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Serum amyloid A induces WISH cell apoptosis[1]

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Key words

serum amyloid A; apoptosis; caspase-3; extracellular signal regulated protein kinase; p38 kinase

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Abstract

Aim: Serum amyloid A (SAA) is an important mammalian acute reactant. Here, we aim to investigate the effect of SAA on apoptosis and its mechanism of action in human amniotic WISH cells. **Methods:** The expression of formyl peptide receptor (FPRL1), which is reported as a SAA receptor, was tested using RT-PCR and ligand binding assay with radio-labeled FPRL1 ligand. The effect of SAA on proliferating cell population was evaluated by thymidine incorporation assay. The protein phosphorylation levels and caspase-3 activity were detected by Western blot assay. **Results:** SAA inhibits thymidine incorporation in human amniotic WISH cells. A SAA-induced decrease of proliferating cell population was accompanied with nuclear condensation and caspase-3 activation in WISH cells, suggesting that SAA induces WISH cell apoptosis. Since FPRL1 has been reported as a SAA receptor, we investigated the effects of several FRPL1 agonists on a proliferating cell population in WISH cells. Among the tested FPRL1 agonists, only SAA induced a decrease of proliferating cell population in WISH cells. On the downstream signaling of SAA, we found that SAA stimulated extracellular signal-regulated kinase and p38 kinase, which were not inhibited by pertussis toxin (PTX), ruling out the role of PTX-sensitive G-proteins. Furthermore a SAA-induced decrease of proliferating cell population was not affected by PTX, suggesting that SAA inhibits WISH cell apoptosis in a PTX-sensitive G-protein-independent manner. A SAA-induced decrease of a proliferating cell population was completely blocked by PD98059 and SB203580, suggesting that mitogen-activated protein kinase activities are essentially required for the process. **Conclusion:** SAA is a novel inducer for WISH cell apoptosis, and the PTX-insensitive pathway is involved in the process.

Introduction

Serum amyloid A (SAA) is an important acute reactant with wide-ranging pathological activities[1,2]. SAA has been reported to induce chemotactic migration in monocytes and neutrophils[3,4]. Some reports have demonstrated that SAA affects cytokine production in several cell types, such as neutrophils and monocytes[5,6]. In terms of SAA cell surface receptors, a member of G-protein coupled receptors called formyl peptide receptor like-1 (FPRL1) has been reported[7]. Although many previous reports have demonstrated the pivotal role of SAA in the modulation of several biological responses, the expression of the SAA receptor in amnion cells, and the role of SAA in these cells, have not been previously studied.

SAA can be dramatically produced against infected conditions[1,2]. Hepatocyte is regarded as a major cell for SAA synthesis, and SAA can be generated by various inflammatory stimuli, including tumor necrosis factor-α[8]. Previously, SAA has been shown to be present in both maternal and fetal plasma in the period around parturition[9]. It has also been reported that ovine allantoic fluid contains high con-

centrations of SAA-like protein[10]. However, the role of SAA in the regulation of reproduction has not been fully investigated.

In this study, we aim to investigate whether the SAA receptor is expressed on human amnion-derived WISH cells and if SAA modulates cellular activity of cells. We also investigated the signaling pathways involved in the SAA-mediated regulation of WISH cell activity.

Materials and methods

Reagents RPMI-1640 medium, dialyzed fetal bovine serum (FBS), and the RT-PCR kit were purchased from Invitrogen Corp (Carlsbad, CA, USA). Enhanced chemiluminescence reagents were from GE Healthcare Bio-Sciences Corp (Piscataway, NJ, USA), and phospho- extracellular signal-regulated protein kinase (ERK) 1/2, phospho-p38 kinase, and ERK2 antibodies were from New England Biolabs (Beverly, MA, USA). Rabbit polyclonal anti-caspase-3 antibodies were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Pertussis toxin (PTX), 2'-amino-3'-methoxyflavone (PD98059), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), and 1-{6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl}-1*H*-pyrrole-2,5-dione (U-73122) were obtained from Calbiochem (San Diego, CA, USA). PD98059, SB203580 and U-73122 were dissolved in dimethyl sulfoxide before being added to cell culture. The final concentrations of dimethyl sulfoxide in culture were 0.1% or less.

Cell culture The WISH human amnion cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The WISH cells were cultured in RPMI-1640 media supplemented with 2 μmol/L *L*-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere. The cells were subcultured twice weekly by trypsinization and seeded in 6-well plates (5×10^5 cells/well).

RT-PCR analysis mRNA was isolated using a QIAshredder and an RNeasy kit (Qiagen, Hilden, Germany). mRNA, M-MLV RT, and pd(N)6 primers (Invitrogen, USA) were used to obtain cDNA. The primers used for the RT-PCR analysis have been reported previously[11]. The sequences of the primer used were as follows; FPR: forward, 5'-CTCCAGTTGGACTAGCCACA-3'; reverse, 5'-CCATCACCCAGGGCCCAATG-3';FPRL1:forward,5'-CTGCTGGTGCT-GCTGGCAAG-3'; reverse, 5'-AATATCCCTGACCCCATCCTCA-3'; FPRL2: forward, 5'-GCCAAGGTCTTTCTGATCC-3'; reverse, 5'-GGTCTGGGCTGAGTCAGGGA-3'. We ran 30 PCR cycles of 94 °C (denaturation, 1 min), 59 °C (annealing, 1 min) and 72 °C (extension, 1 min). PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Ligand binding assay Ligand binding assay was performed as described previously[12]. Radioiodinated Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm) (^{125}I-labeled) was purchased from PerkinElmer Life and Analytical Sciences Inc (Wellesley, MA, USA). Briefly, the WISH cells were seeded at 1×10^5 cells per well into a 24-well plate and cultured overnight. After treating the cells with blocking buffer [33 mmol/L HEPES, pH 7.5, 0.1% bovine serum albumin (BSA) in RPMI-1640 medium] for 2 h, 50 pmol/L of labeled WKYMVm was added to the cells in binding buffer [phosphate buffered saline (PBS) containing 0.1% BSA] in the absence or presence of unlabelled WKYMVm. The mixture was then incubated for 3 h at 4 °C with continuous agitation. The samples were then washed 5 times with ice-cold binding buffer, and 200 μL of lysis buffer (20 mmol/L Tris, pH 7.5, 1% Triton X-100) was added to each well. After incubation for 20 min at room temperature, the lysates were collected and the associated radioactivity was determined using a γ-ray counter[12].

Cellular proliferation assay For SAA treatment, the cells were seeded in 24-well plates, in triplicate, at 5×10^4 cells/well. Complete medium was replaced with serum-free RPMI-1640 medium to starve the cells for 24 h. SAA was added to the cultures to promote growth activity with or without several kinase inhibitors. [^{3}H]Thymidine (1 μCi/mL) was added and incubation continued for 24 h. The medium was then removed and the cells were fixed with 5% cold trichloroacetic acid (TCA) for 15 min. TCA-precipitated material was solubilized in 1 mol/L NaOH for 1 h and neutralized by adding 1 mol/L HCl. [^{3}H]Thymidine uptake was determined by liquid scintillation counting.

Nuclear staining with 4,6-diamino-2-phenylindole (DAPI) The cells were washed with PBS, fixed with cold methanol for 2 min at room temperature, washed with PBS, and stained with DAPI solution (Sigma, USA) for 10 min at room temperature. They were then washed 2 more times with PBS and analyzed under a fluorescence microscope[13].

Western blot analysis The WISH cells were plated in a 6-well plate and treated with SAA at different times. The cells were then washed with cold-PBS, scraped off, and pelleted at 700×*g* at 4 °C. The cell pellet obtained was resuspended in lysis buffer (20 mmol/L HEPES, pH 7.2, 10% glycerol, 150 mmol/L NaCl, 1% Triton X-100, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 10 μg/mL leupeptin, 10 mg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail), cleared by centrifugation, and the supernatant saved as a whole-cell lysate. Proteins (30 μg) were

separated by 10% reducing SDS-PAGE and electroblotted in 20% methanol, 25 mmol/L Tris and 192 mmol/L glycine onto a nitrocellulose membrane. The membrane was then blocked with 5% nonfat dry milk in Tris-buffered saline-Tween 20 (25 mmol/L Tris-HCl, 150 mmol/L NaCl and 0.2% Tween 20), incubated with antibodies for 4 h, washed, and re-incubated for 1 h with secondary antibodies conjugated to horseradish peroxidase. Finally, the membrane was washed and developed using an Enhanced Chemiluminescence (ECL) system.

Statistical analysis The results are expressed as mean±SEM of the number of determinations indicated. Statistical significance of differences was determined by ANOVA. Significance was accepted when $P<0.05$.

Results

Expression of the SAA receptor in WISH cells We investigated whether the cell surface receptor for SAA is expressed on WISH cells. To determine whether the SAA receptor is expressed on WISH cells, we analyzed the mRNA expressions of FPRL1 by semiquantitative RT-PCR. As shown in Figure 1A, WISH cells express SAA receptor, namely, FPRL1. However, WISH cells do not express the other 2 FPR family receptors, FPR and FPRL2 (Figure 1A). We confirmed that the RT-PCR product obtained without the addition of RT did not contain a DNA band (data not shown). We also determined whether FPRL1 is expressed on WISH cells by using ligand binding assay with [^{125}I]WKYMVm, a ligand for FPRL1. As shown in Figure 1B, the addition of various concentrations of [^{125}I]WKYMVm demonstrated the concentration-dependent binding of [^{125}I]WKYMVm to WISH cells (Figure 1B), which was quantified after subtracting nonspecific binding. The addition of molar excess of unlabeled WKYMVm (10 μmol/L) prior to the addition of [^{125}I]WKYMVm reduced this binding. The specific binding of WKYMVm in WISH cells was proven by adding 25–100 pmol/L of [^{125}I]WKYMVm for 5×10^4 cells (Figure 1B). This result indicates that WISH cells express the functional SAA receptor, FPRL1.

SAA decreased proliferating cell population, resulting in apoptosis in WISH cells In order to examine the effect of SAA on the proliferating cell population, we investigated the effect of SAA on [^{3}H]thymidine incorporation in WISH cells. As shown in Figure 2A, the stimulation of WISH cells by several concentrations of SAA for 24 h inhibited [^{3}H]thymidine incorporation in a concentration-dependent manner. The stimulation of WISH cells with 2 μmol/L of SAA inhibited [^{3}H]thymidine incorporation by around half versus the unstimulated control (Figure 2A). To determine whether the growth inhibitory effect of SAA is associated

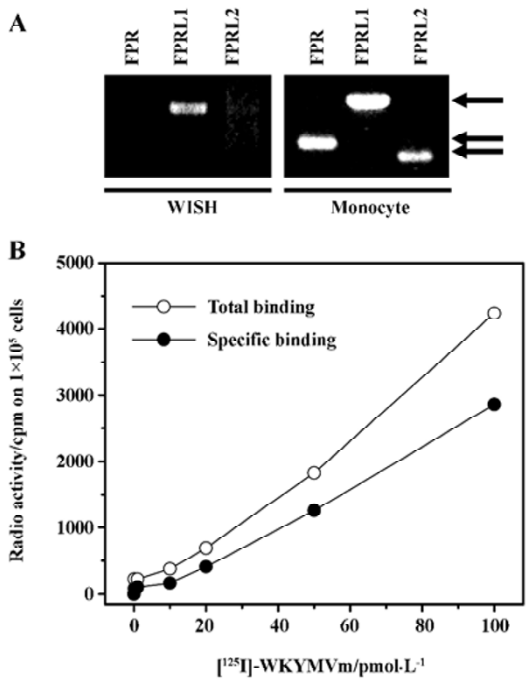

Figure 1. Expression of the SAA receptor in WISH cells. RT-PCR analysis was performed on mRNA isolated from cultured WISH cells. The data presented are representative of 3 independent experiments (A). Several concentrations of [^{125}I]-labeled WKYMVm were incubated with WISH cells for 3 h at 4 ºC in the absence or presence of 10 μmol/L of unlabeled WKYMVm. The quantity of bound [^{125}I]-labeled WKYMVm was determined using a γ-ray counter. The data presented are representative of 3 independent experiments (B).

with apoptosis, we examined WISH cell morphological changes after treatment with SAA. WISH cells growing on culture plates were exposed to 2 μmol/L SAA for 24 h. As shown in Figure 2B, SAA caused significant levels of cell shrinkage and chromatin condensation, thus showing that SAA induces WISH cell apoptosis. As a positive control, hydrogen peroxide (2 mmol/L) also caused WISH cell apoptosis (Figure 2B).

Caspase is a member of a family of proteases that are involved in proteolytic cleavage of cellular proteins during apoptosis[14]. Many reports have shown that apoptosis is accompanied by the activation of caspases, especially caspase-3[14–16]. We studied the effect of SAA on caspase-3 activity. Since caspase-3 is known to be cleaved during its activation process[14,15], we confirmed the effect of SAA on caspase-3 activity by Western blot analysis using an antibody that recognizes caspase-3. Treatment with various concentrations of SAA induced the cleavage of caspase-3, a mark for its activation, in a concentration-dependent manner (Figure 2C). These results coincide with the inhibitory effect

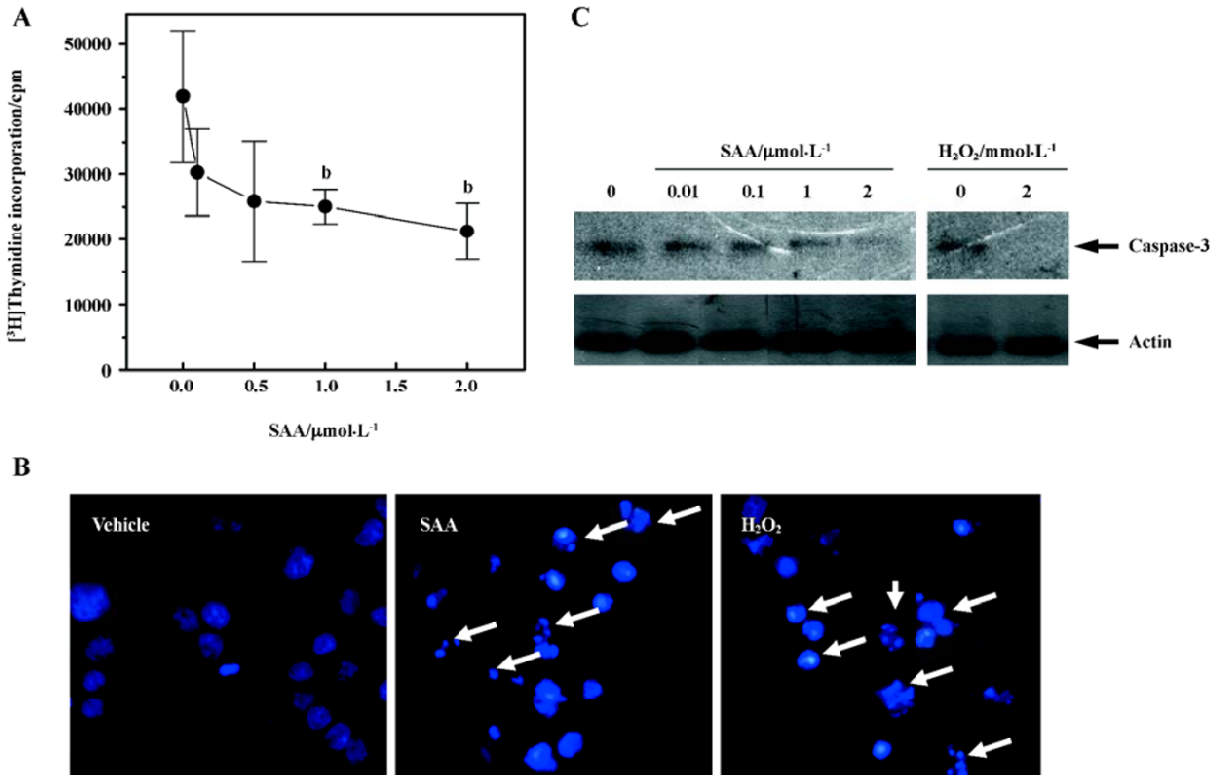

Figure 2. Effect of SAA on the proliferating cell population in WISH cells. The WISH cells (5×10^4) were treated with several concentrations of SAA for 24 h (A). The cells were harvested and [^{3}H]thymidine incorporation was measured. Results are presented as mean±SE of 3 independent experiments (A). $^bP<0.05$ probability levels as compared to the values obtained from the control (0 μmol/L) (A). The cells were treated with vehicle (DW), 2 μmol/L SAA, or 2 mmol/L H_2O_2 for 24 h and then fixed and stained with DAPI. Morphological changes of nuclear chromatin were then viewed under a fluorescence microscope (B). Arrowheads show condensed or fragmented nuclei (B). WISH cells were stimulated with several concentrations of SAA or 2 mmol/L of H_2O_2 for 24 h. Each sample (30 μg of protein) was subjected to 15% SDS-PAGE; caspase-3 (pro-form) was determined by immunoblotting using anti-caspase-3 antibody. The results shown are representative of 3 independent experiments (B,C).

of SAA on thymidine incorporation in WISH cells (Figure 2A).

Among FPRL1 agonists, SAA selectively decreased proliferating cell population in WISH cells Previous reports have demonstrated that SAA binds to FPRL1, an important classical chemoattractant receptor[4,7]. Since in this study SAA decreased proliferating cell population, we also investigated the effect of several FPRL1 agonists on the proliferating cell population in WISH cells. As shown in Figure 3, among the tested FPRL1 agonists, only SAA showed a decrease of proliferating cell population in WISH cells. The other FPRL1 agonists such as WKYMVm[17,18] and Leu-Glu-Ser-Ile-Phe-Arg-Ser-Leu-Leu-Phe-Arg-Val-Met (LESIFRSLLFRVM, MMK-1)[19,20] had no effect on the proliferating cell population in WISH cells (Figure 3), suggesting a SAA-selective response. FPR-selective agonist, formyl-Met-Leu-Phe (fMLF) also did not affect the proliferating cell population in WISH cells (Figure 3).

SAA stimulates mitogen-activated protein kinase in a pertussis toxin-insensitive manner in WISH cells Mitogen-activated protein kinase (MAPK) has been reported to mediate extracellular signals to the nucleus in various cell types[21]. In this study, we examined whether SAA stimulates MAPK by Western blotting with antiphospho-specific antibodies to each enzyme. When the WISH cells were stimulated with 2 μmol/L SAA for different times, the phosphorylation level of ERK transiently increased, showing maximal activity after 2–5 min of stimulation (Figure 4A), and returning to the baseline 30 min after stimulation (Figure 4A). Another important MAPK, p38 kinase, was also transiently phosphorylated by SAA stimulation with kinetics that resembled those of ERK phosphorylation (Figure 4A).

We investigated the role of pertussis toxin (PTX)-sensitive G-protein on SAA-induced ERK phosphorylation.

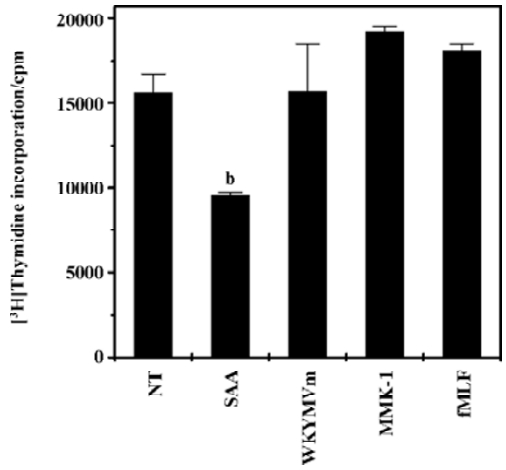

Figure 3. SAA selectively decreases the proliferating cell population in WISH cells. The WISH cells were stimulated with several types of FPR family agonist (2 μmol/L SAA, 1 μmol/L WKYMVm, 1 μmol/L MMK-1 or 1 μmol/L fMLF) for 24 h. The cells were harvested and [^{3}H]thymidine incorporation was measured. Results are presented as mean±SEM of 3 independent experiments. $^bP<0.05$ probability levels as compared to the values obtained from the control (NT, not treated).

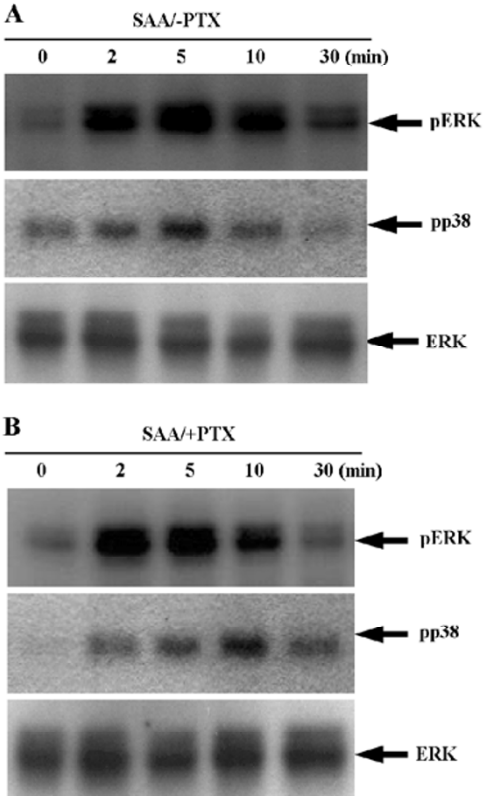

Figure 4. Activation of MAPK by SAA in WISH cells. The WISH cells were pre-incubated in the absence (A) or presence (B) of 100 ng/mL of PTX for 24 h. The WISH cells were stimulated with 2 μmol/L of SAA for various times. Each sample (30 μg of protein) was subjected to 10% SDS-PAGE, and phosphorylated ERK or p38 kinase was determined by immunoblotting using antiphospho-ERK antibody or antiphospho-p38 kinase antibody. The results shown are representative of 3 independent experiments.

Cultured WISH cells were pre-incubated with 100 ng/mL of PTX prior to being stimulated with 2 μmol/L SAA. We found that PTX pretreatment did not block ERK phosphorylation by SAA (Figure 4B), showing that SAA induces ERK phosphorylation in a PTX-insensitive manner. We also examined the effect of PTX on SAA-induced p38 kinase phosphorylation. When the WISH cells were pre-incubated with 100 ng/mL of PTX prior to being stimulated with 2 μmol/L SAA, SAA-induced p38 kinase phosphorylation was not found to be blocked (Figure 4B). These results indicate that SAA stimulates ERK and p38 kinase phosphorylation via a PTX-sensitive G-protein-independent pathway.

Regulation of SAA-induced decrease of proliferating cell population Previously, SAA has been reported to act on cell surface receptor FPRL1, which is mainly coupled to PTX-sensitive G_i proteins[7]. We examined the effect of PTX on the SAA-decreased proliferating cell population in WISH cells. Pre-incubation of WISH cells with 100 ng/mL of PTX for 24 h dramatically inhibited sphingosine-1-phosphate-induced intracellular calcium increase (data not shown), suggesting that PTX completely inhibits G_i-mediated signaling in cells. In the same condition, pretreatment of PTX prior to the addition of SAA for 24 h did not affect the inhibitory activity on the decrease of the proliferating cell population in WISH cells (Figure 5A). The result indicates that SAA decreased the proliferating cell population in a PTX-insensitive manner.

Many studies have demonstrated that p38 kinase is involved in cell proliferation inhibition[22,23]. In this study, we examined the effect of MAPK (ERK and p38 kinase) on SAA-decreased proliferating cell population in WISH cells. As shown in Figure 5B, SAA-inhibited [^{3}H]thymidine incorporation was almost totally inhibited in the presence of PD98059 or in the presence of SB203580. These results suggest that ERK and p38 kinase are essential for SAA-inhibited [^{3}H]thymidine incorporation by WISH cells. However, pre-incubation of WISH cells with 5 μmol/L of U-73122 did not affect the inhibitory effect of SAA on the decrease of the proliferating cell population in WISH cells (Figure 5B), suggesting that phospholipase C is not involved in the SAA-decreased proliferating cell population in WISH cells.

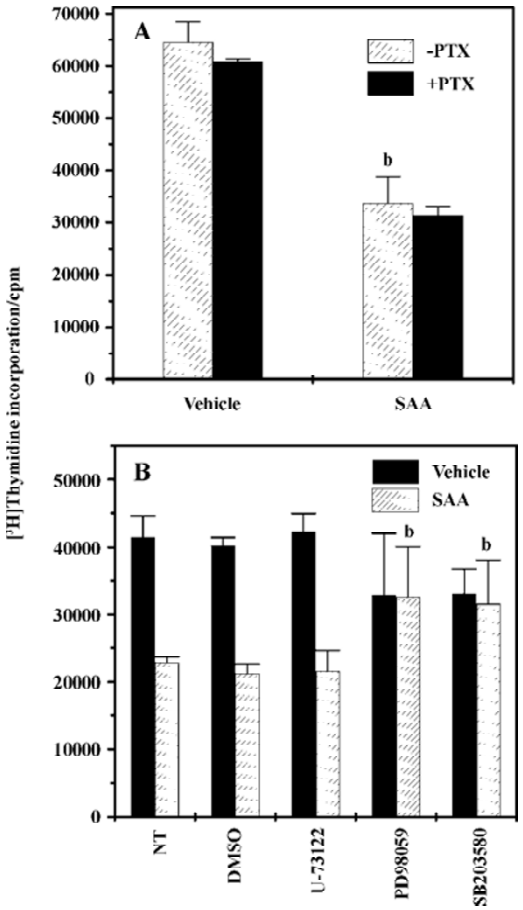

Figure 5. Regulation of SAA-induced decrease of the proliferating cell population. The WISH cells (5×10^4) were treated with 2 μmol/L of SAA for 24 h in the absence or presence of 100 ng/mL of PTX (A). The cells were pre-incubated with DMSO, 5 μmol/L U-73122 (15 min), 10 μmol/L PD98059 (60 min) or 5 μmol/L SB203580 (15 min) prior to being treated with 2 μmol/L of SAA for 24 h (B). The cells were harvested and [^{3}H]thymidine incorporation was measured. Vehicle is distilled water and NT indicates not treated. Results are presented as mean±SEM of at least 3 independent experiments, which were performed in duplicate (A,B). $^bP<0.05$ probability levels as compared to the values obtained from the control (A, vehicle treated; B, DMSO treated).

Discussion

Although many reports have shown that SAA is highly upregulated during infection, its role in amniotic cells has not been investigated. In the present study, we found that human amnion-derived WISH cells express SAA receptor FPRL1, which indicates that SAA may modulate the cellular activity of WISH cells. To identify the functional role of SAA stimulation in WISH cells, we focused on apoptosis. As shown in Figure 2, SAA potently induced apoptosis in WISH cells. Since amnion cell proliferation is important for the maintenance of pregnancy, these data in WISH cells suggests that SAA plays a negative role in the maintenance of pregnancy.

A previous report demonstrated that SAA binds to one of the G-protein coupled cell surface receptor, namely FPRL1[7]. When we performed RT-PCR to examine the expression pattern of the SAA receptor on WISH cells, we found that WISH cells express FPRL1 (Figure 1). According to previous reports, FPRL1 interact with G-proteins of the G_i families[24,25]. In our study, we investigated the effect of PTX, (which specifically inactivates G_i-mediated signaling pathways) on SAA-induced signaling. When WISH cells were pretreated with 100 ng/mL of PTX for 24 h prior to SAA stimulation, SAA-decreased proliferating cell population was not affected (Figure 5A), and SAA-stimulated MAPK (ERK and p38 kinase) activation was not blocked by PTX pretreatment (Figure 4). These results suggest that SAA modulates MAPK activation and leads to a decrease of the proliferating cell population, and that PTX-sensitive G-proteins are not involved in this process in WISH cells. The signaling pathway of the FPR family, including FPRL1, has been extensively studied[24,26]. Among the previous reports, Tsu *et al*, reported that FPR can also couple to G_i1, G_o, and a PTX-insensitive G-protein, G_z, as shown by cotransfection experiments in HEK293 cells. They also demonstrated that in such conditions, G_z can only replace G_i in inhibiting cAMP accumulation, but not in stimulating phospholipase C (PLC)[27]. Keeping in mind that SAA binds to FPRL1 and the fact that SAA decreased proliferating cell population in a PTX-resistant manner, it suggests that SAA may stimulate PTX-insensitive signaling downstream of FPRL1.

To investigate the signal pathway of the effect of the decrease of the proliferating cell population by SAA in WISH cells, we examined the role of MAPK. We observed that SAA stimulated both ERK and p38 kinase activity (Figure 4A). To determine the role of ERK or p38 kinase on the SAA-decreased proliferating cell population, we pretreated WISH cells with 2 different MAPK-selective inhibitors, PD98059 and SB203580 [selective MAPK-kinase (MEK) and p38 kinase inhibitor, respectively]. Since we found that high concentrations of PD98059 (50 μmol/L) and SB203580 (20 μmol/L) affected thymidine incorporation by themselves, we used 10 μmol/L of PD98059 and 5 μmol/L of SB203580. The pre-incubation of WISH cells with PD98059 (10 μmol/L) or SB203580 (5 μmol/L) prior to SAA stimulation blocked the effect on the decrease of the proliferating cell population by SAA (Figure 5B). We also observed that PD98059 (10 μmol/L) and SB203580 (5 μmol/L) blocked SAA-induced MAPK activation (data not shown).This indicates that ERK and p38 ki-

nase play key roles in the SAA-decreased proliferating cell population in amniotic cells. Many previous reports have demonstrated that p38 kinase is essentially required for the induction of cell death in several cell types[22,23]. However, p38 kinase also has been reported to inhibit cell death in certain cell types[28]. In the case of ERK, it also acts as a positive and negative mediator of cell death[29,30]. In our study, we demonstrated that ERK pathway inhibitor, PD98059, strongly blocked the decrease of the proliferating cell population by SAA in WISH cells (Figure 5B). It suggests that ERK is essential for the induction of the SAA-decreased proliferating cell population.

The SAA we used is a recombinant protein SAA which is produced in *Escherichia coli*. Even though the endotoxin content of SAA preparation is negligible (0.1 ng/mg), we further examined the possible contribution of lipopolysaccharide (LPS) on the SAA-induced decrease of the proliferating cell population using a potent inhibitor of LPS (polymyxin B). Pre-incubation of WISH cells with polymyxin B (10 μg/mL) prior to the addition of LPS completely inhibited ERK phosphorylation by LPS, but SAA-induced ERK phosphorylation and the decrease of proliferating cell population were unaffected (data not shown). From the results, we can rule out the possibility of an indirect decrease of the proliferating cell population by SAA, supporting our notion that SAA directly decreases the proliferating cell population in WISH cells via its specific cell surface receptor which is different from FPRL1.

In conclusion, the present study indicates that SAA acts on the SAA-specific receptor FPRL1, resulting in apoptosis in WISH cells which are widely used as a model system for amniotic epithelial cells. However, Kniss *et al* showed that the WISH cell line has been contaminated by Hela cells (a human cervical adenocarcinoma cell line)[31]. Since this study is the first to report on the expression of the SAA receptor on WISH cells, further studies on the pathophysiological and physiological roles of SAA in the maintenance of pregnancy response of primary cultures of human amniotic epithelial cells are required.

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