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Infant Formula Alters Surfactant Protein A (SP-A) and SP-B Expression in Pulmonary Epithelial Cells

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Summary.

Surfactant proteins A (SP-A) and SP-B are critical in the ability of pulmonary surfactant to reduce alveolar surface tension and provide innate immunity. Aspiration of infant milk formula can lead to lung dysfunction, but direct effects of aspirated formula on surfactant protein expression in pulmonary cells have not been described. The hypothesis that infant formula alters surfactant protein homeostasis was tested in vitro by assessing surfactant protein gene expression in cultured pulmonary epithelial cell lines expressing SP-A and SP-B that were transiently exposed (6 hr) to infant formula. Steady-state levels of SP-A protein and mRNA and SP-B mRNA in human bronchiolar (NCI-H441) and mouse alveolar (MLE15) epithelial cells were reduced in a dose-dependent manner 18 hr after exposure to infant formula. SP-A mRNA levels remained reduced 42 hr after exposure, but SP-B mRNA levels increased 10-fold. Neither soy formula nor non-fat dry milk affected steady-state SP-A and SP-B mRNA levels; suggesting a role of a component of infant formula derived from cow milk. These results indicate that infant formula has a direct, dose-dependent effect to reduce surfactant protein gene expression. Ultimately, milk aspiration may potentially result in a reduced capacity of the lung to defend against environmental insults.

Keywords

milk; regulation; aspiration

INTRODUCTION

Pulmonary aspiration in neonates can lead to severe acute respiratory consequences and long-term chronic or recurrent respiratory symptoms.^{1,2} Such exposure may lead to inflammation, airway edema, increased secretions and bronchospasms from mechanical injury, chemical injury, and/or inflammatory responses.^{3,4} Milk aspiration can occur during anesthesia induction, vocal cord dysfunction, or gastroesophageal reflux.^{5–7} Animal models of milk aspiration suggest that acute changes in lung function occur within the first 6 hr after

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milk exposure.^{8,9} Aspiration of both infant formula and breast milk increase the alveolar-arterial (A-a) gradient and decrease dynamic lung compliance.⁸⁻¹¹ While the severity of lung injury is apparently not affected by the degree of acidity of milk,⁹ persistent airway reactivity is caused by repeated aspiration of milk in a rabbit model.¹²

Surfactant associated proteins have been shown to play an important role in lung function by reducing alveolar surface tension, and in preventing lung injury and initiation of immune response.^{13,14} Surfactant protein A (SP-A) is a major surfactant associated protein, constituting approximately 50% of the total surfactant proteins.^{15,16} SP-A is a prominent component of large surfactant lipid aggregates and has a role in the surfactant cycle and function with regards to regulation of uptake and secretion of phospholipids by surfactant producing cells.¹⁷ SP-A is critical for defense of the lung against environmental insults and is an important component of pulmonary innate immunity.¹⁸ The ability of pulmonary surfactant to reduce alveolar surface tension is dependent on the complex of surfactant lipid and the hydrophobic proteins SP-B and SP-C.¹⁹ Reduced levels of these hydrophobic proteins results in reduced lung function and a greater incidence of long-term lung damage.²⁰

While many animal models indicate that milk aspiration can lead to lung dysfunction, direct effects of milk on surfactant homeostasis in pulmonary epithelial cells has not been investigated. SP-A is a quantifiable protein expressed by alveolar epithelial type II cells and nonciliated bronchiolar epithelial (Clara) cells, and it has been speculated that the presence or absence of SP-A in the lung can act as an indicator of the status of surfactant homeostasis.¹⁵ We hypothesized that exposure of pulmonary epithelial cells to infant formula alters surfactant protein homeostasis. The hypothesis was tested in vitro by assessing SP-A and SP-B gene expression in NCI-H441 (human non-ciliated bronchiolar Clara) and MLE15 (mouse alveolar type II) cells transiently exposed to various concentrations of infant formula (Enfamil¹ Premature Lipil¹). We found that steady-state levels of intracellular SP-A protein, SP-A mRNA and SP-B mRNA were reduced in a dose-dependent manner in cells in culture after exposure to infant formula. The effect was specific for milk formula derived from cow milk.

MATERIALS AND METHODS

Infant Formula and Milk Formulations

Enfamil[®] Premature Lipil[®] iron fortified milk-based infant formula (#139201, Mead Johnson and Company, Evansville, IN) and Similac[®] Isomil[®] Advance[®] soy iron fortified infant formula (#56980, Abbott Laboratories, Abbott Park, IL) were used as infant formulas in the study. Carnation[®] Instant Non-fat Dry Milk (Nestlé[®] USA, Wilkes-Barre, PA) was prepared as directed by the manufacturers and filtered sterilized (0.22 µM) before addition to cell media. Cell Culture

NCI-H441 cells, a human non-ciliated bronchiolar epithelial lung adenocarcinoma cell line, were obtained from the American Type Culture Collection (#HTB-174).^{21,22} MLE-15 cells, an immortalized mouse lung epithelial cell line that maintains some morphological and functional characteristics of alveolar epithelial type II cells, were a gift from Dr. C. J.

Bachurski.⁹ Cells were maintained in RPMI-1640 media (MediaTech, Manassas, VA) containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. All incubations were performed in a humidified incubator at 37°C with 5% CO₂.

In Vitro Exposure of Cells to Milk Formula

When cells attained ~80–90% confluence, plates were divided into four groups and exposed to serum free media with milk formula in concentrations of 0%, 0.1%, 1.0%, and 10% (v/v). Cells were exposed for 6 hr, rinsed and incubated in serum-free media. At the end of the 18 hr incubation (24 hr from the start of exposure) cells were collected for protein and RNA analysis.

Determination of Cell Viability

The effect of treatment on cell viability was determined by the method of trypan blue exclusion.²³

Isolation of RNA

RNA was isolated and purified from cells using Trizol Reagent (#15596–026, Invitrogen Corp., Carlsbad, CA) as prescribed by the manufacturer.

Real Time Quantitative RT-PCR Analysis of mRNA

Quantitative real-time RT-PCR assays specific for the human and mouse SP-A and SP-B mRNAs were designed and performed by the Quantitative Genomics Core Laboratory in the Integrative Biology Department at The University of Texas Health Science Center, Houston as previously described.²⁴ Specific quantitative assays for the mRNAs were developed using Primer Express software (Applied Biosystems, Foster City, CA) based on sequences from Genbank at the National Center for Biotechnology Information (NCBI). Table 1 lists the primers used, the sequences, and the characteristics of assay as required by the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines.²⁵

Isolation of Cellular Proteins

Proteins were isolated from cells as described previously.²⁶

Immunoblot Analysis

Prepared culture medium (20 ml) and cellular protein (20 µg) were solubilized in an equal volume loading buffer (0.1 M Tris, pH 7.4, 50 mM DTT, 0.01% bromophenol blue, 2% SDS, and 10% glycerol) and boiled for 5 min. Proteins were resolved on denaturing 4–20% Tris–glycine polyacrylamide gels (#EC6025, Invitrogen). After protein resolution, gels were transferred to Trans-blot nitrocellulose paper (#162–0112, BioRad Laboratories, Hercules, CA) and incubated with rabbit anti-human SP-A antibody (a gift from C.R. Mendel-son, Dallas, TX; 1:1,000) followed by peroxidase-conjugated goat anti-rabbit IgG (1:16,000 dilution) (#A6154, Sigma Chemical, St. Louis, MO). The immune complexes were detected with the ECL Plus Western Blotting Detection System (#RPN2135, Amersham Biosciences Corp, Piscataway, NJ) and quantified on a Storm 840 Phosphorimager (GE Healthcare,

Piscataway, NJ). β -Actin was detected similarly using mouse anti-xenopus antibody (1:5,000 dilution) (#ab6276, Abcam, Cambridge, MA) followed by peroxidase-conjugated rabbit anti-mouse IgG (1:160,000 dilution) (#ab6728 Abcam). SP-A levels were quantified relative to b-actin, the SP-A levels of unexposed cells were normalized as 1, and the SP-A levels in exposed cells expressed relative to control. The final data were normalized and are presented as the molecules of transcript/molecules of normalizer transcript $\times 100$ (= % normalizer transcript).

Statistical Analysis

Statistical analysis of the data was performed by Student's t-test for unpaired means (SigmaPlot Software, Jandel Scientific, San Rafael, CA). Data in all graphs are expressed as means $\pm$ SEM.

RESULTS

Transient Exposure of Lung Bronchiolar Epithelial Cells to Infant Formula Reduces Intracellular Steady-State SP-A Levels in a Dose-Dependent Manner

These investigations were undertaken to determine whether exposure of lung epithelial cells to infant milk formula has a direct effect on surfactant protein expression. NCI-H441 cells, which express SP-A protein in cell culture, were transiently exposed to increasing concentrations of Enfamil® milk-based infant formula as described in the Materials and Methods Section. The effect of exposure on steady-state levels of SP-A protein was determined by Western blot analysis. Typical Western analysis of intracellular SP-A levels is shown in Figure 1A. In Figure 1B are shown the results of analyses in which the ratio of SP-A protein to b-actin protein is determined for each sample and the levels in treated cells are were normalized to levels in untreated cells in each analysis. As can be seen, transient exposure of NCI-H441 cells to cow milk-based infant formula results in a dose-dependent decrease in intracellular SP-A levels.

Transient Exposure of Lung Bronchiolar Epithelial Cells to Infant Formula Reduces Intracellular Steady-State Levels of SP-A and SP-B mRNA in a Dose-Dependent Manner

The results described above indicate that infant formula reduces the steady-state level of SP-A protein. These next phase of these investigations were to determine whether infant formula reduces the steady-state level of surfactant protein mRNA. NCI-H441 cells were transiently exposed to increasing concentrations of cow milk-based infant formula, cellular RNA was isolated and SP-A mRNA quantified by real-time quantitative PCR (qRT-PCR) analysis as described in the Materials and Methods Section. The results of three analyses are shown in Figure 2A. As can be seen, transient exposure of the cells to infant formula results in a dose-dependent decrease in steady-state levels of SP-A mRNA. The change from control in steady-state levels of SP-A mRNA is closely matched to the change in SP-A protein, suggesting that the effect of infant formula is manifested at the level of mRNA transcription and/or stability and not due to degradation of SP-A protein.

Since NCI-H441 cells express another surfactant-associated protein, SP-B, qRT-PCR analysis of the same samples for the presence of SP-B mRNA was performed. As seen in

Figure 2B, steady-state levels of SP-B mRNA are reduced in a dose-dependent manner after transient exposure to infant formula. These results suggest that the effect of exposure of lung epithelial cells to cow milk-based infant formula has a direct effect to reduce expression of the mRNAs of several surfactant proteins.

Viability of Lung Bronchiolar Epithelial Cells Is Unaffected by Transient Exposure to Infant Formula

While the reduction of surfactant protein gene expression by infant formula appears to be directed against transcription or stability of surfactant protein mRNA, the possibility still exists that reduction of the mRNA could be an indirect effect of exposure to reduce cell viability. To address this possibility, trypan blue exclusion analysis was determined on NCI-H441 cells transiently exposed to infant formula. NCI-H441 cells were transiently exposed to increasing concentrations of cow milk-based infant formula as described in the Materials and Methods Section. Trypan blue exclusion analysis indicated that viability of cells exposed to various concentrations of infant formula (0.1%:80% viable, 1.0%:71% viable, 10%:82% viable) was not significantly different from untreated cells (0%:76% viable). These results eliminate the possibility that the effect of cow milk-based infant formula to reduce the steady-state levels of surfactant protein gene mRNA in lung epithelial cells is due to detrimental effects on overall cell physiology.

Transient Exposure of Lung Alveolar Epithelial Type II Cells to Infant Formula Reduces Intracellular Steady-State Levels of SP-A and SP-B mRNA in a Dose-Dependent Manner

While human NCI-H441 cells are used in investigations as a model of lung bronchiolar epithelial cells, mouse MLE-15 cells are used as a model of lung alveolar epithelial type II cells.²⁷ These investigations were undertaken to determine whether the effect of infant milk exposure on surfactant protein gene expression is also manifested in these cells. MLE-15 cells were transiently exposed to increasing concentrations of cow milk-based infant formula, cellular RNA isolated and SP-A and SP-B mRNA quantified as described in the Materials and Methods Section. As seen in Figure 3, transient exposure of alveolar epithelial type II cells to infant formula reduces steady-state levels of SP-A and SP-B mRNA, although the magnitude of the reduction was less. These results suggest that the effect of cow milk-based infant formula to inhibit surfactant protein gene expression can occur throughout the lung in both bronchiolar epithelial cells and alveolar epithelial cells.

Transient Exposure of Lung Bronchiolar Epithelial Cells to Infant Formula Has a Prolonged Effect to Reduce Steady-State Levels of SP-A mRNA While Steady-State Levels of SP-B mRNA Rebound

The findings presented above indicate that transient exposure of lung epithelial cells to 10% cow milk-based infant formula significantly reduces SP-A and SP-B mRNA expression 18 hr after exposure. These investigations were performed to determine whether these effects occur immediately after exposure or after longer periods of time. NCI-H441 cells were transiently exposed to cow milk-based infant formula (10% v/v), cellular RNA isolated and SP-A and SP-B mRNA quantified as described in the Materials and Methods Section at 0, 18, and 42 hr after exposure. Steady-state levels of SP-A and SP-B mRNA relative to b-actin mRNA levels were determined by real-time qRT-PCR analysis using primers specific for

human SP-A and SP-B mRNA. As can be seen in Figure 4, the steady-state levels of SP-A and SP-B mRNA significantly decreased immediately after exposure (6 hr) to infant formula, a trend that continued for 18 hr post-exposure; steady-state SP-A and SP-B mRNA levels decrease significantly compared to levels at 6 hr. At 42 hr post-exposure, steady-state levels of SP-A were increased compared to levels at 18 hr post-exposure. However, levels were still significantly less than levels seen in unexposed cells, suggesting that the effect of transient exposure to reduce SP-A expression is prolonged. On the other hand, steady-state levels of SP-B mRNA increased 10-fold 42 hr after exposure compared to levels 18 hr post-exposure and these levels were twofold higher than those seen in unexposed cells. These results suggest that transient exposure of lung epithelium to cow milk-based infant formula has long-term consequences of SP-A gene expression.

Steady-State Levels of SP-A and SP-B mRNA Are Not Reduced in Lung Bronchiolar Epithelial Cells Exposed to Soy-Based Infant Formula or Non-Fat Dry Milk

Thus far, the research presented has focused on describing a direct effect of cow milk-based infant formula on surfactant protein gene expression in lung epithelium. The next set of investigations were undertaken to determine the specificity of the effect of infant formula on surfactant gene expression; soy-based formula contains no components derived from cow milk, and non-fat dry milk has little fat and has been heat-treated. NCI-H441 cells were transiently exposed to Enfamil® milk-based infant formula, Similac® soy-based infant formula or non-fat dry milk (10% v/v), cellular RNA isolated and SP-A mRNA quantified as described in the Materials and Methods Section. The results of the analyses are shown in Figure 5. As can be seen, cow milk-based infant formula (milk formula) significantly reduces steady-state levels of SP-A and SP-B mRNA while soy-based formula and non-fat dry milk do not have a significant effect SP-A and SP-B mRNA levels compared to untreated cells. In addition, steady-state levels of SP-A and SP-B mRNA were significantly less in cells treated with cow milk-based infant formula compared to soy-based formula and non-fat dry milk. While two infant formulas are made by two different companies, they are essentially identical in content and concentration with regards to carbohydrates, ions, salt, metals, etc., and can be considered interchangeable.²⁸ These results suggest that the effect of cow milk-based formula to reduce surfactant gene expression in lung epithelium is specific for a component in that formula.

DISCUSSION

In these studies, the hypothesis that aspirated infant formula alters pulmonary surfactant protein homeostasis was tested in vitro in lung epithelial cells that express surfactant proteins. Transient exposure (6 hr) of the cells to various concentrations of cow milk-based infant formula resulted in a dose-dependent decrease in steady-state levels of SP-A and SP-B mRNA in human bronchiolar epithelial cells and in murine alveolar epithelial type II cells. This effect was not due to a detrimental effect of the formula on cell physiology. While steady-state levels of SP-B mRNA rebounded 42 hr after exposure; steady-state SP-B mRNA levels were twofold greater than in unexposed cells, steady-state levels of SP-A mRNA remained significantly lower than those seen in unexposed cells, suggesting a long-term effect on surfactant homeostasis in lung epithelial cells to a transient exposure of infant

formula. The inhibitory effect appears to be due to a component found in cow milk-based infant formula; neither soy-based infant formula nor non-fat dry milk elicited the same response as cow milk-based formula. These findings strongly suggest an undefined component of the formula derived from cow milk as a responsible candidate.

Aspiration of foreign material into the respiratory tract is a common occurrence in the neonate. In certain patients with predisposing anatomic and functional disorders, such as tracheoesophageal fistula, GER, or neuromuscular disorders, aspiration may be implicated in the etiology of a variety of pulmonary disorders. Infants and children who aspirate may present with pneumonia, recurrent wheezing, unexplained cough, interstitial lung disease, and even apnea. The association of aspiration of milk and exacerbation and/or development of reactive airway disease has been suggested in the literature.¹² An association between milk aspiration and the development of airway dysfunction has been suggested by clinical and laboratory studies in recent years.^{8–11} The mechanisms by which aspiration induces lung injury are poorly understood.²⁹ Damage or alteration to the surfactant system is known to be associated with decreased lung function.¹⁵ In this regard, a decrease in SP-A levels have been reported among patients with ARDS, traumatic parenchymal lung injury, bacterial pneumonia, and in patients with interstitial lung disease.^{3,4} These studies were undertaken to determine the possibility of a direct effect of infant formula on lung cell physiology as a mechanism. The present findings indicate that a component of the infant formula has a direct effect on epithelial cells to alter surfactant gene expression. Such a direct effect of milk and infant formula has been reported previously in which exposure of murine 3T3–L1 pre-adipocytes to these agents in vitro (10% v/v) results in increased expression of differentiation-dependent adipocyte mRNAs.³⁰ Interestingly, Similic® did not elicit this response.

It is important to realize that by its design, this study was performed in the absence of the normal inflammatory cell milieu of the lung. Not only do pulmonary epithelial cells produce SP-A and SP-B, but they also secrete a range of cytokines, such as intracellular adhesion molecule (ICAM)-1 interleukin (IL)-6 and IL-8, tumor necrosis factor (TNF- α) and RANTES, potent molecules involved in defense of the lung.^{31–33} In addition, IL-6, IL-8, and TGF- α are known to have effects on surfactant protein biosynthesis.³⁴ It is possible that infant formula contains cytokines that act on the cells in culture or that the components of the formula induce expression of cytokines from the cells that act in an autocrine fashion to regulate surfactant protein gene expression. While it is known that breast milk contains these cytokines,³⁵ the processing of cow milk-based infant formulas are generally believed to destroy bioactive components such as cytokines and chemokines.³⁶ This suggests that if the mediator of surfactant protein regulation in the presence of infant formula is a cytokine(s), then it must be expressed from the epithelial cell in response to the formula. This study in no way replicates the complex interactions that takes place when pulmonary inflammatory cells are exposed to aspirated infant formula.

Pulmonary surfactant is a barrier material of the lungs and has a dual role in lowering the alveolar surface tension and in participating in innate immune defense of the lung.^{13,14} Surfactant is composed of approximately 90% lipids and 10% proteins. While there are four surfactant specific proteins (SP-A, SP-B, SP-C, and SP-D), SP-A is the most abundant, has

been shown to facilitate surfactant function and has been implicated in surfactant regulation at the levels of secretion and reuptake.^{17,37–39} In addition, SP-A has a role in the structural organization of surfactant.¹⁶ The hydrophobic surfactant protein SP-B is indispensable in the ability of surfactant to reduce alveolar surface tension and is critical in the synthesis and structural organization of surfactant.²⁰ The findings of this study indicates that transient exposure of the lung epithelial cells that express and secrete surfactant proteins has profound, immediate, and long-term consequences in the steady-state levels of these critical proteins. Ultimately, these changes may affect the reduce ability of the lung to function efficiently.

In these studies, the choice was made not to acidify the infant formula. Although there is an expectation of acidic pH in aspirated gastric material, previous studies have shown that the effect of infant formula on lung function is independent of pH.^{9,10} These results clearly demonstrate an effect of infant formula on surfactant protein homeostasis that is completely independent of pH. However, aspirated material contains undefined gastric contents, such as gastric acid and other secretions, in addition to infant formula. Since these were not tested in our study, their potential effects remain unknown with regard to clinical aspiration syndrome. In fact, there may be an additive effect of the individual gastric components that results in a pronounced clinical manifestation of aspiration syndrome. Since these studies were limited to infant formula, the results may not indicate direct causation of formula with regard to clinical syndromes.

The results of this study clearly indicate that transient exposure of pulmonary epithelial cells to infant formula causes a decrease in steady-state levels of SP-A and SP-B mRNA within 24 hr. Continued incubation of the exposed cells in the absence of infant formula suggests that the reduction in expression of the surfactant proteins begins to reverse (Fig. 4), albeit at different rates. At 42 hr post-exposure, SP-A expression is significantly higher than the levels at 18 hr post-exposure, but expression is still significantly lower than levels seen in unexposed cells. On the other hand, steady-state levels of SP-B mRNA are significantly higher 42 hr post-exposure compared to both exposed and unexposed cells. It is known that transcription of the genes that encode all four surfactant proteins have common regulatory elements and factors.⁴⁰ However, there are factors specific to each individual gene, and the mechanisms that mediate post-transcriptional regulation of surfactant protein mRNA are widely divergent between the genes.⁴¹ It is understandable that expression of these genes rebound after transient exposure of the cell to an environmental insult, the cell may be responding in defense of the lung (SP-A) or in replenishing damaged components necessary for proper lung function (SP-B).

With regards to long-term effects of infant formula exposure, observations in rabbit bronchial muscle in vitro suggest that airway injury from milk aspiration early in development may persist with functional abnormalities increasing, even after the initial airway insult has ceased.⁴² Such is the case in this study; transient exposure to infant formula elicits a change in surfactant protein homeostasis up to 42 hr after exposure. As reported by Chin et al.,¹⁰ the type of infant formula makes a difference in the severity of functional change (A-a gradient and dynamic compliance) after exposure (Enfalac® ≈ HBM > Soy > Control). In these studies, the effect of infant formula on surfactant protein gene

expression is restricted to cow milk-based infant formula. Since non-fat dry milk did not have an effect on surfactant protein gene expression, the fat or lipid component of cow milk becomes very suspect as a causative agent in this phenomenon. However, the processing of non-fat dry milk may inactivate a protein component of the cow milk in that formulation that is not inactivated during production of infant formula.

These studies suggest that in addition to surfactant inactivation by aspiration, there may be an intracellular change in the lung epithelium which could augment or prolong lung dysfunction by altering homeostasis of surfactant proteins. Determination of the components of cow milk-based formula that are the cause of this decreased SP-A and SP-B expression is beyond the scope of this current study, but is important in the next step in understanding the pathogenesis for initial and sustained lung injury, and may help to influence clinical practice in such patients who are prone to have a higher likelihood of aspiration injury.

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Fig 1.

Expression of SP-A protein in NCI-H411 cells after transient exposure to various concentrations of infant formula. NCI-H441 cells were transiently exposed to infant formula for 6 hr, and intracellular and extracellular proteins were isolated 18 hr post-exposure. A: Representative Western blot of intra-cellular protein for the presence of SP-A and β -actin. B: SP-A levels were quantified relative to β -actin, and the SP-A levels of unexposed cells were normalized as 1. Shown are the SP-A levels from three experiments (N = 9); * P 0.02 from control, ** P 0.01 from control.

Fig 2.

Expression of SP-A and SP-B mRNA in NCI-H441 cells after transient exposure to various concentrations of infant formula. NCI-H441 cells were transiently exposed to infant formula for 6 hr, and RNA was isolated 18 hr post-exposure. Levels of SP-A mRNA or SP-B mRNA were determined relative to β -actin mRNA, and the SP-A or SP-B mRNA levels of unexposed cells were normalized as 1. A: Steady-state SP-A mRNA levels relative to unexposed cells from three experiments (N = 10); # P = 0.05 from control, * P = 0.01 from control. B: Steady-state SP-B mRNA levels relative to unexposed cells from three experiments (N = 10); * P = 0.01 from control.

Fig 3.

Expression of SP-A and SP-B mRNA in MLE-15 cells after transient exposure to various concentrations of infant formula. Murine alveolar epithelial (MLE-15) cells were transiently exposed to the various concentrations of infant formula for 6 hr. Eighteen hours post-exposure RNA was isolated and quantified by real-time qRT-PCR. Levels of SP-A mRNA or SP-B mRNA were determined relative to β -actin mRNA, and the SP-A or SP-B mRNA levels of unexposed cells were normalized as 1. A: Steady-state SP-A mRNA levels relative to unexposed cells from two independent experiments (N = 10); * P < 0.01 from B: Steady-state SP-B mRNA levels relative to control. unexposed cells SP-B mRNA levels relative to unexposed cells from two independent experiments (N = 10); * P < 0.01 from control.

Fig 4.

Temporal expression of SP-A and SP-B mRNA in NCIH411 cells after transient exposure to 10% infant formula. NCIH411 cells were transiently exposed to 10% infant formula for 6 hr. RNA was isolated immediately after exposure (0 hr), 18 and 42 hr post-exposure, and quantified by real-time qRT-PCR. Levels of SP-A mRNA or SP-B mRNA were determined relative to b-actin mRNA, and the SP-A or SP-B mRNA levels of unexposed cells were normalized as 1. A: Steady-state SP-A mRNA levels relative to unexposed cells from two independent experiments ($N = 10$); * $P = 0.02$ relative to unexposed cells, # $P = 0.04$ relative to 18 hr exposed samples. B: Steady-state SP-B mRNA levels relative to unexposed cells from two independent experiments ($N = 10$); * $P = 0.02$ relative to cells, # $P = 0.04$ relative to unexposed to 18 hr exposed samples.

Fig 5.

Expression of SP-A and SP-B mRNA in NCI-H441 cells after transient exposure to various infant formulas. NCI-H441 cells were transiently exposed to various infant formulas indicated (10% v/v) for 6 hr. RNA was isolated immediately 18 hr post-exposure, and quantified by real-time qRT-PCR. Levels of SP-A mRNA or SP-B mRNA were determined relative to β -actin mRNA, and the SP-A or SP-B mRNA levels of unexposed cells were normalized as 1. A: Steady-state SP-A mRNA levels relative to unexposed cells from two independent experiments ($N = 10$); * $P = 0.02$ relative to unexposed cells, # $P = 0.02$ relative to infant formula exposed samples. B: Steady-state SP-B mRNA levels relative to unexposed cells from two independent experiments ($N = 10$); * $P = 0.02$ relative to unexposed cells, # $P = 0.04$ relative to infant formula exposed samples.

TABLE 1—

Characteristics of the primers and specific assays used in real-time quantitative RT-PCR analysis of the mRNA

Human Surfactant Protein A			
Gene Symbol:	hSFTPA	438(+) CACTTTGTGATGCCAATTCAGGAG	PCR amplicon length = 69 bases
Accession Number:	NM_005411	500(-) TTCCTTGGGACAGCAATGC	LOD (RT-PCR) = 210 copies
		461(+) FAM-CATGTGCCAGAGCAGGGCGGC-BHQ1	PCR Efficiency = 97%
			Assay completely within Exon 6
Human Surfactant Protein B			
Gene Symbol:	hSFTPB	216(+) TGAGGACATCGTCCACATCC	PCR amplicon length = 79 bases
Accession Number:	NM_000542	289(-) CCAGGAACTTCTCTCATCGTGT	LOD (RT-PCR) = 185 copies
		242(+) FAM-AGATGGCCAAGGAGGCCAATTTCC-BHQ1	PCR Efficiency = 95%
			Assay crosses the Exon 3/4 junction
Mouse Surfactant Protein A			
Gene Symbol:	mSFTPA	690(+) CTTTGGCCTTCACCCCTCTTC	PCR amplicon length = 72 bases
Accession Number:	NM_023134	759(-) CCGGCACAACTTCTGTGCC	LOD (RT-PCR) = 200 copies
		711(+) FAM-TGACTGTGTGCTGGCATCAAGTGC-BHQ1	PCR Efficiency = 98%
			Assay completely within Exon 6
Mouse Surfactant Protein B			
Gene Symbol:	mSFTPB	567(+) GCAACAGCTCCCCAATCC	PCR amplicon length = 64 bases
Accession Number:	NM_147779	629(-) TGAACCGCTTGATCAGAGT	LOD (RT-PCR) = 230 copies
		608(-) FAM-CTGCAAGCCAGCAGAGGGCAG-BHQ1	PCR Efficiency = 100%
			Assay crosses the Exon 4/5 junction
Human β-Actin			
Gene Symbol:	Hactb	1040(+) CCTTGGCACCACGAC	PCR amplicon length = 71 bases
Accession Number:	NM_001101	1110(-) GCCGATCCACACGGAGTAC	LOD (RT-PCR) = 205 copies
		1052(+) FAM-ATCAAGATCATTCCTCTCTGAGCGC-BHQ1	PCR Efficiency = 99%
			Assay crosses the Exon 5/6 junction
Mouse β-Actin			
Gene Symbol:	mACTB	1037(+) TCTGGCTCTAGCACCATGA	PCR amplicon length = 74 bases
Accession Number:	NM_007393	1108(-) -CCACCGATCCACACAGAGTACT	LOD (RT-PCR) = 200 copies
		1059(+) FAM-ATCAAGATCATTCCTCTCTGAGCGC-BHQ1	PCR Efficiency = 97%
			Assay crosses the Exon 5/6 junction

LOD = lowest limits of detection.

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