect cells. The cells were washed once more with above DMEM/F-12 before resuspension in complete culture medium, which was DMEM/F-12 supplemented with 5 µg/ml insulin, 10 µg/ml transferrin, 0.5 µg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 1×10^{-7} mol/L retinoic acid, 0.5 mg/ml bovine serum albumin (all from Sigma, USA), 5% fetal bovine serum (Gibco-BRL, USA), and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). About 1×10^7 cells/cm² were plated in culture flasks coated with rat tail collagen and grown at 37°C in 5% CO₂ air. Experiments were performed in first-passages from primary culture and were repeated with different isolations of cells.

1.2 Plasmids

Tcf (T cell factor) luciferase reporter plasmids (pGL3-OT and pGL3-OF, improved versions of pTOPFLASH and pOPFLASH [16]) were generous gifts from Dr. Bert Vogelstein (The Sidney Kimmel Comprehensive Cancer Center, the Johns Hopkins Medical Institutions, USA). Each construct harbors an Xho1 fragment containing three copies of wild type (CCTTTGATC, pGL3-OT) or mutant (CCTTTGGCC, pGL3-OF) human Tcf-4 binding site cloned into pGL3-Basic plasmid (Promega, USA). Before our experiments, aliquots of plasmids were introduced into human embryonic kidney cells (HEK293) for confirming their expression.

1.3 Cell fractionation and extracts

Cells were allowed to attach for 24~30 h at a density of about 5×10⁴ cells/cm² in 25 cm² culture flasks, then incubated with fresh medium containing lithium chloride (LiCl, Armesco, USA) at indicated concentrations or 10 mmol/L NaCl as control. After 12 or 24 h, cells were washed with phosphate-buffered saline (PBS), collected by trypsinization (0.1% trypsin + 0.02% EDTA, 1:1) and lysed in buffer (50 mmol/L Tris-HCl, pH 8.0, 0.1% Triton X-100, 0.1% SDS, 50 mmol/L sodium fluoride, 1 mmol/L sodium vanadate) plus protease inhibitors (10 µg/ml each of aprotinin, leupeptin, pepstatin (all from Sigma) and 1 mmol/L phenylmethylsulfonyl fluoride) to obtain whole cell protein. Lysate was cleared by centrifugation and protein concentration was determined by BCA kit (Pierce, USA). For cell fractionation into cytoplasmic and nuclear extracts, cells was pelleted and lysed with NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Pierce) plus protease inhibitors as instructions from the manufacturer.

1.4 Western blotting

Equal amount of protein (for detection of phosphorylated GSK3, loading 10 µg of whole cell proteins each
lane; for β-catenin, 20–30 µg of cytoplasmic protein and 10–15µg of nuclear protein; for involucrin, 75 µg of whole cell lysate) was subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting according to standard method. The first antibodies we used were: anti-involucrin (1:150, NeoMaker, USA), anti-β-catenin (1:1 000, Santa Cruz, USA) and anti-phosphorylated GSK3 (1:500, Ser-21-GSK3α/Ser-9-GSK3β, Cell Signaling, USA). Membranes were visualized with ECL (Enhanced chemiluminescence) substrate (Santa Cruz) and exposed to X-ray films (Fuji, Japan).

1.5 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (including RNA from pig esophagus epithelium for positive control) was isolated using TRIzol reagent (Life Technologies, USA). Reverse transcription was performed using M-MLV and oligo-dT₁₅ (Promega) according to the manufacturer’s instruction. Equal volume of RT product was subjected to PCR. Primers for pig small proline-rich protein (SPRP) were picked up based on published sequence (gene accession number: M88166). The forward primer was 5’-AAC CAT GTG TCC CCA AAA C -3’, and backward primer was 5’- CTT CTG CTT GGT CTT CTG CT -3’, generating a 200 bp fragment. β-actin cDNA was amplified as internal control using forward primer: 5’-GAC TAC AGC TTC ACC ACC AC-3’, and backward primer: 5’- TAC TCC TGC TTG ATC CAC -3’, with a 498 bp product. The amplification conditions were 35 cycles of 94ºC, 30 s; 55ºC, 30 s; 72ºC, 40 s for SPRP and 20 cycles of 94ºC, 30 s; 55ºC, 30 s; 72ºC, 40 s for β-actin. PCR product was run on 2% agarose gel containing 0.5 µg/ml ethidium bromide. Optical density of the product was quantified with Kodak Digital Science 1D System (Kodak, USA).

1.6 Transient transfection and luciferase reporter assay

Transient transfection was carried out using Lipofectamine 2000 (Life Technologies, USA) according to the recommendations from the manufacturer and a method described by Tucker et al.[17] with minor modification. About 2×10⁵ cells (initially seeded cell number) in each well of 12-well plate were transfected with 0.5 µg pGL3-OT or pGL3-OF including 0.1 µg pCH110 (β-galactosidase expression plasmid) for monitoring transfection efficiency. 10 mmol/L LiCl was added 18 h after transfection and further incubated for 12 or 24 h. Luciferase reporter assay and β-galactosidase assay were preformed using commercial kits as directed by the manufacturer (Promega). Luciferase activity was read using Lumat LB 9507 luminometer (EG&G BERTHOLD, Germany), and normalized for β-galactosidase activity (OD420).

1.7 Statistical analysis

Data were expressed as mean ± SD. Statistical differences between values from different groups were determined by Student’s t test (SPSS 10.0 software). Significance was set at P<0.05.

2 RESULTS

2.1 Change of morphology and growth of pig airway epithelial cells (PAEC) after lithium treatment

Under phase contrast microscopy, most of the cells treated with 10 mmol/L LiCl for 12 h did not manifest distinct changes in growth and shape, whereas after 24 h, the treated cells showed delayed confluence and adopted a more spread and flatten appearance with widen cell-cell interspaces (Fig.1B), compared with spindle to round and stereo shape of closely adherent control cells (Fig.1A). These phenomena were more apparent when LiCl concentration was increased to 20 mmol/L (Fig.1C). However, when fresh medium without LiCl was added, cells resumed rapid growth and the widen interspaces disappeared within hours, a shift consistent with the reversible inhibitory effect of LiCl on GSK3[13]. 5 mmol/L LiCl treatment did not alter growth characteristics of PAEC.

2.2 Lithium enhanced expression of squamous differentiation markers

The above results showed that lithium was able to induce a more squamous phenotype of PAEC. We next examined the effect of lithium on the expression of squamous differentiation markers. Expression of involucrin protein and small proline-rich protein (SPRP) mRNA was determined by Western blotting and RT-PCR, respectively. Freshly isolated PAEC did not express involucrin, whereas the protein can be faintly detected in the first passage of the cells. Elevation of protein level was observed after 10 mmol/L LiCl treatment for 12 h, and more markedly after 24 h treatment (Fig.2). A similar reaction was also shown in SPRP mRNA level (Fig.3A, B).

2.3 Lithium induced inhibitory phosphorylation of GSK3

Lithium inhibits GSK3 in two ways: competing for Mg²⁺ binding and increasing inhibitory phosphorylation of GSK3 (Ser-21-GSK3α/Ser-9-GSK3β)[14]. To provide evidence for direct inhibition of GSK3 activity, we examined phosphorylated GSK3 using phospho-specific antibody and West-
ern blotting. The results showed that the levels of phosphorylated GSK3 were low in control cells, but notably rose in lithium treated cells and peaked at 24 h after incubation with 10 mmol/ml LiCl (Fig.4).

2.4 Lithium induced nuclear translocation of β-catenin and activated β-catenin/Tcf signaling

Inhibition of GSK3 by lithium has been documented to stabilize cytoplasmic β-catenin and to enhance nuclear translocation of the protein, which binds with Tcf/Lef (lymphoid enhancer factor) to form a transcriptional complex and to promote the expression of target genes.[6,7] Therefore, we investigated if induction of squamous differentiation of PAEC by lithium was due to activation of β-catenin/Tcf pathway. Cells were fractionated and cytoplasmic and nuclear extracts were subjected to Western blotting to examine β-catenin expression following LiCl treatment. We observed only a slight elevation of β-catenin level in cytoplasmic fractions of PAEC after LiCl treatment, but nuclear β-catenin did increase in a time- and dose-dependent manner, with considerable accumulation of β-catenin in nuclei when cells were incubated with 5 or 10 mmol/L LiCl for 24 h (Fig.5).

We then assessed if nuclear translocation of β-catenin activated β-catenin/Tcf signaling. Two luciferase reporter plasmids, pGL3-OT and pGL3-OF, which contain respectively wide type and mutant human Tcf-4 binding sequences, were transfected into PAEC. Transfected cells were subsequently treated with 10 mmol/L LiCl followed
by luciferase assay in cell lysates. LiCl treatment for 12 h did not augment pGL3-OT transcription (Fig. 6A), probably because of minor increase in nuclear β-catenin levels (Fig. 5), whereas incubation with LiCl for 24 h increased the luciferase activity of pGL3-OT significantly (Fig. 6B). Conversely, LiCl treatment decreased or did not alter pGL3-OF (control vector) transcription (Fig. 6A, B). These results demonstrated that lithium activated β-catenin/Tcf signaling in PAEC. However, activation of β-catenin/Tcf signaling was lagged behind the increased expression of squamous differentiation markers (Fig. 2, 3), which was observed after 12 h of 10 mmol/L LiCl treatment, suggesting that the later event may not result from the former.

3 DISCUSSION
In this investigation, we demonstrated that treatment of primary cultured PAEC with lithium, an established potent inhibitor of GSK3, was able to induce a squamous morphology of PAEC, and to enhance expression of squamous cell differentiation markers. These results suggest that GSK3 is highly likely to be involved in squamous differentiation of PAEC.

Interestingly, we further observed that elevated expression of squamous cell differentiation markers occurred before the activation of β-catenin/Tcf signaling. Miyoshi et al. has shown that transgenic expression of dominant negative GSK3β (dnGSK3β) in mouse mammary epithelium resulted in squamous metaplasia, which was believed to be due to stabilization of β-catenin by dnGSK3β and promote Wnt signaling[9]. Our data was not consistent with their argument, but support the proposal that β-catenin/Tcf signaling may be cell-specific[9,18] and the consequences of β-catenin stabilization may depend on the targeted cell type and also on the immediate environment of the cell[10]. However, based on our limited research, we cannot exclude the role of β-catenin/Tcf signaling in squamous differentiation of airway epithelial cells. Activation of β-catenin/Tcf signaling has been observed in squamous lung cancer cell lines[19], and in proliferative bronchiolar lesions including basal-cell hyperplasia and squamous metaplasia in idiopathic pulmonary fibrosis[20]. Therefore, the role of β-catenin signaling in airway epithelium differentiation remains to be elucidated.

Small proline-rich protein and involucrin are protein precursors of epidermal cross-linked envelope[21]. Both of human small proline-rich protein and involucrin genes contain AP-1 binding sites in their regulation regions[22,23]. GSK3 is a primary kinase involved in negative regulation of AP-1 function by phosphorylating and decreasing the DNA-binding activity of c-Jun and other Jun proteins[7]. Lithium has been demonstrated to activate AP-1-luciferase reporter in Xenopus embryos and in neuronal cells[13,24], and to increase AP-1 activity in cultured cells and in rat brain[24,25]. Currently, it is unclear that lithium also functions in this way in PAEC and increases the expression of small proline-rich protein mRNA and involucrin protein levels.

Though lithium is a highly selective GSK3 inhibitor, it has other molecular targets other than GSK3[26], thus further researches are needed to confirm that GSK3 is directly involved in squamous differentiation of airway epithelial cells. Furthermore, although primary culture closely resembles to its in vivo counterpart, they are not physiologically identical. It is essential to investigate the role of GSK3 in squamous metaplasia of airway epithelium in vivo.

Squamous differentiation of airway epithelial cell is a common phenomenon in chronic inflammatory injury/repair process of airway epithelium but also a precancerous lesion with unclear mechanisms. Our data suggest that GSK3 may play a role in the maintenance of mucociliary
phenotype, and in the injury/repair process and carcinogenesis of airway epithelium.

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ACKNOWLEDGEMENTS: The authors thank Dr. Bert Vogelstein for the generous gifts of plasmids.

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