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NOTE

Immunology

Lipopolysaccharide and lipoteichoic acid enhance serum amyloid A3 mRNA expression in murine alveolar epithelial cells

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ABSTRACT. Serum amyloid A (SAA) is an acute-phase protein indicative of inflammation. In murine colonic epithelial cells, lipopolysaccharide (LPS), a gram-negative bacterial antigen, strongly enhanced mRNA expression of SAA3, but not SAA1 or SAA2, suggesting that SAA3 might respond to bacterial infection in other epithelia. We examined SAA1/2 and SAA3 mRNA expression in murine alveolar epithelial cells exposed to LPS or the gram-positive bacterial antigen, lipoteichoic acid (LTA), using real-time PCR. LPS enhanced SAA3 mRNA expression at lower concentrations than did LTA, whereas SAA1/2 mRNA expression was not enhanced by either LPS or LTA. These results suggest that SAA3 expression is enhanced in lung epithelium upon bacterial infection as part of innate immunity, with higher sensitivity to LPS than to LTA.

KEY WORDS: epithelium, innate immunity, lung, serum amyloid A, serum amyloid A3

Serum amyloid A (SAA) is a precursor protein of amyloid A (AA). SAA production is increased in AA amyloidosis in various animal species and in chronic inflammatory disorders, such as rheumatoid arthritis and juvenile inflammatory arthritis, in humans [11]. SAA is a major acute-phase protein and serves as a diagnostic marker of inflammation in animals and humans [3]. In mice, four SAA isoforms (SAA1–4) have been identified [16]. SAA1 and SAA2 are predominantly produced in the liver and are the main circulating isoforms in plasma [16]. During inflammation, the plasma concentration of SAAs, mainly SAA1, dramatically increases up to 1,000-fold and SAA1 is converted to AA fibrils [11, 16]. In contrast, SAA3 is mainly expressed in extrahepatic organs, such as the intestine and lungs [10, 16]. SAA3 protein has been detected in the colon surfaces of conventional, but not germ-free mice [12]. Lipopolysaccharide (LPS), a major membranous antigen of gram-negative bacteria such as *Escherichia coli*, strongly enhanced mRNA expression of SAA3, but not SAA1 or SAA2 in murine colonic epithelial CMT-93 cells [12, 13]. Furthermore, treatment with recombinant SAA3 protein increased mucin 2 (MUC2) mRNA expression in CMT-93 cells [13]. MUC2 is the major component of the mucus layer in the colon that protects epithelial cells from pathogens [8]. These findings suggest that SAA3 contributes to innate immunity in the murine colon, leading to the hypothesis that SAA3 also contributes to host defenses in other types of epithelium, such as lung epithelium. Therefore, in this study, we aimed to examine whether murine SAA3 level is enhanced by bacterial antigens in alveolar epithelium, using two murine alveolar epithelial cell lines.

Murine type-II alveolar epithelial MLE-15 cells transfected with the simian virus 40 large tumor antigen gene under transcriptional control of a promoter region from the human surfactant protein C (SP-C/SV40) gene [17] were kindly provided by Dr. Whitsett (Cincinnati Children's Hospital Medical Center, Cincinnati, OH, U.S.A.). The cells were maintained in HITES medium [1] with slight modification; the HITES medium comprised Roswell Park Memorial Institute (RPMI) 1640 medium (RPMI-1640, 189-02145, Wako, Osaka, Japan) and the supplements listed in Table S1. Murine type-II alveolar epithelial T7 cells [2] were purchased from the European Collection of Authenticated Cell Cultures (07021402, ECACC, Salisbury, U.K.) and were maintained in Dulbecco's modified Eagle's medium (DMEM, 044-29765, Wako) with the supplements listed in Table S1.

MLE-15 and T7 cells were seeded in 6-well plates at 4–7 × 10^5 cells/well and were incubated in RPMI-1640 and DMEM, respectively, for 13 ± 1 hr before experiments. After incubation, the cells were rinsed with sterile phosphate-buffered saline (PBS) and treated with LPS from *E. coli* O111:B4 (115K4092, Sigma, St. Louis, MO, U.S.A.) or lipoteichoic acid (LTA) from *Bacillus*

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Table 1. Primers used for quantitative real-time PCR

Target	Sequence (5′-3′)	Reference
SAA1/2 F	CTGCCTGCCAAATACTGAGAGTC	[4]
SAA1/2 R	CCACTTCCAAGTTCCTGTTTATTAC	
SAA3 F	GCTGGCCTGCCTAAAAGATACTG	[4]
SAA3 R	GCATTTCACAAGTATTTATTCAGC	
SP-A F	TCCTGGAGACTTCCACTACCT	[15]
SP-A R	CAGGCAGCCCTTATCATTCC	
SP-B F	CTGCTTCCTACCCTCTGCTG	[15]
SP-B R	CTTGGCACAGGTCATTAGCTC	
SP-C F	ATGGACATGAGTAGCAAAGAGGT	[15]
SP-C R	CACGATGAGAAGGCGTTTGAG	
MUC5AC F	CCATGCAGAGTCCTCAGAACAA	[6]
MUC5AC R	TTACTGGAAAGGCCCAAGCA	
MUC5B F	GCTGCTGTTACTCCTGTGAAAAAG	[6]
MUC5B R	TGACCTCTGTCTCACAGCCCTTA	
GAPDH F	TGCACCACCAACTGCTTAG	This study
GAPDH R	GGATGCAGGGATGATGTTC	

SAA, serum amyloid A; SP, surfactant protein; MUC, mucin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

subtilis (L3265-5MG, Sigma), which are gram-negative and -positive bacterial membranous antigens, respectively. The cells were incubated with LPS and LTA diluted to 0 to 100 μg/m*l* in RPMI-1640 for MLE-15 cells or DMEM for T7 cells at 37°C for 2 hr. For temporal mRNA expression analysis, MLE-15 cells were treated with 10 μg/m*l* of LPS or LTA in RPMI-1640 at 37°C and collected for mRNA expression analysis at 2, 4, 6, 8, or 12 hr. PBS-treated cells were included as a control. The cells were collected from the plates using cell lifters and washed with PBS. Total RNA was extracted immediately using an RNeasy Mini kit (74106, Qiagen, Hilden, Germany) following the manufacturer's instructions. The RNA was quantified using a NanoDropLite spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.) and stored at −80°C until use. Contaminating DNA was eliminated by treatment with DNaseI (18068-015, Invitrogen, Carlsbad, CA, U.S.A.) and cDNA was synthesized using PrimeScript RT Master Mix (RR036A, Takara, Kusatsu, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was carried out in 96-well plates using 300 nmol each of forward and reverse primers, 10 *n*g of cDNA, and PowerUp SYBR Green Master Mix (A25742, Applied Biosystems, Foster City, CA, U.S.A.) on a StepOnePlus thermal cycler (Applied Biosystems). Thermal cycling was carried out for 2 min at 5°C and 2 min at 95°C, followed by 40 cycles at 95°C for 3 sec and 60°C for 30 sec. Specific primers (Table 1) were used to investigate mRNA expression of SAA1/2, SAA3, surfactant protein (SP)-A, B, and C, and mucin 5AC (MUC5AC) and MUC5B, which are antibacterial proteins found in the lungs [5, 9]. The SAA1/2 primers [4] detect both SAA1 and SAA2, as the sequences of mouse SAA1 and SAA2 are highly similar. Target mRNA expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and fold changes relative to control levels were determined using the ΔΔCt method [18]. Specific amplification was verified by melting curve analysis of the amplification products at the end of each PCR. All experiments, except temporal mRNA expression analysis in MLE-15 cells, were replicated at least three times. Results are expressed as the mean ± standard deviation. Data were analyzed using one-way analysis of variance and Tukey's *post-hoc* tests. $P<0.05$ was regarded significant.

In MLE-15 cells, SAA3 mRNA expression was significantly enhanced ($P<0.01$) under all concentrations of LPS (0.01–100 μg/m*l*) (Fig. 1A). LTA also enhanced SAA3 mRNA expression, but at higher concentrations (1–100 μg/m*l*) than LPS. LTA enhanced SAA3 mRNA expression in a dose-dependent manner up to 10 μg/m*l* (Fig. 1B). In contrast, SAA1/2 mRNA expression was not significantly enhanced by LPS or LTA (Fig. 1A and 1B). In T7 cells, SAA3 mRNA expression was enhanced under a low concentration of LPS (0.01 μg/m*l*), whereas SAA1/2 mRNA expression was not enhanced (Fig. 2A). LTA also enhanced SAA3 mRNA expression, but again at a higher concentration than LPS. LTA slightly enhanced SAA1/2 mRNA expression, which was not as high as SAA3 mRNA expression (Fig. 2B). The fact that LPS enhanced SAA3 mRNA expression in MLE-15 and T7 cells at lower concentrations than did LTA suggests that type-II alveolar epithelial cells are more sensitive to LPS than to LTA.

Temporal mRNA expression analysis indicated that both LPS and LTA enhanced SAA3 mRNA expression in a time-dependent manner, but the increase induced by LPS was greater than that induced by LTA. mRNA expression of SAA1/2, SP-A, B, and C, and MUC5AC and MUC5B was not enhanced by LPS or LTA (Fig. S1A and S1B). These results suggest that SAA3 is expressed as a primary host defense protein against bacterial infection. The relationship between SAA3 and the expression of other polypeptides in response to bacterial antigenic challenge remains to be elucidated. In line with our findings, MUC2 mRNA expression in murine colon was not significantly increased by *E. coli* or LPS [13]. Upregulation of MUC2 expression by SAA3 reportedly is involved in the activation of nuclear factor κB (NFκB) via TLR4 [14]. Thus, it is possible that SAA3 acts as an immunostimulator in respiratory and gastrointestinal epithelium to protect animals from bacterial infection. As we analyzed gene expression only *in vitro*, to fully elucidate the relationship between SAA3 and innate immunity, it is necessary to investigate whether recombinant SAA3 protein induces the expression of proteins involved in innate immunity, such as NFκB, TLR4, MUCs,

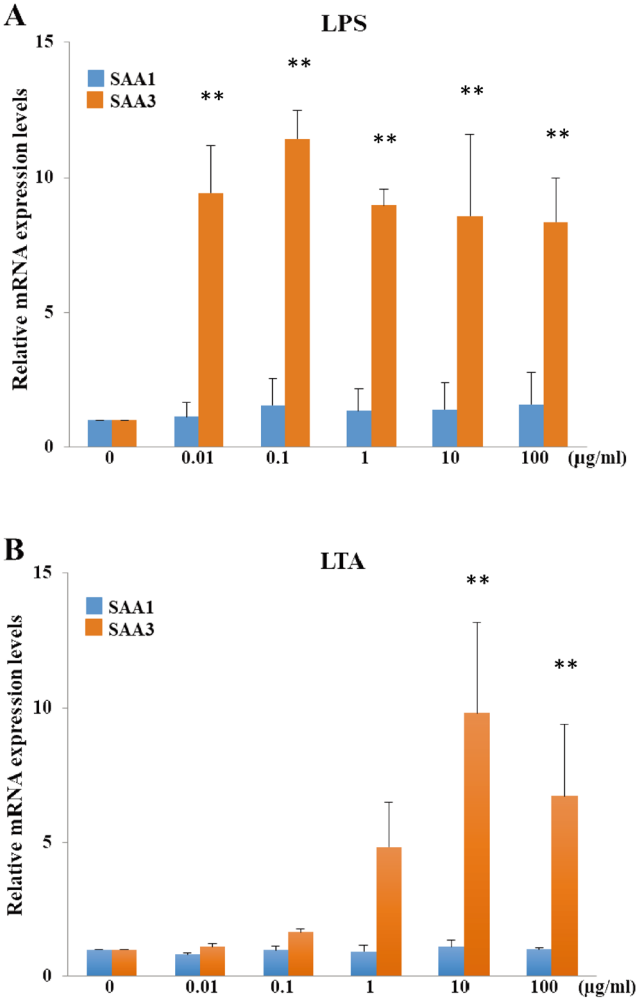

Fig. 1. Comparison of mRNA expression induced by lipopolysaccharide (LPS) and lipoteichoic acid (LTA) in MLE-15 cells. MLE-15 cells were treated with 0–100 μg/m*l* LPS (A) or LTA (B) at 37°C for 2 hr. MLE-15 cells treated with phosphate-buffered saline (PBS) served as a control. mRNA expression of SAA1/2 and SAA3 was normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA expression and compared with that in control cells assumed to have 0 μg/m*l* expression. Data are the mean plus standard deviation from three independent experiments. ***P*<0.01.

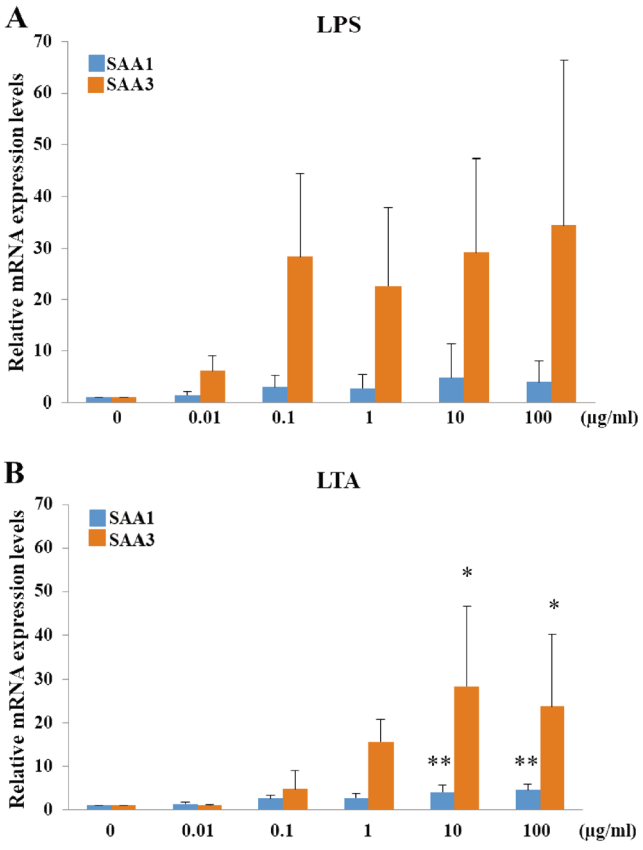

Fig. 2. Comparison of mRNA expression induced by lipopolysaccharide (LPS) and lipoteichoic acid (LTA) in T7 cells. T7 cells were treated with 0–100 μg/m*l* of LPS (A) or LTA (B) at 37°C for 2 hr. T7 cells treated with phosphate-buffered saline (PBS) served as a control. mRNA expression of SAA1/2 and SAA3 was normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA expression and compared with that in control cells assumed to have 0 μg/m*l* expression. Data are the mean plus standard deviation from three independent experiments. **P*<0.05, ***P*<0.01.

SPs, and hyaluronan, which protect epithelial cells from apoptosis [7] in murine alveolar epithelium. Our findings contribute to the understanding of the function of SAA3 in epithelia.

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