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Role of neuregulin-1β in dexamethasone-enhanced surfactant synthesis in fetal type II cells

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Running title: NRG1β stimulates surfactant phospholipid synthesis

Abstract

It is well established that glucocorticoids elevate the production of fibroblast-pneumocyte factor (FPF), which induces type II cells to synthesize surfactant phospholipids. FPF, however, has not been identified and it is not clear whether it is a single factor or a complex mixture of factors. In this study it has been shown that, when lung fibroblasts are exposed to dexamethasone, the concentration of neuregulin-1β (NRG1β) in conditioned medium is elevated 2-fold ($p < 0.05$), even though NRG1β gene expression is unaffected. This, together with the finding that exposure of type II cells to NRG1β directly stimulates by 3-fold the rate of phospholipid synthesis ($p < 0.05$), suggests that NRG1β is a component of FPF that promotes lung development.

Introduction

The fetal lung undergoes extensive physiological and biochemical maturation prior to birth in preparation for its postnatal function as an organ for gas exchange. Pulmonary surfactant, a substance that reduces surface tension and prevents alveolar collapse, is produced by type II pneumocytes in the developing lung [1,2]. Any reduction in the ability of type II cells to produce surfactant leads to neonatal respiratory distress syndrome (NRDS) [1]. Synthesis of the major phospholipid component of surfactant, phosphatidylcholine, was shown to be stimulated by glucocorticoids [3] even though they have no effect when directly applied to type II cells. However, when lung fibroblasts were cultured in the presence of glucocorticoids, the resultant fibroblast-conditioned media (FCM) is known to stimulate surfactant production in cultured type II cells [4]. This indirect effect of glucocorticoids upon surfactant phospholipid synthesis was attributed to a fibroblast-derived peptide, termed fibroblast-pneumocyte factor (FPF) [5]. It was subsequently reported that purified FPF was able to directly stimulate the synthesis of surfactant-associated phospholipids [6]. Even though several peptides have been shown to have properties similar to FPF [7-9], the chemical nature of FPF is still not certain.

Torday *et al.* [7] have shown that leptin, a 16 kDa peptide expressed by fetal lung fibroblasts, stimulates *de novo* synthesis of surfactant phospholipids in type II cells. Coupled with the observation that dexamethasone, which is known to stimulate production of FPF, also stimulated expression of leptin mRNA, they concluded that leptin might be FPF [7]. However, neuregulin-1β (NRG1β) has also been shown to play a major role in the development and maturation of the fetal lung. Dammann *et al.* [8] provided evidence that the stimulation of surfactant synthesis in type II cells by media previously conditioned by fibroblasts in the presence of dexamethasone can be mimicked by NRG1β and inhibited by antibodies raised against this peptide.

Neuregulins are part of the epidermal growth factor (EGF) family [10], and interact with several members of the ErbB family of receptors [11]. NRG1β is known to have a role in the early stages of lung development [12], and ErbB receptors are distributed throughout the developing lung [12,13]. Dammann *et al.* showed that dexamethasone, a steroid used to stimulate production of FPF by fibroblasts, also regulated levels of ErbB receptors in the fetal lung [14]. Zscheppang *et al.* used small interfering RNA to show that ErbB4 regulates surfactant phospholipid synthesis in the fetal rat type II cells [15].

Surfactant production is also induced by keratinocyte growth factor (KGF) [16-18], otherwise known as fibroblast growth factor 7. This peptide is a product of lung mesenchymal cells and stimulates both the synthesis of the major component of surfactant, disaturated phosphatidylcholine, as well as the expression of the SP-A, -B and –C genes in a dose-dependent manner [17]. However, although KGF has many of the properties of FPF, its size (28 kDa) [19] is much larger than the 5-15 kDa ascribed to FPF [20]. Thus, in this study the focus was on the specific effects of leptin and neuregulin-1β on surfactant phospholipid synthesis and secretion in cultured fetal type II pneumocytes.

From the above it is clear that NRG1β performs an important role in this system, although the exact details of the mechanism have not been elucidated. In this study we have examined the effect of NRG1β on surfactant phospholipid synthesis over a range of concentrations and exposure times, and have also compared the effects of NRG1β and leptin on this synthesis. In addition, the effect of dexamethasone on NRG1β production by fibroblast cells, including mRNA expression and direct quantification using liquid chromatography-mass spectrometry (LC-MS), was examined.

Materials and Methods

Animals

Nineteen-day pregnant rats of the Wistar strain of *Rattus norvegicus* (22-day gestation period) were supplied by the Animal Resource Centre (Murdoch, Australia). The presence of sperm in a vaginal smear after overnight mating indicated conception had occurred and was designated day zero. Experiments complied with National Health and Medical Research Council guidelines, and were approved by the university's Animal Ethics Committee.

Materials

Eagle's minimal essential medium (MEM) and newborn bovine serum (NBCS) were obtained from Thermo Fisher Scientific (Waltham, USA). Radiolabelled compounds were supplied by GE Healthcare (Little Chalfont, UK). HPLC grade acetonitrile was obtained from Burdick and Jackson (Muskegon, USA) and water was obtained from a Milli-Q system (Millipore Corp., Billerica, USA). Lyophilized recombinant human neuregulin-1β (heregulin-1β, 7.5 kDa) (PeproTech, Rocky Hill, USA) was reconstituted in Milli-Q water. The SV total RNA isolation and OneStep RT-PCR kits used for RT-PCR reactions were purchased from Promega Corporation (Madison, WI, USA) and Qiagen Sciences (Germantown, MD, USA), respectively. All other reagents were supplied by Sigma Aldrich (St. Louis, USA).

Preparation of media

Eagle's minimal essential medium was reconstituted according to the manufacturer's specifications, supplemented with 0.2% $NaHCO_3$ and adjusted to pH 7.4. To this was added L-glutamine, penicillin G and streptomycin sulfate to final concentrations of 2.6 mM, 100 IU/mL and 135 μM, respectively. The medium was then sterilized by filtration through a 0.22 μm filter and amphotericin was added to a final concentration of 3.2-3.6 μg/mL. The medium was then supplemented to include 10% charcoal-treated NBCS.

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Isolation of fibroblasts and type II pneumocytes

Pregnant rats (19 days gestation) were asphyxiated with CO_2 and fetuses delivered by Caesarean section. The fetal lungs were removed, minced and incubated with collagenase (0.05 $IU.mL^{-1}$) for 20 minutes in a 37°C shaking water bath, as previously described [21,22]. After filtration through two layers of sterile French voile, the cells were centrifuged at 20 g for 2 min and the pellet resuspended in serum-free medium. This suspension was plated (6 lungs per plate) onto 6 cm diameter culture plates (Corning Life Sciences, Lowell, USA) and incubated for 30 minutes at 37°C in a humidified CO_2 incubator to allow adhesion of fibroblasts. Non-adhering cells were removed by gentle swirling and the medium replaced with serum-containing media. The non-adhering cells were used to isolate type II pneumocytes, according to the method of Dobbs *et al.*[23]. The type II cell medium was changed after 24 and 72 h. When the cells had been cultured for 3 days the cultures were nearly confluent and consisted predominantly of differentiated type II cells, each containing numerous lamellar bodies and being capable of both synthesis [21] and secretion [22,24] of surfactant phosphatidylcholine.

Preparation of fibroblast-conditioned medium

Confluent fibroblast cultures were considered free of contamination from type II cells as judged by microscopy. Media was removed from the plates and replaced with serum-free medium supplemented with either dexamethasone in propylene glycol or vehicle (control). Plates were returned to the incubator for a further 24 hours, after which FCM was collected and stored at -80°C. Prior to assay, each FCM was thawed and heat-treated at 65°C for 60 minutes to remove known inhibitory factors [5]. After cooling to room temperature, they were filtered through 0.22 μm filters and diluted 1:4 with serum-free medium.

Assay of synthesis of surfactant phospholipids

The medium from confluent type II pneumocyte cultures was removed and replaced with 3.0 mL of either diluted FCM supplemented with the corresponding additives or serum-free media supplemented with the indicated concentrations of heregulin-1β or leptin. After 21 h of culture, serum-free medium containing [methyl-^{3}H]-choline chloride (final concentration 1.0 μCi/mL) was added and the plates further incubated for the indicated times. After this final incubation, incorporation of [^{3}H]-choline into cellular surfactant phospholipid (expressed as dpm/μg DNA) was determined as described by Sen and Cake [21]. The radiolabelled material produced using this method was previously validated using TLC and shown to consist predominantly (> 85%) of phosphatidylcholine (PC), of which disaturated PC was the major component [22].

RT-PCR quantification

The RT-PCR primers for the GAPDH and NRG1β mRNA assays were designed to include a splice junction between the forward and reverse primers thereby providing the means of distinguishing between the products of mRNA and genomic DNA based on the size of the products. Each of the primers were checked for melting temperature (T_M) and cross reactivity with other sequences using Primer3 and BLAST software, respectively. Agarose electrophoresis of the RT-PCR products demonstrated that neither primer dimer formation nor interference with GAPDH primers were evident. The forward and reverse primer sequences chosen for the GAPDH and NRG1β RT-PCR assays are shown below:

Primer	Size (bp)	Forward primer sequence	Reverse primer sequence
GAPDH	207	5'-agacagccgcatcttcttgt-3'	5'-cttgccgtgggtagagtcat-3'
NRG1β	196	5'-acgactgggaccagccatc-3'	5'-tctggtagagttcctccgctttg-3'

GAPDH and NRG1β TaqMan probes, which had melting temperatures approximately 10ºC higher than either of the forward and reverse primers, were designed with an Iowa Black®

quencher at the 3' end and FAM (green) or Cy5 (red) covalently attached at the 5' end, respectively. The sequence of these probes is shown below:

Probe	Dye	TaqMan probe sequnce
GAPDH	FAM	5'(FAM)-ccgtgtgaacggatttggccgtatc-(IABkFQ) 3'
NRG1β	Cy5	5'(Cy5)-tggcaacgatcaccagtaaactcatttgg-(IAbRQSp) 3'

Total cellular RNA was extracted using a SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA). All pipette tips and microcentrifuge tubes used were certified nuclease-free. Following extraction, RNA extracts were stored at -70°C until quantification. RNA quantification of each extract was made using an Agilent 2100 Bioanalyzer with an Agilent RNA 6000 Pico kit (Agilent Technologies Inc., Santa Clara, CA, USA). The extracts were diluted such that an aliquot containing 5 ng of mRNA was added to each RT-PCR.

To measure relative gene expression, QIAGEN OneStep RT-PCR was undertaken using a QIAGEN Rotor-Gene Q6000 (QIAGEN, Hilden, Germany). For each RNA extract, a PCR reaction was set-up in a UV-sterilised, RNase-free PCR tube (Axygen - Fisher Biotec, Perth, Western Australia), containing forward and reverse primers as well as the TaqMan probes for GAPDH and NRG1β. It was established that an annealing temperature of 57ºC provided the best specificity and the highest yield of product. Thus, the reaction mixtures were incubated at 50°C for 30 minutes followed by denaturation at 95ºC for 15 minutes. This was followed by 40 cycles using the following parameters; 94°C for 30 sec; 57°C for 30 sec and 72°C for 60 sec. The reactions were then held at 72°C for 10 minutes. The RT-PCR products were then analysed for the expression of NRG1β relative to GAPDH using the method of Pfaffl [25].

Liquid chromatography-mass spectrometry (LC-MS)

Mass spectrometry was undertaken using an Agilent Classic series ion trap mass spectrometer. The MS was operated in positive ionization mode using electrospray ionization.

Nebulizer pressure was set at 35 psi and drying gas was set at 10 L/min of N_2 and 325°C. Standards and samples were infused directly into the MS at 5 µL/min using a KD Scientific 100 syringe pump (KD Scientific, Holliston, USA). Two identifier ions were selected: m/z 1068.5 ($[M+7H]^{7+}$), and 1246.7 ($[M+6H]^{6+}$). MS/MS was performed on m/z 1068.5, and this generated fragment ions, of which m/z 958.3 (a_{26}^{3+}), 1028.7 (y_{26}^{+}) and 1047.3 were selected.

To allow quantification, an Agilent 1100 capillary HPLC (Agilent Technologies) was coupled to the ion trap MS. The analytical column used was an Agilent Zorbax 300SB-C_{18} capillary column with dimensions of 100 mm x 0.3 mm with 3.5 µm particle size. Mobile phase solvents consisted of (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. Flow rate was set at 5 µL/min with a gradient as follows (A:B): 0 min – 90:10, 10 min - 90:10, 25 min – 0:100, 35 min – 0:100. Standard NRG1β eluted at a retention time of 12.5 minutes, and a standard curve for NRG1β was prepared in triplicate over the range of 0.01 to 1.33 nM. This curve showed a very strong correlation (R = 0.998).

Data analysis

Statistical analyses were performed using SPSS Statistics for Windows 21.0. Repeated measures ANOVA [26] was used to analyze the time-course experiment and for ascertaining differences between 3 or more experimental conditions. If significance was determined in the later case, the ANOVA was followed by Dunnett pairwise comparisons of the various groups. LC-MS analysis was undertaken using Agilent ChemStation for LC 3D (Rev. B.09.03) and Bruker-Daltonik MSD Trap Control software (v. 5.1) (Bruker-Daltonik, Bremen, Germany).

Results and Discussion

Effect of fibroblast-conditioned medium on surfactant phospholipid synthesis

Six pairs of control and dexamethasone-treated FCM were assayed for their effect on surfactant phospholipid synthesis in cultured type II pneumocytes. FCM generated in the

presence of 100 nM dexamethasone enhanced by 24% the rate of surfactant phospholipid synthesis by type II cells when compared to that measured in control cultures (Figure 1A; $p < 0.05$). Previously, this increase has been ascribed to the generation of FPF, which is produced by lung fibroblasts and acts on type II pneumocytes to enhance surfactant phospholipid synthesis [6,27]. Dammann *et al.* [8] observed that 1 and 10 nM NRG1β were able to mimic the effect of FPF, suggesting that it may be involved in the FPF response.

In order to quantify the amount of NRG1β present, the same six pairs of control and dexamethasone-treated FCM that were used above were also analysed by LC-MS. The mean NRG1β concentration in the control FCM was 2.65 ± 0.23 nM, while in the test FCM it was 5.55 ± 0.19 nM (Figure 1B). These findings indicate that fibroblast cells produce and secrete NRG1β in the absence of dexamethasone stimulation, but that its concentration was more than two-fold higher when the fibroblasts were exposed to dexamethasone ($p < 0.001$). This observation may account for the finding of Dammann *et al.* [8] that NRG1β antibody is able to block the enhanced rate of surfactant synthesis in type II cells that had been exposed to FCM.

It is important to note that the effects of dexamethasone on expression and activation of ErbB receptors in fetal mouse type II cells were distinct from those induced by mature (d18) FCM [14]. Thus, in order to ascertain whether the glucocorticoid-induced increase in NRG1β mimics natural maturation processes, additional experiments should be conducted to determine the NRG1β concentration in media conditioned by lung fibroblasts isolated from fetal rats of different gestation ages.

Effect of NRG1β on surfactant phospholipid synthesis

When type II cells were incubated with NRG1β in the range 0 - 26.67 nM for 21 hours, the quantity of labelled surfactant phospholipid synthesised after 6 hours was higher in those cells exposed to the peptide. Synthesis was highest when cells were exposed to 2.67 nM NRG1β, at

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3648 ± 239 dpm/μg DNA, compared to 1045 ± 137 dpm/μg DNA in control cells. Similar NRG1β dose-response curves have been seen with other cell types [28,29]. The increased level of surfactant phospholipid synthesis in cells exposed to either 2.67 or 6.67 nM NRG1β (3269.6 ± 616.4 dpm/μg DNA) was significantly different from that in the controls (more than 3-fold higher; $p < 0.05$) (Figure 2A). It is important to note that the control cells in this experiment were incubated with MEM$^+$ whereas those in the experiment reported in Figure 1A were incubated with FCM, which contains approximately 0.66 nM NRG1β (after being diluted 1:4) together with any other components of FPF (Figure 1B). It is thus not surprising that the level of phospholipid synthesis is much lower in those cells maintained in MEM$^+$ and that the observed response to NRG1β is greater.

The response to NRG1β was reduced at higher concentrations of the ligand, and it is likely that the ErbB receptor to which NRG1β binds was down-regulated at these concentrations. Although the mechanism by which this down-regulation might occur in type II cells is unknown, Cao *et al.* [30] have shown that neuregulin induces both ubiquitination and degradation of the ErbB3 receptor in MCF-7 cells. If neuregulin-induced degradation of the ErbB receptor also occurs in type II cells this could explain the decline in the response at higher concentrations of NRG1β. In the current study, the commercially available form of NRG1β (heregulin-1β) was used whereas the form used in the study by Dammann *et al.* [8] is not known. Despite this, as well as the fact that the type II cells were derived from rats and mice, respectively, elevated phospholipid synthesis by these cells was evident at similar NRG1β concentrations.

The quantity of radiolabelled surfactant phospholipids synthesised by type II pneumocytes in the 2, 4 and 6 hours following 21 hours incubation with 6.67 nM NRG1β was enhanced when compared with that which occurred in cells grown in the absence of NRG1β (Figure 2B). The control value increased from 795.6 ± 293.1 dpm/μg DNA at 2 hours to 1888.6 ± 195.6

dpm/μg DNA at 6 hours, while NRG1β-treated cells increased from 1219.4 ± 208.8 dpm/μg DNA at 2 hours to 2469.6 ± 184.1 dpm/μg DNA at 6 hours. The difference between control cultures and NRG1β-treated cells was significant ($p < 0.05$) and, as expected, there was also evidence of an increase in the rate of phospholipid synthesis with time ($p < 0.01$).

In order to understand the mechanism(s) by which NRG1β exerts its effect it needs to be recognized that, in mice and rats, the response of lung cells to this peptide is mediated via ErbB receptors [14,15,31]. Using a small interfering RNA (siRNA), which targeted the ErbB4 gene and silenced ErbB4 receptor activity in cultured 19-day fetal type II pneumocytes, Zscheppang *et al.* [15] demonstrated that the resulting down-regulation of the ErbB4 receptor caused a diminished rate of surfactant phospholipid synthesis. Their conclusion that the response of these cells to NRG1β is mediated through ErbB4 receptor activation was supported by the observation that exposure of both fetal and adult rat lung epithelial cells to NRG1β resulted in increased phosphorylation of the ErbB4 receptor [13,32].

NRG1β mRNA expression

RT-PCR analysis of the level of expression of the NRG1β gene in cultured fetal lung fibroblasts following exposure to dexamethasone showed no significant difference with either 20 or 50 nM dexamethasone (Table 1). The fold change in NRG1β mRNA levels, relative to that of GAPDH, after exposure to 20 nM dexamethasone ranged from 0.93-fold at 4 hours to 0.73-fold at 24 hours, while with 50 nM dexamethasone, changes ranged from 0.95-fold at 4 hours to 1.10-fold at 24 hours. Preliminary experiments conducted after 2 and 6 h exposure to the steroid also showed no significant elevation of NRG1β gene expression. The absence of a significant increase in NRG1β gene expression following exposure to dexamethasone suggests that the steroid does not mediate its effects via an elevated rate of transcription of the NRG1β gene. It is well known that at least 15 different isoforms of NRG1β are produced from a single gene, and that these forms can include transmembrane and secreted proteins

[33]. Given that neuregulins are produced as transmembrane precursors, which are considered to generate diffusible ligands when subjected to cleavage [34,35], it is possible that dexamethasone stimulates the rate of cleavage of the neuregulin precursors. In this context it is relevant that glucocorticoids enhance proteolytic activity in both muscle cells and thymocytes [36,37]. Alternatively, dexamethasone may induce secretion of pre-formed NRG1β followed by new NRG1β synthesis more than 24 h later. Thus, any transient increase in NRG1β gene expression may occur 24 h or more after exposure to dexamethasone.

Comparison of NRG1β and leptin effects on surfactant phospholipid synthesis

Previous studies have shown that leptin, which is expressed by lipofibroblasts during rat lung development, has many of the characteristics of FPF [7,38]. Leptin, at both 1.24 (5245.8 ± 906.1 dpm/µg DNA) and 3.09 nM (5691.0 ± 533.4 dpm/µg DNA), showed a significant increase in surfactant phospholipid synthesis compared to the control (3183.8 ± 349.9 dpm/µg DNA) ($p < 0.05$ and $p < 0.01$, respectively), but not at 6.17 nM (4405.4 ± 174.4 dpm/µg DNA). Similar results were obtained by Torday *et al.* [7] although, in their study, there was no decline in the response to the higher leptin concentration. When one compares the response of these cells to leptin (Figure 3) with that of NRG1β (Figure 2A) it is apparent that both peptides stimulate surfactant phospholipid synthesis by approximately the same extent and at a similar peptide concentration (i.e. ~ 3 nM). On the basis of its size (16 kDa) and it having many of the same characteristics as FPF, Torday *et al.* [7] suggested that leptin may be FPF. However, as NRG1β has a molecular weight within the range given for FPF (5-15 kDa [20]), and is produced by lung fibroblasts [8], particularly in response to glucocorticoids as shown in this study, it too shares many of the attributes of FPF. Given the strong evidence of a role for each of these in the pulmonary surfactant system, it is possible that FPF consists of both agents.

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Figure legends

Figure 1: Exposure of lung fibroblasts to dexamethasone enhances both surfactant phospholipid synthesis in type II cells and the concentration of NRG1β in the conditioned media.

Cultured fetal rat lung fibroblasts were exposed to 100 nM dexamethasone (☐) or propylene glycol (control, ☐) for 24 hours. The conditioned media was analysed (A) for its ability to stimulate phosphatidylcholine synthesis in cultured fetal type II pneumocytes, or (B) for the concentration of NRG1β (nM), calculated using ion trap mass spectrometry. The results are depicted as the mean ± SEM of six separate experiments. Any result that is significantly different from that seen in cells exposed to FCM (without dexamethasone) are denoted with asterisks (* $p < 0.05$; ** $p < 0.01$).

Figure 2: Effect of NRG1β on surfactant phospholipid synthesis.

After three days in culture, fetal rat type II pneumocytes were exposed to (A) the indicated concentrations of NRG1β or vehicle and (B) 6.67 nM NRG1β (●) or vehicle (control, ■). After 21 h exposure to the peptide, the extent of incorporation of [^{3}H-methyl]-choline chloride into surfactant phospholipids was determined after a further incubation of (A) 6 h or (B) 2, 4 and 6 h. The results are depicted as the mean ± SEM of four separate experiments. Any significant differences from the controls (vehicle) are denoted with an asterisk (* $p < 0.05$).

Figure 3: Effect of NRG1β and leptin on surfactant phospholipid synthesis.

Cultured fetal rat type II pneumocytes were exposed to MEM$^+$ containing 1.24, 3.09 and 6.17 nM leptin (☐) or an equivalent volume of vehicle (control, ☐). After a 21-hour exposure, the cells were further exposed to 1.0 μCi/mL [^{3}H-methyl]-choline chloride for 4 hours prior to determining the amount of cellular radiolabeled surfactant phospholipids. The results are depicted as the mean ± SEM of four separate experiments. Any significant differences from the control (vehicle) are denoted with asterisks (* $p < 0.05$; ** $p < 0.01$).

Table 1: Expression of the NRG1β gene in lung fibroblasts after exposure to dexamethasone.

Cultured fetal rat lung fibroblasts were exposed to MEM$^+$ containing 20 or 50 nM dexamethasone or an equivalent volume of propylene glycol (vehicle). After the indicated exposure time, the cells were subjected to RNA extraction and the mRNA concentration of these extracts quantified. The level of NRG1β mRNA was determined using multiplex qPCR and the data represent the mean ± SEM of the indicated number of independent fibroblast cultures.

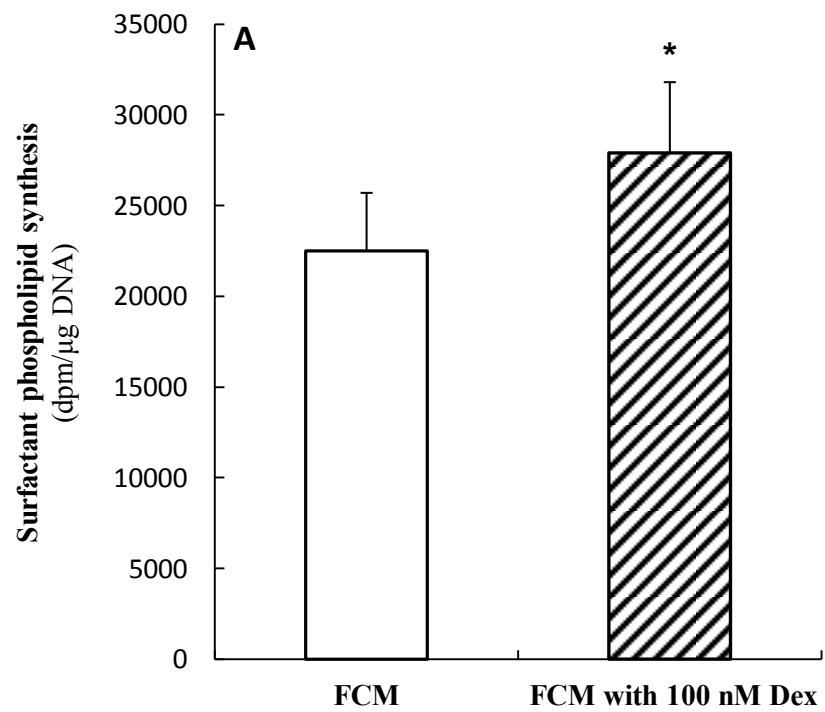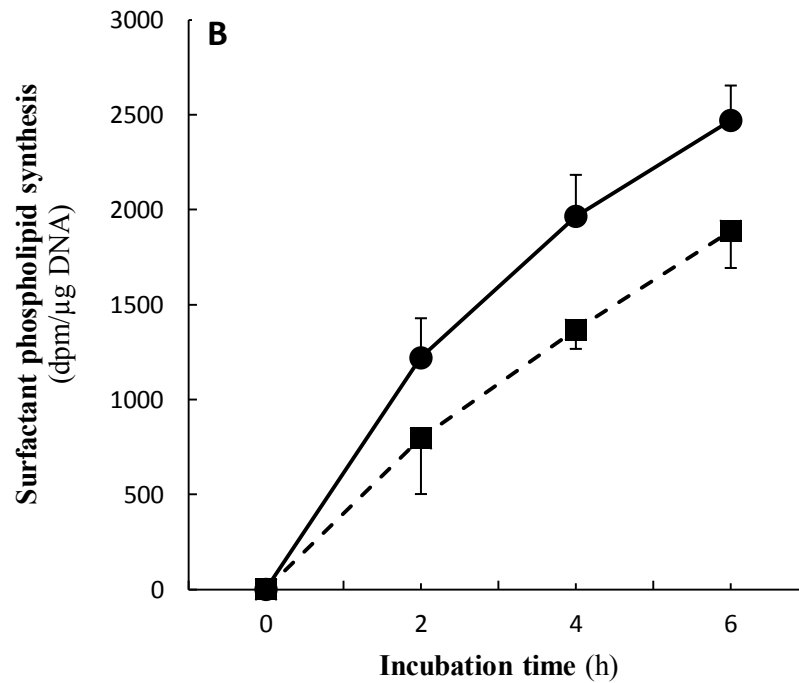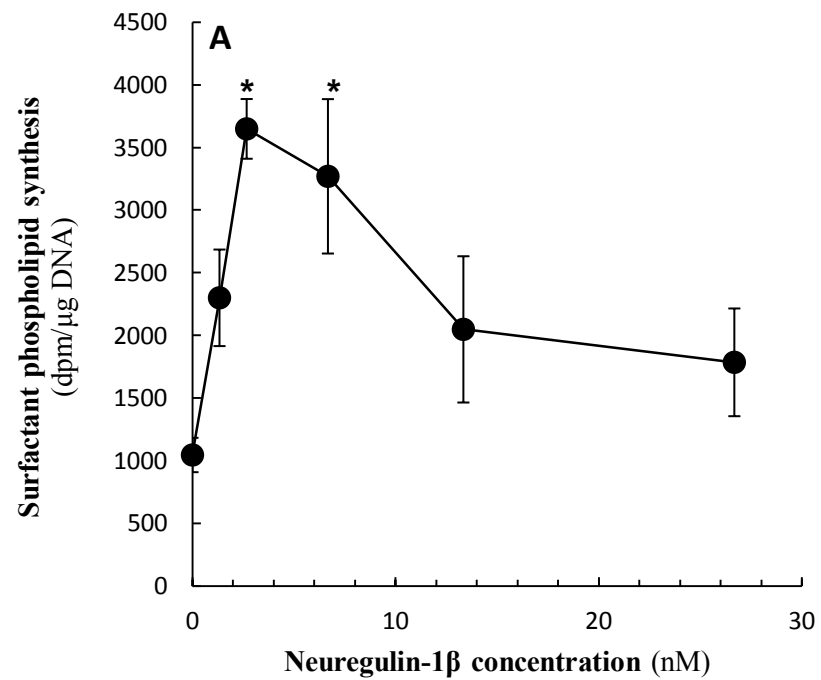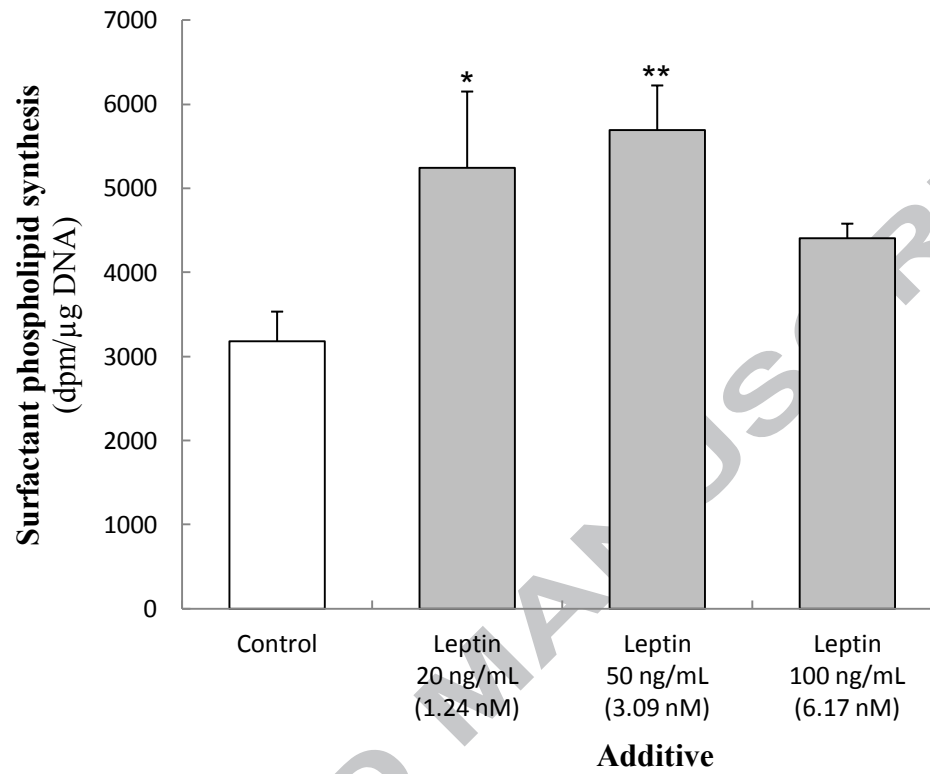| Exposure time	NRG1β gene expression in lung fibroblasts after exposure to dexamethasone (relative to a control value of 1.00)	
	20 nM dexamethasone	**50 nM dexamethasone**
4 hours	0.93 ± 0.33 (5)	0.95 ± 0.41 (5)
8 hours	0.89 ± 0.15 (6)	1.01 ± 0.35 (7)
24 hours	0.73 ± 0.42 (3)	1.10 ± 0.80 (3)

Figure 1
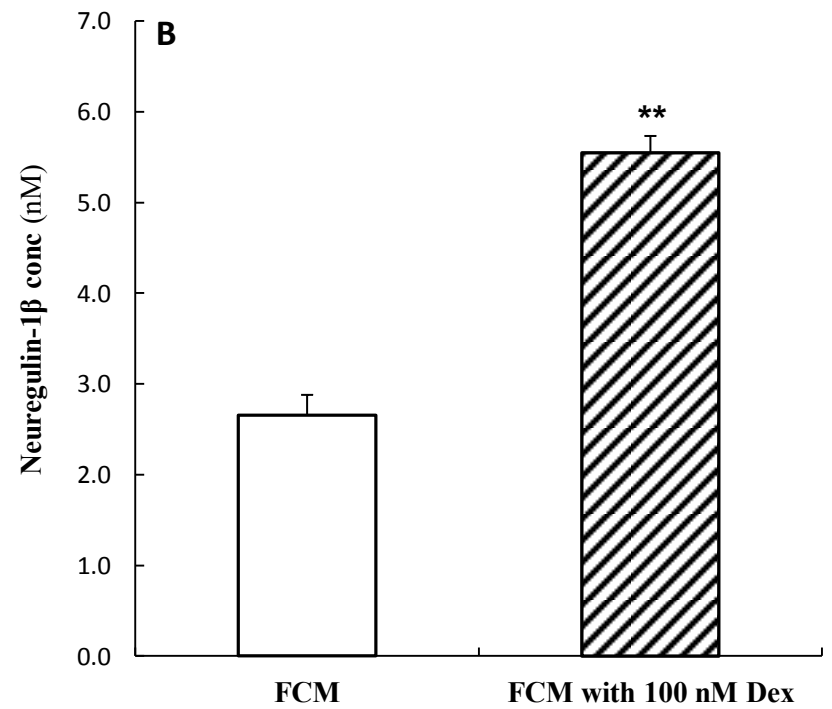
Figure 2

Figure 3

Highlights

- Dexamethasone elevates neuregulin-1β concentration in fibroblast-conditioned media.

- This effect is not the result of increased neuregulin-1β gene expression.

- Surfactant phospholipid synthesis in type II cells is stimulated by neuregulin-1β.

- It is concluded that neuregulin-1β is a likely component of fibroblast-pneumocyte factor.