Dexamethasone Increases β_2 -Adrenoceptor-Regulated Phosphatidylcholine Secretion in Rat Alveolar Type II Cells

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ABSTRACT—Insufficient production of pulmonary surfactant in alveolar type II cells is relevant to many lung diseases. To cure its deficiency, glucocorticoid is commonly used in clinical areas. In the present study, we investigated the effect of dexamethasone on the secretion of phosphatidylcholine, a major phospholipid of pulmonary surfactant, in a primary culture of rat alveolar type II cells. Dexamethasone had no effect on the basal secretion rate of phosphatidylcholine. Dexamethasone augmented both the phosphatidylcholine secretion and the cyclic AMP formation increased by terbutaline. Furthermore, dexamethasone increased the number of β -adrenoceptors and mRNA expression of β_2 -adrenoceptors in type II cells. These findings indicate that dexamethasone increases pulmonary surfactant secretion through an enhancement of β_2 -adrenoceptor gene expression.

Keywords: Alveolar type II cell, Glucocorticoid, β -Adrenoceptor, Pulmonary surfactant, RNase protection assay

Insufficient production of pulmonary surfactant in alveolar type II cells can lead to the respiratory distress syndrome in newborn infants because the surfactant reduces surface tension at the air-liquid interface in the lung and provides alveolar stability. In addition, surfactant deficiency or abnormality is also believed to contribute to the pathophysiological changes observed in adult lung diseases (1, 2). To repair the insufficient surfactant in the lung, glucocorticoids seem to be the most effective among the few useful drugs. For example, glucocorticoids increase enzymatic activities of surfactant phospholipid synthesis; i.e., choline-phosphate cytidyltransferase and fatty acid synthetase activities (3). Furthermore, dexamethasone increases synthesis of surfactant proteins, SP-A, SP-B, SP-C and SP-D (4, 5). These findings focused on the effect of glucocorticoid on the synthesis of pulmonary surfactant in alveolar type II cells. However, there are very few findings that a glucocorticoid can affect the secretion of pulmonary surfactant.

Based on recent observations, glucocorticoid is likely to affect both secretion and synthesis by independent mechanisms in alveolar type II cells. Phorbol esters increase pulmonary surfactant secretion but decrease its synthesis (6). Additionally, in feline tracheal submucosal glands, glucocorticoid inhibited glycoconjugate secretion

without a significant alteration in synthesis (7). Therefore, we examined the effect of dexamethasone on the secretion of phosphatidylcholine, which is a major phospholipid of pulmonary surfactant in a primary culture of rat alveolar type II cells. Especially, we have focused on the effect on β -adrenergic agonist-induced surfactant secretion, since we had assumed that surfactant secretion may be increased by glucocorticoids through the enhancement of β -adrenoceptor gene expression (8).

MATERIALS AND METHODS

Materials

Dexamethasone and terbutaline sulfate were purchased from Sigma (St. Louis, MO, USA). Methyl- ^3H choline chloride (specific activity: 2.22–3.33 TBq/mmol) was from New England Nuclear (Boston, MA, USA), ^{125}I -iodocyanopindolol (ICYP) (specific activity: 74 TBq/mmol) and $[\alpha\text{-}^{32}\text{P}]$ cytidine 5'-triphosphate (CTP) (specific activity: 30 TBq/mmol) were from Amersham (Amersham, UK). Fetal bovine serum was from JHR Bioscience (Lenexa, KS, USA). RU-486 was a kind gift from Dr. H. Okamura, Medical School of Kumamoto University.

Alveolar type II cells isolation and culture

Alveolar type II cells were isolated from the lungs of

adult pathogen-free male Wistar rats (180–200 g), as described previously (9). Briefly, trypsin was used to dissociate the cells from the lung tissues. The resultant cell suspension was incubated on rat IgG-coated plastic petri dishes for 30 min to remove the non-type II cells. The isolated alveolar type II cells were suspended at 1.0×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamate, 100 units/ml penicillin, 100 μ g/ml streptomycin and 10% fetal bovine serum that was charcoal-dextran treated for depletion of glucocorticoids. This procedure routinely yielded 1.0×10^7 cells/rat. The cells were dispensed onto plastic culture dishes at a density of 4×10^5 cells/cm² and cultured in 5% CO₂/95% air at 37°C. In all experiments, nonadherent cells were removed from the dishes by washing with DMEM after 24-hr cultivation.

Phosphatidylcholine secretion

Secretion of phosphatidylcholine, the major component of pulmonary surfactant, by cultured alveolar type II cells was determined as follows: The cells were cultured with 74 kBq/ml [methyl-³H]choline for 24 hr. After this cultivation, cells were rinsed with fresh serum- and antibiotics-free DMEM to remove [³H]choline and unattached cells. These [³H]choline labeled and washed cells were incubated for 90 min with or without terbutaline, one of the most widely used β -adrenoceptor agonists for the phosphatidylcholine secretion assay (10, 11). At the end of this incubation period, the medium was aspirated off, and the cells were lysed with 2 ml ice-cold 0.05% Triton X-100 solution. Lipids were extracted from both cells and medium with the method of Folch et al. (12), and the radioactivity of the lipids was measured with a liquid scintillation counter. The secretion rate was expressed as the amount of [³H]phosphatidylcholine in the medium after the 90-min incubation, as a percentage of that in the cells plus medium.

To assess cellular integrity, the activity of lactate dehydrogenase (LDH) in the cells and medium was measured with an LDH Kit-S (Nippon Shoji Co., Ltd., Osaka). The LDH activity released into the medium did not exceed 1% of the total cell content in all experiments.

Intracellular cyclic AMP

Intracellular cyclic AMP was measured by the method of Brown and Longmore (13). The incubation medium was aspirated at various times after the addition of test agents, and the cells were extracted with 0.1 N HCl. The extracts were immediately frozen, lyophilized and stored at -70°C. The sample was reconstituted and acetylated, and the cyclic AMP content was determined as described by the radioimmunoassay kit manufacturer.

Radioligand receptor binding

Cultured alveolar type II cells were washed as described above, scraped and suspended in lysis buffer (1 mM Tris, 5 mM EDTA). Cells were then disrupted with a teflon homogenizer (15 sec $\times$ 5 times). The homogenate was centrifuged at $1,000 \times g$, for 10 min at 4°C, and the supernatant was then pelleted by spinning at $100,000 \times g$, for 20 min at 4°C. The resulting pellet was resuspended in an isotonic buffer (20 mM Tris, 12.5 mM MgCl₂, 1.5 mM EDTA, 154 mM NaCl; pH 7.5), frozen in liquid nitrogen, and stored at -80°C until use. Sample protein was determined by the method of Bradford (14). Radioligand binding assay was performed as described by Jones et al. (15). The density and affinity of β -adrenoceptor were assessed from saturation isotherms with the use of 12.5–800 pM [¹²⁵I]ICYP. Non specific binding was determined in the presence of 0.1 mM (-)isoprenaline. All studies were performed at 37°C for 80 min in assay buffer (12 mM Tris, 7.5 mM MgCl₂, 92.4 mM NaCl, 0.9 mM EDTA, 0.6 mM ascorbic acid with 0.4% BSA; pH 7.4) containing 50 μ g of crude membrane proteins. The incubation was terminated by rapid filtration through Whatman GF/C glass-fiber filters, followed by 3×5 ml washes with Tris-saline, using a Brandel cell harvester (Biomedical Research and Development Laboratories, Inc., Gaithersburg, MD, USA). The filters were subjected to liquid scintillation spectrometry. Specific binding was calculated by subtracting nonspecific binding from total binding. The maximal binding capacity (B_{max}) and dissociation constant (K_D) were obtained from individual experiments using the computer program Radlig (Biosoft, Cambridge, UK).

β -Adrenoceptor mRNA levels

Total cellular RNAs were extracted from cultured alveolar type II cells by the acid guanidinium thiocyanate-phenol-chloroform method (16). Extracted total RNAs were checked for integrity by staining with ethidium bromide following electrophoretic separation in agarose gel containing formaldehyde. Only undegraded samples were used.

The levels of β_1 - and β_2 -adrenoceptor mRNAs were determined by the RNase protection method as described previously (17). A 172-bp fragment of β_1 -adrenoceptor cDNA, corresponding to nucleotides +774 to +945 of the coding region (18), and a 183-bp fragment of β_2 -adrenoceptor cDNA, nucleotides -1 to +182 (19), were subcloned into pBluescript with a standard method. A 224-nucleotide-long radiolabeled antisense RNA probe for β_1 -adrenoceptor and a 251-nucleotide-long probe for β_2 -adrenoceptor were produced and [α -³²P]CTP labeled from these pBluescript constructs by means of an in vitro transcription kit (Stratagene) according to the supplier's

recommendations.

Total cellular RNA (10 μ g) was hybridized with either β_1 - or β_2 -adrenoceptor radiolabeled antisense RNA probes (1×10^5 cpm) in 80% deionized formamide, 100 mM sodium citrate, 300 mM sodium acetate and 1 mM EDTA for 24 hr at 60°C. After this hybridization, samples were tenfold diluted with a buffer containing 10 mM Tris (pH 7.5), 5 mM EDTA, 300 mM NaCl, 2.5 U/ml RNase A and 100 U/ml RNase T, and were then incubated at 37°C for 30 min.

After this digestion, the RNase-resistant hybrids were precipitated with 7.5% trichloroacetic acid for 10 min on ice and collected by vacuum filtration on Whatman GF/C glass-fiber filters. The filters were washed with 7.5% trichloroacetic acid, dried, and subjected to liquid scintillation spectrometry. The levels of β_1 - and β_2 -adrenoceptor mRNA were quantified by use of a standard curve generated by plotting the amounts of sense RNA, produced by *in vitro* transcription, versus the amount of RNase-resistant hybrids formed by prior hybridization of sense RNA with a radiolabeled antisense RNA probe.

Analyses of results

Data are reported as the mean $\pm$ S.E.M. Duncan's multiple-range test was used for statistical analysis. $P < 0.05$ was considered to be significant.

RESULTS

To examine the effect of glucocorticoid on pulmonary surfactant secretion, we cultured adult rat alveolar type II cells in the presence or absence of dexamethasone for 24 hr. In the cells treated with 1 μ M of dexamethasone, the basal secretion rate of [3 H]phosphatidylcholine ($0.42 \pm 0.06\%$) was similar to that in control cells treated with 0.01% of ethanol. However, terbutaline-increased secretion was significantly greater in the cells treated with dexamethasone than in control cells. The maximal response to terbutaline (10 μ M) was 2 times greater in the dexamethasone-treated cells (Fig. 1A). The enhancement of dexamethasone was concentration-dependent (Fig. 1B).

To examine whether the enhancement is due to an augmentation of β -adrenoceptor functions, propranolol was added 30 min before terbutaline application (Fig. 2). Propranolol blocked the terbutaline-induced secretion in dexamethasone-treated cells as well as in control cells. Furthermore, we measured intracellular cyclic AMP levels after the addition of terbutaline. As shown in Fig. 3, terbutaline caused a rapid rise of cyclic AMP level in the control cells. The rise of cyclic AMP was significantly enhanced in the dexamethasone-treated cells.

To examine the influence of dexamethasone on β -adrenoceptor number, we determined ICYP binding

Fig. 1. Effect of dexamethasone on terbutaline-induced [3 H]phosphatidylcholine secretion by alveolar type II cells. Rat alveolar type II cells were cultured for 24 hr with vehicle (0.01% ethanol, ○), 1 μ M (●) (A) or the indicated concentration (B) of dexamethasone. Following washing of the cells, the indicated concentration (A) or 10 μ M (B) of terbutaline were added and incubated for 90 min. Secretion is reported as the amount of [3 H]phosphatidylcholine (PC) in the medium expressed as the percentage of that in cells plus medium at the end of the incubation period. Each point represents the mean $\pm$ S.E.M. from 5 different experiments. * indicates a significant difference from the vehicle-treated group at $P < 0.05$.

Fig. 2. Effect of propranolol on terbutaline-induced [3 H]phosphatidylcholine secretion by dexamethasone-treated alveolar type II cells. Rat alveolar type II cells were cultured for 24 hr with (■) or without (□) 1 μ M of dexamethasone. Following washing of the cells, 10 μ M of terbutaline was added and the incubated for 90 min. Propranolol was added 30 min prior to the application of terbutaline. Secretion is expressed as the amount of [3 H]phosphatidylcholine in the medium as the percentage of that in cells plus medium at the end of the incubation period. Each bar represents the mean $\pm$ S.E.M. from 4 different experiments. * and # indicate a significant difference from the control and from the terbutaline-treated group at $P < 0.05$, respectively.

Fig. 3. Effect of dexamethasone on terbutaline-induced cyclic AMP formation in alveolar type II cells. Rat alveolar type II cells were cultured for 24 hr with (▲, ●) or without (△, ○) 1 μ M dexamethasone. Following washing of the cells, 10 μ M terbutaline (●, ○) was added and the incubated for the indicated periods. Intracellular cyclic AMP was extracted and determined with a radioimmunoassay kit. Each point represents the mean $\pm$ S.E.M. from 5 different experiments. * indicates a significant difference from the vehicle-treated group at $P < 0.05$.

Table 1. Effect of dexamethasone on β -adrenoceptor number in alveolar type II cells

	K_D (pM)	B_{max} (fmol/mg protein)
Vehicle	44.46 ± 3.18	111.79 ± 1.80
Dexamethasone	37.94 ± 7.22	$201.64 \pm 9.61^*$

The number of β -adrenoceptors was analyzed by [125 I]iodocyanopindolol binding. Each value represents the mean $\pm$ S.E.M. from 4 different experiments. * indicates a significant difference from the vehicle-treated group at $P < 0.05$.

capacity (B_{max}) and its affinity (K_D) in alveolar type II cells (Table 1). The maximal binding capacity of [125 I]ICYP was increased to approximately twofold by the treatment with dexamethasone. However, the affinity of ICYP binding was not changed (Table 1).

Furthermore, we examined the steady state levels of mRNA for β_1 - and β_2 -adrenoceptors both in dexamethasone-treated cells and control cells by the RNase protection assay. In the control cells, the steady state levels of mRNA for β_1 - and β_2 -adrenoceptors were 0.66 ± 0.06 and 1.81 ± 0.17 amol/total cellular RNA, respectively. The level of β_2 -adrenoceptor mRNA was increased by the treatment with dexamethasone in a concentration-dependent manner (Fig. 4). In addition, the increase of β_2 -adrenoceptor mRNA level by dexamethasone was significantly suppressed by the simultaneous

Fig. 4. Effect of dexamethasone on steady state levels of mRNA for β_1 - and β_2 -adrenoceptors. Rat alveolar type II cells were cultured for 24 hr with or without 1 μ M dexamethasone. Total cellular RNA was extracted from cultured cells. The levels of mRNA for β_1 - (□) and β_2 -adrenoceptors (■) were determined by the RNase protection assay. Each bar represents the mean $\pm$ S.E.M. from 5 different experiments. * indicates a significant difference from the vehicle-treated group at $P < 0.05$.

Fig. 5. Effect of RU-486 on β_2 -adrenoceptor mRNA expression increased by dexamethasone. RU-486 (10 μ M) was added to alveolar type II cells simultaneously with the addition of dexamethasone (Dex., 1 μ M). After 24 hr cultivation, the total cellular RNA was extracted and the mRNA level for β_2 -adrenoceptor was determined by the RNase protection assay. Each bar represents the mean $\pm$ S.E.M. from 4 different experiments. * and # indicate a significant difference from the vehicle-treated group and the dexamethasone-treated group at $P < 0.05$, respectively.

application of RU-486, a glucocorticoid receptor antagonist (Fig. 5). In contrast, the β_1 -adrenoceptor mRNA level was not significantly affected by dexamethasone treatment.

DISCUSSION

The major purpose of this study was to determine whether glucocorticoid has any effects on phosphatidylcholine secretion from alveolar type II cells. Our findings have shown here that dexamethasone clearly enhances the terbutaline-induced secretion. In good agreement with the effect on secretion, dexamethasone treatment augmented the increase of intracellular cyclic AMP induced by terbutaline. In addition, the number of β -adrenoceptors and the steady state level of β_2 -adrenoceptor mRNA in alveolar type II cells were increased by the treatment with dexamethasone. It is, therefore, likely that dexamethasone increased the β_2 -adrenoceptor number based on enhancement of the mRNA expression and that the cyclic AMP formation and phosphatidylcholine secretion were enhanced by the increase of receptor number. Previous work had demonstrated that intracellular cyclic AMP is the important second messenger for phosphatidylcholine secretion induced by β -adrenoceptor agonists (9, 10, 13). In this study, the basal levels of phosphatidylcholine secretion and intracellular cyclic AMP were not affected by dexamethasone-treatment. In addition, terbutaline-induced secretion in dexamethasone-treated cells were com-

pletely blocked by propranolol as well as in control cells. Therefore, we assumed that the enhancement of surfactant secretion in dexamethasone-treated cells is based on the enhancement of β -adrenergic signaling systems. We may exclude other possibilities that dexamethasone may enhance surfactant secretion with alterations of the protein processing or a cell membrane property necessary for secretion (20, 21) because the basal secretion rate (Fig. 1) and the increased secretion by phorbol myristate acetate, another type of secretagogue, were not affected by dexamethasone treatment (data not shown).

Dexamethasone increased the mRNA level for only β_2 -adrenoceptor without changing that for β_1 -adrenoceptor. In our previous study, the mRNA levels for β_1 - and β_2 -adrenoceptors corresponded to the data obtained with receptor binding analysis (17). Furthermore, in differentiating 3T3-L1 cells, the changes of β -adrenoceptor numbers were a reflection of changes in the level of mRNA (22). Therefore, we assumed that dexamethasone-induced enhancements in alveolar type II cells were based on enhancement of β_2 -adrenoceptor mRNA transcription rate through the activation of glucocorticoid receptor. As support for this idea, it has been shown that dexamethasone increases the transcription rate of β_2 -adrenoceptor mRNA in human and rat lung tissues (23, 24). In the 5' flanking region of the β_2 -adrenoceptor gene, four glucocorticoid-responsive elements (GREs) in rats (25) and five GREs in humans and mice have been identified (26, 27); GRE forms a complex with glucocorticoid receptors to activate or suppress gene expressions (28). In contrast, only one GRE has been found in the 5' flanking region of the β_1 -adrenoceptor genes of the three species (29–31). The difference in the number of GREs between β_1 - and β_2 -adrenoceptor genes may explain the selective enhancement by dexamethasone against β_2 -adrenoceptor expression in rat alveolar type II cells.

The phosphatidylcholine secretion induced by a β -adrenergic agonist is controlled by a cascade consisting of receptors, G-proteins, adenylate cyclases and cyclic AMP-dependent protein kinases. In our preliminary study, the surfactant secretion induced by a adenylate cyclase activator (forskolin) slightly increased in the dexamethasone-treated cells. Therefore, it is possible that dexamethasone may enhance mRNA expression for not only β_2 -adrenoceptors but also other regulatory proteins in this cascade, although we did not examine the gene expression of other proteins in this study. For example, Michel et al. showed that dexamethasone treatment sensitized cyclic AMP formation by altering the adenylate cyclase catalyst in lymphocytes (32).

In conclusion, the present study revealed that dexamethasone enhanced phosphatidylcholine secretion and that the enhancement may be, at least in part, due to an

increase of β_2 -adrenoceptor mRNA expression. In agreement with our findings, an in vivo study showed that β_2 -adrenoceptor mRNA selectively increased in the lungs when dexamethasone subcutaneously administered to rats (24). Recently, we found that glucocorticoid suppressed the gene expression of mucins in human tracheal epithelial cells (33). Therefore, we consider that glucocorticoids may exert their effects on lung diseases not only by anti-inflammatory actions but also by both suppression of airway mucus production and enhancement of pulmonary surfactant secretion.

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