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ORIGINAL ARTICLE

Interleukin-1β Induces Intracellular Serum Amyloid A1 Expression in Human Coronary Artery Endothelial Cells and Promotes its Intercellular Exchange

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Abstract— Serum amyloid A (SAA) is an acute-phase protein with important, pathogenic role in the development of atherosclerosis. Since dysfunctional endothelium represents a key early step in atherogenesis, we aimed to determine whether induced human coronary artery endothelial cells (HCAEC) modulate SAA1/2/4 expression and influence intracellular location and intercellular transport of SAA1. HCAEC were stimulated with 1 ng/ml IL-1β, 10 ng/ml IL-6, and/or 1 μM dexamethasone for 24 h. QPCR, Western blots, ELISA, and immunofluorescent labeling were performed for detection of SAA1/2/4 mRNA and protein levels, respectively. In SAA1 transport experiments, FITC- or Cy3-labeled SAA1 were added to HCAEC separately, for 24 h, followed by a combined incubation of SAA1-FITC and SAA1-Cy3 positive cells, with IL-1β and analysis by flow cytometry. IL-1β upregulated SAA1 (119.9-fold, $p < 0.01$) and SAA2 (9.3-fold; $p < 0.05$) mRNA expression levels, while mRNA expression of SAA4 was not affected. Intracellular SAA1 was found mainly as a monomer, while SAA2 and SAA4 formed octamers as analyzed by Western blots. Within HCAEC, SAA1/2/4 located mostly to the perinuclear area and tunneling membrane nanotubes. Co-culturing of SAA1-FITC and SAA1-Cy3 positive cells for 48 h showed a significantly higher percentage of double positive cells in IL-1β-stimulated (mean ± SD; 60 ± 4%) *vs.* non-stimulated cells (48 ± 2%; $p < 0.05$). IL-1β induces SAA1 expression in HCAEC and promotes its intercellular exchange, suggesting that direct communication between cells in inflammatory conditions could ultimately lead to faster development of atherosclerosis in coronary arteries.

KEY WORDS: serum amyloid A; IL-1β; atherosclerosis; human coronary artery endothelial cells; intercellular transport.

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INTRODUCTION

New evidence has emerged reinforcing previous reports [1, 2] on the effects of chronic inflammation on atherogenesis and strengthening the inflammatory hypothesis in coronary artery disease [3, 4]. Specifically, the Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) by Ridker et al. [3] was designed to directly test the inflammatory hypothesis of

atherothrombosis. This trial included patients with a history of myocardial infarction and persistent proinflammatory response (high sensitivity C-reactive protein (hsCRP) levels above 2 mg/ml) who were receiving canakinumab (a fully human monoclonal antibody that neutralizes IL-1β) or placebo. Patients treated with canakinumab had reduced vascular inflammation and lower rate of recurrent cardiovascular events with significantly reduced levels of inflammatory parameters hsCRP and IL-6 but without changes in lipid levels, suggesting that IL-1β might be one of the targets for atheroprotection treatment [3].

Activated immune cells, such as macrophages and T-helper cells (Th1), in atherosclerotic plaques produce many cytokines, among them, IL-1β and in turn, IL-6 with elevated major acute-phase proteins, such as CRP and serum amyloid A (SAA), sensitive nonspecific markers of inflammation [5]. Serum levels of SAA have been reported to correlate with severity of atherosclerosis [6, 7] and can be utilized as a predictor of mortality following acute myocardial infarction [8, 9]. SAA is not only a risk factor for atherosclerosis, but also an active participant in atherogenesis, since it can directly accelerate the progression of atherosclerosis in ApoE$^{-/-}$ mice [10]. Importantly, SAA was reported to play causal roles in atherosclerosis by enhancing monocyte and lymphocyte recruitment, directly stimulating foam cell formation, associating with HDL/LDL particles and compromising reverse cholesterol transport, among other processes [7]. In addition, SAA also induces matrix metalloproteinases, as well as increases biglycan synthesis and influences retention of lipoproteins by vascular proteoglycans [11].

The *SAA* gene family is highly conserved [12] and includes four different genes, clustered on chromosome 11p15.1, encoding for SAA1, SAA2, SAA3, and SAA4 [13, 14]. Human *SAA1* and *SAA2* genes share a 93% nucleotide identity and probably originate from gene duplication during evolution [12, 15]. Mature SAA1 and SAA2 are small globular and inducible proteins (~ 12 kDa), 104 amino acids in length, previously termed acute SAA (A-SAA) [16]. The promotor regions of their genes include binding sites of nuclear transcription factors NF-κB and NF-IL-6, which upregulate SAA expression upon stimulation with proinflammatory cytokines such as IL-1β, IL-6, and TNF-α [17, 18]. The third gene *SAA3* was previously thought to be a pseudogene; however, its mRNA expression was later reported in human mammary gland epithelial cells [19]. The *SAA4* gene does not contain any NF-IL-6 binding sites and comprises of eight additional codons in comparison to *SAA1* and *SAA2*. Due to the octapeptide insertion, the mature SAA4 protein (~ 14 kDa) consists of 112-amino acids and shares only 52% identity with SAA1/2 [20, 21]. The octapeptide insertion also generates a N-linked glycosylation site responsible for the co-existence of glycosylated and un-glycosylated forms of SAA4 in human serum [20]. Under physiological conditions, SAA4, as an apolipoprotein, represents more than 90% of total SAA in serum [22]; however, its function is currently still unclear. In contrast to SAA1/2, sera levels of SAA4, previously termed constitutive SAA (C-SAA), do not increase significantly during inflammation [23, 24].

In cellular studies, Lakota et al. reported in 2007 on greater sensitivity of human coronary endothelial cells (HCAEC) (as opposed to human umbilical vein endothelial cells (HUVEC)) to SAA stimulation, important for inflammation [25]. Later, Wang et al. showed that SAA (at clinically relevant concentrations) induced dysfunction of both porcine coronary arteries and HCAECs through molecular mechanisms involving eNOS downregulation, oxidative stress, and activation of JNK and ERK1/2, as well as NF-κB [26]. Zhang et al. recently observed that SAA induced the upregulation of the matrix synthesis related genes, such as collagen I and elastin in vascular smooth muscle cells and also increased their proliferative and migratory ability, which could consequently lead to participation in atherosclerosis [27].

A dynamic synthesis pattern has been described for SAA [11] executed largely through *de novo* synthesis in the liver within a relatively short period of time (24 h) during the acute-phase response. This yields highly elevated circulatory levels, followed by resolution to physiological concentrations. On the other hand, local SAA mRNA expression was also found in numerous normal and diseased tissues and cells, including atherosclerotic plaques, carotid artery endothelial and smooth muscle cells [28], as well as within ruptured plaques [29]. HCAEC were also reported to be responsive to stimulation by SAA, exerting strong positive feedback onto its own mRNA expression [30]. High expression of A-SAA in cultured smooth muscle cells under IL-1- and IL-6-mediated conditions suggested that SAA expression in the atherosclerotic lesion may be induced by paracrine or autocrine mechanisms, in response to events that stimulate cytokine production by vascular cells and macrophages [28].

While IL-1β is the strongest inducer of A-SAA in hepatocytes [31], as well as in human choriocarcinoma cell lines [32], it may act synergistically with IL-6, to greatly increase A-SAA synthesis [31]. Although synthetic glucocorticoids exhibit immunosuppressive and anti-inflammatory actions *in vivo*, they paradoxically (in combination with proinflammatory cytokines) augment the

expression of A-SAA in hepatocytes [33], as well as in non-hepatic cells, such as synoviocytes [34] and KB epithelial cells [35]. Till now, it was unclear whether IL-1β and IL-6, in the presence or absence of dexamethasone, may affect SAA1/2/4 mRNA and protein expression in HCAEC.

One previous study [36] indicating immunofluorescent analysis of intracellular SAA expression in non-stimulated HCAEC revealed its colocalization with microtubules, prominent perinuclear location, and intense labeling of SAA in thin cellular protrusions, extending from cells. SAA presence was also reported in membrane nanotubes bridging neighbouring cells, with occasional detection in dilatations of nanotubes termed gondolas [36]. In fact, tunneling membrane nanotubes (TNTs) between HCAEC may provide open communication channels between cells. While intercellular communication is essential for tissue homeostasis, specific cell functions, and responses to external cues, dysregulation of cell-cell communication is implicated in different pathologies, such as neurodegenerative diseases and malignoma [37, 38]. Numerous factors can be transported *via* TNTs, such as proteins, Ca^{2+}, microRNAs, mitochondria, or even lipid droplets [37, 39, 40].

The aims of this study were to elucidate if HCAEC stimulation with IL-1β and IL-6, in the presence/absence of dexamethasone, can modulate the expression of SAA1/2/4 at the mRNA and/or protein levels. We also aimed to determine the intracellular location of different SAA forms in HCAEC and examine whether SAA1 can be transported between cells in a uni- or bilateral manner.

MATERIALS AND METHODS

Materials

Lyophilized human recombinant IL-1β (Invitrogen, Frederick, MD, USA) was spun down, reconstituted in cell culture grade sterile water to a stock concentration of 100 ng/ml, and stored at − 80 °C until used at a final concentration of 1 ng/ml. Lyophilized human IL-6 was purchased from PeproTech (EC, Ltd., London, UK), spun down and reconstituted according to the manufacturer's instructions in cell culture grade sterile water to a stock concentration of 100 ng/ml, and stored at − 80 °C until used at a final concentration of 10 ng/ml. Dexamethasone (Krka, Novo Mesto, Slovenia; stock concentration of 4 mg/ml) was used at a final concentration of 1 μM.

Cell Culture and Stimulation

HCAEC were purchased from Lonza (Walkersville, MD, USA). Cells were seeded into 75 cm^2 flasks, followed by 6-well plates (TPP, Trasadingen, CH) and cultured in EGM-2M medium (Lonza) containing 5% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere and 5% CO_2. Prior to experiments, cells were incubated in serum free media for 2 h. For experiments, subconfluent (80–85% confluency) cell cultures in serum-free medium were used between passages 4 and 6 plated in 6-well plates, with an addition of IL-1β (1 ng/ml), and/or IL-6 (10 ng/ml), and/or dexamethasone (1 μM) in different combinations for 24 h.

Cell viability was determined by detaching the cells from wells, using trypsin/EDTA, collecting them by centrifugation for 5 min, 300×g at room temperature (RT), and resuspending them in 100 μl of flow cytometry buffer (2 mM EDTA and 0.5% BSA in dPBS) and adding 10 μl propidium iodide (Miltenyi Biotec, Bergisch Gladbach, DE) to each well. Fluorescence was then measured using flow cytometer (MacsQuant, Miltenyi Biotec).

Quantitative PCR

Total RNA from HCAEC was isolated using RNeasy Plus Universal Mini kit (Qiagen, Hilden, DE) following manufacturer's instructions. The purity and amount of RNA were determined by measuring the OD at 260 and 280 nm. One microgram of total RNA was transcribed into cDNA with Reverse Transcription System (Promega, Madison, USA) and RT-PCR was performed in the Thermal cycler (Applied 144 Biosystems, Foster City, USA). HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) with SAA-specific primers (Integrated DNA Technologies, Coralville, USA) as shown in Table 1, were used for performing quantitative PCR (qPCR) with StepOne (Applied 154 Biosystems, Paisley, UK). For normalization of data, GAPDH (Integrated DNA Technologies) was used as endogenous control. Gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Cell lysates were prepared from HCAEC with RIPA buffer containing Halt protease inhibitors (Pierce, Rockford, USA) and protein concentration subsequently determined with the Bio-Rad protein assay (Bio-Rad, Munchen, DE). The lysate was mixed with loading buffer and loaded onto a 10% SDS-PAGE gel in equal protein concentrations. Following electrophoresis, transfer to nitrocellulose

Table 1. Primers Used in qPCR Analysis

Primer name	Primer sequence	Fragment size (bp)	Reference
SAA1-forward	5′-CTG CAG AAG TGA TCA GCG-3′	237	[41]
SAA1-reverse	5′-ATT GTG TAC CCT CTC CCC-3′		
SAA2- forward	5′-CTG CAG AAG TGA TCA GCA-3′	262	[41]
SAA2- reverse	5′-ATT ATA TGC CAT ATC TCA GC-3′		
SAA4- forward	5′-GTC CAA CGA GAA AGC TGA GG-3′	164	[42]
SAA4- reverse	5′-AGT GAC CCT GTG TCC CTG TC-3′		
GAPDH-forward	5′-TGT AGT TGA GGT CAA TGA AGG G-3′	143	
GAPDH-reverse	5′-ACA TCG CTC AGA CAC CAT G-3′		

membrane was preformed (100 V, 250 mA, 1 h). The membrane was dried, blocked in TBS buffer with 5% milk, for 1 h, at RT and incubated with primary mouse anti-SAA1 (MyBiosource, San Diego, USA), diluted 1:100 in blocking buffer; or rabbit anti-SAA2 (ProteinTech, Chicago, USA), diluted 1:250; or rabbit anti-SAA4 antibodies, diluted 1:500 (Sino Biological, Wayne, USA) for 2 h, at RT. Secondary anti-mouse-HRP (Abcam, Cambridge, UK) or anti-rabbit-HRP antibodies (Santa Cruz Biotechnology, Dallas, USA) were used at dilutions of 1:1000 in blocking buffer. Detection was performed using femtoluminol (Thermo Fisher Scientific, Hemel Hempstead, UK) with G:Box (Syngene, Cambridge, UK).

SAA ELISA

SAA protein levels in supernatants and lysates of treated and untreated HCAEC were measured by commercially available ELISA kit (Abcam, Cambridge, UK), following the manufacturer's instructions. Briefly, 100 μl of standards and cell supernatants or lysates (containing the same concentration of proteins) were added into appropriate wells. After 2.5 h incubation at RT, 100 μl of biotinylated anti-SAA detection antibody was added to each well. Following 1 h incubation at RT, 100 μl HRP-streptavidin solution was added and incubated for 45 min at RT. Subsequently, 100 μl tetramethylbenzidine one-step substrate reagent was added and incubated for an additional 30 min at RT. After adding 50 μl of stop solution, the absorbance was read at 450 nm with a microplate reader (Tecan, Groening, Austria). The concentrations of SAA were calculated from standard curves.

Fluorescent Labeling of Cells

HCAEC grown on coverslips were permeabilized with 3.7% formalin in PEM buffer (100 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, 0.2% Triton X-100) for 5 min at 37 °C, then washed 3× in PBS and fixed in 3.7% paraformaldehyde in PBS for 5 min, at 37 °C. After washing in PBS (5×), cells were blocked with 1% BSA/PBS for 30 min, at 37 °C. For SAA1, SAA2, SAA4, and tubulin immunolabeling, HCAEC were incubated with mouse anti-SAA1 (1:25; MyBiosource), rabbit anti-SAA2 (1:100; Proteintech), rabbit anti-SAA4 (1:100; Sino Biological), and rabbit (Abcam) or mouse (Invitrogen) anti-α-tubulin antibodies, all diluted 1:100. After overnight incubation with primary antibodies and washing in PBS, appropriate secondary antibodies conjugated with Alexa Fluor 488 or 555 (1:300; Invitrogen, Frederick, MD, USA) were added to the cells and incubated for 1 h, at 37 °C. For labeling of F-actin filaments, fluorescein phalloidin was used (Invitrogen). After washing in PBS, the coverslips overgrown with cells were embedded with Prolong Diamond antifade mountant containing the nuclear stain diamidinophenylindole diacetate (DAPI) (Invitrogen) and observed with a fluorescence microscope (Nikon Eclipse TE 300 and AxioImager Z.1, Carl Zeiss).

SAA1 Transfer Experiment

Human recombinant SAA1 (PeproTech, UK) was fluorescently labeled using FITC or Cy3 Fast Conjugation Kits (Abcam), as per manufacturer's instructions.

Subconfluent HCAEC, seeded into 6-well plates were incubated in fresh medium supplemented with 5% FBS, containing a mixture of 6 μg/ml fluorescently labeled SAA1 (either SAA1-FITC or SAA1-Cy3) and 6 μg/ml non-labeled SAA1 for an initial time period of 24 h. After incubation, the medium was removed and cells were detached using trypsin/EDTA, neutralized by trypsin neutralization solution, collected by centrifugation for 5 min, 300×g at RT, and resuspended in 0.5 ml fresh media with 5% FBS. Cells stimulated with

differentially labeled SAA1 proteins were mixed in equal concentration (1:1), added to a fresh 12-well plate, and further incubated for 4, 24 or 48 h with or without stimulation with IL-1β (1 ng/ml). Afterwards, cells were collected by trypsinization and centrifugation, washed, resuspended in 100 µl of flow cytometry buffer, and analyzed by flow cytometry (Miltenyi Biotec). Analysis of flow cytometry data was performed using FlowLogic (Flow Cytometry Analysis Package, version 700.0a).

Statistical Analysis

Data are presented as mean ± standard deviation (SD) of three biological replicates unless otherwise stated. Means were compared among the treated and control groups using one-way ANOVA and Tukey's Multiple Comparison Test. All data were analyzed using the GraphPad Prism 5.03 software. All tests were two tailed and p values of < 0.05 were regarded as statistically significant.

RESULTS

Il-1β Stimulates mRNA Expression of SAA1 and SAA2

We could observe a statistically significant elevation of SAA1 mRNA expression in IL-1β-stimulated HCAEC as compared to control. HCAEC showed elevated SAA1 expression as a mean increase of 119.9-, 92.8-, 117.2-, and 166.6-fold above control, when treated with IL-1β, IL-1β in combination with IL-6, IL-1β in combination with IL-6 and dexamethasone, or IL-1 β in combination with dexamethasone, respectively. No effect in SAA1 expression was observed when HCAEC were stimulated with IL-6 or dexamethasone alone or with combination of both without IL-1β (Fig. 1a). Similarly, SAA2 also exhibited increased mRNA expression in IL-1β-treated HCAEC; however, the expression of SAA2 mRNA was lower as compared to SAA1. There was a 9.3-fold increase in the mean expression of SAA2 when cells were treated with IL-1β *vs.* untreated cells. Cell treatment with IL-1β in combination with IL-6, dexamethasone, or IL-6 and dexamethasone showed 10.6-, 10.9-, and 13.4-fold increases, respectively, in SAA2 mRNA levels as compared to untreated HCAEC (Fig. 1b). Similarly to SAA1, IL-6 or dexamethasone alone or in combination, without the presence of IL-1β, had no effect on SAA2 synthesis. No difference in mRNA expression of SAA4 was observed between treated and untreated HCAEC, showing that SAA4 is constitutively expressed and not affected by stimulation with proinflammatory cytokines or glucocorticoids (Fig. 1c). Cell viability was not affected by the treatments (between 90 and 97% viable under both treated and untreated conditions (data not shown)).

Unchanged Protein Levels of SAA in Lysates of HCAEC

In order to confirm the presence of intracellular SAA protein within HCAEC, ELISA was used (Fig. 2). No difference was observed in protein levels between control (untreated) and IL-1β-, IL-6-, or dexamethasone-treated HCAEC. Secondly, we sought to determine SAA levels in supernatants from control and treated HCAEC. Surprisingly, A-SAA levels were too low to be detected by the assay used (limit of detection was 0.41 ng/ml), even in IL-1β-treated cells.

Western Blot Analysis

To confirm the results of ELISA in cell lysates, we also performed SDS-PAGE followed by a Western blot of untreated HCAEC (background) and IL-1β-treated HCAEC. Again, no difference in signal was observed between untreated and IL-1β-treated cells. Interestingly, SAA1 was found mainly as a monomer (band at 12 kDa), while SAA2 mainly formed octamers (bands at around 100 kDa). SAA4 was present in the form of monomers, dimers, and octamers (Fig. 3).

Intracellular Localization of SAA1/2/4 in HCAEC

In order to determine the intracellular localization of different SAA forms within HCAEC, fluorescent labeling and immunolabeling of SAA1, SAA2, SAA4, nucleus, F-actin filaments, and tubulin were performed in untreated and IL-1β-, IL-6-, or dexamethasone-stimulated cells.

A strong signal for all three SAA forms was observed in untreated, as well as treated, HCAEC with no difference in localization of immunoreactions inside the cells (data not shown). All further fluorescent immunolabeling was therefore performed only in unstimulated HCAEC. SAA1, SAA2, and SAA4 all showed predominantly perinuclear localization with a signal also seen in the nuclear space. A clear separation of all, SAA1, SAA2, and SAA4, from F-actin filaments was observed, when performing double immunolabeling of SAA with phalloidin (Fig. 4). We confirmed previously observed colocalization with microtubules [36] by double immunolabeling of SAA forms

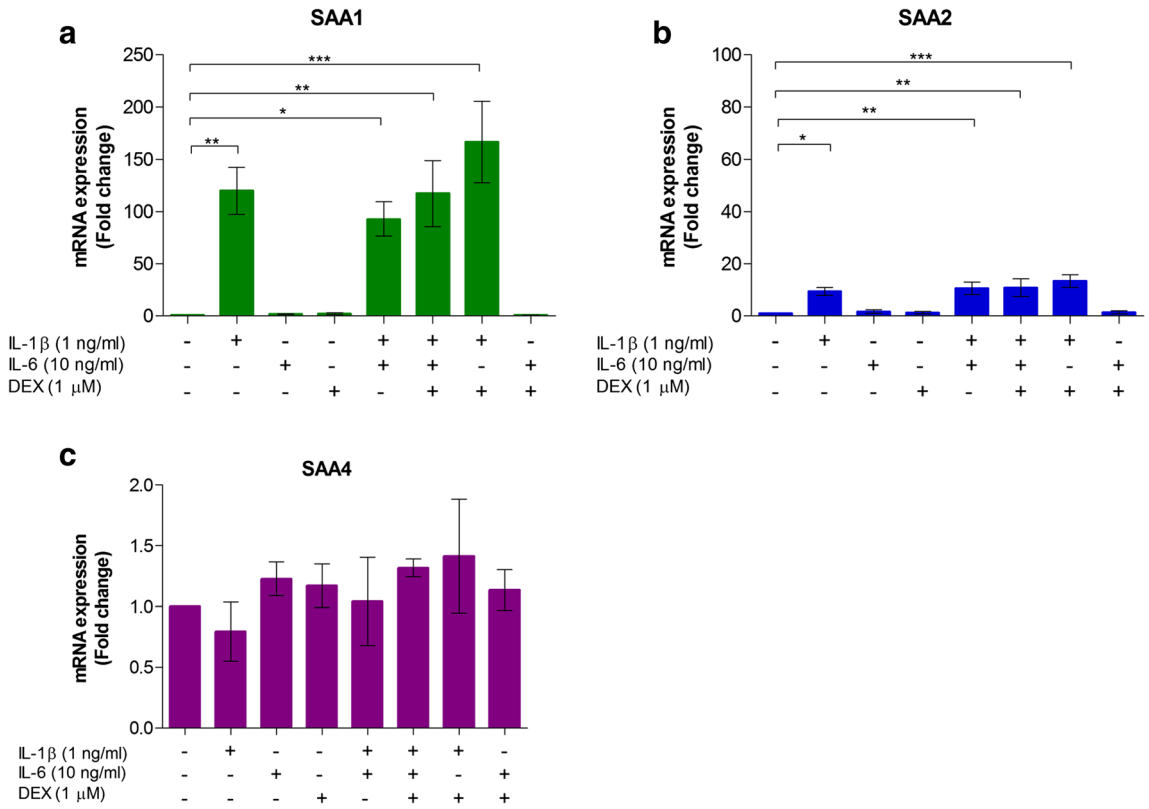

Fig. 1. Relative mRNA expression of SAA1 (**a**), SAA2 (**b**), and SAA4 (**c**) normalized with GAPDH in IL-1β-, IL-6- and dexamethasone-treated *vs.* untreated HCAEC. Shown is mean ± SD of four independent experiments for each condition. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

with anti-tubulin antibodies (Fig. 4) and using different specific anti-SAA1/2/4 antibodies compared to the previous study. However, large amounts of SAA1/2/4 also seemed to be unattached to any of the labeled structures. In addition, a strong signal for SAA1 and SAA4 and to smaller extent also for SAA2 was observed in nanotubes, extending from the cells, connecting cells to one another (Fig. 5).

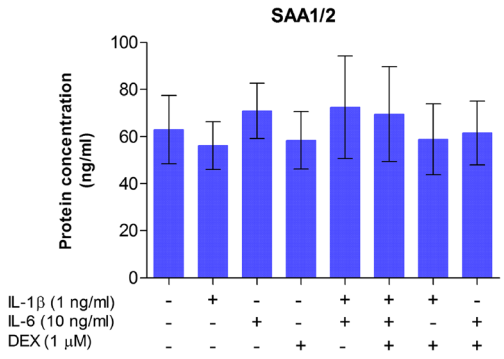

Fig. 2. SAA1/2 concentration in cell lysates of IL-1β-, IL-6-, and dexamethasone-treated *vs.* untreated HCAEC. Shown is mean ± SD of three independent experiments for each condition.

Increased Intercellular Transport of SAA1 Occurs in IL-1β-Stimulated HCAEC

Flow cytometry analysis of the mixture of SAA1-FITC- and Cy3-labeled HCAEC revealed that cells at time point 0 (after the first 24 h incubation and before co-culture) are positive for either SAA1-FITC or SAA1-Cy3 protein, but not for both labeled proteins simultaneously. After 4 h of co-incubation, only a few cells were positive for both fluorescent protein variants (mean ± SD percentage of double positive cells in three replicates was 6.3 ± 0.8 and 3.5 ± 0.9 in untreated *versus* IL-1β-treated HCAEC, respectively). While at 24 h, one third of cells (22.0 ± 7.0 and 27.5 ± 7.2 in untreated and IL-1β-treated HCAEC, respectively) already exhibited double positivity for both SAA1-FITC and SAA1-Cy3. A significantly higher percentage of double

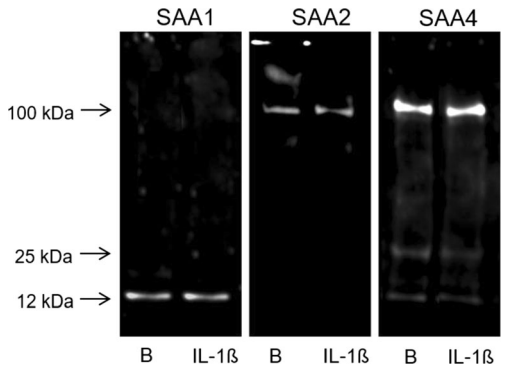

Fig. 3. Western blots from untreated and IL-1β-treated HCAEC. Cell lysates from HCAEC were blotted onto nitrocellulose membranes and overlayed with antibodies against SAA1, SAA2, and SAA4. Indicated are SAA monomers (MW ~12 kDa), dimers (~25 kDa). and octamers (~100 kDa). Shown is a representative blot from two independent blots performed.

positive cells was observed after 24 h, as compared to 4 h co-culture ($p < 0.05$ for untreated and $p < 0.01$ for IL1-β-treated HCAEC). Interestingly, at 48 h of co-culture, a significantly higher percentage of double positive HCAEC treated with IL-1β (60.0 ± 4.3) as compared to background (47.9 ± 1.8; $p < 0.05$) was observed (Fig. 6), suggesting that IL-1β promotes SAA1 transport from cell to cell at later time points.

DISCUSSION

This is the first study to report the upregulation of mRNA expression of both SAA1 and SAA2 by IL-1β in HCAEC, while confirming the constitutive expression of SAA4, unchanged upon stimulation. SAA1 exhibited approximately 10-fold higher levels of mRNA expression than SAA2 following IL-1β stimulation of HCAEC (as compared to background), which was also observed for human mesenchymal stem cells, osteosarcoma cell lines [43], KB epithelial cells [35], and THP-1 monocytic cell lines [44]. This is in line with observations that SAA1 is the predominant SAA protein expressed under inflammatory conditions [45, 46]. With the development of newer methodology, it is now possible and important to discriminate between SAA1 and SAA2, because they can have distinct functions despite their high sequence identity [47]. One of the most important distinctions is that SAA1 predominates in the formation of AA amyloid deposits, found in reactive AA amyloidosis in humans and mice [48–50].

When hepatocytes were treated with IL-1β in combination with IL-6, they acted synergistically to greatly increase SAA1/2 synthesis [31]. On the contrary, this was not the case for HCAEC in our study, where IL-6 alone or in combination with IL-1β did not have an effect on SAA expression. The expression of SAA1/2 by cytokines alone and/or in combination may differ greatly depending on tissue and cell type and specific receptors expressed on these cells [35]. Endothelial cells do not express IL-6 receptor (IL-6R) and are known to require soluble IL-6R to be able to response to IL-6 stimuli [51, 52]. It would therefore be interesting to see whether the addition of exogenous soluble IL-6R and stimulation with IL-6 would increase SAA1/2 production in HCAEC.

Conflicting data exist for glucocorticoid (specifically dexamethasone)-induced SAA expression in endothelial cells. In one study, only a slight increase in SAA1/2 mRNA levels was observed when HUVEC were stimulated with IL-1β and this response was augmented by dexamethasone and IL-6 [53]. In contrast, another study found that neither A-SAA nor C-SAA was expressed in HUVEC, not even after stimulation with IL-1β and/or dexamethasone [54]. This is in contrast with our results in HCAEC, where IL-1β greatly increased SAA1, and to a lesser extent, SAA2 expression, while dexamethasone alone was not able to upregulate SAA1/2 expression in HCAEC and also, did not act in concert with IL-1β. HCAEC have previously been shown to exhibit increased responsiveness to both inflammation and coagulation, compared to HUVEC, and thus could account for greater susceptibility of coronary arteries to IL-1β stimulation [30] and development of atherosclerosis.

In the present study, we discovered that protein levels of A-SAA in HCAEC do not correlate with mRNA levels, indicating that SAA protein upregulation has to be tightly controlled to prevent over-increased levels during inflammation. The concentration of SAA1/2 in HCAEC lysates reached 50–70 ng/ml (as measured by ELISA), lending support to local extrahepatic synthesis. However, in contrast to mRNA levels, we could not observe any difference in intracellular SAA1/2 protein concentrations between untreated and IL-1β-treated HCAEC, as determined by ELISA and confirmed by fluorescent immunolabeling and Western blots.

The discrepancy between high SAA1/2 mRNA levels as opposed to their protein levels in stimulated HCAEC could be explained by a variety of transcriptional, posttranscriptional, and translational mechanisms. Despite high levels of SAA mRNA in human hepatic cells, translation may not always be proportionally higher [55]. Thus, SAA synthesis can also be regulated post-transcriptionally,

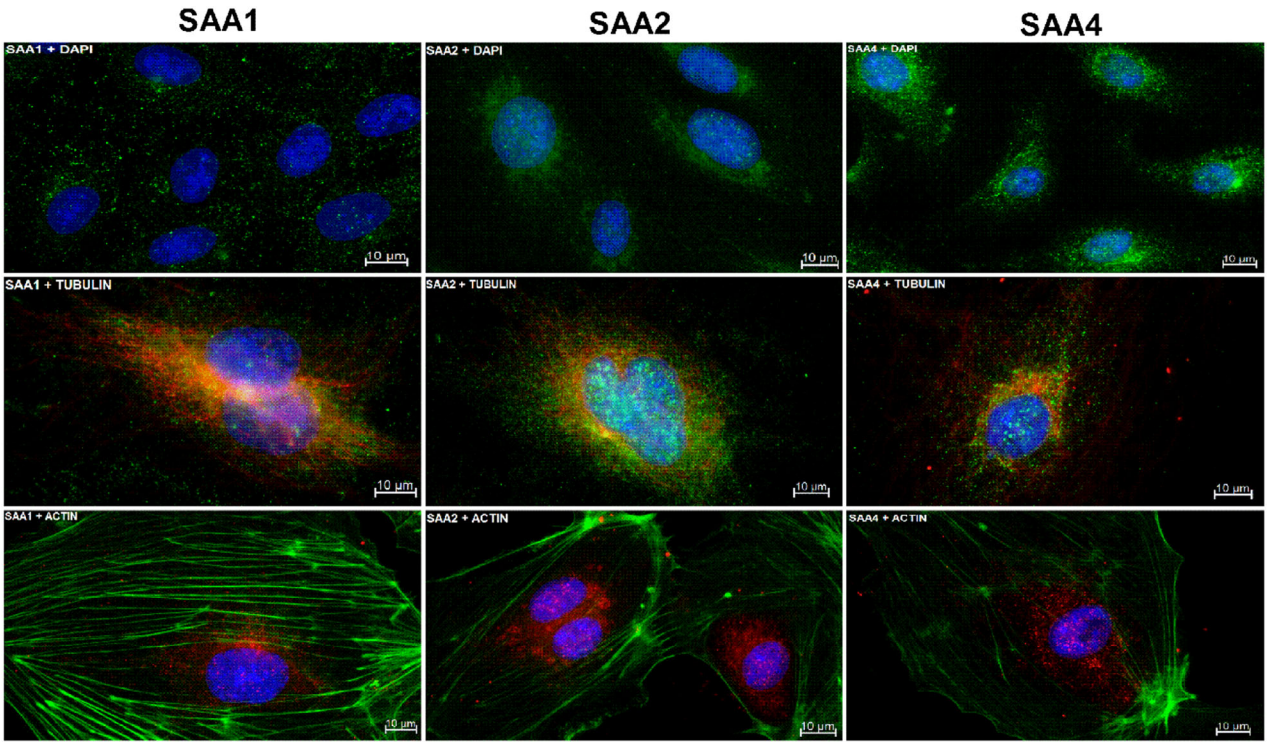

Fig. 4. Intracellular location of SAA isoforms in unstimulated HCAEC by fluorescence microscopy. SAA1 (left panel), SAA2 (middle panel), and SAA4 (right panel) show predominantly perinuclear location, with a signal also in the nuclear space. SAA1/2/4 were immunolabeled with appropriate antibodies against SAA1, SAA2, and SAA4 (green fluorescence in combination with tubulin or red fluorescence in combination with actin). α-tubulin was immunolabeled with anti-α-tubulin antibodies (red fluorescence), F-actin filaments were labeled with fluorescein phalloidin (green fluorescence), and nuclei were labeled with DAPI (blue fluorescence). Shown is a representative experiment of three independent experiments performed.

specifically, following induction by IL-1 and IL-6, SAA mRNA poly (A) tail has been reported to undergo a gradual and homogeneous reduction in length and subsequent decrease in SAA mRNA in hepatoma cell lines. This could account for downregulating the A-SAA protein synthesis in its *in vitro* response to cytokine treatment [56]. Regulation at the posttranscriptional level can also be mediated by microRNAs, small noncoding RNAs that bind target mRNA to prevent protein production by cleavage of target mRNA with subsequent degradation or translation inhibition [57]. Currently, there are no known effects of miRNA to modulate SAA expression; however, miR-377-5p levels were found to be positively correlated with SAA circulating levels in tumor necrosis factor receptor-associated periodic syndrome [58]. Since our SAA1/2/4 transcripts (Fig. 1) do not encompass full length, coding regions, it is probable that no proteins will be produced from them, as observed also by our ELISA results (Fig. 2). While the location and function of these SAA1/2/4 transcripts in HCAEC are currently unknown, it would be interesting in the future to evaluate whether they could be involved in regulation of certain processes, such as inflammation and cardiovascular disease pathology. Recently, reports have emerged that long noncoding RNA (MALAT1) may regulate inflammation in endothelial cells in response to glucose and that SAA is a target of MALAT1 [59]. IL-6-induced MALAT1 was also shown in murine cardiomyocytes to enhance TNF-α expression, at least partly *via* SAA3 [60]. So, it would be intriguing to determine the roles and intracellular location of noncoding transcripts of SAA themselves.

Multimerization of SAA (prevalently hexamers) has previously been shown on immunoblots for expressed and purified murine SAA2.2, while urea-induced denaturation yielded monomers [61]. More recently, Villapol et al. identified both SAA1 monomers and higher molecular weight SDS-resistant SAA1 species in mouse liver homogenates after ultracentrifugation [62]. Similarly, we identified SDS-resistant multimers in HCAEC for SAA2 and SAA4, while SAA1 was prevalently monomeric (Fig. 3).

Since we could not observe any differences in intracellular levels of SAA1/2 between treated and untreated

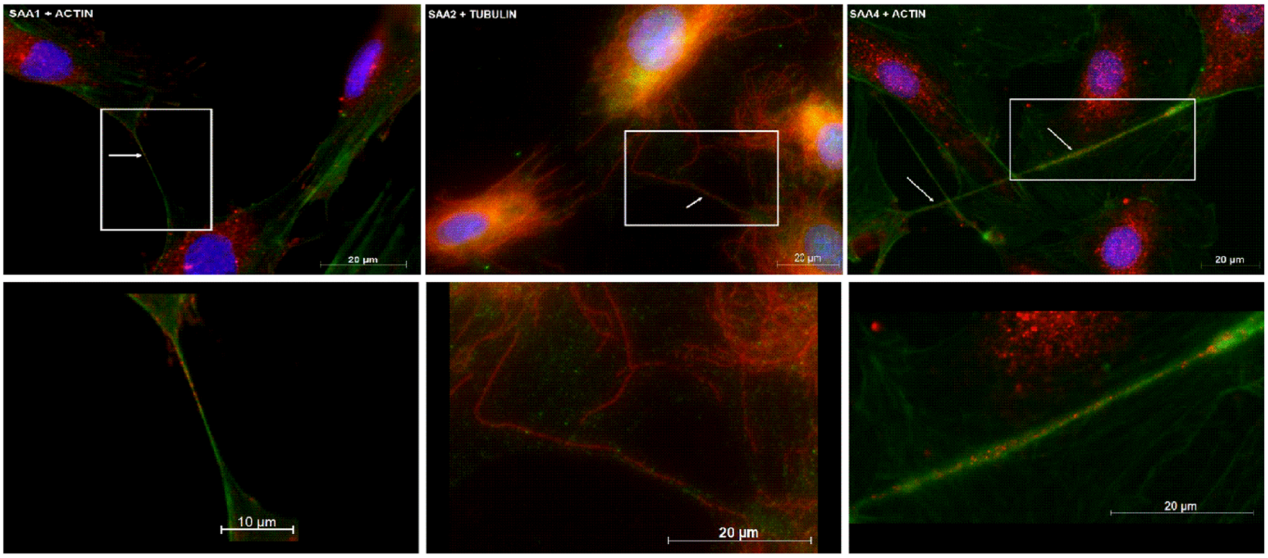

Fig. 5. Intercellular transfer of SAA1, SAA2, and SAA4 *via* tunneling membrane nanotubes in HCAEC by fluorescence microscopy. SAA1/2/4 (white arrows) were detected in tunneling membrane nanotubes, interconnecting cells. SAA isoforms were labeled with appropriate anti-SAA antibodies targeting SAA1/SAA4 (red fluorescence) and SAA2 (second row, green fluorescence). α-tubulin was immunolabeled with anti-α-tubulin antibodies (second row, red fluorescence) and F-actin filaments were labeled with fluorescein phalloidin (green fluorescence). Nuclei were labeled with DAPI (blue fluorescence). Shown is a representative experiment of three independent experiments performed.

HCAEC, we speculated that SAA is released into the supernatants. However, we could not detect released SAA protein by ELISA (at a low detection limit of 0.41 ng/ml) following a 24 h incubation of HCAEC in culture. This led us to further explore possible differences in intracellular location of SAA1, SAA2, and SAA4 in HCAEC using immunolabeling and fluorescence microscopy. While SAA1 was found to be dispersed over the cell, SAA2 was organized in lines, especially in the perinuclear space. Since SAA1 and SAA2 are more than 90% identical, this observation could be due to the differential quarternary structure. Indeed, when performing Western blots from HCAEC lysates, we could observe that SAA1 was predominantly found as a monomer, while SAA2 associated to form octamers as well. In mice, SAA1.1 and SAA2.2 (six residue differences) show different biochemical properties, specifically oligomerization and fibrillation *in vitro*. Mouse SAA2 can refold *in vitro* into an octamer and a hexamer while mouse SAA1 refolds into a mixture of oligomers, specifically dodecamers, tetramers, and monomers [63, 64]. High-resolution X-ray crystallography of lipid-free human variant SAA1 reported that native SAA1.1 exists as a hexamer and refolds mainly into trimers that become monomers at lower concentrations; however, the 3D crystal structure for human SAA2 form has yet to be resolved [65].

The detection of SAA1 in TNTs, bridging neighbouring cells and offering cargo traffic from one cell to another, led us to analyze potential SAA1 transport between cells. In the current report, the exchange of SAA1 between cells was bilateral, increasing steadily over 48 h, with IL-1β promoting SAA1 transport from cell to cell, presumably through direct contact, as previously indicated in cultured macrophage cell lines [66].

This report is the first (to our knowledge) to identify TNTs between HCAEC. We found SAA associated with thin, actin filaments containing nanotubes, as well as thicker, microtubule containing TNTs. Both cytoskeletal elements inside TNTs are probably important as tracks for motor proteins transporting various types of cargo from cell to cell. Communication between non-treated cells in a primary cell culture, such as HCAEC, might represent a signal of well-being and establishment of "intercellular homeostasis," while an inflammatory signal, such as IL-1β, accelerates SAA1 exchange, leading presumably to increases in cellular activity. It would be of interest in the future to block IL-1β, with a specific monoclonal antibody, such as canakinumab, and determine its effect on both TNTs, as well as the rate of SAA1 exchange.

This direct communication between cells that are not juxtapositioned but are positioned further away could

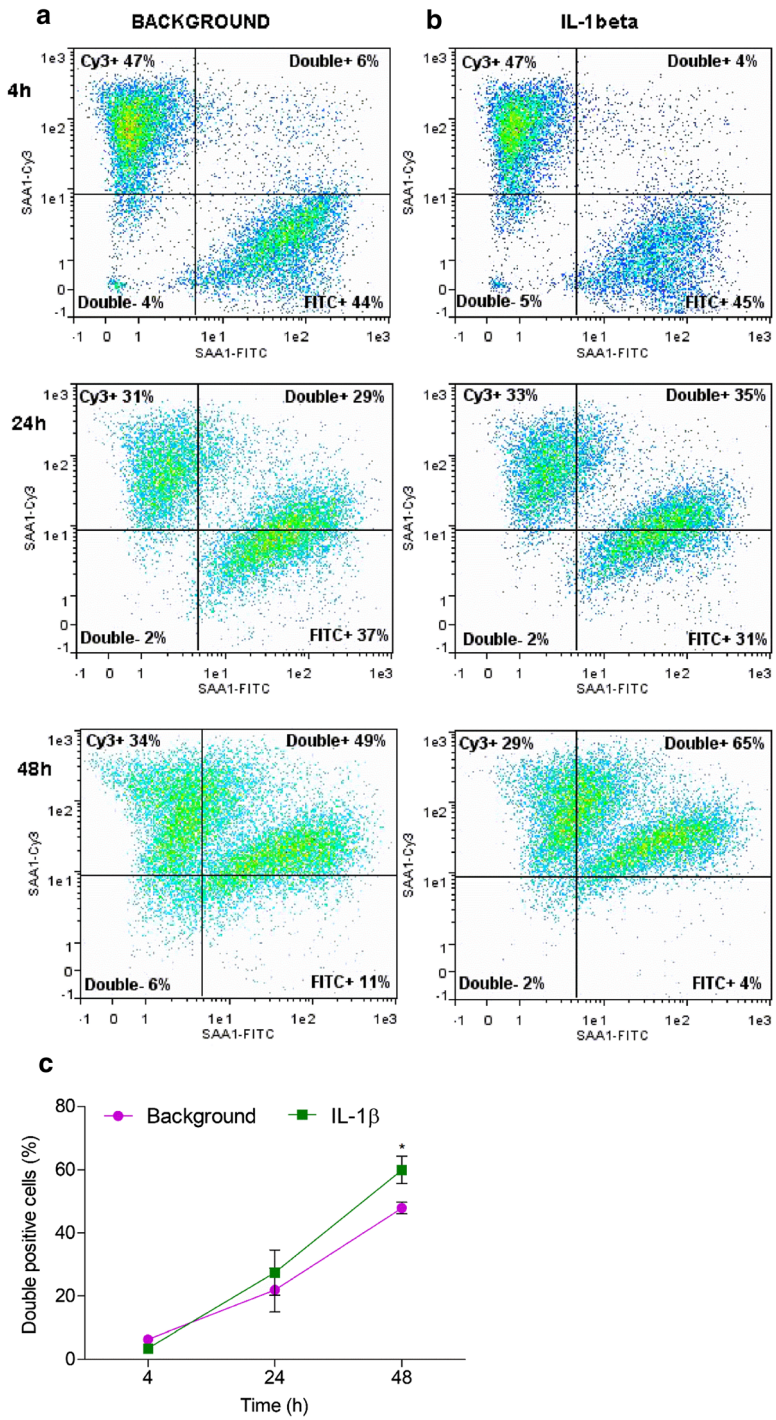

Fig. 6. Cell-to-cell transfer of SAA1 by flow cytometry in the presence or absence of IL-1β. HCAEC were preincubated for 24 h with 6 μg/ml non-labeled SAA1 and 6 μg/ml fluorescently labeled SAA1 (either SAA1-FITC or SAA1-Cy3). Equal numbers of SAA1-FITC and SAA1-Cy3 labeled HCAEC (10^5 cells/well) were then co-cultured for different periods of time (4 h, 24 h, 48 h) in the absence (**a**) or presence of IL-1β (**b**). The vertical and horizontal lines represent thresholds of HCAEC that were considered as SAA1-FITC and SAA1-Cy3 positive. (**c**) Shown are the mean ± SD percentages of SAA-FITC and SAA1-Cy3 double positive cells (from three independent experiments). *$p < 0.05$ of IL-1β-stimulated HCAEC as compared to background.

contribute to a uniform cellular response, and in inflammatory conditions, might promote potential susceptibility of HCAEC to atherogenesis and ultimately, faster development of atherosclerosis in coronary arteries.

AUTHOR'S CONTRIBUTION

TK, SSS, and AE designed the experiments, acquired and analyzed the data, and wrote the manuscript. TK, KMP, and KL performed the experiments. KL, SČ, and SSS coordinated the study. All authors participated in critical discussion of the data and drafting the manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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