

Pulmonary and Systemic Induction of SAA3 After Ventilation and Endotoxin in Preterm Lambs

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ABSTRACT

Serum amyloid A (SAA), an acute phase reactant (APR) protein, is induced in liver during systemic inflammation. Serum amyloid A3 (SAA3), an isoform of SAA, is induced in both liver and extra hepatic sites in response to proinflammatory stimuli such as cytokines. Previously, we showed a modest increase in plasma cytokine levels in a preterm lamb model of lung injury. The study objective was to determine the relative contributions of lung and liver to the acute phase response during postnatal lung injury. Preterm (130d) and near term (141d) newborn lambs (term = 150d) were randomized to either no ventilation (controls), ventilation + intratracheal (IT) endotoxin (endo) or ventilation + IT saline. A group of near term lambs were exposed to ventilation + IV endotoxin. In the lungs, ventilation alone increased SAA3 mRNA 3- and 13-fold while ventilation + IT endotoxin increased SAA3 mRNA 64 and 366-fold above controls in preterm and near term lambs, respectively. In the liver, SAA3 mRNA was induced by ventilation alone (three-fold) and ventilation + IT endotoxin (45-fold) above controls in both

preterm and near term animals. Ventilation + IV endotoxin caused the highest increase in SAA3 mRNA (212-fold) in the liver of near term animals. A different isoform, identified as SAA-Liver inducible was maximally induced in liver by ventilation alone with minimal further response to endotoxin. Lung SAA3 mRNA expression was detected primarily in airway epithelium, bronchial glands, perichondrium of bronchial cartilage and vascular smooth muscle cells. Our experiments show rapid induction of an APR gene in lung in response to proinflammatory stimuli. (*Pediatr Res* 58: 1204–1209, 2005)

Abbreviations

APR, acute phase reactant
BAL, bronchoalveolar lavage
IT, intratracheal
IV, intravenous
SAA, serum amyloid A
SAA-Li, serum amyloid A-liver inducible

The acute phase of the systemic inflammatory response is characterized by the hepatic synthesis and increased plasma concentrations of acute phase reactants (APR) such as heat shock proteins, C reactive protein (CRP), and the serum amyloids (1–3). The serum amyloid A family of proteins is one of the major APR whose plasma levels can increase up to 1000-fold in response to inflammation (4). Several isoforms of the SAA protein are conserved across species. The liver is the main source of SAA1 and SAA2 isoforms whereas SAA3 isoform expression is increased in liver as well as extra hepatic tissues (4–7). We identified SAA3 as an endotoxin inducible gene in the fetal lung using subtraction hybridization of endotoxin *versus* saline exposed lung mRNA (8). No information is

available regarding the biology of SAA3 in preterm newborn animals exposed to postnatal lung injury induced by mechanical ventilation, oxygen or endotoxin.

Mechanical ventilation and pulmonary infections frequently cause lung injury in preterm infants. Mechanical ventilation contributes to neonatal morbidity and mortality (9,10) by causing lung inflammation with the induction of the pro-inflammatory cytokine cascade (11–13). Intratracheal endotoxin induces both lung inflammation and a systemic inflammatory response in preterm and term ventilated lambs (14). Whether there is induction of a lung acute phase reactant response in these models is not known. Furthermore, the occurrence of a systemic acute phase reactant response on lung exposure to pro-inflammatory stimuli is not well characterized. We hypothesized that SAA3 mRNA expression would increase in the preterm newborn lung postnatally exposed to pro-inflammatory stimuli. We, therefore, examined the effect of mechanical ventilation with or without endotoxin exposure on SAA3 mRNA induction in preterm and near term lamb lungs and contrasted the lung responses to the liver responses. In

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addition, we characterized the sites of induced expression of SAA3 mRNA in lung.

METHODS

Animals and ventilator strategy. The tissue samples from the preterm (130d GA) and near term lambs (141d GA) used for these experiments were previously used for traditional assessments of lung inflammation (14). The protocols were approved by the Animal Care and Use Committee of the Cincinnati Children's Hospital Research Foundation. The lambs were delivered by cesarean section and randomized to receive either 10 mg/kg of endotoxin (*Escherichia coli* 055:B5; Sigma Chemical Co. Chemicals, St. Louis, MO) or identical volumes of saline intratracheally at delivery before the first breath. The preterm animals received endotoxin mixed with 100 mg/kg surfactant (Ventecut; Byk Gulden, Konstanz, Germany) containing 2% recombinant human SP-C in phospholipids (15). To characterize the systemic response to endotoxin, another group of near term animals was given intravascular endotoxin (5 μ g/kg) at delivery. The lambs were ventilated for 6 h using time-cycled, pressure limited infant ventilators (Sechrist Industries, Anaheim, CA). The initial ventilator settings included fraction of inspired oxygen (F_{iO_2}): 1.0; inspiratory time: 0.6 s; positive end-expiratory pressure (PEEP): 4 cm H_2O and a peak inspiratory pressure (PIP) sufficient to obtain a target tidal volume (V_T) of about 10 mL/kg. The PIP was limited to 35 cm H_2O to decrease the risk of pneumothorax. Subsequently, to minimize lung injury, the ventilatory pressure (PIP-PEEP) and F_{iO_2} were adjusted to achieve a target $PaCO_2$ of 45–55 mm Hg and a PaO_2 of 150–200 mm Hg. Higher airway pressures were needed for the preterm compared with the near term animals to maintain the target $PaCO_2$ values (14). Control groups of gestation-matched lambs were not ventilated. Comparisons were made between treated animals at 6 h of age and the unventilated control groups killed at birth with an overdose of IV pentobarbital.

Sample collection. Lung tissues from the right lower lung lobe and liver tissues were snap-frozen for RNA and protein analysis. The right upper lung lobe was inflation fixed at 30 cm H_2O in 4% paraformaldehyde and used for *in situ* hybridization. Bronchoalveolar lavage (BAL) was performed on the left lung using 0.9% NaCl at 4°C until full distension and repeated five times. Plasma was analyzed at 4 h after delivery for SAA levels since a previous study demonstrated pro-inflammatory cytokine expression at this time period (14).

SAA3. Riboprobe. The sheep SAA3 cDNA clone [pGEMT-sSAA3(8C3)] (8) was cut with Fok I and a 420 nucleotide antisense riboprobe was synthesized using T7 RNA polymerase. This riboprobe produced a 282 nucleotide protected fragment with SAA3 as shown in Fig. 1A.

RNA quantitation. Total RNA was isolated using a modified Chomczynski method from lung and liver tissue (16). Ten micrograms of RNA were used for RNase protection assays with the antisense SAA3 riboprobe depicted in Fig. 1A. Solution hybridization was performed in 80% deionized formamide, 0.4M NaCl, 2 mM EDTA and 0.04M PIPES, pH 6.6 using a molar excess of [32 P] UTP labeled probes for 16h at 55°C. Single stranded RNA was digested with RNase A/T1 (Pharmingen, San Diego, CA). RNase was inactivated and the protected RNA was precipitated using RNase inactivation buffer (Ambion, Austin, Texas) and ethanol. The probe for L32, a ribosomal protein mRNA, was included in each assay as an internal standard (8). Protected fragments were resolved on 6% polyacrylamide 8 M urea gels, visualized by autoradiography and quantified on a Phosphor Imager using Image Quant version 1.2 software (Molecular Dynamics, Sunnyvale, CA). To ensure accurate comparisons between large numbers of samples in different groups, the same set of ventilated only animals were used as "standards" in each experiment. Thus the SAA3 mRNA comparisons in each gel were made relative to the ventilated group. A correction factor was then used to account for experimental variations between gels. For ease of depiction and graphical presentation, the values for the unventilated control group of both preterm and near term animals were arbitrarily set to equal 1.

In situ hybridization. *In situ* hybridizations of paraffin-embedded sections were performed using [35 S]UTP labeled probes as previously described (17). The plasmid psSAA(8c3) was digested with *NotI* for antisense probe (T7 polymerase; Promega) and with *NcoI* for sense probe (SP6 polymerase; Promega) synthesis. For hybridization, the sense and the antisense probes were diluted in hybridization buffer to a final concentration of 4.7×10^4 cpm/ μ L and incubated at 57°C. After hybridization, the sections were washed at high stringency because of the high GC content (55%) of the riboprobe. A wash buffer containing 50% formamide, $2 \times$ SSC and 10 mM DTT for 30 min at 65°C was used. The sections were treated with RNase A/T1 to reduce nonspecific binding and subsequently washed in a descending series of SSC solutions ending in a 15 min wash at room temperature in $0.1 \times$ SSC/1 mM DTT. Tissue sections were photographed under dark-field illumination and

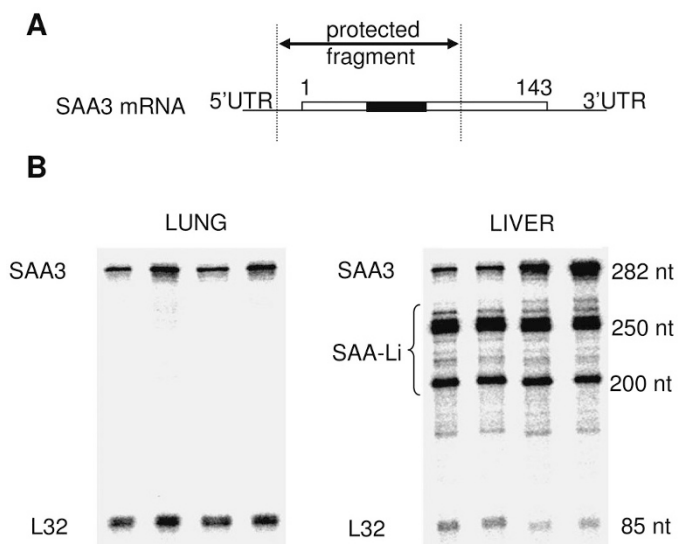


Figure 1. Sheep SAA mRNA isoforms. A) Schematic representation of SAA3 riboprobe in relation to the mRNA. The SAA3 riboprobe protected a 282 nucleotide (nt) fragment which encompassed portions of the 5' untranslated region (UTR) and the coding sequence of the SAA3 mRNA including a conserved region present among all SAA isoforms (shaded region). The amino acid sequence of the conserved region is DKYFHARGNYDAAQRGPG-GVWAA. The numbers 1 through 143 represents the amino acids of the translated SAA3 protein. B) Representative RNase protection assays of SAA3 and serum amyloid A-Liver inducible (SAA-Li) isoforms using 10 μ g total RNA from lung and liver of 4 IV (IV) endotoxin-exposed near term animals. SAA3 was detected in both lung and liver whereas SAA-Li isoforms were detected only in the liver.

then counterstained with hematoxylin for morphologic analysis. Controls for specificity of riboprobe binding included lung tissues from preterm and near-term lambs ventilated with no endotoxin exposure and a homologous (sense) probe.

ELISA. The concentration of SAA proteins in the plasma and lung homogenates of near term animals and the bronchoalveolar lavage of preterm animals was measured using a sandwich ELISA kit (Tridelata Diagnostics, New Jersey). The SAA isoform specificity of the ELISA assay is not known. An antibody specifically binding to SAA3 is not available.

Statistics. All values were expressed as means \pm SEM. Analysis of variance (ANOVA) was used for comparison of differences between groups with Student-Newman-Keuls test used for *post hoc* analysis. The nonparametric Mann-Whitney test was used for two group comparisons. Significance was accepted at $p < 0.05$.

RESULTS

Detection of SAA isoforms. Using the SAA3 riboprobe (Fig. 1A), a single protected fragment (282 nucleotides) corresponding to the full-length SAA3 probe was detected in lung samples (Fig. 1B). In contrast, in the liver, in addition to the 282 nucleotide protected fragment, two other fragments of approximately 250 and 200 nucleotides in length were detected. These additional protected fragments, closely related to SAA3 but different in expression pattern and size, were designated as SAA-Liver inducible (SAA-Li) in this manuscript.

SAA3 mRNA expression in preterm lambs. SAA3 mRNA increased approximately 3-fold in the lung and liver of ventilated preterm animals compared with unventilated controls (Fig. 2). Compared with controls, SAA3 mRNA increased 64-fold in the lung and 44-fold in the liver in response to intratracheal endotoxin and ventilation.

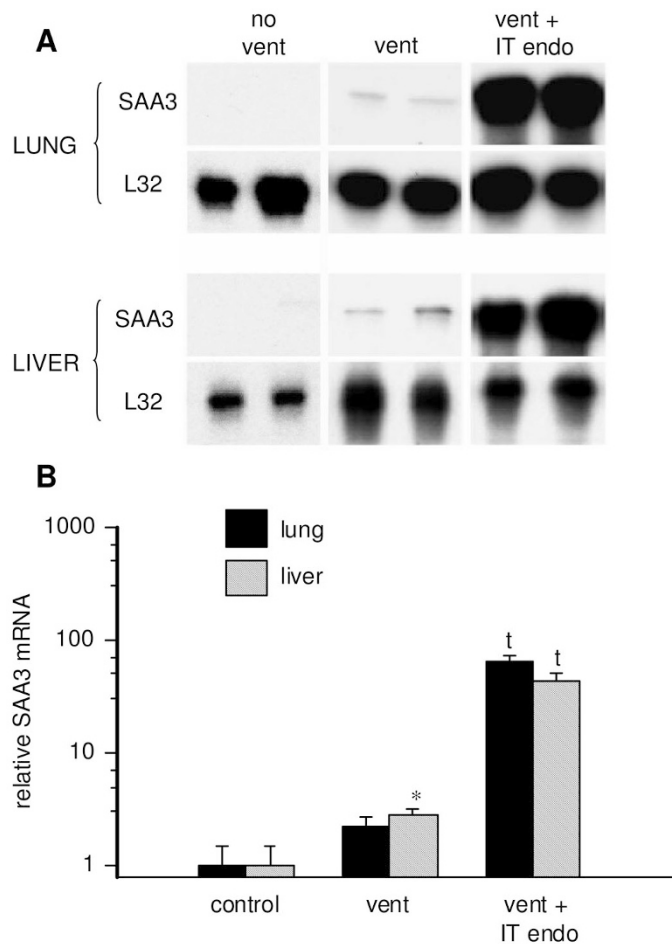


Figure 2. SAA3 mRNA induction in preterm (130d) lamb lung and liver. *A*) Representative RNase protection assays of SAA3 using 10 μ g total RNA from lung and liver of two animals per group. *B*) Quantitation of induction of SAA3 mRNA in response to ventilation and ventilation (vent) + endotoxin (endo) using a log scale (Y-axis). Control animals were not ventilated. SAA3 mRNA values were normalized to L32. The mean SAA3 mRNA signal in the control animals was given a value of 1 and the levels in the treatment groups are expressed as mRNA values relative to controls. Ventilation + intratracheal endotoxin (IT endo) caused the highest increase in SAA3 mRNA in both lung and liver. (* $p < 0.05$ vs. control; $p = 0.056$ for the lungs of vent group vs. control; $^t p < 0.001$ vs. other groups in lung or liver; $n = 5$ animals for control and 4–5 animals for the treatment groups; IT, intratracheal).

SAA3 mRNA expression in near term lambs. In response to ventilation alone, SAA3 mRNA increased almost 13-fold in the lung while liver SAA3 mRNA increased 3-fold when compared with unventilated controls (Fig. 3). On exposure to ventilation and intratracheal endotoxin, SAA3 mRNA increased 366-fold in the lung compared with controls while there was a 42-fold induction of SAA3 mRNA in the liver. Exposure to ventilation and IV endotoxin resulted in a 212- and 139-fold induction of SAA3 mRNA in liver and lung, respectively, compared with controls.

SAA-Li mRNA expression in liver of near term lambs. Due to similarity in the magnitude of induction of the 250 and 200 nucleotide SAA-Li fragments, the quantitation of only the 250 nucleotide fragment was reported (Fig. 4). When compared with unventilated controls, SAA-Li mRNA increased 10-fold in the liver of ventilated near term animals. Exposure of these

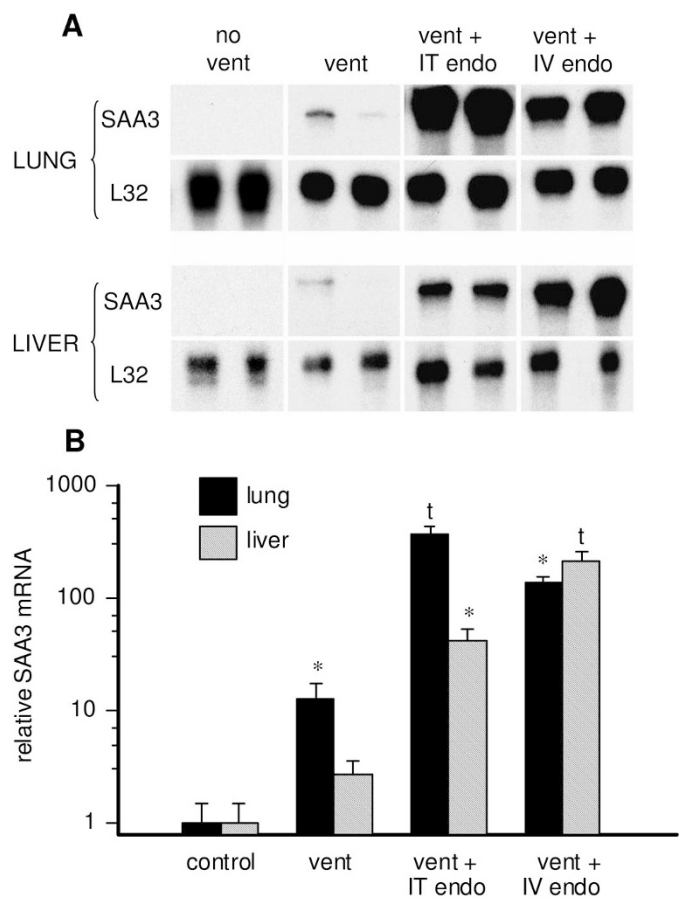


Figure 3. SAA3 mRNA induction in near term (141d) lamb lung and liver. *A*) Representative RNase protection assays of serum amyloid A3 (SAA3) using 10 μ g total RNA from lung and liver of two animals per group. *B*) Quantitation of induction of SAA3 mRNA in response to ventilation (vent) and ventilation + endotoxin (endo) shown on a log scale (Y-axis). Control animals were not ventilated. SAA3 mRNA values were normalized to L32. The mean SAA3 mRNA signal in the control animals was given a value of 1 and the levels in the treatment groups are expressed as mRNA values relative to controls. Ventilation caused a significant increase in SAA3 mRNA in the lung relative to controls. Ventilation + intratracheal endotoxin (IT endo) caused the highest increase in SAA3 mRNA expression in the lung relative to all other lung groups. Ventilation + IT endotoxin significantly increased SAA3 mRNA expression in the liver relative to controls. Ventilation + IV endotoxin produced the highest increase in SAA3 mRNA in the liver relative to all other liver groups. (* $p < 0.05$ vs. control; $^t p < 0.001$ vs. other lung or liver groups; $n = 5$ –6 animals for control and 4–6 animals for the treatment groups; IT, intratracheal; IV, intravenous).

ventilated animals to either intratracheal or IV endotoxin did not produce significant further induction of SAA-Li mRNA in the liver.

Sites of SAA3 expression in lung after IT endotoxin. Ventilation alone induced a low level of expression of SAA3 mRNA in the saline treated near term animals in the bronchial epithelium and vascular smooth muscle (Fig. 5A,B). Exposure to ventilation and intratracheal endotoxin resulted in prominent expression in the bronchial epithelium, bronchial glands and arterial smooth muscle cells (Fig. 5C,D). A high level of expression was also detected in the bronchial epithelium, bronchial glands, and arterial smooth muscle cells in response to ventilation and IV endotoxin (Fig. 5E,F). The perichondrial cells of bronchial cartilage had prominent SAA3 mRNA ex-

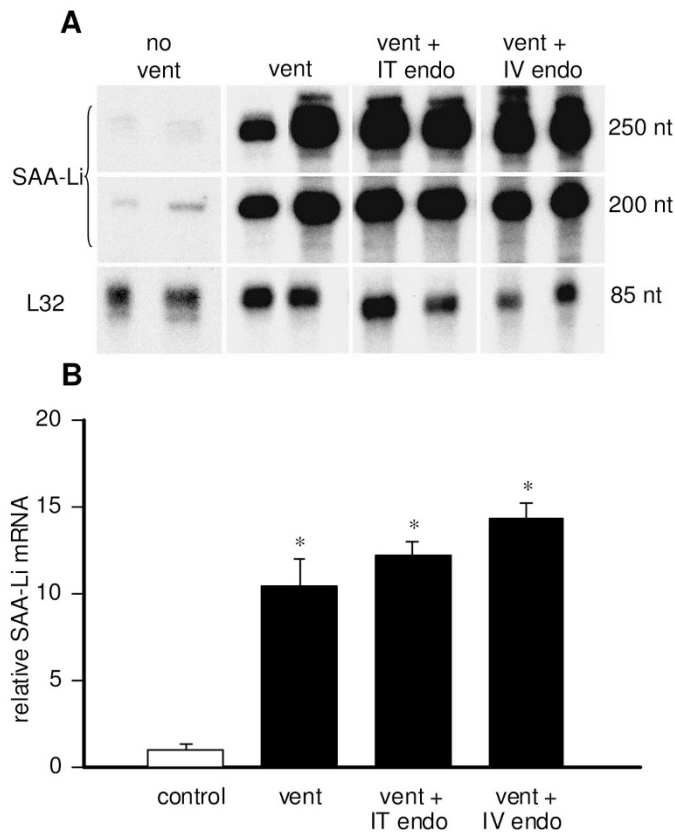


Figure 4. SAA-Li mRNA induction in liver from near term lambs. *A*) Representative RNase protection assays of serum amyloid A-Liver inducible (SAA-Li) using 10 μ g total RNA from liver of two animals per group. *B*) Quantitation of induction of SAA-Li mRNA in response to ventilation (vent) and ventilation + endotoxin (endo). Control animals were not ventilated. The SAA-Li mRNA values were normalized to L32. The mean SAA-Li mRNA signal in control animals was given a value of 1 and the levels in the treatment groups are expressed as mRNA values relative to controls. The SAA3 mRNA expression in the treatment groups was significantly increased relative to controls. (* $p < 0.001$ vs. control; $n = 6$ animals for control and 4–6 animals for the treatment groups; IT, intratracheal; IV, intravenous; nt, nucleotides).

pression (Fig. 5E,F). This was detected in the lungs of ventilated lambs exposed to either IV or intratracheal endotoxin. The controls had no expression of SAA3 mRNA (Fig. 5G,H). Sporadic expression of SAA3 mRNA was also detected in neutrophils but this expression was much lower than the levels detected in the bronchial epithelium, bronchial glands, perichondrial cells and arterial smooth muscle cells. The pattern of expression was similar for the preterm lambs (data not shown).

SAA protein quantitation. SAA protein was not detectable in the plasma of unventilated or ventilated near term animals using an ELISA kit of unknown SAA specificity. Intratracheal endotoxin induced plasma SAA concentration to 1474 ± 165 ng/mL in the near term animals whereas IV endotoxin caused plasma SAA levels of 2928 ± 589 ng/mL. The SAA protein was below detectable levels in the lung homogenates or BAL fluid obtained from near term or preterm lambs exposed to ventilation and endotoxin.

DISCUSSION

In this study designed to evaluate lung acute phase reactant response, we demonstrate that SAA3 mRNA is robustly in-

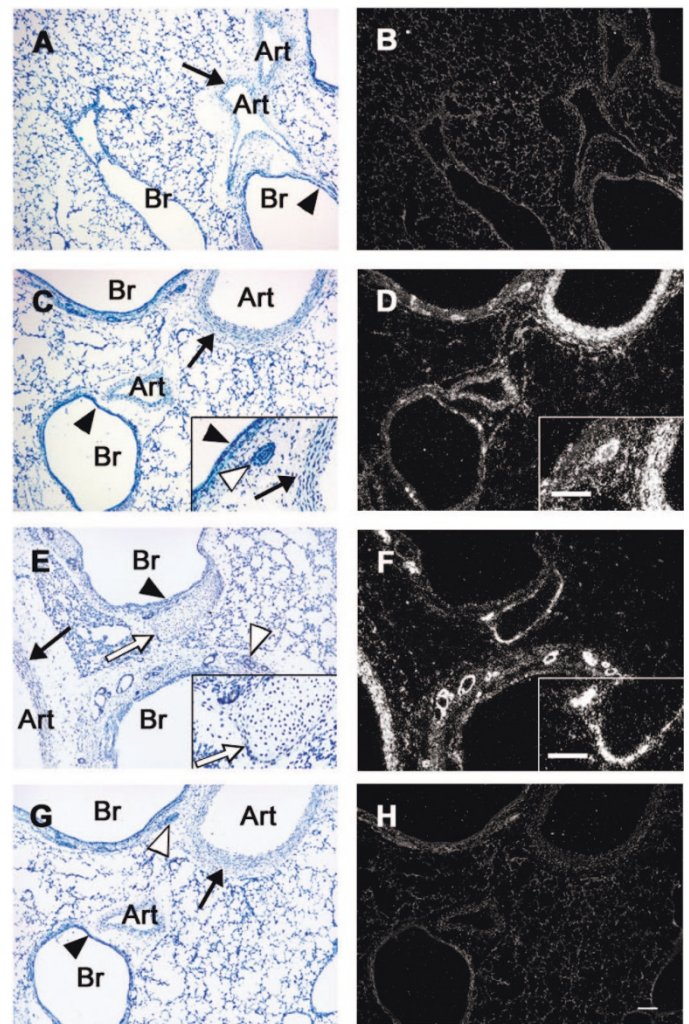


Figure 5. SAA3 mRNA expression in endotoxin-exposed lung. *In situ* hybridization was performed with [35 S] labeled antisense sheep SAA3 riboprobe on lung sections from near term lambs. (A,B) ventilation + IT saline, (C,D) ventilation + IT endotoxin, (E,F) ventilation + IV endotoxin, (G,H) ventilation + IT endotoxin with sense [35 S] labeled SAA3 riboprobe (controls). Intense focal expression was seen in the bronchial glands (open arrowhead), bronchial epithelium (closed arrowhead), perichondrial cells of bronchial cartilage (open arrow) and arterial smooth muscle (closed arrow). Insets in C, D, E and F show higher magnification. A, C, E and G are bright-field images and B, D, F and H are corresponding dark-field images (bar represents 100 μ m).

duced postnatally in the lung of both preterm and near term lambs in response to ventilation and endotoxin administration. Regardless of the route of endotoxin administration (intratracheal (IT) or IV), SAA3 mRNA expression was induced in the bronchial epithelium, bronchial glands, perichondrium of bronchial cartilage and vascular smooth muscle of the lung. These experiments suggest that significant induction of expression of the acute phase reactant SAA3 mRNA occurs in the neonatal lung in response to pro-inflammatory stimuli.

The SAA protein family, as described in humans and mice, consists of the acute phase isoforms SAA1, SAA2 and SAA3 and the constitutively expressed SAA4 (18). The SAA3 riboprobe used in our study protected a single full length fragment in the lung which corresponded to SAA3 mRNA. However, in the liver, in addition to this fragment, two other smaller

protected fragments were detected. These fragments likely represent other SAA isoforms that are inducible (liver-inducible) because they were detected in the liver and not lung and were induced by ventilation and endotoxin exposure. We have designated these fragments as SAA-Li (SAA-liver inducible) mRNA in this manuscript. Definitive identification of these isoforms could not be performed because the mRNA sizes of SAA1, 2 and 3 are similar and full length mRNA sequences of ovine SAA isoforms are not available. However, our data demonstrates the differential expression of SAA isoforms in the lung and liver. The "noninjurious" ventilator strategy used in this study caused only a mild increase in liver SAA3 mRNA in the near term animals in response to mechanical ventilation alone. However, a more impressive induction of the SAA-Li isoforms was detected in these animals demonstrating a significant systemic acute phase reactant (APR) response to mechanical ventilation.

The liver is the main source of expression of SAA1 and SAA2 whereas SAA3 is produced principally in extra hepatic sites (4,6). Expression of SAA3 mRNA was previously demonstrated in the lungs of adult mice following lipopolysaccharide (LPS) injection (6,7). We found that sheep SAA3 mRNA expression was induced both in the lung and the liver whereas SAA-Li mRNA expression was induced in liver but not in the lung. As expected, a systemic pro-inflammatory stimulus (IV endotoxin) caused a more prominent SAA3 mRNA induction in liver than lung, while a pulmonary inflammatory stimulus (intratracheal endotoxin) caused a more prominent lung SAA3 mRNA expression than liver expression. The increased oxygen exposure in the mechanically ventilated animals can cause oxidative lung injury. This may have contributed to the increased SAA3 mRNA expression detected in these animals. These results indicate that the lung and liver of the developing sheep both participate in the APR response, with the magnitude of the response in each organ dependent on the type and route of pro-inflammatory stimulus.

The SAA proteins are apolipoproteins, which during the acute phase response bind to HDL fraction 3 (HDL₃) (4,19–21) and stimulate the recruitment of inflammatory cells (22,23) and the release of pro-inflammatory cytokines (24). Although the precise function of amyloid A proteins is unknown, they are thought to play an important role in modulating the innate host defense against inflammatory agents and are involved in cholesterol transport and metabolism (19,25–27). In humans, SAA3 has been postulated to be a pseudogene in some studies (28,29), although in another study the human SAA3 gene was expressed in mammary gland epithelial cells in response to either prolactin or LPS (30). It is not known if the different isoforms have distinct functions. A number of alleles have been reported for some isoforms thus making the SAA family a large and complex array of proteins that may be multifunctional (18). Because SAA3 mRNA expression is induced in inflammatory models of the developing sheep lung, the presence of this isoform expressed in the lung of preterm humans may warrant further investigation.

In this study, SAA3 expression was detected in bronchial epithelium, bronchial glands, perichondrial cells of bronchial cartilage, vascular smooth muscle and, to a lesser extent, in

inflammatory cells. Perichondrial cell and vascular smooth muscle expression of SAA3 were not detected in our previous study of antenatal endotoxin exposure (8). It is possible that the different design of this study, which involved exposure of the lamb lungs to mechanical ventilation, increased partial pressures of oxygen, higher pulmonary blood flow and intratracheal or IV endotoxin, preferentially induced SAA3 mRNA in the perichondrium and vascular smooth muscle.

Pro-inflammatory cytokines, in particular IL-1, IL-6 and tumor necrosis factor alpha (TNF- α) stimulate SAA synthesis during the adult acute phase response (19,31,32). We previously reported increased lung mRNA expression of these cytokines by 2h in ventilated preterm lambs compared with unventilated fetal controls (11). In addition, we demonstrated that exposure to ventilation and IT endotoxin causes a modest increase in lung IL-1 β and IL-6 mRNAs in preterm and near term lambs (23- and 13-fold respectively) (14). We now show a much more robust expression of SAA3 mRNA in the lungs of these animals (366 fold increase). The SAA-Li isoform was induced in the liver by ventilation and oxygen exposure with minimal incremental response to endotoxin suggesting that different SAA isoforms have different mechanisms of induction.

A limitation of this study is the lack of a specific anti-SAA3 antibody. Plasma SAA levels increased after ventilation and IT or IV endotoxin within 4h of exposure. This plasma SAA was not of lung origin since the BALF and lung homogenate SAA3 was not detected by the commercially available antibody used.

In summary, we demonstrated selective lung expression of SAA3 mRNA in preterm lambs, rapid induction in the lung in response to both mechanical ventilation and endotoxin stimulation as well as a systemic acute phase reactant response.

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