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Research Paper

Decreased Biosynthesis of Lung Surfactant Constituent Phosphatidylcholine Due to Inhibition of Choline Transporter by Gefitinib in Lung Alveolar Cells

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Purpose. We investigated whether gefitinib, an anticancer agent, inhibits phosphatidylcholine (PC) biosynthesis and choline uptake by alveolar epithelial type II cells.
Materials and Methods. Uptake of choline and PC biosynthesis were examined *in vitro*, using human alveolar epithelia-derived cell line A549 and rat alveolar type (AT) II cells as models.
Results. Gefitinib reduced the incorporation of [^{3}H]choline into PC in A549 and rat ATII cells. The uptake of [^{3}H]choline by A549 and rat ATII cells was concentration-dependent, and the Km values were 15.0 and 10–100 μM, respectively. The uptake of [^{3}H]choline by A549 and rat ATII cells was weakly Na^+-dependent, and inhibited by hemicholinium-3. RT-PCR revealed expression of choline transporter-like protein (CTL)1 and organic cation transporter (OCT)3 mRNAs in both cells. The choline uptake by A549 and rat ATII cells was strongly inhibited by gefitinib with the IC_{50} value of 6.77 μM and 10.5 μM, respectively.
Conclusions. Our results demonstrate that gefitinib reduces PC biosynthesis via inhibition of cellular choline uptake by A549 and rat ATII cells, which is mainly mediated by CTL1, resulting in abnormality of lung surfactant that can be one of mechanisms of the interstitial lung disease associated with gefitinib.

KEY WORDS: alveolar type II; choline; gefitinib; lung toxicity; phosphatidylcholine.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide. Non-small cell lung cancer accounts for 80% of lung cancer, and is usually associated with a poor prognosis (1). Gefitinib is an oral selective inhibitor of epidermal growth factor receptor tyrosine kinase (EGFR-TK), and has received approval in the US, Japan and a number of other countries for the treatment of advanced non-small cell lung cancer. The most commonly reported toxicities associated with gefitinib are mild and self-limiting, such as rash, skin toxicities and diarrhea. Although gefitinib was recognized as a relatively safe oral anticancer agent, accumulating evidence has demonstrated that gefitinib has been associated with severe interstitial lung disease (ILD) (2,3). On the other hand, in the Iressa Survival Evaluation in Lung cancer (ISEL) study, events of the ILD were found, but there was no significant difference between the gefitinib and placebo groups (4). Accordingly, the involvement of gefitinib in ILD that occurs during gefitinib treatment is still unclear. The median time to onset of ILD associated with gefitinib was 24 days in Japan. In addition to normal onset of ILD, sudden onset of ILD only 2 or 4 days after exposure to gefitinib has been reported in elderly patients (78 or 75 years old) with lung cancer (2,5). The mechanism(s) of sudden onset of ILD associated with gefitinib treatment is unknown.

Phosphatidylcholine (PC) is synthesized within alveolar type (AT) II epithelia and secreted into the alveolus as the major phospholipid component of lung surfactant, which is essential to establish normal breathing. Interference with surfactant synthesis is recognized as a hallmark of the neonatal respiratory distress syndrome, and is to some degree a component of acute and chronic lung disease (6,7). PC synthesis in all eukaryotic cells occurs predominantly through the cytidine 5′-diphosphocholine (CDP-choline) pathway in which three enzymes, choline kinase, phosphocholine cytidilyltransferase and cholinephosphotransferase, are involved. However, the uptake mechanism of choline by ATII cells, which could be considered the first step of the CDP-choline pathway, has not been elucidated yet.

Choline transport is classified into two major categories based on the degree of affinity for choline and the Na^+-dependence (8). Na^+-dependent high-affinity choline transport is localized at cholinergic nerve terminals, where it contributes to the synthesis of acetylcholine, and is antagonized by low

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ABBREVIATIONS: ATII, alveolar type II; CDP, cytidine 5′-diphosphocholine; CTL, choline transporter-like protein; EGFR-TK, epidermal growth factor receptor tyrosine kinase; HC-3, hemicholinium-3; ILD, interstitial lung disease; MPP^+, 1-methyl-4-phenylpyridinium; NMG, N-methylglucamine chloride; OCT, organic cation transporter; PC, phosphatidylcholine; siRNA, small interfering RNA.

concentrations of a choline analogue, hemicholinium-3 (HC-3). A low-affinity and Na^+-independent system is found widely in various tissues, and this system primarily supplies choline for the synthesis of PC and other phospholipids in the cellular membrane (8). The organic cation transporter (OCT)2 has been proposed as a low-affinity choline transporter across the ventricular membrane of the choroid plexus (9), and two major higher-affinity choline transporters belonging to different protein families have been cloned. The first, CHT1, exhibits all the characteristics of the canonical high-affinity choline transporter associated with cholinergic nerve terminals (10,11). The second, CTL1, belongs to a new family of choline transporter-like (CTL) proteins. CTL1-mediated transport exhibits only a weak sodium dependence (12) and CTL1 is expressed in several neuronal populations, including motorneurones and oligodendrocytes (13).

In order to elucidate the mechanism of ILD associated with gefitinib completely, a step-by-step approach is required. Thus, we focused on the lung surfactant as one of factors. We hypothesized that gefitinib inhibits the uptake of choline by human ATII cells, and that the resultant reduction of PC synthesis causes a lung surfactant disorder, because PC is a major constituent of the surfactant. In the present study, we examined whether gefitinib inhibits PC biosynthesis and choline transport in primary-cultured rat ATII cells and human alveolar epithelia-derived cell line A549, which has the characteristics of type II cells, including production of surfactant protein and lipids (14,15). Furthermore, we characterized the mechanism of choline transport in A549 and primary-cultured rat ATII cells.

MATERIALS AND METHODS

Chemicals

Gefitinib (ZD1839) (Fig. 1) was provided by AstraZeneca (Macclesfield, UK). Stock solutions were prepared in dimethyl sulfoxide and stored at −20°C. [Methyl-^{3}H]choline chloride was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). HC-3, 1-methyl-4-phenylpyridinium (MPP^+), histamine, corticosterone, and dipalmitoyl-D-α-phosphatidylcholine were purchased from Sigma-Aldrich (St. Louis, MO). Choline chloride was obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals and reagents were commercial products of reagent grade.

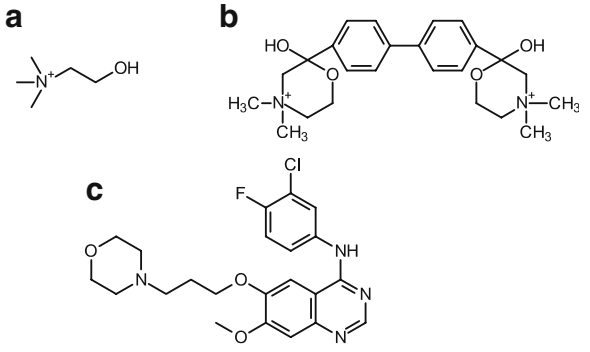

Fig. 1. Chemical structures of choline (**a**), hemicholinium-3 (**b**) and gefitinib (**c**).

Isolation of Rat ATII Cells

ATII cells were isolated from adult Wistar rat lung according to the procedure of Dobbs *et al*. (16). Briefly, lungs of anesthetized rats were cleared of blood by perfusion and removed from the thorax. After lavage, the lungs were instilled with 15 ml of elastase (4.3 U/ml, Wako Pure Chemicals), incubated at 37°C for 20 min and minced with scissors in a mixture of 5 ml of fetal bovine serum (FBS) and 4 ml of 250 μg/ml deoxyribonuclease (Sigma-Aldrich). The cell suspension was sequentially filtered through nylon gauze (150-, 41- and 15-μm mesh size) and centrifuged at 130 g for 10 min. The resultant cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) and panned on an IgG-coated bacteriological plastic dish at 37°C for 1 h. The unattached cells were centrifuged and suspended in DMEM supplemented with 10% FBS, 139 μg/ml streptomycin, 70 μg/ml penicillin and 10 μg/ml gentamycin. The type II cells were more than 80% of total cells by evaluation with the modified Papanicolaou stain, throughout the experiment (17).

Cell Culture

Human lung adenocarcinoma A549 cells (ATCC-CCL185) were routinely grown at 37°C in F12-K medium (Dainippon Pharmaceutical, Osaka, Japan) supplemented with 10% FBS in a 5% CO_2 humidified incubator.

Uptake of Choline

A549 cells (5.0×10^4 cells/well) and rat ATII cells (4.0×10^5 cells/well) were seeded into normal and fibronectin coated 24-well plates (BD Biosciences, Bedford, MA) and grown for 72 and 20 h, respectively. Uptake was initiated by adding 0.25 ml of transport buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM Hepes, adjusted to pH 7.4) containing radiolabeled and unlabeled substrates after the cells had been washed twice and preincubated with transport buffer alone for 15 min. To investigate the effect of preincubation with gefitinib, a preincubation time of 60 min with transport buffer alone or containing gefitinib was used. The solvent concentration in the final transport solution did not exceed 0.5% (v/v). For the evaluation of membrane potential dependence, uptake of [^{3}H]choline was measured in the presence of increasing and decreasing concentrations of KCl (6 to130 mM) and NaCl (125 to 1 mM), respectively, at pH 7.4 for designated times. To measure the effect of Na^+, sodium chloride in the incubation buffer was replaced with N-methylglucamine (NMG) and lithium chloride. In case of the replacement from Na^+ to NMG^+, chloride concentration was adjusted by hydrochloric acid to afford same chloride concentration as that in the normal transport buffer. The pH of the transport buffer was varied by adjusting the concentrations of Mes, Hepes and Tris to obtain the desired pH. The uptake was terminated at designated times by adding 0.5 ml of ice-cold transport buffer after removal of the incubation buffer. The cells were washed twice with 0.5 ml of ice-cold transport buffer, solubilized in 1 N NaOH, and kept for 1 h at 37°C. Aliquots were transferred to scintillation vials after neutralization with HCl. The radioactivity associated with the cells and incubation

buffer was measured in a liquid scintillation counter (TRI-CARB 2500TR, Perkin Elmer, Boston, MA or LSC-5100, Aloka, Tokyo, Japan) after adding 2 ml of scintillation fluid. The remaining 25 μl of cell lysate was used to determine the protein concentration by the method of Lowry or Bradford with bovine serum albumin as a standard.

Biosynthesis of Phosphatidylcholine

The incorporation of [^{3}H]choline into PC was carried out as described previously (15). A549 cells (2.0×10^5 cells/well) and rat ATII cells (7.0×10^6 cells/well) were seeded into normal and fibronectin-coated 6-well plates (BD Biosciences) and grown for 72 and 20 h, respectively. Cells in each well were incubated with 3–5 μCi/ml [methyl-^{3}H]choline chloride in transport buffer after they had been washed twice and preincubated with transport buffer alone or containing gefitinib at 37°C for 60 min. The solvent concentration in the final transport solution did not exceed 0.5% (v/v). They were washed three times with ice-cold transport buffer, scraped off the plate into CH_3OH/H_2O (2/1, v/v), and briefly sonicated for the determination of apparent uptake of [^{3}H]choline. Cellular lipids were extracted according to Bligh and Dyer (18). The chloroform phase was dried under nitrogen, and PC was dissolved in the development solvent of $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (100/50/14/7) and separated on silica gel chromatographic plates (Kieselgel 60 F_{254}, Merck) with the same solvent used for the determination of the incorporation of [^{3}H]choline into PC. The Rf values of PC were determined from the locations of spots of authentic PC visualized with iodine vapor.

RNA Extraction and RT-PCR

Cultured A549, primary-cultured rat ATII cells and Calu-3 (a human bronchial epithelial cell line) cells, rat liver, and rat kidney were washed with sterile PBS and total RNA was extracted with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA from human tissues of lung (64092-1), trachea (636541), liver (64099-1) and kidney (64097-1) were purchased from Clontech (Palo Alto, CA). After deoxyribonuclease treatment, total RNA of A549, Calu-3, and tissues was reverse-transcribed using an RNA PCR kit (Takara Shuzo Co., Ltd., Kyoto, Japan) according to the manufacturer's instructions. For conversion of total RNA to cDNA, a reaction mixture (10 μl) was prepared containing 2.5 U of reverse transcriptase avian myeloblastosis virus (Takara Shuzo Co., Ltd., Kyoto, Japan), 1 x RT-PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 5 mM $MgCl_2$, 1 mM deoxynucleotide triphosphates, 25 pmol of random 9-mer primers, 10 U of ribonuclease inhibitor, and 1 μg of total RNA. The reaction was carried out at 55°C for 60 min. RT (reverse transcription) was terminated by heating the reaction mixture to 99°C for 5 min, followed by rapid chilling at 4°C. RT reaction mixtures, including cDNA products, were stored at −20°C until used. A single cDNA produced from total RNA was amplified by PCR with primers for OCT1, OCT2, OCT3, CTL1, CTL2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), rOct1, rOct2, rOct3, rCTL1 and rGAPDH. Specific oligonucleotide primer pairs for human OCT1, OCT2, OCT3, GAPDH, rOct1, rOct2, rOct3, rCTL1, and rGAPDH were synthesized as listed in Table I. Specific oligonucleotide primer pairs for CTL1 (QT00075838) and CTL2 (QT00081214) were purchased from Qiagen (Basel, Switzerland). The expected product sizes from OCT1, OCT2, OCT3, CTL1, CTL2, GAPDH, rOct1, rOct2, rOct3, rCTL1 and rGAPDH specific primers were 197, 599, 455, 138, 95, 676, 481, 246, 266, 678, and 985 bp, respectively. For PCR amplification of cDNA, the PCR was allowed to proceed for 25 to 35 cycles in 25-μl aliquots of reaction mixture containing 1×polymerase reaction buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$), 0.2 mM deoxynucleotide triphosphates, 0.625 U of EX *Taq* DNA polymerase (Applied Biosystems, Foster City, CA), 50 and 20 ng of human and rat cDNA, respectively, and 5 pmol of the specific primers. The following temperature program was used: initial denaturation (94°C) for 2 min, 94°C for 30 s, 52°C/human, 58°C/rat for 30 s, 72°C for 30 s for 25–35 cycles, and a final extension at 72°C for 10 min. A two-step temperature program was applied for the amplification of rGAPDH: initial denaturation (94°C) for 2 min, 94°C for 30 s, 68°C for 2 min for 25 cycles, and a final extension at 68°C for 7 min. Aliquots (10 μl) of amplified cDNA products were separated by electrophoresis using 1–1.5% agarose gels. Gels were stained with ethidium bromide and visualized under UV light.

CTL1 Small Interfering RNA (siRNA) and siRNA Transfection

Three kinds of siRNA (SI00342468, SI00342475 and SI00342482) targeted to different regions of hCTL1 gene and negative control siRNA AlexaFluor 488 used as negative control (1022563) were purchased from Qiagen (Basal, Switzerland). A549 cells (5.0×10^4 cells/well) were seeded

Table I. Specific Oligonucleotide Primer Pairs

Transporter	Forward (5′–3′)	Reverse(5′–3′)	Ref
OCT1	CGATCATGTACCAGATGGCC	TCTTCATCCCTCCAACACGAC	(36)
OCT2	GATTTCTTCTACTCTGCCCTGGTT	GGATTTCTACTTTTGGTCTTGCTG	(37)
OCT3	GACAAGAGAAGCCCCCAACCTGAT	CACTAAAGGAGAGCCAAAAATGTC	(37)
GAPDH	ACTGGCGTCTTCACCACCAT	TCCACCACCCTGTTGCTGTA	(38)
rOct1	GATCTTTATCCCGCATGAGC	CTTCTGGGAATCCTCCAAGT	
rOct2	CATCGAGGATGCCGAGAA	ACAGACCGTGCAAGCTAC	
rOct3	TCAGAGTTGTACCCAACGACATT	TCTGCCACACTGATGCAACT	
rCTL1	GGCAGTGCTGTTCAGAATGA	ACAGGAAGCAATGAGCGACT	(39)
rGAPDH	TGAAGGTCGGTGTGAACGGATTTGGC	CATGTAGGCCATGAGGTCCACCAC	

into normal 24-well plates (BD Biosciences) and grown for 24 h. Three μl of HyperFect Reagent (Qiagen), 100 μl of F12-K FBS free medium and siRNA (25 nM) were mixed, incubated at room temperature for 5 min and added to cells. After 96 h, cells were used for analysis and experimentation. A549 cells transfected with siRNA were washed with sterile PBS and total RNA was extracted with Isogen reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. After deoxyribonuclease treatment, total RNA (1 μg) of A549 cells was reverse-transcribed using Improm-II™ reverse transcriptase (Promega, Madison, WI) and oligo (dT). A mixture of 1 μl of oligo (dT) (0.5 μg/μl) and total RNA (1 μg) was incubated at 70°C for 5 min, then rapidly chilled at 4°C for 5 min. For conversion of total RNA to cDNA, the reaction mixture (20 μl) was prepared containing the mixture of oligo (dT) and total RNA, 4 μl of 5×RT buffer, 2.4 μl of 25 mg $MgCl_2$, 4 μl of 2.5 mM dNTP mix, 0.5 μl of Rnasin (1 U/μl), 1 μl of Improm-II reverse transcriptase and 3.1 μl of diethylpyrocarbonate-treated water. The reaction was carried out at 25°C for 5 min, 42°C for 60 min and 70°C for 15 min. RT reaction mixtures, including cDNA products, were stored at −20°C until used. A single cDNA produced from total RNA was amplified by quantitative PCR with FullVelocity SYBER Green QPCR Master Mix (Strategene, Tokyo, Japan KK) and specific primers for CTL1 (QT00075838, Qiagen) and GAPDH (forward: '5-GAAGGTGAAGGTCGGAGT-3' and reverse: '5-GAAGATGGTGATGGGATTTC-3'). A two-step temperature program was applied for the amplification of hCTL1 and GAPDH: initial denaturation (95°C) for 10 min, then 95°C for 15 s, 60°C for 60 s for 50 cycles.

Kinetic Analyses

Uptake was expressed as the cell-to-medium ratio (μl/mg protein) obtained by dividing the uptake amount by the concentration of substrate in the incubation buffer. Specific uptake was obtained by subtracting the uptake at 4°C from the uptake at 37°C. Kinetic parameters were obtained using the following equation (Eq. 1):

$$v = \frac{V_{max} \times S}{K_m + S} \quad (1)$$

where v is the uptake velocity of the substrate (pmol/30 s/mg protein), S is the substrate concentration in the medium (μM), K_m is the Michaelis constant (μM) and V_{max} is the maximum uptake rate (pmol/30 s/mg protein). Fitting was performed by the nonlinear least-squares method using a computer program (WinNonlin program, Pharsight, USA). The half-inhibitory concentration (IC_{50}) of inhibitors was obtained by examining their inhibitory effects on the uptake of choline based on Eq. 2.

$$\frac{C}{M_{+I}} = \frac{C}{M} \Big/ \left(1 + \left(\frac{1}{IC_{50}}\right)^H\right) \quad (2)$$

where C/M and C/M_{+I} represent the uptake clearance in the absence and presence of inhibitor, respectively, H is the slope factor and I is the concentration of inhibitor. IC_{50} values were estimated by the nonlinear least-squares method using a computer program (WinNonlin program, Pharsight, USA).

Statistical Analysis

Student's t-test was used to assess the significance of differences between two sets of data. Differences were considered to be statistically significant when p was <0.05.

RESULTS

Inhibitory effect of gefitinib on PC biosynthesis in A549 and primary-cultured rat ATII cells. To examine whether gefitinib reduces PC biosynthesis in A549 and primary-cultured rat ATII cells, the effect of gefitinib on the CDP-choline pathway was examined. In both A549 and rat ATII cells, [^{3}H]choline was incorporated into PC in a time-dependent manner over 3 h (Fig. 2a and d). Twenty and 50 μM gefitinib reduced the apparent uptake of [^{3}H]choline to 53.6±2.36 and 70.7±4.50% in A549 and rat ATII cells, respectively, compared to the values in the absence of gefitinib (Fig. 2b and e). In addition, the radioactivity in the chloroform phase from A549 and rat ATII cells, containing only PC, was decreased by 20 and 50 μM gefitinib to 57.7±7.33 and 64.1±2.56%, respectively (Fig. 2b and e). Furthermore, preincubation of 20 and 50 μM gefitinib with those cells for 60 min exhibited stronger reductions of apparent uptake and incorporation into PC of [^{3}H]choline (uptake by the cells: 47.9±3.11 and 11.1±0.794%; PC biosynthesis: 30.5±7.90 and 1.44±0.131% for A549 and rat ATII cells, respectively) (Fig. 2c and f). These results demonstrated that gefitinib inhibits the apparent uptake of choline and the subsequent biosynthesis of PC, and its effect is significantly increased when the cells are pretreated with the drug.

Characteristics of [^{3}H]choline transport in A549 and primary-cultured rat ATII cells. [^{3}H]Choline was taken up linearly by A549 cells up to 1 min, and the uptake showed temperature dependence (37°C: 40.2±4.12 μl/30 s/mg, 20°C: 21.5±1.57 μl/30 s/mg, 4°C: 8.12±1.00 μl/30 s/mg). The initial uptake of choline by A549 cells was studied over the concentration range from 0.1 to 50 μM. The Eadie-Hofstee plot showed a single saturable component and the apparent K_m and V_{max} values were 15.0±1.15 μM and 314±18.2 pmol/30 s/mg, respectively. Similarly, [^{3}H]choline was taken up linearly by rat ATII cells up to 5 min and the initial uptake at 2 min was temperature-dependent (37°C: 54.1±3.25 μl/2 min/mg, 4°C: 16.7±3.42 μl/2 min/mg). The temperature-dependent [^{3}H]choline uptake was almost completely inhibited by unlabeled 100 μM choline, showing that the uptake is saturable ([^{3}H]choline alone (1 μM): 37.4±3.25 μl/2 min/mg, with unlabeled choline 10 μM: 18.9±3.37 μl/2 min/mg, 100 μM: 1.66±2.73 μl/2 min/mg). Decrease of Na^+ and K^+ in the incubation media reduced the uptake of [^{3}H]choline by A549 cells (Fig. 3a, b and d). The uptake of [^{3}H]choline by A549 cells was found to be markedly increased by changing the extracellular pH from 5.5 to 8.5 (Fig. 3c). [^{3}H]Choline uptake by rat ATII cells was weakly reduced by replacement from Na^+ to NMG^+ in the incubation media as well as A549 cells (Fig. 3e). The uptake of [^{3}H]choline was not affected by corticosterone, histamine or MPP^+, which are substrates and

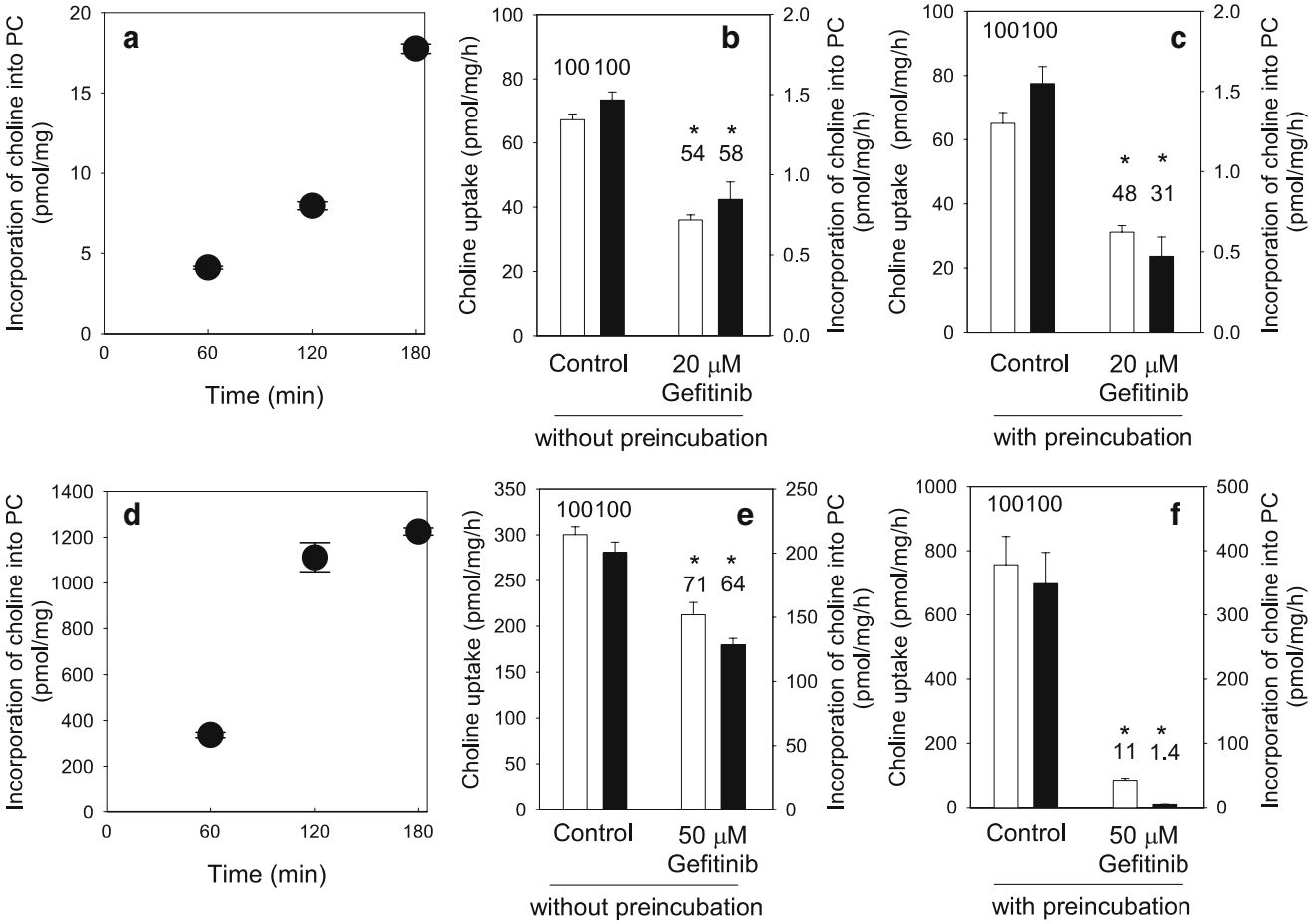

Fig. 2. Time profile of incorporation of [^{3}H]choline into PC in A549 cells (**a**) and rat primary-cultured ATII cells (**d**), and inhibitory effect of gefitinib on the apparent uptake and incorporation of [^{3}H]choline into PC by A549 (**b** and **c**) and rat primary-cultured ATII cells (**e** and **f**) with (**c** and **f**) or without preincubation (**b** and **e**). The concentration of [^{3}H]choline was 0.1 μM. (**b**, **c**, **e**, **f**) *Open* and *filled bars* indicate the apparent uptake by the cells and PC in the chloroform phase (which corresponds to incorporation into PC), respectively. Values above bars are percentages of apparent uptake and PC in the chloroform phase in the absence of gefitinib. The inhibitory effect of gefitinib on the incorporation of [^{3}H]choline into PC by A549 and rat ATII cells was measured at 37°C for 1 h. The preincubation of A549 and rat ATII cells with gefitinib for 1 h was carried out prior to the PC biosynthesis. * $p<0.05$ compared with control. Each point and bar represents the mean ± S.E. from at least 3 wells.

inhibitors of human OCT1, 2, and 3, whereas HC-3, a choline analogue, inhibited the [^{3}H]choline uptake by A549 and rat ATII cells in a concentration-dependent manner with IC_{50} values of 9.48±1.06 μM (Fig. 4a) and 79.9±17.9 μM (Fig. 4b), respectively.

Identification of transporters expressed in A549 and primary-cultured rat ATII cells. To investigate what transporter molecules are expressed in A549 and primary-cultured rat ATII cells and to compare the expression profile in A549 and rat ATII cells with those in human bronchial epithelial Calu-3 cells, lung, trachea, liver and kidney and in rat lung, liver and kidney (Fig. 5), the expression of transporter mRNAs was examined by RT-PCR. The experimental conditions used for RT-PCR were validated by demonstrating the formation of RT-PCR products of the expected sizes for OCTs and CTLs with total RNAs of liver and kidney from rat and human as positive controls. Under these conditions, RT-PCR did not show the expression of human or rat OCT2 in A549, Calu-3, lung, trachea, rat ATII cells and or rat lung. In rat ATII cells, very weak mRNA expression of OCT1 was observed. The expression of human and rat OCT3 was observed in all tissues examined, except for human liver. The expression of human CTL1 mRNA was confirmed in all tissues, but the expression levels in trachea, liver and kidney were very low. The expression of rat CTL1 mRNA was observed in all tissues examined. Although mRNA of CTL2 was found in Calu-3, lung, trachea, liver and kidney, it was not detected in A549 cells.

Effect of hCTL1 siRNA on hCTL1 mRNA expression and [^{3}H]choline uptake in A549 cells. We investigated whether hCTL1 knockdown induced any change in hCTL1 mRNA expression and [^{3}H]choline transport in A549 cells (Fig. 6a and b). Firstly, we checked whether the negative control siRNA from Qiagen was suitable as a negative control for hCTL1. The negative control siRNA did not decrease hCTL1 mRNA expression compared with that in the cells transfected only with the transfection reagent, at 48, 72 and 96 h after transfection (data not shown). Thus, we used this siRNA as the negative control in the present study. The hCTL1 mRNA expression level and [^{3}H]choline uptake by A549 cells were determined 96 h after siRNA transfection in A549 cells. The expression of hCTL1 mRNA in A549 cells

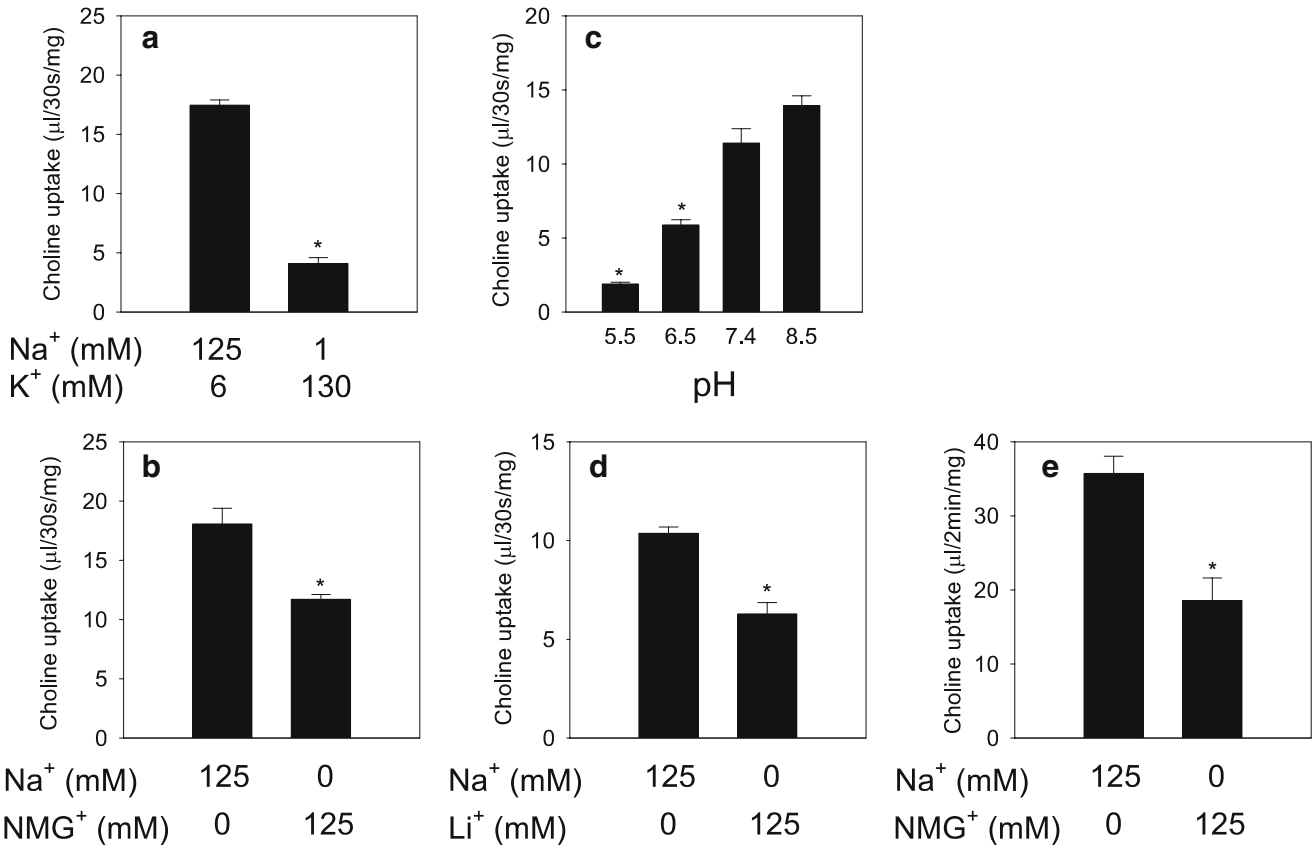

Fig. 3. Effect of membrane potential (**a**), Na^+ (**b**, **d** and **e**), and pH (**c**) on the uptake of [^{3}H]choline by A549 cells and rat AT II cells. The substrate concentration was 0.1 μM. Transporter-mediated uptake of [^{3}H]choline in A549 and rat ATII cells was calculated by the subtraction of uptake at 4°C from that at 37°C for 30 s and 2 min, respectively. **a** Uptake of [^{3}H]choline was measured in A549 cells in the presence of increasing and decreasing concentrations of KCl (6–130 mM) and NaCl (125–1 mM), respectively, at pH 7.4. **b** and **d** Uptake of [^{3}H]choline was measured in A549 cells (**b** and **d**) and rat ATII cells (**e**) in Na^+-free buffer. In Na^+-free buffer, the NaCl in the incubation buffer was replaced with N-methylglucamine (NMG) and lithium chloride at pH 7.4. **c** Uptake of [^{3}H]choline was measured in A549 cells in the various pH of the incubation buffer varied by adjusting the concentrations of Mes, Hepes and Tris. * $p<0.05$ compared with the uptake in normal incubation buffer at pH 7.4. Each bar represents the mean ± S.E. from at least 3 wells.

and the [^{3}H]choline uptake by A549 cells were decreased by the transfection of three different siRNAs to 38.6–66.4% and 34–50%, respectively, compared with the values obtained with the negative control siRNA. To examine whether transporter(s) other than CTL1 are involved in choline transport in A549 cells, we investigated the effect of HC-3 on the choline uptake by negative control and hCTL1 siRNA-treated A549 cells. CTL1 mRNA expression was decreased to about 15% of that in negative control siRNA-treated A549 cells. The choline uptake in negative control siRNA-treated A549 cells was significantly inhibited by HC-3 but that in CTL1 siRNA-treated A549 cells was hardly inhibited (Fig. 7).

Inhibitory effect of gefitinib on [^{3}H]choline transport in A549 and primary-cultured rat ATII cells. The inhibitory effect of gefitinib on the initial uptake of [^{3}H]choline was evaluated in A549 and primary-cultured rat ATII cells. Gefitinib strongly inhibited [^{3}H]choline uptake by A549 and rat ATII cells with IC_{50} values of 19.9±2.07 and 85.8±13.1 μM, respectively (Fig. 8a and b). When A549 and rat ATII cells were preincubated with various concentrations of gefitinib for 60 min, the inhibitory effect of gefitinib on the choline uptake was significantly increased, and the IC_{50} values became 6.77±0.604 and 10.5±0.728 μM, respectively (Fig. 8a and b). We assessed the interaction between gefitinib and choline uptake in A549 cells with and without preincubation of A549 cells with gefitinib. In the absence of preincubation of A549 cells with gefitinib, the plots of the choline uptake in the absence and presence of gefitinib almost intersected at the horizontal axis (Fig. 9a). On the other hand, when the cells were preincubated with gefitinib, the plots did not intersect at the horizontal axis (Fig. 9b). The kinetic parameter (Km and Vmax) values without and with preincubation are shown in Table II.

DISCUSSION

Gefitinib is an anticancer agent that acts as a selective inhibitor of EGFR-TK. Gefitinib has been associated with sudden and normal onset of severe ILD with the low incidence of 1% worldwide (19). However, the molecular mechanisms of both sudden and normal onset of ILD associated with gefitinib treatment remain largely uncharacterized.

PC is synthesized from choline within ATII epithelia and secreted into the alveolar lumen via lamellar bodies as the major phospholipid component of lung surfactant, which is essential to establish normal breathing. ABCA3 recognizes

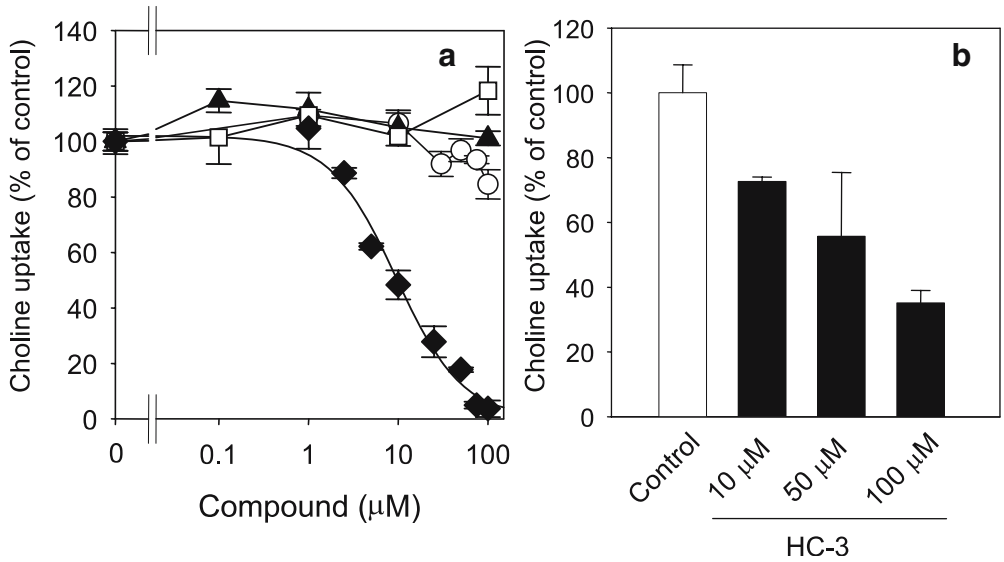

Fig. 4. Effect of various compounds on the uptake of [^{3}H]choline by A549 (**a**) and rat ATII cells (**b**). The substrate concentration was 0.1 μM. Transporter-mediated uptake of [^{3}H]choline by A549 and rat ATII cells was expressed as the difference between the uptakes at 37 and 4°C. Data are shown as percentages of the transporter-mediated uptake of [^{3}H]choline in the absence of inhibitors. *Squares*, *triangles*, *circles* and *diamonds* represent the uptake of [^{3}H]choline in the presence of histamine, corticosterone, MPP^+ and HC-3, respectively. *Solid lines* represent the fitted curves obtained by nonlinear regression analysis. Each point represents the mean ± S.E. from at least 3 wells.

PC as a substrate and transports PC into lamellar bodies in ATII cells (20,21). Mutation of ABCA3 is reported to cause neonatal respiratory distress syndrome and ILD (22), and ABCA3 knock-out mice die within an hour after birth (23). It is considered that the cause of the neonatal respiratory distress syndrome and death of the mice is the lack of secretion of PC into alveolar space due to the decreased ABCA3 activity. Furthermore, changes in PC content are significantly correlated with loss of surface activity in patients with ILD (24).

To elucidate the mechanism of ILD associated with gefitinib, we focused on lung surfactant synthesis and tested the hypothesis that gefitinib reduces PC biosynthesis via inhibition of cellular choline uptake, resulting in abnormality of lung surfactant. We also examined that choline transport in A549 and primary-cultured rat ATII cells, and found that it is mediated by a weakly Na^+-dependent transporter, which has transport characteristics similar to those of human and rat CTL1.

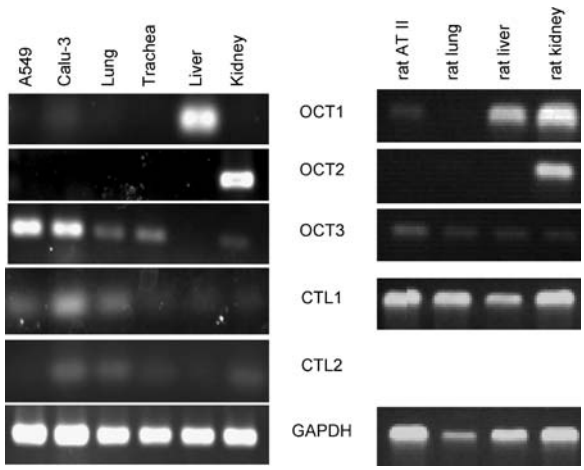

Fig. 5. Expression of several transporter mRNAs in A549 cells, Calu-3 cells, lung, trachea, liver, kidney, primary-cultured rat ATII cells, rat lung, rat liver and rat kidney. The reactions using specific primer sets were described in the text. PCR products were separated by electrophoresis in 1–1.5% agarose gel and ethidium bromide was used to visualize bands.

Firstly, we investigated whether gefitinib impairs the biosynthesis of PC in A549 and rat ATII cells. The apparent uptake and incorporation into PC of externally added [^{3}H]choline by A549 and rat ATII cells was markedly reduced by 20 and 50 μM gefitinib (Fig. 2b and e). Twenty and 50 μM gefitinib reduced the apparent uptake of [^{3}H]choline by A549 and rat ATII cells to about 50 and 70%, which was comparable to the values of 57.7 and 64.1% for incorporation into PC in the respective cells. These results suggested that 20 and 50 μM gefitinib may not affect three enzymes involved in the CDP-choline pathway but choline uptake. In rats, the total radioactivity related to [^{14}C]gefitinib in lung did not change greatly up to 6 h (25). To check the effect of gefitinib existing in lung for hours on the incorporation of choline into PC, we evaluated the inhibitory effects of gefitinib after a 60 min preincubation of A549 and rat ATII cells with gefitinib. The incorporation of choline into PC in A549 and rat ATII cells was more strongly inhibited by gefitinib than those in the absence of preincubation (Fig. 2c and f). The inhibitory effect was also observed on the apparent uptake of [^{3}H]choline by both cells. These observations with or without preincubation suggest that the choline uptake process largely regulates the incorporation of choline into PC in A549 and rat ATII cells.

To characterize the choline transport regulating PC biosynthesis in A549 and rat ATII cells, we performed choline uptake studies by both cells. [^{3}H]Choline was taken up into A549 cells in a time-, temperature- and concentration-

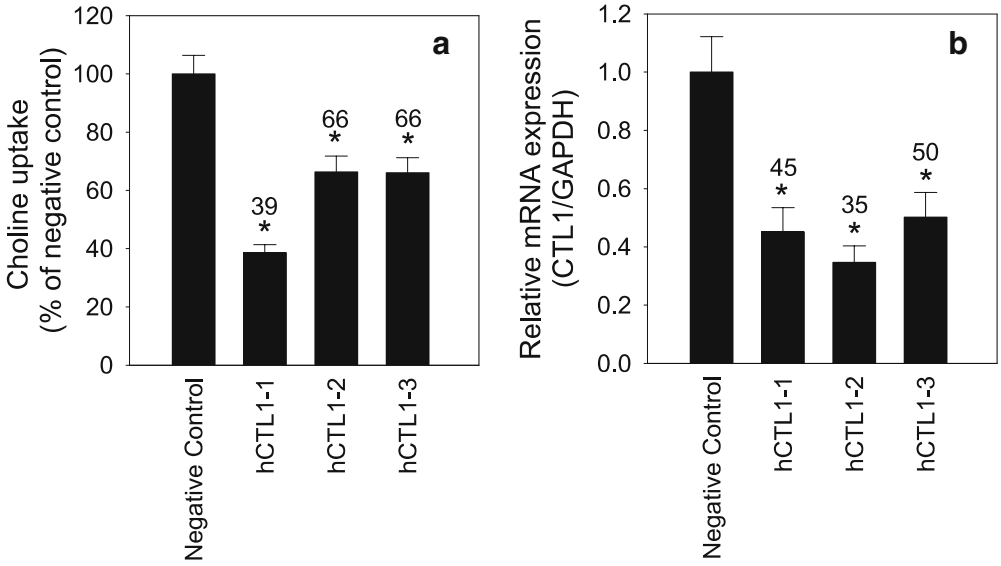

Fig. 6. Effect of hCTL1 siRNA on [^{3}H]choline uptake (**a**) and hCTL1 mRNA expression (**b**) in A549 cells. [^{3}H]Choline uptake (0.1 μM) was evaluated at 30 s. Transporter-mediated uptake of [^{3}H]choline was expressed as the difference between the uptakes at 37 and 4°C. Quantitative RT-PCR using specific primer sets were done as described in the text. The histogram shows hCTL1 mRNA knockdown data as the hCTL1/GAPDH ratio. Values above bars are percentages of choline uptake and CTL1 mRNA in the negative control siRNA-treated A549 cells. Each point represents the mean ± S.E. of at least 3 wells. * $p<0.05$ compared with the negative control siRNA.

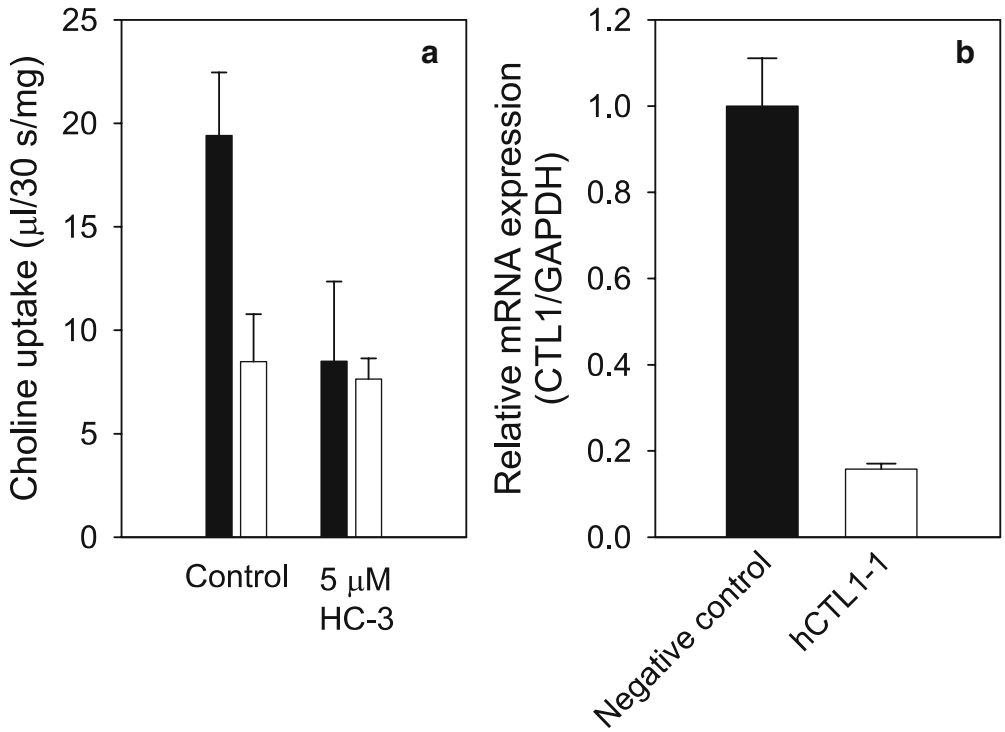

Fig. 7. Effect of HC-3 on the uptake of [^{3}H]choline by negative control siRNA- and CTL1 siRNA-treated A549 cells. The substrate concentration was 0.1 μM. **a** Transporter-mediated uptake of [^{3}H]choline by A549 cells was expressed as the difference between the uptakes at 37 and 4°C. The *filled* and *open bars* represent choline uptake by negative control and CTL1 siRNA-transfected A549 cells, respectively. **b** Quantitative RT-PCR using specific primer sets were done as described in the text. hCTL1 mRNA knockdown data was shown as the hCTL1/GAPDH ratio. Each point represents the mean ± S.E. from at least 3 wells.

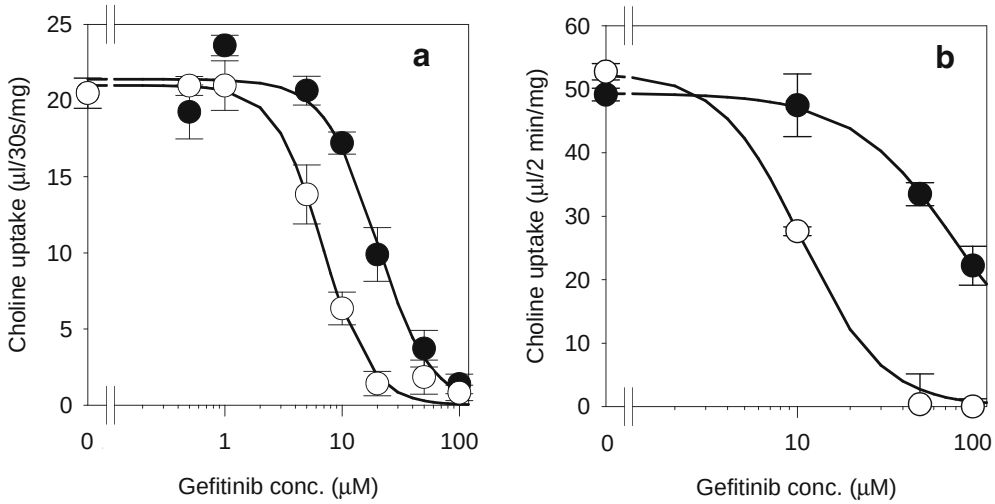

Fig. 8. Inhibitory effect of gefitinib on [^{3}H]choline uptake by A549 (**a**) and rat primary-cultured ATII cells (**b**) with or without preincubation. Transporter-mediated uptake of [^{3}H]choline was expressed as the difference between the uptakes at 37 and 4°C. *Closed* and *open circles* represent the transporter-mediated uptake of [^{3}H]choline in the absence and presence of preincubation of A549 and rat ATII cells with gefitinib, respectively. [^{3}H]Choline (0.1 μM) was incubated with various concentrations of gefitinib (0.5–100 μM for A549 cells and 10–100 μM for rat ATII cells) for 30 s and 2 min, respectively. The *solid line* represents the mean ± S.E. from at least 3 wells.

dependent manner, demonstrating that the uptake is transporter-mediated. For further characterization of [^{3}H]choline transport, the time of 30 s was used for the evaluation of initial uptake in A549 cells. The K_m value (15 μM) in A549 cells is about 10 times higher than that of CHT1 (1.6 μM) (26), and comparable to those of CTL1 and OCT2 (53–102 μM) (9,27). The uptake of [^{3}H]choline by A549 cells showed membrane potential-, pH-, and weak Na^+-dependence (Fig. 3). These transport properties of choline uptake in A549 cells are similar to those of OCTs and CTLs (27,28). A choline analogue, HC-3, reduced the choline uptake by A549 cells with the IC_{50} value of 9.48 μM, whereas corticosterone, histamine and MPP^+, which are substrates and inhibitors of OCT1, 2, and 3, were not inhibitory up to 100 μM (Fig. 4). RT-PCR analysis revealed the expression of OCT3 and CTL1 mRNAs in A549 cells (Fig. 5). Based on the results that the IC_{50} value of HC-3 for choline transport in A549 cells was much higher than that for CHT1-mediated choline transport (5 nM) (26), that OCT3 substrate and/or inhibitors did not inhibit choline uptake by A549 cells, and that OCT3 hardly recognizes choline as the substrate (9), the involvements of CHT1 and OCT3 in choline transport in the cells were considered to be negligible. The three different siRNAs targeted to the three different regions of the hCTL1 gene reduced both hCTL1 mRNA expression and choline transport activity in A549 cells to about 50%, indicating that the contribution of hCTL1 to transporter-mediated choline uptake in A549 cells is at least 50% (Fig. 7). On the basis of the

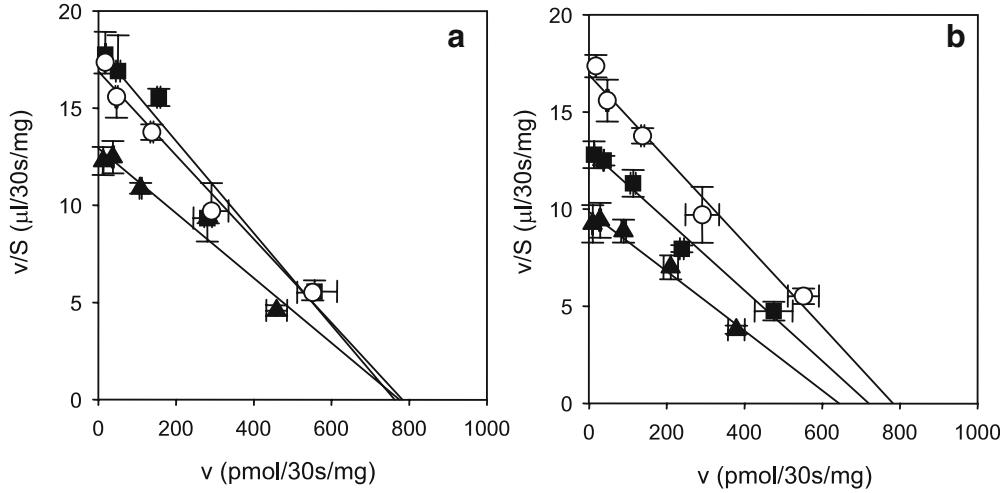

Fig. 9. Eadie–Hofstee plots of the interaction between choline and gefitinib without (**a**) or with (**b**) preincubation of A549 cells. Concentration dependent uptake of [^{3}H]choline (1–100 μM) was examined at 30 s in the absence (*circles*) or presence of 3 μM (*squares*) and 10 μM (*triangles*) gefitinib. Transporter-mediated uptake of [^{3}H]choline was expressed as the difference between the uptakes at 37 and 4°C. The *solid line* represents the fitted curve. Each point represents the mean ± S.E. from at least 3 wells.

Table II. Kinetic Parameters of Choline Uptake by A549 Cells in the Presence and Absence of Gefitinib

Gefitinib	Preincubation (−)		Preincubation (+)	
	Km[a]	Vmax[b]	Km[a]	Vmax[b]
+ 0 μM	43.4±6.54	717±61.6	43.4±6.54	717±61.6
+ 3 μM	39.1±6.37	688±70.1	52.4±6.10	681±46.9
+ 10 μM	56.2±6.48	732±53.3	70.0±10.6	650±62.3

[a] (μM), [b] (pmol/30 s/mg)

transport properties, inhibitory effects of various compounds on [^{3}H]choline transport in A549 cells, effect of hCTL1 siRNA and the transporter molecules identified by RT-PCR, we consider that choline transport in A549 cells is mainly mediated by CTL1, which is known to be membrane potential-, pH-dependent and weakly Na^+-dependent, and to have affinity for choline and HC-3 (27). In CTL1 siRNA-treated A549 cells, 5 μM HC-3, which is a concentration close to IC_{50} of HC-3 on CTL1-mediated choline transport, did not exhibit the inhibitory effect on choline uptake compared to that observed in negative control siRNA-treated A549 cells. This result indicates that CTL1 mainly contributes to the uptake of choline, while the involvement of transporter(s) other than CTL1 cannot be excluded in choline transport by A549 cells. Further experiments will be required to identify the transporter molecule(s) involved.

The mechanism of choline uptake into rat ATII cells was reported previously (29). In this study, we confirmed that the profile of choline uptake in our primary-cultured rat ATII cells was similar to that previously reported. [^{3}H]Choline was taken up by rat ATII cells time-dependently for up to 5 min. The initial uptake of choline evaluated at 2 min showed weak Na^+-dependence. The Km value of choline uptake by rat ATII cells was predicted to be 10–100 μM and the IC_{50} of HC-3 for the choline uptake by rat ATII cells was estimated to be 79.9 μM. These results are comparable with reported data (29). Furthermore, CTL1 mRNA expression in primary-cultured rat ATII cells was confirmed. Accordingly, we consider that choline transport in rat ATII cells is mainly mediated by a transporter which is similar to that in A549 cells and to CTL1, as reported previously (29).

In human lung, the mRNA expression of CTL1, CTL2 and OCT3 was confirmed, whereas CTL1 and OCT3 mRNAs, but not CTL2 mRNA, were found in A549 cells. There is a discrepancy in CTL2 mRNA expression between human lung and A549 cells. This discrepancy can be explained by the fact that the lung is composed of many types of cells, and only 2 to 5% of the alveolar space is occupied by ATII cells (30). In addition, the mRNA expression patterns of the transporters examined were similar in human and rat. Considering the occupancy of ATII cells in human lung, and the transporter expression pattern and transport properties of choline in rat ATII cells, A549 cells seem to have a similar choline transport system to intact human pulmonary ATII cells.

Finally, we further characterized the inhibitory effect of gefitinib on choline transport in A549 and rat ATII cells. Gefitinib inhibited [^{3}H]choline uptake with the IC_{50} values of 19.9 μM in A549 cells and 85.8 μM in rat ATII cells (Fig. 8). Furthermore, preincubation of A549 and rat ATII cells with gefitinib reduced the IC_{50} values to 6.77 and 10.5 μM, respectively (Fig. 8). The interaction between gefitinib and choline was kinetically studied. The Eadie–Hofstee plots revealed that the modes of inhibition of choline uptake into A549 cells by gefitinib were competitive and mixed type for A549 cells with and without preincubation with gefitinib, respectively. Gefitinib has a similar chemical structure to choline (Fig. 1). Based on the inhibitory kinetics by gefitinib with and without preincubation, it is considered that CTL1 has at least two binding sites (possibly extracellular and intracellular or intramembrane), and the concentrations at these sites are both important determinants of the inhibitory effect on CTL1 activity. Preincubation also had a similar effect on the extent of inhibition of PC biosynthesis. Accordingly, it is considered that the reduction of PC biosynthesis caused by the preincubation of A549 and rat ATII cells with gefitinib is caused largely by the reduction of choline uptake process.

The maximum plasma total concentration of gefitinib observed at a clinically relevant dose is 0.5 to 1 μM or higher, but the plasma concentration shows inter-individual variability amounting to 6- to 10-fold among patients (31–34). Considering that about 90% of gefitinib in blood is bound to plasma proteins, that the total radioactivity of gefitinib is concentrated in the lung with a ratio higher than 10, and that the total radioactivity in lung does not change greatly for up to 6 h in rats (25), the concentration of gefitinib in the lung can be estimated to be about 1 μM, and this level may be maintained in the lung for several hours. This gefitinib concentration in lung is close to the IC_{50} value of 6.77 μM obtained in the present study. When CYP3A4-mediated gefitinib metabolism, which is a major clearance pathway of gefitinib, is inhibited by the potent CYP3A4 inhibitor itraconazole, the gefitinib plasma concentration is increased (35). For cancer therapy, gefitinib will be co-administered with a range of other drugs. If the major metabolic pathway and the transport process of gefitinib are inhibited by co-administered drugs and/or altered by genetic polymorphisms of the involved enzymes and transporters, the unbound gefitinib concentration in the lung may exceed the IC_{50} value, resulting in impaired PC biosynthesis that may lead to abnormality of lung surfactant. Accordingly, it is probable that sudden onset of ILD associated with gefitinib is due to an abnormality of the production of lung surfactant.

In conclusion we demonstrated that gefitinib reduces PC biosynthesis via the inhibition of choline uptake by A549 and primary-cultured rat ATII cells. We also showed that choline transport by A549 and rat ATII cells is mainly mediated by CTL1, which is a membrane potential-, pH-dependent and weakly Na^+-dependent transporter. These results may have implication for the one of mechanisms of drug-induced lung toxicity.

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