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Mechanisms responsible for surfactant changes in sepsis-induced lung injury

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ABSTRACT: Pulmonary surfactant is altered in sepsis, and these changes contribute to the predisposition of septic lungs to subsequent insults, ultimately leading to acute lung injury. Specifically, the total amount of surfactant is lower in sepsis, mainly due to decreased small aggregate (SA) surfactant pools. The amount of large aggregate (LA) surfactant is not altered.

To evaluate the mechanisms responsible for these alterations, trace doses of tritium-labelled dipalmitoylphosphatidylcholine (^{3}H-DPPC)-labelled LA were instilled intratracheally into adult rats 20 hrs after caecal ligation and perforation (CLP) or sham surgery. Animals were sacrificed at 0, 1 and 4 h after instillation and recovery of ^{3}H-DPPC in alveolar macrophages (AM), LA and SA was measured. In separate *in vitro* experiments, AM isolated from CLP/sham rats were incubated with LA or SA isolated from normal animals to evaluate the uptake of these aggregates into the AM.

Results showed increased surfactant radioactivity associated with AM of CLP animals compared with sham animals both *in vivo* and *in vitro*. In addition, more ^{3}H-DPPC label remained in LA forms in the CLP animals *in vivo* compared with sham.

These findings indicate that differences in surfactant aggregate uptake and large aggregate conversion occur in septic lungs, resulting in changes in surfactant pools.

KEYWORDS: Lung injury, sepsis, surfactant

Pulmonary surfactant is a mixture of phospholipids and proteins that reduces surface tension at the air–liquid interface of the alveoli and thus prevents alveolar collapse during end-exhalation [1]. The surfactant system is altered both quantitatively and functionally in patients with acute lung injury (ALI) [2–6]. Furthermore, these observed impairments of pulmonary surfactant contribute to lung dysfunction. Unfortunately, the results of clinical trials evaluating exogenous surfactant administration in patients with ALI have been disappointing, in part due to the complexity and severity of the injury at the time of treatment [7–9]. Although earlier interventions would seem appropriate, a better understanding of surfactant alterations at this stage of the disease is required to justify this approach. Recent studies have shown that changes in the alveolar surfactant subfractions occur in animals and humans with less severe lung injury, while spontaneously breathing [10–12].

Within the alveoli, surfactant is present in a functional large aggregate (LA). During respiration, the LA are converted into nonfunctional smaller vesicles called small aggregates (SA) [13]. While the amounts of these aggregates remain stable within the normal lung, in severely injured lungs, the relative percentage of LA is decreased [3, 14]. Interestingly, recent findings have shown an increase in the percentage of large aggregate forms at earlier stages of injury. For example, the current authors have shown that both rats and mice with a mild lung injury induced by sepsis had an increase in the percentage of LA, which was due to a decrease in SA pools within the airspace [10, 15]. The mechanisms responsible for these specific changes are unknown, but did not involve surfactant protein A (SP-A) nor inducible nitric oxide synthase [15, 16].

The current authors hypothesised that the alterations of endogenous surfactant, occurring at earlier stages of sepsis, were due to an increased uptake of surfactant by alveolar macrophages and/or a decreased conversion of large into small aggregates. To test this hypothesis, the current authors determined the fate of intratracheally injected tritium labelled dipalmitoylphosphatidylcholine (^{3}H-DPPC) LA in rats after caecal ligation and perforation (CLP). In addition, the uptake of radiolabelled aggregates by alveolar macrophages (AM) isolated from CLP and sham animals was evaluated *in vitro*.

MATERIALS AND METHODS

Preparation of radiolabelled aggregates

LA were isolated *via* differential centrifugation of lung lavage obtained from normal adult rats. The

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LA were then labelled with ^{3}H-DPPC and the association of the radiolabel with the LA was verified with the use of sucrose density centrifugation, as previously described [17]. Radiolabelled SA were obtained *via* surface area cycling of aliquots of labelled LA [18]. Briefly, aliquots of labelled LA were isolated and cycled at 40 rev·min^{-1}, at 37°C, for 180 min. SAs were obtained in the supernatant of a 15 min, 40,000×*g* centrifugation, which were quantified by measuring phospholipids-phosphorus *via* the method of DUCK-CHONG [19] after chloroform extraction [20], and by measurement of radioactivity by scintillation counting.

Animal procedure

The animal procedures were performed according to the guidelines of the Canadian Council on Animal Care and were approved by the University Animal Care Committee. Sepsis was induced *via* the CLP procedure in adult rats as described previously [10]. Briefly, male Sprague-Dawley rats (350–450 g) were anaesthetised and the right external jugular vein and right carotid artery were cannulated with PE50 tubes. Both catheters were then routed subcutaneously to the back of the neck and attached to a three-fluid-channel (22-gauge) swivel system that allowed the rat to have an unlimited movement within the cage. A laparotomy was performed and animals randomised to the CLP procedure had the distal one-third section of the caecum ligated, punctured twice with a 16-gauge needle and compressed to extrude bowel contents into peritoneum. The caecum was then returned to the abdominal cavity and the incision was closed with 2.0 sutures. Sham animals underwent identical procedures after induction of anaesthesia, including catheter placement and laparotomy, but the CLP procedure was not performed.

After surgery, the rats were placed in plastic cages to recover. All animals received a continuous infusion of sterile saline at 7.5 mL·kg^{-1}·h^{-1} containing 2 μg·mL^{-1} of fentanyl for analgesia *via* the venous catheter. The arterial catheter was infused with sterile heparinised saline (1 U·mL^{-1}) at 1 mL·h^{-1} to maintain patency. Arterial blood gases were measured on an Acid-Base Laboratory 500 blood gas analyser (Radiometer, Copenhagen, Denmark). Arterial lactate levels were measured *via* a YSI 2300 STAT plus glucose/lactate analyser (Yellow Springs Instruments, Yellow Springs, OH, USA). Mean arterial pressure (MAP) and heart rate (HR) were recorded *via* a pressure transducer attached to the arterial line. Respiratory rate (RR) was also recorded. All of these parameters were measured at 4 and 20 h post surgery.

In vivo *metabolism of large aggregates*

At 20-h post sham or CLP surgery, the rats were re-anaesthetised intravenously with ketamine (10–20 mg·kg^{-1}) and xylazine (0.5–1 mg·kg^{-1}). After sedation was induced, the neck incision was reopened and the trachea was exposed. The animal was placed on a board at a 45° angle, and a trace dose of ^{3}H-DPPC-labelled LA (0.5 μCi·kg^{-1} body weight in 0.5 mL of saline, 0.5 μCi=0.125 mg phospholipids) was intratracheally instilled through a 22.5-gauge needle. After closing the incision with 2.0 sutures, the rats were then placed back into their cages and allowed to recover until sacrifice. Groups of animals were sacrificed at 0, 1, and 4 h post LA radiolabelled instillation *via* an overdose of sodium pentobarbital (110 mg·kg^{-1}) followed by transection of the abdominal aorta.

After sacrifice, the lungs were lavaged five times with saline, as previously described [10]. The total lavage fluid was then centrifuged at 150×*g* for 10 min to obtain the cellular pellet. The 150×*g* cell pellet was suspended in 1 mL of saline with aliquots of this suspension taken for cell counting *via* a haemocytometer, and for cell differential counting on a cytocentrifuge slide stained by the HemaColor® (EM Science, Gibbstown, NJ, USA). After washing twice with saline, the remaining cell pellet was subsequently resuspended in 200 μL lysis buffer (150mM NaCl, 50mM $NaPO_4$, 2mM EDTA, and 0.5% Nonidet P-40) for scintillation counting for radioactivity recovery. In the present study, cell differential counts demonstrated that the cell population isolated from the lung lavage of both CLP and sham rats was >98% macrophages. Therefore, the radioactivity measurements in the cell pellets were regarded as that attributed totally by the AM. The 150×*g* supernatant of each animal's lavage was then centrifuged at 40,000×*g* for 15 min, yielding a supernatant containing the SA subfraction of alveolar surfactant. The 40,000×*g* pellet was suspended in saline and defined as the LA fraction.

Immediately after the lavage procedure, the lung tissue was removed and homogenised in 15 mL saline, with aliquots extracted by the method of BLIGH and DYER [20]. The radioactivity of the lung homogenate extract together with aliquots of the alveolar cell suspension (150×*g* pellet), the 150×*g* supernatant (total surfactant), both LA and SA fractions as well as an aliquot of the administered radiolabelled input sample were all processed for scintillation counting. The total recovery of administrated radioactivity was expressed as the sum recovered in alveolar cells, total surfactant, and lung homogenate relative to the administered input sample.

To measure surfactant pool sizes in the CLP and sham animals at the 20-h time point after surgery, aliquots from the total surfactant, LA and SA fractions were extracted and phospholipid levels were evaluated using a modification of the Duck-Chong phosphorous assay [19, 20]. Total protein in the 150×*g* supernatant (total surfactant) sample was determined by the microbicin-choninic acid method (Pierce, Rockford, IL, USA) with bovine serum albumin used as the standard.

In vitro *uptake of aggregates by alveolar macrophages*

Separate groups of CLP/sham animals were prepared as described above and killed 20 h after surgery with their lungs subsequently lavaged. The cell pellet was isolated by centrifuging the lavage at 150×*g* for 10 min, and washed twice with ice-cold saline. The cells were then counted on a haemocytometer and viability was determined *via* trypan blue exclusion. Under the conditions employed, neither sepsis nor the sham surgical procedure affected the viability of lavage cells. The isolated cells (>98% macrophages) were resuspended in Dulbeco's Modified Eagle's Medium (Gibco Invitrogen, Burlington, On, Canada) with 10% foetal bovine serum at a concentration of 1×10^6 cells·mL^{-1}. Aliquots of normal, fresh ^{3}H-DPPC labelled LA or SA as described in the preparation of radiolabelled aggregates above, were added to this cell suspension at a concentration of 10 μg phospholipid·mL^{-1}. The cells were then incubated at 37°C for 60 min with gentle

agitation. Lipid uptake of the AM was stopped by washing and centrifuging the cells three times with ice-cold saline. After the third centrifugation, the cell pellet was suspended in lysis buffer for measurement of radioactivity via scintillation counting.

Statistics

Data are presented as means±SE. Values among groups were analysed using a two-way ANOVA followed by the unpaired Newman-Keuls t-test. A probability level of $p<0.05$ was considered statistically significant.

RESULTS

Physiological parameters of caecal ligation and perforation/sham rats

There were no differences in body weights between the CLP and sham rats (422±7 g *versus* 423±10 g, n=15 per group). At the 4-h time point, post surgery, there were no significant differences in arterial blood gases (arterial oxygen tension: P_{a,O_2}; carbon dioxide arterial tension: P_{a,CO_2}; pH), alveolar-arterial oxygen tension difference (P_{Aa,O_2}), arterial lactate levels, MAP, respiratory rate (RR) and HR between the two groups (table 1). These data indicate that all animals adequately recovered from the surgical procedures up to 4-h post surgery. Data shown at 20 h represent the values recorded immediately prior to the animals being re-anaesthetised for intratracheal instillation of the labelled LA. Consistent with previous studies in both rats and mice undergoing similar CLP procedures, significantly lower P_{a,O_2}, MAP, and significantly higher P_{Aa,O_2}, HR, RR, and arterial lactate levels were observed in the CLP rats, compared with the sham group (table 1) [10, 15, 16]. Consistent with these changes, representing a relatively mild lung injury, were previous studies showing that there were no changes in static lung compliance in the CLP group at 20 h as measured by pressure–volume curves [10, 15, 16].

TABLE 1 Physiological parameters for rats at 4 and 20 h post surgery

	Sham		CLP	
	4 h	20 h	4 h	20 h
P_{a,O_2} mmHg	95.5±1.6	94.5±1.3	93.2±2.2	82.7±3.4**,#
P_{a,CO_2} mmHg	33.2±0.7	36.9±0.8#	34.1±1.0	39.9±1.4#
O_2 saturate %	95.4±0.6	95.8±0.6	94.3±0.7	92.5±0.7**
P_{Aa,O_2}	12.5±1.4	8.8±1.2	14.5±2.0	17.9±3.3*
MAP mmHg	118±2	124±2	114±3	116±3*
HR beats·min^{-1}	343±8	338±7	335±7	372±14*,#
RR breaths·min^{-1}	91±2	94±2	92±1	106±4**,#
Lactate mmol·L^{-1}	0.61±0.03	0.62±0.03	0.69±0.02	1.0±0.07**,#

Data presented as mean±SD, n=15. CLP: caecal ligation and perforation; P_{a,O_2}: arterial oxygen tension; P_{a,CO_2}: carbon dioxide arterial tension; P_{Aa,O_2}: alveolar-arterial oxygen tension difference; MAP: mean arterial pressure; HR: heart rate; RR: respiratory rate. *: $p<0.05$, **$p<0.01$ *versus* sham; #: $p<0.01$ *versus* 4-h time point.

Alveolar surfactant pools, cells and total protein

The number of AM recovered from the CLP rats was not significantly different from that recovered from sham animals 20 h post surgery (4.7±0.5 *versus* 5.3±0.7×10^6 cells·lung^{-1}) or at various time points after intratracheal instillation of the trace dose of radiolabelled LA (data not shown). Figure 1 shows the total surfactant and aggregate pool sizes for CLP and sham rats 20 h post surgery. Both total surfactant and SA pool sizes were significantly lower in CLP *versus* sham groups ($p<0.01$), but LA pools were not different. Similar results were observed for samples obtained after the trace dose of labelled LAs were instilled (data not shown). Total protein levels in lung lavage were similar in CLP (17.6±2.9 mg·kg^{-1}) and sham (17.7±2.3 mg·kg^{-1}) groups at all time points, indicating minimal leak of plasma proteins into the airspace at this early and relatively mild phase of sepsis-induced lung injury.

Tritium dipalmitoylphosphatidylcholine recovery in vivo

Immediately after the intratracheal instillation of radiolabelled LA at the 20-h time point after either the CLP or sham procedure (time 0 h, fig. 2), the total recovery of the radiolabel in the lung (lavage+lung tissue+alveolar macrophages) relative to the input sample was 70.5±3.2 and 72.1±2.7% in the CLP rats and sham rats, respectively. This suggests that some of the instilled radiolabel was lost during the instillation and processing procedures. The recovery of the radiolabel in the lavage and lung tissue, as shown in figure 2a, is expressed as a percentage of the radioactivity measured at the 0-h time point. Although recovery in lavage decreased and recovery in tissue increased over time after instillation, there were no statistically significant differences noted between the CLP and sham groups in the radiolabel recovery. Figure 2b shows that the radioactivity recovered in AM normalised to cell number. The radioactivity associated with the alveolar macrophages isolated from the CLP rats was significantly higher than the activity associated with the macrophages isolated from the sham rats. This was statistically significantly high at the 1-h ($p<0.05$,) and 4-h time points ($p<0.01$) after ^{3}H-DPPC LA was instilled.

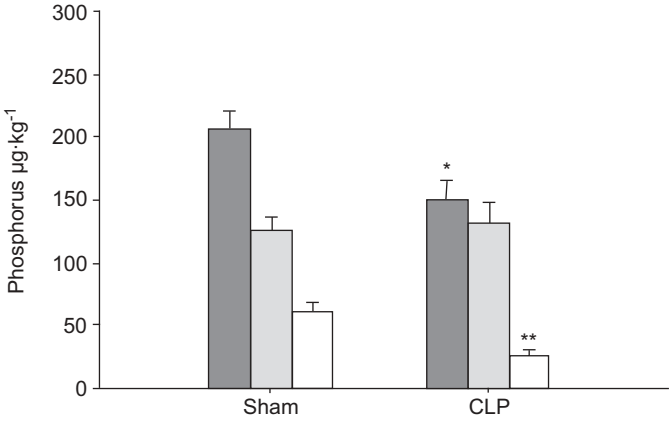

FIGURE 1. Analysis of phosphorus-phospholipid in alveolar lavage of caecal ligation and perforation (CLP)/sham group rats 20-h post surgery. Animals were sacrificed, lungs were lavaged and surfactant was isolated and analysed. ■: total surfactant; ▒: large aggregate; □: small aggregate. Results are shown as means±SE, n=8; *: $p<0.05$; **: $p<0.01$ *versus* the sham group.

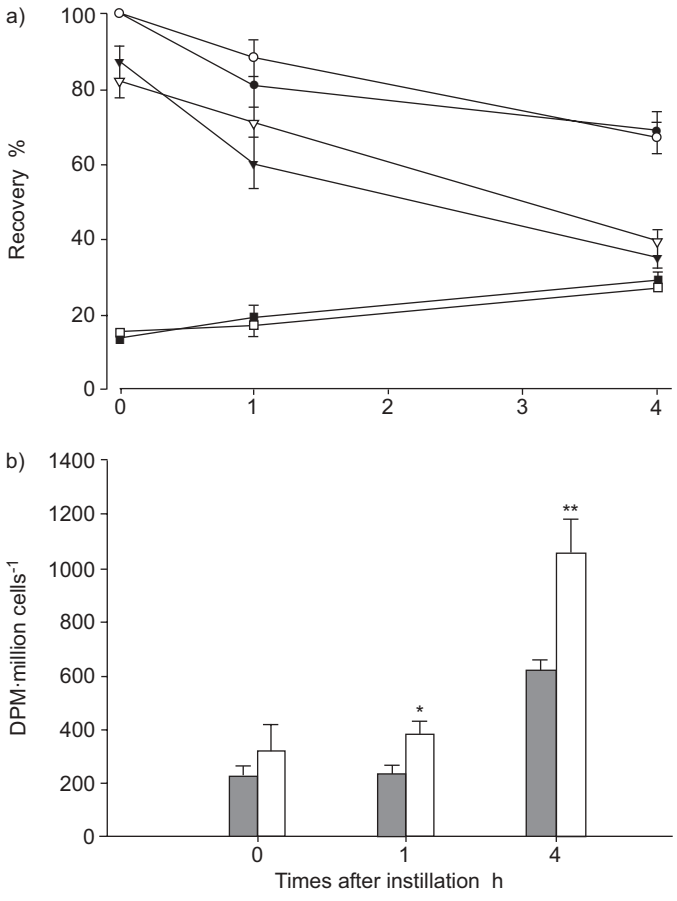

FIGURE 2. Recovered radioactivity in the lung of caecal ligation and perforation (CLP)/sham rats after the intratracheal instillation of tritium-labelled dipalmitoylphosphatidylcholine (^{3}H-DPPC) large aggregates (LA). ^{3}H-DPPC-labelled LA was intratracheally instilled into the lung of sham/CLP rats at 20 h post surgery. Animals were then killed and lungs were lavaged at 0-, 1-, and 4-h time points after instillation (n=5 per time point per group). a) The per cent recovery of radioactivity in alveolar total surfactant (TS), lung tissue relative to the recovery of total lung at 0 h was shown. ●: sham rat total lung; ○: CLP rat total lung; ▼: sham rat lavage TS; ▽: CLP rat lavage TS; ■: sham rat lung tissue; □ CLP rat lung tissue. b) The recovered radioactivity in alveolar macrophages was represented as disintegrations per minute (DPM) per million cells. Results are presented as means±SE, n=5. ■: sham rats; □: CLP rats. *: p<0.05; **: p<0.01 *versus* sham group at corresponding time point.

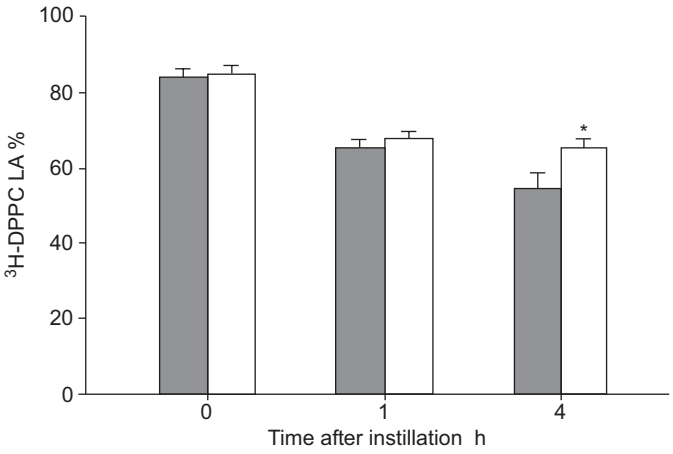

FIGURE 3. The per cent recovery of radiolabel in the large fraction of lung lavage after instillation. Animal groups and treatments were the same as described in figure 2. Alveolar large aggregate (LA) and small aggregate fractions were separated by centrifugation and radioactivity was counted. Results are means±SE, n=5. ^{3}H-DPPC: titrium-labelled dipalmitoylphospatidylcholine. ■: large aggregate; □: small aggregate*: p<0.05 *versus* sham group at corresponding time point.

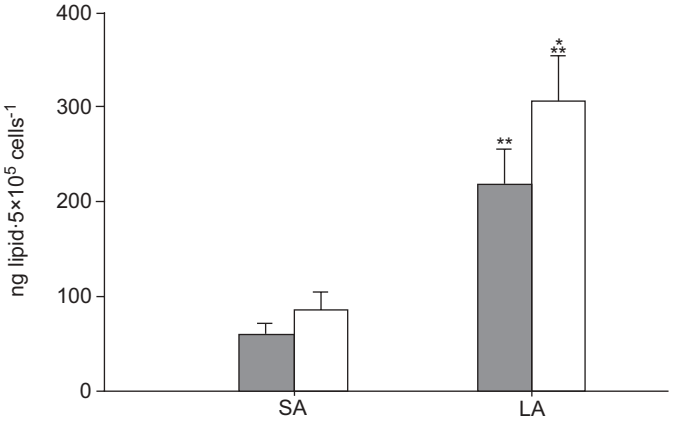

FIGURE 4. *In vitro* uptake of small aggregates (SA) and large aggregates (LA) fractions by alveolar macrophages from caecal ligation and perforation (CLP) or sham rats after 20 h. The isolated cells were incubated with ^{3}H-DPPC-labelled LA or ^{3}H-DPPC-labelled SA at a concentration of 10 μg phospholipids·mL^{-1} for 60 min at 37°C. Results are means±SE, n=7. ■: sham; □: CLP. **: p<0.01 *versus* SA uptake; *: p<0.05 *versus* sham.

Figure 3 shows the per cent recovery of ^{3}H-labelled LA in the lavage obtained from both CLP and sham animals at 0, 1 and 4 h after instillation. There was progressively less of the ^{3}H-DPPC recovered as LA over time (0–4 h) in both groups, although at the 4-h time point, a significantly higher per cent recovery of LA was observed in the CLP group compared with the corresponding sham group (p<0.05).

Tritium dipalmitoylphosphatidylcholine uptake in vitro

Figure 4 shows the results of the *in vitro* experiments involving the incubation of normal radiolabelled aggregates with AM isolated from either sham or CLP animals. The amount of radiolabelled LA associated with AM after 60 min of incubation with either sham or CLP AM was significantly greater (p<0.01) than the association of SA with these cells. Furthermore, the AM isolated from CLP rats had significantly more LA associated with these cells after 60 min than the macrophages isolated from sham animals.

DISCUSSION

Although mortality in patients with acute respiratory distress syndrome/acute lung injury has declined over the last decade, it remains high, in the order of 30–60% [21]. The most common and lethal cause of this disorder is systemic sepsis arising from the gut [22–24]. An important factor contributing to the particularly high mortality associated with sepsis-induced lung injury is the complexity and severity of the disease at the time of treatment. Pulmonary involvement at earlier stages

of sepsis has not been extensively evaluated, although recent studies using the CLP models of sepsis have shown specific alterations of the endogenous surfactant system when lung dysfunction was relatively mild [10, 15]. In the present study, the total amount of surfactant and the percentage of LAs was higher in the septic *versus* sham lungs, mainly due to decreased SAs pool sizes. The total amount of remaining functional forms of LA surfactant was not altered.

To examine the metabolism of surfactant, trace doses of radioactive LAs were instilled into septic rats. The greater radioactivity remaining in LA forms in the CLP animals suggests that there was decreased conversion from LA to SA in the septic rats. In addition, AM from CLP rats were associated with significantly greater amounts of the instilled radiolabel both *in vivo* and *in vitro*. It is concluded that both increased uptake of surfactant metabolites *via* AM and decreased conversion of LA into SA were responsible for the specific alterations of surfactant observed (*i.e.* decreased SA pools) at this early phase of sepsis-induced lung injury.

The current authors specifically focused on surfactant aggregate metabolism in these animals since these alterations of alveolar surfactant occur relatively quickly after the induction of lung injury. In contrast to the situation in severely injured lungs, the relatively mild lung injury observed in these spontaneously breathing septic animals was associated with no changes in LA pool sizes but a decrease in SA. This resulted in an increased percentage of LA in these spontaneously breathing animals, a finding also reported in a human study evaluating spontaneously breathing children with respiratory infections [12]. The current authors speculate that these changes in the endogenous surfactant system represent a compensatory response of the host to the initial insult. In the current study, more ^{3}H-label remained within the airspace in LA forms for the CLP animals compared with sham animals; this is consistent with the concept that decreased conversion of LA into SA is responsible for the altered amounts of surfactant aggregates in the septic lung. One mechanism through which these changes in aggregate conversion may occur is *via* decreases in the tidal volumes of the CLP animals. At this early stage of lung injury, spontaneously breathing animals have an increased respiratory rate accompanied by an increase in carbon dioxide tension levels. Although the tidal volumes were not measured in these animals, these changes would suggest that the tidal volumes were decreased in the CLP animals. Previous studies have shown that tidal volumes, but not respiratory rates were positively correlated with the conversion of LA into SA [17]. Lower tidal volumes in this setting would, therefore, tend to preserve surfactant in the functional LA forms due to smaller phasic changes in alveolar surface area. Moreover, given the fact that there was relatively little protein leaking into the airspace of these animals, increased protease activity, another major factor driving aggregate conversion, was an unlikely contributor to the aggregate changes observed.

The importance of AM in surfactant metabolism is evident from studies involving transgenic mice deficient in granulocyte macrophage colony stimulating factor [25, 26]. These animals have marked increases in alveolar surfactant due to the inability of the macrophage to degrade surfactant components. The current authors showed that within the injured lungs of the CLP rats, AM were associated with greater amounts of the instilled ^{3}H-DPPC LA compared with sham groups. In addition, macrophages isolated from the septic rats took up more surfactant lipids *in vitro* compared with the macrophages from sham animals. Mechanisms responsible for these differences in macrophage activity are unknown, but may be related to increased levels of macrophage inflammatory protein-2 and other inflammatory mediators that are observed in CLP animals.

Although AMs are thought to mainly take up SA forms within the airspace [13, 27], the current *in vitro* results showed that greater amounts of LAs were associated with the macrophages compared with SAs. However, it should be noted that *in vitro* conditions, such as the specific localisation of cells with surfactant, as well as the concentrations of surfactant used, may differ from the situation *in vivo*.

There are some limitations to the present study. These include the fact that tidal volumes in these animals were not measured and it could only be speculated that they were decreased in the CLP group. Measuring tidal volumes in spontaneously breathing animals is technically difficult, and the current authors feel that the assumption is valid given the changes observed in other physiological parameters. Another limitation is that only the macrophages were focused upon in these studies although type II cells and other inflammatory cells recruited to lungs at later stages of injury, such as neutrophils, may also contribute to surfactant changes. Finally, the current authors did not address the role of the surfactant proteins in this study. SP-A has been shown to influence surfactant metabolism, although previous studies, carried out in the authors' laboratories, showed that transgenic mice deficient in SP-A had similar outcomes as wild-types, including surfactant changes, when undergoing the CLP procedure. Evaluation of the other surfactant proteins, particularly their role in treatment strategies for sepsis-induced lung injury, will be the focus of future studies.

In conclusion, the present study suggests that the early changes in surfactant in sepsis-induced lung injury are related to altered surfactant metabolism, including decreased conversion of large aggregates into small aggregates and an increased uptake of surfactant into alveolar macrophages. The authors speculate that these changes may represent a protective response of the host to the primary lung insult by preserving alveolar surfactant in large aggregate forms. However, further studies are required to determine if these changes are indeed beneficial or potentially detrimental in the long term. A greater understanding of the importance of the surfactant changes occurring at the various stages of lung injury, and in particular the earlier stages, is required so that optimal treatment strategies can be developed with the aim of mitigating progressive lung dysfunction.

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