Effects of intratracheal instillation of TNF-α on surfactant metabolism

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Salome, Ronald G., Diann M. McCoy, Alan J. Ryan, and Rama K. Mallampalli. Effects of intratracheal instillation of TNF-α on surfactant metabolism. J. Appl. Physiol. 88: 10-16, 2000.—Tumor necrosis factor-α (TNF-α) has been shown to play an integral role in the pathogenesis of the acute respiratory distress syndrome. This disorder is characterized by a deficiency of alveolar surfactant, a surface-active material that is composed of key hydrophobic proteins and the major lipid disaturated phosphatidylcholine (DSPC). We investigated how TNF-α might alter DSPC content in rat lungs by instilling the cytokine (2.5 µl) intratracheally for 10 min and then assaying parameters of DSPC synthesis and degradation in alveolar type II epithelial cells, which produce surfactant. Cells isolated from rats given TNF-α had 26% lower levels of phosphatidylcholine compared with control. TNF-α treatment also decreased the ability of these cells to incorporate [3H]choline into DSPC by 45% compared with control isolates. There were no significant differences in the levels of choline substrate or choline transport between the groups. However, TNF-α produced a 64% decrease in the activity of cytidylyltransferase, the rate-regulatory enzyme required for DSPC synthesis. TNF-α administration in vivo also tended to stimulate phospholipase A2 activity, but it did not alter other parameters for DSPC degradation such as activities for phosphatidylcholine-specific phospholipase C or phospholipase D. These observations indicate that TNF-α decreases the levels of surfactant lipid by decreasing the activity of a key enzyme involved in surfactant lipid synthesis. The results do not exclude stimulatory effects of the cytokine on phosphatidylcholine breakdown.

alveolar type II epithelial cells; cytidylyltransferase respiratory distress syndrome; tumor necrosis factor-α

PULMONARY SURFACTANT IS A HETEROGENOUS MATERIAL CONSISTING OF KEY HYDROPHOBIC PROTEINS AND LIPIDS THAT LINE THE ALVEOLAR SURFACE AND PREVENT ALVEOLAR COLLAPSE (26). DISATURATED PHOSPHATIDYLCHOLINE (DSPC) IS THE MAJOR SURFACE-ACTIVE LIPID COMPONENT OF SURFACTANT THAT IS PRODUCED BY THE ALVEOLAR TYPE II EPITHELIAL CELL (26). DEFICIENCY OF DSPC IS AN IMPORTANT FEATURE OF THE NEONATAL RESPIRATORY DISTRESS SYNDROME (RDS), LEADING TO WIDESPREAD ATELECTASIS, VENTILATORY IMPAIRMENT, AND GAS-EXCHANGE ABNORMALITIES. IN RDS, SURFACANT DEFICIENCY IS PRIMARILY THE RESULT OF LUNG IMMATURITY, AS INSUFFICIENT SURFACANT IS SYNTHESIZED AND SECRETED INTO THE ALVEOLAR LUMEN BY THE TYPE II CELL (26). SURFACANT DEFICIENCY ALSO OCCURS IN THE ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS), A CONDITION THAT COMMONLY OCCURS SECONDARY TO SEPSIS (10, 16). IN SEPSIS-INDUCED ARDS, SURFACANT DEFICIENCY APPEARS TO RESULT FROM SEVERAL INJURIOUS FACTORS THAT MIGHT INHIBIT SURFACANT PRODUCTION OR ALTER ITS FUNCTIONAL PROPERTIES (16).

TUMOR NECROSIS FACTOR-α (TNF-α) IS A SMALL POLYPEPTIDE CYTOKINE RELEASED BY ALVEOLAR MACROPHAGES AND OTHER CELL TYPES. IT HAS DIVERSE BIOLOGICAL EFFECTS, INCLUDING LEUKOCYTE ACTIVATION, INDUCTION OF FEVER, APOPTOSIS, AND CELL LYSIS (3). IN SEPSIS-INDUCED ARDS, TNF-α HAS BEEN IMPLICATED AS A MAJOR FACTOR FOR INDUCING ACUTE LUNG INJURY (29). TNF-α APPEARS TO HAVE HIBITATORY EFFECTS ON PARAMETERS OF SURFACANT METABOLISM. FOR EXAMPLE, IN VITRO, TNF-α INHIBITS THE PRODUCTION OF THE KEY SURFACANT-ASSOCIATED PROTEINS, SUCH AS SP-A AND SP-B (33). IN ARDS PATIENTS, ELEVATED LEVELS OF TNF-α MAY ALSO BE PRIMARILY RESPONSIBLE FOR DECREASING THE LEVELS OF SURFACANT PHOSPHOLIPID. ONE MAJOR MECHANISM BY WHICH TNF-α MIGHT DECREASE SURFACANT PHOSPHOLIPID IS BY STIMULATING THE ACTIVITY OF ENZYMES INVOLVED IN PHOSPHATIDYLCHOLINE BREAKDOWN (13, 16, 17, 23, 27, 35).

In addition to phosphatidylcholine degradation, the mass of this lipid is also tightly governed in cells by biosynthesis. The sequential steps required for phosphatidylcholine synthesis involve cellular uptake of choline, phosphorylation of choline by choline kinase (CK, EC 2.7.1.32), conversion of cholinephosphate to CDP-choline by cytidylyltransferase (CT, EC 2.7.7.15), and finally, generation of phosphatidylcholine by choline-phosphotransferase (CPT, EC 2.7.8.2).

To date, prior studies have not directly evaluated whether TNF-α alters surfactant lipid synthesis in vivo or in vitro. Recent studies showing that TNF-α suppresses [14C]glucose incorporation into DSPC in human adult type II cells led us to hypothesize that the inhibitory actions of TNF-α on surfactant phospholipid metabolism are mediated by inhibition of phosphatidylcholine synthesis (1, 2, 32). Studies using incorporation of radiolabeled precursors such as [14C]glucose or choline into surfactant lipids are limited, because differences in incorporation rates could be regulated at several steps, such as cellular transport of these labeled substrates, limitations of substrate pool sizes, and enzymatic sites within the biosynthetic pathway. Interpretation of studies using [14C]glucose incorporation into phosphatidylcholine are also difficult because

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glucose transport at the cell surface, interestingly, is also regulated by TNF-α (6). Thus, to test our hypothesis, we administered TNF-α intratracheally into rats and measured the sequential metabolic steps involved in surfactant synthesis and degradation in primary alveolar type II epithelial cells.

MATERIALS AND METHODS

Materials. The phospholipids, choline, cholinephosphate, and the lactic dehydrogenase kit were purchased from Sigma Chemical (St. Louis, MO). Human tumor necrosis factor-α (1 ng = 1.1 × 10⁶ activity units) was obtained from Endogen (Minneapolis, MN). All solvents were of Optima grade (Fisher Chemical). Silica LK5D (250 mm × 20 cm) TLC plates were purchased from Whatman International (Maidstone, UK). DMEM and choline-free medium were obtained from the University of Iowa Tissue Culture and Hybridoma Facility (Iowa City, IA). All radiochemicals were purchased from DuPont-New England Nuclear Chemicals (Boston, MA). Cell numbers were determined using a Coulter Z1 dual particle counter (Coulter, Miami, FL).

Animals and tissue preparation. Adult male Sprague-Dawley rats weighing 250–300 g were obtained from Sasco (Boston, MA). Rats were anesthetized with phenobarbital sodium (75 mg ip). Each experiment consisted of two control and two TNF-α-treated animals. The trachea was intubated with a 20-gauge plastic catheter, and animals immediately received either 0.5 ml of diluent or 2.5 µg of TNF-α intratracheally. Ten minutes after cytokine treatment, the animals were killed and the lungs were lavaged by instilling eight 3-ml volumes of normal saline. Lung tissue was enzymatically digested, and adult type II alveolar epithelial cells were isolated by cell filtration and differential adherence. After 4 h later as described previously (18), purity of the type II cells was >90% as assessed by using tannic acid staining. Cells were sonicated briefly in buffer A (150 mM NaCl, 50 mM Tris, 1.0 mM EDTA, 2 mM dithiothreitol, 0.025% sodium azide, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) at 4°C before enzyme analysis. In separate studies, alveolar type II epithelial cells were cultured in DMEM with the inclusion of 10% FCS for 24 h.

Lung measurements. Dry lung weight was measured by heating tissue at 80°C under 4 mmHg for 24 h by using a vacuum oven (29). Wet lung weight was also determined to assess for pulmonary edema.

Cell viability. As an overall measure of cell death, the release of lactic dehydrogenase was measured in lavage fluid. Alveolar type II cell viability was further assessed by Trypan blue exclusion. Cells were also pulsed with 1 µCi of [methyl-3H]thymidine to assess metabolic uptake of the nucleotide. After a 24-h pulse, the medium was removed, and 1 ml of cold 5% trichloroacetic acid was added to the cells for 30 min on ice. The cells were rinsed three times with water, and 1 ml of 0.33 N NaOH was added. The cells were scraped from the dishes and spun to pellet the crude membrane and debris, and an aliquot of supernatant was taken for scintillation counting.

Phosphatidylcholine and DSPC analysis. Lipids were extracted from equal amounts of protein from cells by using the method of Bligh and Dyer (5). The lipids were dried under nitrogen gas, applied in 50 µl of chloroform-methanol (2:1) to silica LK5D plates, and developed in chloroform-methanol-petroleum ether-acetic acid-boric acid [40:20:30:10:1.8, vol/vol] (21)]. After each plate was dried in a fume hood, the sample lanes and phospholipid standard lanes were briefly exposed to iodine vapors. Samples that comigrated with phosphatidylcholine standard were scraped from the silica gel and quantitatively assayed for phosphorus content (18). In other studies, the phosphatidylcholine samples were reacted with osmium tetroxide and run in the second dimension (21). The levels of DSPC were then quantitated by using scintillation counting or the phosphorus assay (18).

Choline transport and choline pool sizes. Choline transport was assessed by rinsing cells in choline-free medium and puncturing the cells in this medium overnight. Cells were then pulsed for 4 h with 1 µCi [methyl-3H]choline chloride at 37°C, after which the medium was discarded, cells rinsed thrice, and 0.1 N NaOH was applied to the cell monolayers (7). Cells were scraped into 15-ml plastic tubes, centrifuged, and the cell pellet resuspended and sonicated briefly in buffer A. An aliquot was then taken for scintillation counting.

Choline mass was assayed as a modification of the enzymatic procedure described by Post et al. (24). Each reaction contained 250 µg of protein residue, 100 mM glycylglycine (pH 9.2), 4 mM MgCl₂, 6 mM ATP, 4 µCi [γ-32P]ATP, and 0.1 units CK in a final volume of 200 µl. The reaction was terminated with the addition of 200 µl of cold ethanol after 1 h at 37°C, and the mixture was added to a 1 × 6-cm column prefilled with AG1-X8 resin. The choline phosphate product was eluted with three 1-ml volumes of 0.1 M ammonium bicarbonate and the resulting effluent taken for scintillation counting. The radioactivity of choline phosphate was compared with a standard curve of choline to determine choline mass.

Enzymes of phosphatidylcholine synthesis. The activity of CK was assayed as described (11). The reaction mixture (0.1 ml volume) contained 100 mM Tris-HCl buffer (pH 8.0), 10 mM magnesium acetate, 0.016 mM [14C]choline (specific activity ~7,000 dpm/nmol), 10 mM ATP, and 50–100 µg of cell sample. After a 1-h incubation at 37°C, the reaction was terminated with 0.2 ml of cold 50% trichloroacetic acid. Twenty-microliter aliquots of the mixture were spotted on Whatman 3MM paper, and choline metabolites were resolved by using paper chromatography as described (11). The spots that comigrated with the radiolabeled standard, choline phosphate, were cut and used for scintillation counting.

The activity of CT was determined by measuring the rate of incorporation of [methyl-14C]phosphocholine into CDP-choline by using a charcoal extraction method (21). All assays were performed without the inclusion of a lipid activator in the reaction mixture. Enzyme-specific activity is expressed as picomoles per minute per milligram of protein. One picomole per minute of activity represents 1 pmol of the product, CDP-choline, synthesized per minute and is equivalent to 1 micromol of enzyme activity.

The activity of CPT was assayed as described (22). Each reaction mixture contained 50 mM Tris-HCl buffer (pH 8.2), 0.1 mg/ml Tween 20, 1 mM 1,2-dioleoylglycerol, 0.8 mM phosphatidylglycerol, 0.5 mM [14C]CDP-choline (specific activity 1,110 dpm/nmol), 5 mM dithiothreitol, 5 mM EDTA, 10 mM MgCl₂, and 30–40 µg of sample. The lipid substrate was prepared by combining appropriate amounts of 1,2-dioleoylglycerol (1 mM) and phosphatidylglycerol (0.8 mM) in a test tube, drying under nitrogen gas, and brief sonication before addition to the assay mixture to achieve the final desired concentration. The reaction proceeded for 1 h at 37°C and terminated with 4 ml of methanol-chloroform-water (2:1:7, vol/vol). The remainder of the assay was performed exactly as described (22).

Enzymes of phosphatidylcholine hydrolysis. Direct exogenous assays for the acidic, calcium-independent phospholipase A₂ (PLA₂) and phosphatidylcholine-specific phospholipase C (PC-PLC) were conducted (8). The reactions were linear up to 500 µg of protein in the reaction mixture.
Phospholipase D (PLD) activity was also measured exactly as described (15).

Statistical analysis. The data are expressed as means ± SE. Statistical analysis was performed by using Student's t-test or the ANOVA with a Bonferroni adjustment for multiple comparisons.

RESULTS

Cell viability. TNF-α can induce cell death by apoptosis, and high doses (>75 μg) of cytokine can also induce cell necrosis (31). Thus we assayed parameters of cellular viability (Table 1). Recent studies demonstrate that low doses of intratracheal TNF-α (5 μg) do not induce alveolar type II epithelial cell apoptosis (20). Cell viability in the present studies was 87 ± 2 and 86 ± 3% in the control and TNF-α-treated groups, respectively, as determined by Trypan blue exclusion immediately after cell isolation. Preliminary studies demonstrated no histological evidence of lung cell injury (data not shown). Finally, because TNF-α has also been shown to alter cell proliferation, we assayed tritiated thymidine uptake as an overall measure of DNA synthesis and metabolic activity (Table 1). Indeed, no significant differences were observed for thymidine uptake, and the yield of type II cells was also comparable between the two groups.

Lavage analysis. Total lavage cell counts and levels of lactic dehydrogenase tended to be higher in the group of animals given TNF-α compared with controls; however, these values were not significantly different (Table 2). No significant differences in lavage protein concentration, total protein recovery, or lung weights were observed between the two groups. However, TNF-α administration decreased DSPC levels by 35% relative to control (Table 2, n = 3, P ≤ 0.05). Thus these results suggest that low doses of TNF-α decrease surfactant lipid content in vivo without significantly inducing alveolar inflammation.

Phosphatidylcholine and DSPC analysis. Intratracheal instillation of TNF-α significantly decreased the mass of phosphatidylcholine (Fig. 1A). After TNF-α instillation, the content of phosphatidylcholine in type II cells decreased from 30.7 ± 1.5 nmol/mg protein (control) to 22.7 ± 2.5 nmol/mg protein (TNF-α, P < 0.05). To determine whether the decrease in phosphatidylcholine mass was due to a decrease in phospholipid synthesis, type II cells were pulsed with [methyl-3H]choline and incorporation into DSPC, a more specific marker of surfactant lipid, was assessed. TNF-α significantly decreased incorporation of the label into DSPC by 45% (P = 0.001, Fig. 1B). These results suggest that TNF-α substantially reduces the biosynthesis of surfactant lipid in the lung by primary type II cells.

Choline transport and choline pool sizes. Differences in choline incorporation into DSPC observed between the two groups could be attributed to a decrease in choline transport or greater intracellular pools of unlabeled substrates for DSPC, such as choline, in TNF-α cells compared with controls (24, 30). To address these possibilities, we first cultured the cells in the presence of choline-free medium overnight and then pulsed the cells with [3H]choline in the same medium for 4 h. Cellular uptake of [3H]choline was then measured. Consistent with prior studies (6), TNF-α-treated cells analyzed shortly after isolation exhibited a 30% lower activity for [3H]choline transport relative to control cells, but these differences were not statistically significant (data not shown). To confirm whether there were differences in the pool size of choline, we directly assayed the concentration of choline in freshly isolated type II cells. In three separate studies, we did not detect significant differences between the groups in the mass of choline (15 ± 2.3 nmol/mg protein [control] vs. 12.5 ± 1.5 nmol/mg protein [TNF-α]).

Enzymes of phosphatidylcholine synthesis. To confirm whether TNF-α inhibits surfactant lipid synthesis, we assayed the activities of enzymes within the CDP-choline pathway, the principal pathway for phosphatidylcholine synthesis. There were no significant effects of the cytokine on CK activity, the first committed step in the pathway, or on CPT, the terminal enzyme involved in phosphatidylcholine synthesis. TNF-α sig-

### Table 1. The effect of intratracheal tumor necrosis factor-α on rat alveolar type II epithelial cell yield and viability

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>TNF-α</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell yield, ×10^6</td>
<td>34 ± 9</td>
<td>33 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>Cell protein, μg/dl</td>
<td>4.66 ± 1.6</td>
<td>3.49 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Cell viability, %Trypan blue exclusion</td>
<td>87 ± 2</td>
<td>86 ± 3</td>
<td>NS</td>
</tr>
<tr>
<td>[3H]thymidine uptake, dpm/2×10^6 cells</td>
<td>2560 ± 606</td>
<td>3155 ± 1105</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. Adult male rats were given tumor necrosis factor-α (TNF-α) (2.5 μg) intratracheally. The animals were killed 10 min later. Bronchoalveolar lavage fluid was obtained, and alveolar type II epithelial cells were isolated by enzymatic digestion and differential adherence. Data are representative of four separate experiments; each experiment consisted of four rats. Statistical analysis was performed using Student's t-test. NS, not significant.

### Table 2. Analysis of rat lung lavage following intratracheal TNF-α administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>TNF-α</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells, ×10^6</td>
<td>2.89 ± 0.66</td>
<td>3.57 ± 0.35</td>
<td>NS</td>
</tr>
<tr>
<td>Protein First lavage concentration, (μg/ml)</td>
<td>0.141 ± 0.03</td>
<td>0.116 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>Total protein recovery, mg (8 lavages)</td>
<td>4.72 ± 0.21</td>
<td>3.78 ± 1.27</td>
<td>NS</td>
</tr>
<tr>
<td>LDH, U/ml</td>
<td>28.3 ± 11</td>
<td>38.3 ± 6.0</td>
<td>NS</td>
</tr>
<tr>
<td>Lung weight, g</td>
<td>2.19 ± 0.14</td>
<td>2.15 ± 0.74</td>
<td>NS</td>
</tr>
<tr>
<td>Wet</td>
<td>0.188 ± 0.01</td>
<td>0.203 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Dry</td>
<td>1206 ± 182</td>
<td>790 ± 144</td>
<td>&lt;0.05</td>
</tr>
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</table>

Values are means ± SE. Adult male rats were administered TNF-α (2.5 μg) intratracheally. Bronchoalveolar lavage fluid was then analyzed. Data are representative of lavage analysis performed separately in four control and four TNF-α-treated rats. Statistical analysis was performed using Student's unpaired t-test. LDH, lactic dehydrogenase; DSPC, disaturated phosphatidylcholine.
The activities for each enzyme are expressed as pmol·min⁻²⁻³H]choline chloride. Cellular lipids were extracted, phosphatidylcholine resolved by using TLC, and the methyl moieties were separated by using TLC. These results indicate that the primary effect is to block the conversion of cholinephosphate to CDP-choline at the rate-limiting step within the phosphatidylcholine biosynthetic pathway.

Table 3. Effect of intratracheal TNF-α on the activity of enzymes involved in phosphatidylcholine synthesis in alveolar type II epithelial cells

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control</th>
<th>TNF-α</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline kinase</td>
<td>33 ± 6</td>
<td>29 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>Cytidylyltransferase</td>
<td>990 ± 310</td>
<td>360 ± 80</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cholinephosphotransferase</td>
<td>442 ± 46</td>
<td>425 ± 41</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. Adult rats were given TNF-α (2.5 µg it) and 10 min later animals were killed. The activity of enzymes involved in phosphatidylcholine synthesis was assayed in isolated alveolar type II epithelial cells 4 h later. The results represent data obtained from four separate experiments (choline kinase, n = 3). Each experiment consisted of two control and two TNF-α-treated animals. The activities for each enzyme are expressed as pmol·min⁻¹·mg cellular protein⁻¹. Statistical analysis was performed using Student’s t-test.

Fig. 1. Effect of tumor necrosis factor-α (TNF-α) on phosphatidylcholine content and disaturated phosphatidylcholine. Adult rats were given TNF-α (2.5 µg) or diluent (0.5 ml saline) intratracheally and were killed 10 min later. Alveolar type II epithelial cells were isolated 4 h later. The results represent data obtained from four separate experiments (choline kinase, n = 3). Each experiment consisted of two control and two TNF-α-treated animals. The activity of enzymes involved in phosphatidylcholine synthesis was assayed in isolated and 10 min later animals were killed. Alveolar type II epithelial cells were isolated from rats given diluent or various amounts of intratracheal TNF-α. Ten minutes after cytokine administration, animals were killed. Alveolar type II epithelial cells were analyzed for the activities of major phospholipases by using direct exogenous substrate assays. As shown in Table 4, TNF-α tended to increase PLA₂ activity by nearly 41%, although these effects did not achieve statistical significance (Table 3). Additional dose-response studies showed that 0.75–10 µg of intratracheal TNF-α administration were all effective in significantly reducing CT activity compared with control (Fig. 2). An overall trend toward a dose-response effect for TNF-α was observed; however, no significant differences in activity were seen between groups of animals receiving ≥1 µg of cytokine (Fig. 2). When TNF-α was instilled for longer periods of time, the cytokine also inhibited enzyme activity. For example, after a 30-min instillation, TNF-α decreased CT activity by 46%. Preliminary studies using similar conditions revealed that another cytokine elevated in ARDS, interleukin-1β, did not substantially reduce CT activity after intratracheal administration (data not shown). These results indicate that the primary effect of TNF-α is to block the conversion of cholinephosphate to CDP-choline at the rate-limiting step within the phosphatidylcholine biosynthetic pathway.

Fig. 2. Effect of intratracheal TNF-α on cytidylyltransferase activity in rat alveolar type II epithelial cells. Adult alveolar type II epithelial cells were isolated from rats given diluent or various amounts of intratracheal TNF-α. Ten minutes after cytokine administration, animals were killed. Alveolar type II epithelial cells were then isolated 4 h later, and cytidylyltransferase activity was assayed. Enzyme activity is expressed as pmol·min⁻¹·mg cellular protein⁻¹. For each dose of TNF-α used in the experiments, at least 3 animals were used (at the 2.5 µg TNF-α dose, n = 14). Data are expressed as means ± SE. *Values for control vs. TNF-α-treated groups were significant (P < 0.05) at all doses of cytokine using ANOVA.
reached statistical significance. Other studies revealed no effect of the cytokine on either PC-PLC or PLD activities. These results do not exclude the possibility that stimulation of PLA2 activity might also contribute to the negative effects of TNF-α on lung DSPC content.

**DISCUSSION**

Sepsis-induced ARDS is associated with surfactant deficiency and remains an important cause of mortality in hospitalized patients (28). The pathophysiological mechanisms underlying lung injury in ARDS remain complex. Neutrophils or alveolar macrophages recruited to the lung actively participate in producing lung edema and injury, in part, by releasing a variety of inflammatory mediators. Some of these mediators, including oxygen radicals and phospholipases, have been shown to decrease the functional pool of surfactant by directly inactivating its surface activity; these mediators can also decrease surfactant synthesis by injuring alveolar epithelium (16, 28). Perhaps more importantly, many of the manifestations of sepsis-induced lung injury can be reproduced in vivo by infusion of cytokines, such as TNF-α (28). The deleterious effects of TNF-α can also be attenuated in animal models by blocking antibodies to this cytokine (4). We focused our studies, therefore, on this cytokine because it appears to be a key participant in sepsis-associated ARDS. To date, prior studies have not directly investigated effects of TNF-α on surfactant lipid metabolism. In this work, we demonstrate that intratracheal TNF-α decreases surfactant phosphatidylcholine levels. Moreover, a novel observation from these studies is that the reduced levels of phospholipid are the result of TNF-α inhibiting the activity of the rate-limiting enzyme required for surfactant phospholipid synthesis, CT, rather than significant effects of the cytokine on stimulation of phospholipase activity.

One concern for these studies is that our biochemical observations might be attributed to significant cellular injury to alveolar epithelium after TNF-α treatment. Indeed, there is evidence that intratracheal cytokine administration to the animal model can induce considerable alveolar inflammation. By using one-fifth the dose of TNF-α used in this study, Ulich et al. (31) showed in rats that a neutrophilic inflammatory infiltrate persists up to 48 h after intratracheal cytokine (50 × 10⁶ U) treatment, and at higher doses (75 µg of TNF-α) Fuchs et al. (9) demonstrated significant bronchovascular edema. Interestingly, neither of these studies reported that intratracheal TNF-α produced alveolar epithelial cell death. Our results also show that the type II cell isolates were highly viable and metabolically active after TNF-α exposure (Table 1). In related studies, we have shown that alveolar type II epithelial cells do not undergo apoptosis after low-dose intratracheal TNF-α administration (20). Collectively, these observations indicate that potential cytotoxic effects of TNF-α on alveolar epithelium are not a major contributor to the findings in this study.

Most prior studies in other systems suggest that TNF-α lowers phosphatidylcholine content by increasing phospholipid degradation (13, 16, 17, 23, 27, 35). On the basis of these in vitro data, these effects of the cytokine are presumably an early event in the setting of sepsis-induced lung injury (13, 27). In sepsis, bacterial lipopolysaccharide (LPS) triggers the release of TNF-α from alveolar macrophages and subsequently initiates an inflammatory cascade leading to diffuse lung injury (16, 28). One event in this cascade is the activation of phosphatidylcholine-specific phospholipases that appear to be an important effector mechanism by which LPS and TNF-α stimulate surfactant breakdown. In this study, we observed a trend toward an increase in PLA2 activity, but we did not detect substantial increases in the other phospholipases. Nevertheless, because these enzymes are often activated within seconds to minutes after TNF-α treatment, it is possible that phospholipase activation was at least partly responsible for decreased alveolar and type II cell surfactant levels observed in these studies. Thus we suspect that this pathway is also physiologically relevant in vivo (Table 3).

Unlike surfactant phospholipid catabolism, changes in phosphatidylcholine biosynthesis usually occur over hours in vivo (34). In this study, we performed analysis of TNF-α effects on the biosynthetic pathway in cells several hours after cytokine administration. We observed that TNF-α exposure produces a substantial decrease in incorporation of radiolabeled choline precursor into DSPC in type II cells. Because TNF-α produced a modest decrease in choline transport as described previously in other systems (6, 12), this could partly explain the decrease in choline incorporation into DSPC. However, a greater effect of TNF-α in these studies was to significantly inhibit the conversion of cholinephosphate to CDP-choline at the CT step. The fact that TNF-α inhibited both choline incorporation into DSPC and CT activity clearly indicates that the cytokine downregulates the surfactant lipid biosynthetic pathway. The mechanisms by which TNF-α inhibits CT activity could be attributed to regulation of protein synthesis, changes in enzyme phosphorylation state, or induction of lipid inhibitors for the enzyme (14). With regard to the latter mechanism, we have recently shown that intratracheal TNF-α stimulates the generation of sphingolipids in the lung (20); some of these
lipids have been shown to be elevated in acute lung injury and inhibit CT function and phosphatidylcholine synthesis directly (19, 25). Thus, although there was only transient exposure of the lung to TNF-α, it is possible that the cytokine triggered a series of inflammatory events leading to the generation of these inhibitory lipids that decrease surfactant production.

In summary, studies to date indicate that TNF-α inhibits both surfactant apoprotein and phospholipid metabolism in alveolar type II epithelial cells. Effects of the cytokine on lipid metabolism include rapid acceleration of phosphatidylcholine turnover as supported by data in prior in vitro studies (27). However, our studies also show that an important complementary mechanism for TNF-α is to negatively impact overall synthetic capacity of phospholipid in type II cells. The significance of this latter mechanism on steady-state levels of lung DSPC content is more likely to be realized long-term after cytokine exposure; however, these biochemical effects are physiologically important because inhibitory effects on synthesis would undermine the ability of these cells to compensate for accelerated surfactant turnover observed initially. Future studies directed at determining how phosphatidylcholine production (and specifically CT) are inhibited by TNF-α might lead to new strategies targeted at antagonizing the cytokine’s effect on surfactant synthesis. Such studies might, in turn, lead to newer therapeutic interventions designed to minimize sepsis-associated acute lung injury.

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