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CIGARETTE SMOKE EXTRACT, KALLIKREIN-6 AND APROTININ REGULATE PRODUCTION OF SOLUBLE VCAM-1 AND ICAM-1 IN HUMAN CAROTID ENDOTHELIAL CELLS

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ABSTRACT: Atherosclerosis is a cardiovascular disease that affects the integrity of the arteries and the flow of blood. Indicators of atherosclerosis are, but not limited to, increased expression of Cell Adhesion Molecules (CAMs) on the vascular endothelium, recruitment of serum monocytes (MO) to the endothelium, transmigration of MO across the endothelial layer, maturation of MO to macrophages, plaque formation, and vessel occlusion and rupture. Monocyte adhesion is regulated by the balance between membrane (mCAM) and soluble (sCAMs). We found that Kallikrein-6 (KLK6) cleaves membrane Vascular Cell Adhesion Molecule-1 (mVCAM-1) on vascular endothelial cells, and hypothesized that this proteolytic cleavage event may affect the balance between relative levels of mCAM and sCAM. Since cigarette smoking increases the risk of developing atherosclerosis, we hypothesized that serum extracts of smoke increase the presence of mCAMs and decrease the abundance of sCAMs, thus increasing the likelihood of MO binding to the endothelium. Furthermore, we conjectured that smoking may increase the incidence of plaque formation by affecting an mVCAM-1 protease, leading to decreased sVCAM-1. We determined that in Human Carotid Endothelial Cells (HCEC) (1) Lipopolysaccharide and Tumor Necrosis Factor-alpha increased mVCAM-1 and mICAM-1 (2) cigarette smoke extract (CSE) decreased sVCAM-1, but not sICAM-1, production in time- and dose-dependent manners, (3) KLK6 increased sVCAM-1 and sICAM-1 production in time- and dose-dependent manners, (4) aprotinin, a serine protease inhibitor, decreased sVCAM-1, but not sICAM-1, production in a dose-dependent fashion, and (5) KLK6 reversed aprotinin inhibition of sVCAM-1, but not sICAM-1, production.

KEYWORDS: Atherosclerosis, Vascular Cell Adhesion Molecule-1, Endothelial Cells, Kallikrein-6, Cigarette Smoke.

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1. INTRODUCTION

The integrity of the vascular wall indicates the health of an artery and the circulatory system in general [1,2]. Many soluble serum components, such as cytokines and hormones, impact the integrity of the arterial endothelium [3]. Physical, biochemical and exogenous factors can adversely affect the luminal walls of the arteries and thereby activate sequelae of events that are detrimental to the integrity of the endothelium and vasculature [4]. Atherosclerotic plaque is initiated in part by decreased rolling of serum monocytes (MO) on the vascular endothelial surface, transmigration of MO through the endothelium and maturation of tissue MO to mature macrophages (MΦ); all of these are precursors to greater pathological conditions, resulting in the production of cytokines and chemokines. Many internal pathophysiologic conditions such as hyperinsulinemia and inflammatory agents play integral roles in arterial inflammation and atherosclerosis [5,6], contributing to the remodeling of the endothelium, and cardiovascular disease (CVD). Contaminants from the external environment can also affect the vasculature. Soluble contaminating constituents from cigarette smoke are introduced to the cardiovascular system via the pulmonary circulation [7] and can negatively impact the vascular endothelium. Although smoking has been linked to lung disease [8,9], many soluble chemical components of cigarette smoke have been found in the vascular serum as well [10]. These soluble extracts of smoke may have deleterious effects on the cardiovascular tissues and have been associated with increased plaque formation and cardiovascular disease [11]. Cellular Adhesion Molecules (CAMs) such as Vascular Cellular Adhesion Molecule-1 (VCAM-1) and Intercellular Adhesion Molecule-1 (ICAM-1) are expressed on the surface of arterial endothelial cells [12]. Both CAMs interact with ligands on the surface of monocytes (MO) and mediate the adhesion of these serum-based cells to the arterial endothelium [13]. During inflammation, serum monocytes bind to the vascular endothelium, transmigrate the arterial wall and embed themselves into the intimal and medial layers. Subsequently, these monocytes transform into mature macrophages (MΦ) and secrete cytokines and chemokines. In turn, these inflammatory molecules contribute to the recruitment of additional leukocytes, lipids, and other molecules, ultimately resulting in remodeling of the vascular wall and vascular plaque formation [14]. Membrane CAMs (mVCAM-1 and mICAM-1) are cleaved at the endothelial surface by what appears to be an endothelial cell protease, yielding soluble forms of CAMs (sCAMs); that is, sVCAM-1 and sICAM-1, respectively. Previous studies have suggested that sCAMs may advance

the progression of CVD [15]. Since MO bind to the endothelial surface via the CAMs [16], we were interested in investigating the regulation of the production of mCAMs and sCAMs by intrinsic and extrinsic factors. We report here preliminary findings on the regulation of production of sVCAM-1 and sICAM-1 in the endothelium of Human Carotid Endothelial Cells in the presence and absence of the serine protease Kallikrein-6 (KLK6) and the serine protease inhibitor aprotinin.

2. MATERIALS AND METHODS

Primary human carotid endothelial cells (HCEC) and MesoEndo Cell Growth Medium (MGM) were obtained from Cell Applications, Inc. (San Diego, CA). Tumor Necrosis Factor-alpha (TNFα) was obtained from PeproTech (Rock Hill, NJ), Lipopolysaccharide (LPS) was from Sigma-Aldrich (St. Louis, MO) and Cigarette Smoke Extract (CSE) was obtained from Murty Pharmaceuticals, Inc. (Lexington, KY). CSE was reconstituted in dimethyl sulfoxide (DMSO,Sigma-Aldrich [St. Louis, MO]) to a stock concentration of 10 mg/mL. APC-conjugated anti-human ICAM-1, PE-conjugated anti-human VCAM-1, and FITC-conjugated anti-human CD14 antibodies were obtained from BD Pharmingen (San Jose, CA). Kallikrein-6 (KLK6) was from Fisher Scientific (Waltham, MA). All general lab reagents were purchased from Sigma-Aldrich (St. Louis, MO). Leupeptin was from Sigma Aldrich and aprotinin was from Fisher Scientific.

Flow Cytometry Analyses

HCEC were cultured in MGM at 37°C, 5% CO2 atmosphere in 6-well tissue culture dishes (Fisher Scientific) until confluent. Prior to stimulation of HCEC, spent growth medium was removed, cells were washed twice with PBS and fresh growth medium was added to the cells. Cells were introduced to various concentrations of analytes for indicated times. After stimulation, cell-free supernatants were collected and stored at -80°C for analysis by ELISA. The cells were washed twice with 2 mL of 1X PBS (Gibco, Waltham VA), and after the final PBS wash was aspirated, 0.5 mL of Cell Dissociation Solution Non-Enzymatic (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. After incubating the cells at 37˚C and 5% CO2 for 30 min, 1 mL of 1% Bovine Serum Albumin (BSA, Sigma-Aldrich) in PBS was added to the cells and the cells were gently triturated into single cell suspensions. The cells were transferred to 5 mL Falcon polystyrene round bottom tubes (Fisher Scientific) and centrifuged at 500 x g for 5 min. After aspirating the supernatants, the cells were resuspended in 3 mL 1% BSA, pelleted at 500 x g by centrifugation, and the supernatants were removed by aspiration. The cells were resuspended in 200 µL of 1% BSA, 4 µL of PE-conjugated anti-VCAM-1 and 4 µL of APC-conjugated anti-ICAM-1 antibodies. Cells were incubated in the dark for 30 min at 4°C. The cells were washed twice with 3 mL 1% BSA and resuspended in 200 µL of 1% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA). Cells were diluted with an additional 300 µL of PBS and analyzed using flow cytometry. The experiments were run on a BD LSRII (BD Biosciences, San Jose, CA). Mean Fluorescence Intensity (MFI) and gating percentages (part of data analysis) were performed using BD FACSDiva v6

software.

ELISA Analyses

Cell-free supernatants were collected from cells exposed to CSE, lipopolysaccharide (LPS) and Tumor Necrosis Factor-alpha (TNFα) and stored at -80°C until analysis for quantification. Soluble Vascular Cellular Adhesion Molecule-1 (sVCAM-1) and soluble Intercellular Adhesion Molecule-1 (sICAM-1) were measured using a multiplex array chemiluminescence ELISA assay (Aviva Systems Biology, San Diego, CA.).

Data Analyses

Data were analyzed by either unpaired Student's *t* test (two groups) or ANOVA with subsequent Tukey posttest (several groups) as indicated. A "P" value of less than 0.05 was considered significant. Results represent the mean ± Standard Error of the Mean (SEM) of four or more independent experiments.

3. RESULTS AND DISCUSSION

We previously demonstrated that HCEC produced significantly greater amounts of mVCAM-1 than mICAM-1 in the presence of CSE in time- and dose-dependent experiments [17]. In the current study, we observed significantly ($P < 0.05$) increased amounts of mCAMs and sCAMs in the presence of the pro-inflammatory stimuli LPS and TNFα (Fig 1).

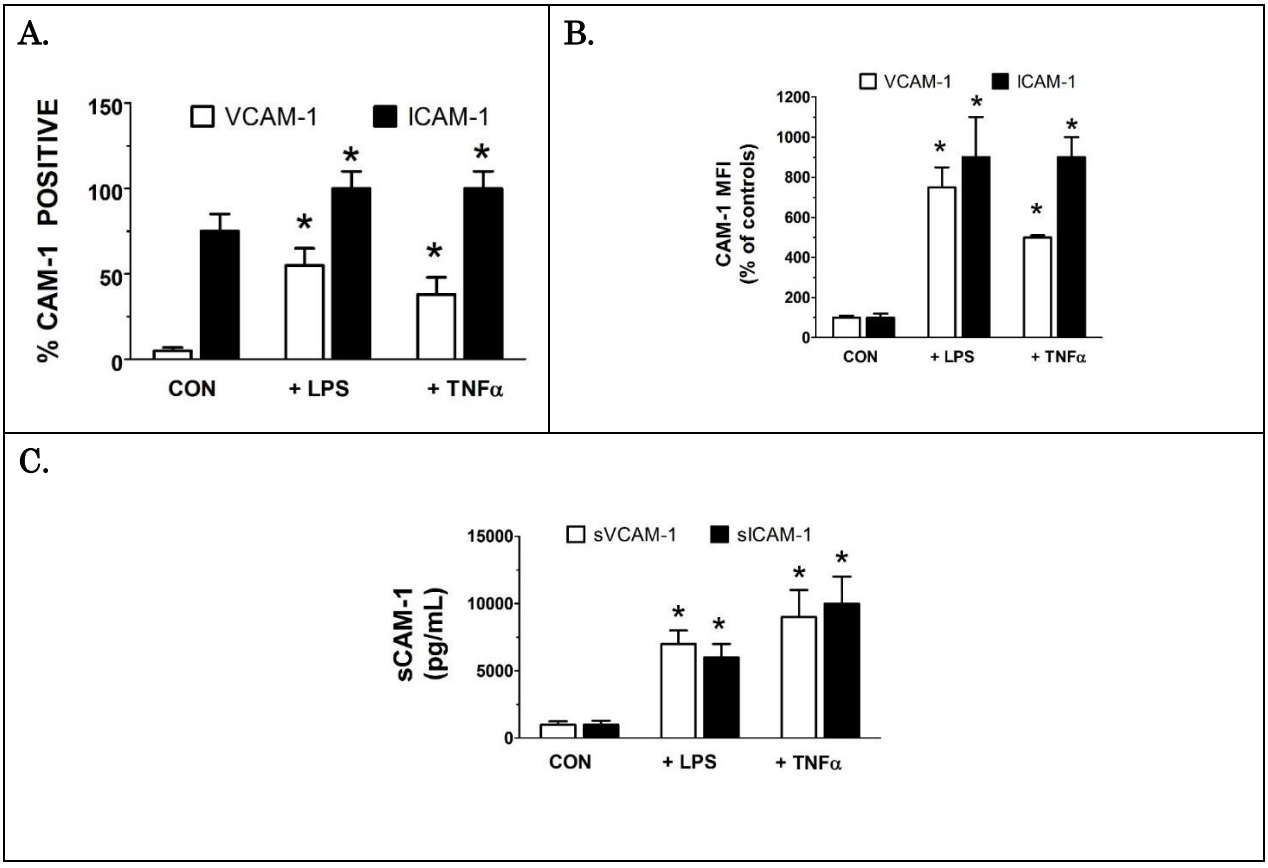

Figure 1: Lipopolysaccharide (LPS) and Tumor Necrosis Factor-alpha (TNFα) increase the production of membrane VCAM-1 and ICAM-1 compared to negative controls. (A) % mCAM-1 positive and **(B)** mCAM-1 MFI (% of controls) of mVCAM-1 and mICAM-1 were determined by

flow cytometry in the absence and presence of LPS (1μg/mL) and TNFα (20 ng/mL). at 24 h. *, P < 0.05 vs negative controls (CON). **(C)** sVCAM-1 and sICAM-1 were determined by ELISA in the absence or presence of LPS (1 μg/mL) and TNFα (20 ng/mL). *, P < 0.05 vs negative controls (CON).

Next, in CSE dose response studies, we observed decreased sVCAM-1 (Fig 2A), but not sICAM-1 (Fig 2B), production over 24 h. In time course experiments where HCEC were incubated for 24h in the absence or presence of CSE, we observed increasing levels of sVCAM-1 (Fig 2C) and sICAM-1 (Fig 2D) over time in HCEC cultures without CSE. In contrast, we measured significant (P < 0.05) decreases in production of sVCAM-1 in the presence of CSE compared to those expressed in its absence (Fig 2C). We did not observe this phenomenon with sICAM-1 (Fig 2D).

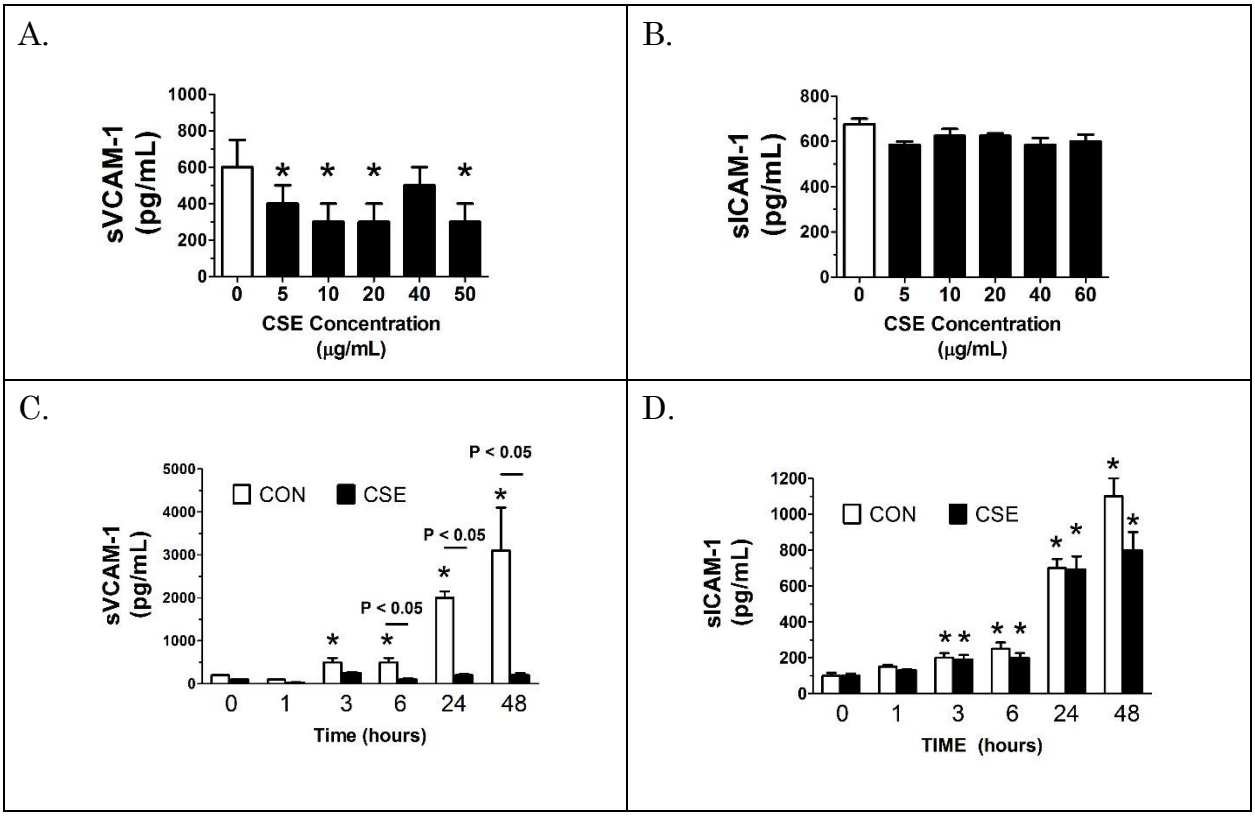

Figure 2: Cigarette Smoke Extract (CSE) decreases sVCAM-1, but not sICAM-1, in a dose-dependent and time-dependent manner. Human Carotid Endothelial Cells (HCEC) were incubated for 24 h in medium without or with indicated concentrations of Cigarette Smoke Extract (CSE). sVCAM-1 and sICAM-1 supernatant concentrations were determined by ELISA. Amounts of sVCAM-1 **(A)** and sICAM-1 **(B)** are expressed in pg/mL and represent the mean ± SEM.

*, P < 0.05 versus negative controls; n = 4. In time-course assays, HCEC were incubated without or with 50 μg/mL of CSE for indicated times. Cell-free supernatants were collected and sCAMS were determined by ELISA. Amounts of sVCAM-1 **(C)** and sICAM-1 **(D)** are expressed in pg/mL and represent the mean ± SEM. *, P < 0.05 versus negative controls; n = 4. CSE concentration was 10 μg/mL.

Since the production of sVCAM-1 from CSE-treated HCEC was inhibited as compared to sVCAM-

1 of negative controls, and there were no differences in expression of sICAM-1 from CSE-treated versus non-treated controls, we hypothesized that CSE disrupts a key enzyme produced by endothelial cells that cleaves mVCAM-1, but not mICAM-1, to produce sVCAM-1 only. We used RNA array analysis of gene expression in HCEC to determine downregulation of genes that occurred in the presence of CSE compared to the absence of CSE. We determined that the expression of several serine proteases, including some members of the Kallikrein gene family, decreased significantly in HCEC treated with CSE for 24 hours compared to that seen in cells not challenged with CSE (data not shown). To confirm that a serine protease is a likely mVCAM-1 processing protease, we performed *in vitro* inhibition assays to confirm these results. HCEC were stimulated with 500 ng/mL LPS for 18h in the absence or presence of two broad serine protease inhibitors, leupeptin (10 μg/mL) and aprotinin (50 KIU/mL). Incubation of HCEC with LPS increased expression of both mVCAM-1 and sVCAM-1. Addition of leupeptin and aprotinin resulted in increased mVCAM-1 (Fig 3A and 3B) and decreased sVCAM-1 (Fig 3C).

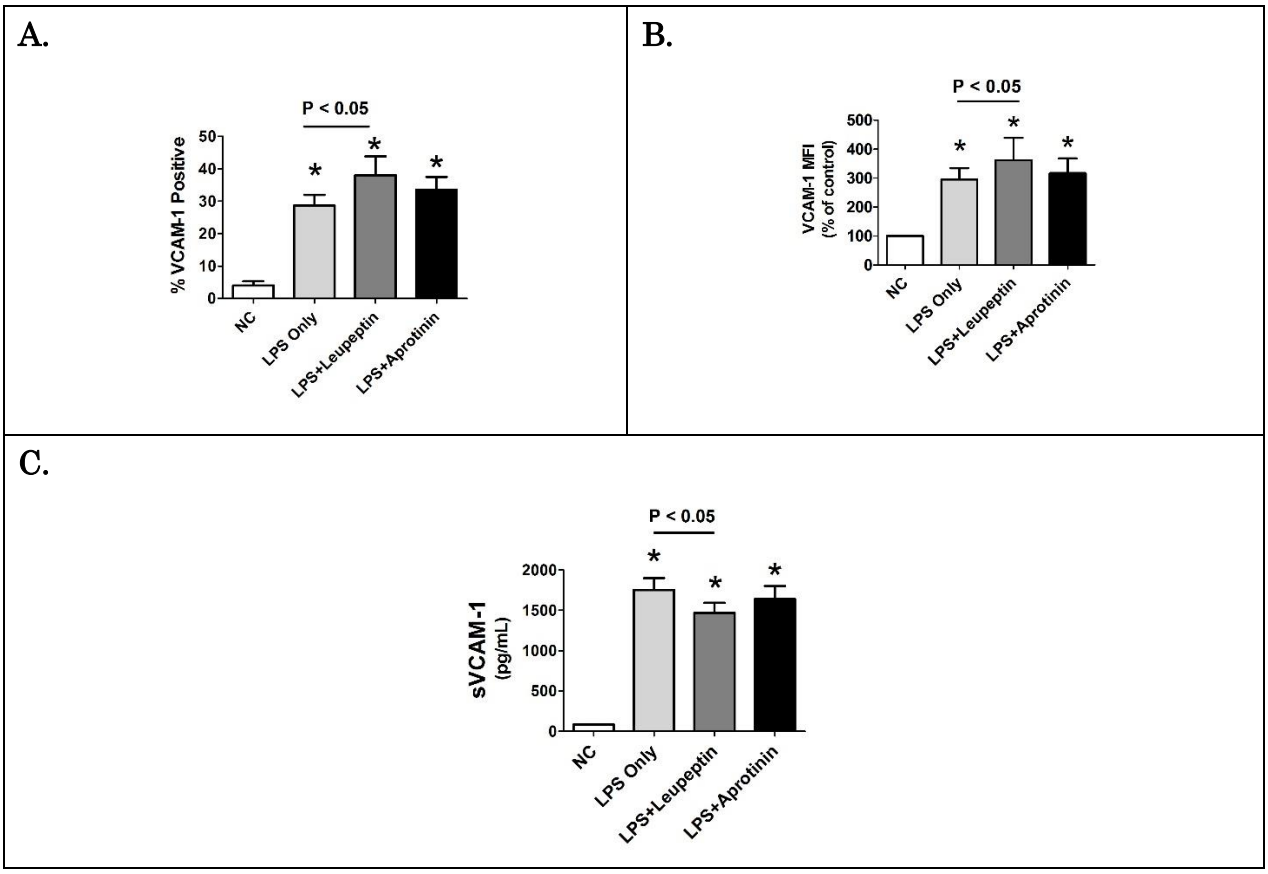

Figure 3: Serine protease inhibitors increase mVCAM-1 and decrease sVCAM-1 in HCEC cultures.

HCEC were incubated in medium with or without 500 ng/mL bacterial lipopolysaccharides (LPS) and with or without protease inhibitors for 18h. **(A)** % mVCAM-1 positive and **(B)** mVCAM-1 MFI (% of controls) were determined by flow cytometry. *, $P < 0.05$ vs negative controls (NC). **(C)** The amounts of sVCAM-1 in the cell culture medium were determined by ELISA and compared to negative controls. Amounts of sVCAMs are expressed in pg/mL and represent the mean ± SEM. *, $P < 0.05$ versus negative controls (NC). n = 4.

After confirming that a serine protease was a logical candidate for the mVCAM-1 processing protease, and that gene expression of several members of the kallikrein family was affected by CSE, we focused our experiments on the effects of kallikrein on VCAM-1 expression. One member of the Kallikrein family, Kallikrein-6 (KLK6), has been shown to cleave the E-cadherin ectodomain in epithelial cells [18] and the amyloid precursor protein (APP), a transmembrane protein glycoprotein [19]. Since KLK6 expression was inhibited by CSE (as shown by RNA array analyses, data not shown) and can cleave protein ectodomains, we narrowed our search for a putative sVCAM-1 protease to KLK6. Using ELISA measurements to determine changes in sVCAM-1 expression, we noted that adding exogenous KLK6 to HCEC significantly ($P < 0.05$) increased sVCAM-1, but not sICAM-1, expression in a dose-dependent manner (Fig 4).

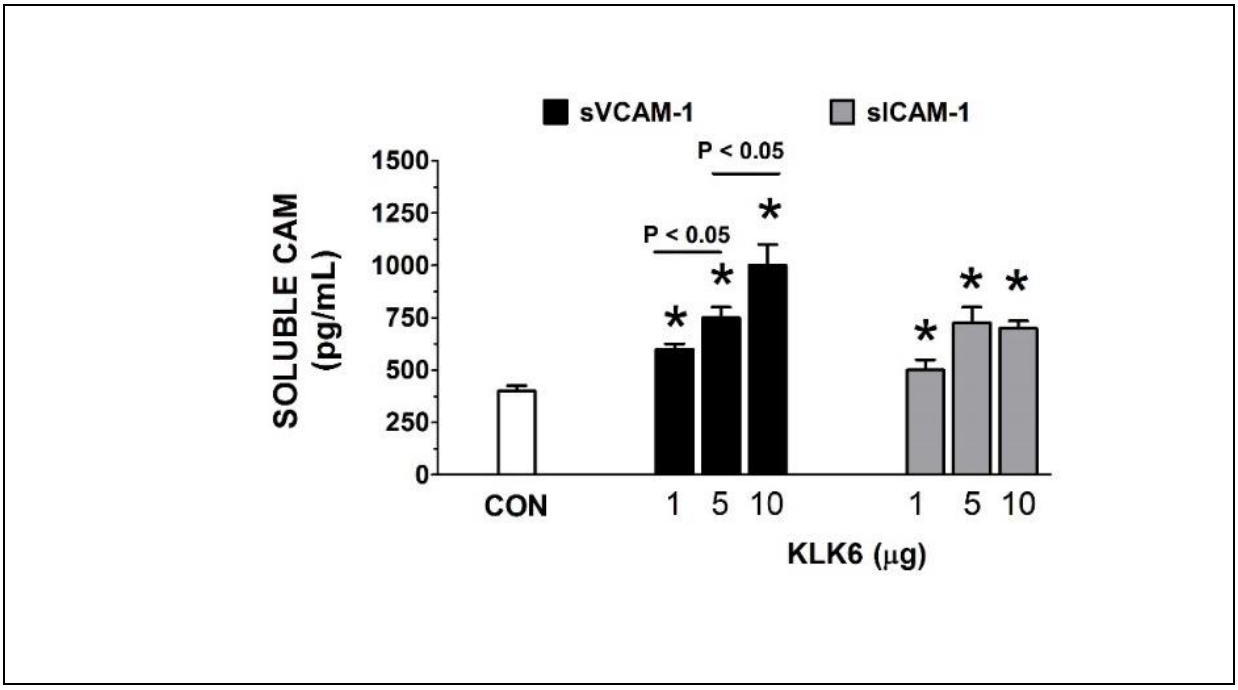

Figure 4: Kallikrein-6 (KLK6) increases the production of sVCAM-1, but not sICAM-1 in a dose-dependent manner. HCEC were incubated in medium with indicated concentrations of KLK6 for 24h. The amounts of sVCAM-1 and sICAM-1 in the cell culture medium were determined by ELISA and compared to negative controls. Amounts of soluble CAMs are expressed in pg/mL and represent the mean ± SEM. *, $P < 0.05$ versus negative controls (CON); n = 4

Since KLK6 is a serine protease, we hypothesized that aprotinin, a serine protease inhibitor known to inhibit Kallikrein activity, would inhibit KLK6 amplification of sVCAM-1 production. We performed dose-response studies on sVCAM-1 production in the absence and presence of the serine protease KLK6 and the serine protease inhibitor aprotinin (Fig 5). In the presence of aprotinin alone, sVCAM-1 significantly ($P < 0.05$) decreased in production compared to negative controls after a 24h incubation (Fig 5A). KLK6 increased sVCAM-1 production at 24 hours in a dose responsive manner (Fig 5B). However, in the presence of aprotinin (100 KIU), KLK6 stimulated production of sVCAM-1 was inhibited at all three doses of KLK6.

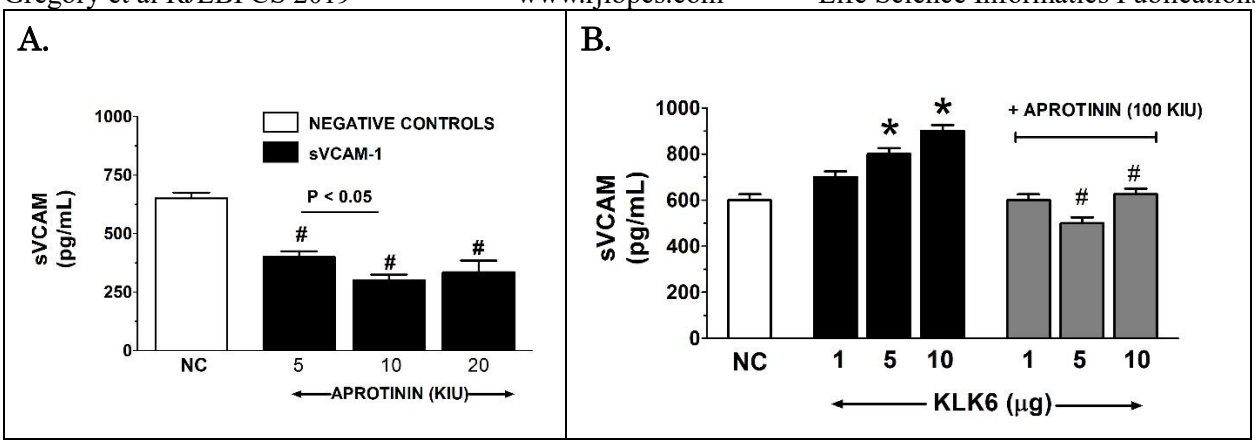

Figure 5: Aprotinin decreases the amounts of sVCAM-1 in the absence and presence of KLK6. **(A)** HCEC were incubated in medium for 24h with indicated concentrations of aprotinin in the absence of CSE. Soluble VCAM-1 was detected by ELISA. Data are expressed in pg/mL of sVCAM-1 and represent the mean ± SEM. #, $P < 0.05$ vs negative controls (NC). **(B)** HCEC were incubated in medium with indicated amounts of KLK6 and without or with aprotinin 100 Kallikrein Inhibitory Units (KIU). Data are expressed in pg/mL of soluble VCAM-1 and represent the mean ± SEM. *, $P < 0.05$ vs negative controls (NC). #, $P < 0.05$ vs positive control at same concentration of KLK6. n = 4.

We next measured changes in the amounts of sVCAM-1 expressed over time in the absence or presence of KLK6 and aprotinin (Fig 6). HCEC were incubated in medium without or with KLK6 (5 μg/mL) and aprotinin (10 KIU) for 0 to 48 hours. Supernatants were collected from cell cultures at designated times and sVCAM-1 and were determined by ELISA. Concentrations of sVCAM-1 from negative controls (NC) significantly ($P < 0.05$) increased in a time-dependent manner (Fig 6). Compared to negative controls, exposure to aprotinin alone resulted in a significant ($P < 0.05$) decrease in sVCAM-1 at 48 h. sVCAM-1 production significantly ($P < 0.05$) increased with time in the presence of KLK6. Interestingly, KLK6 plus aprotinin-treated HCEC exhibited a decrease in sVCAM-1 production at 6 hours as compared to KLK6 alone at 6 hours, but not at 24h and 48h, suggesting that aprotinin was no longer active at the later time points.

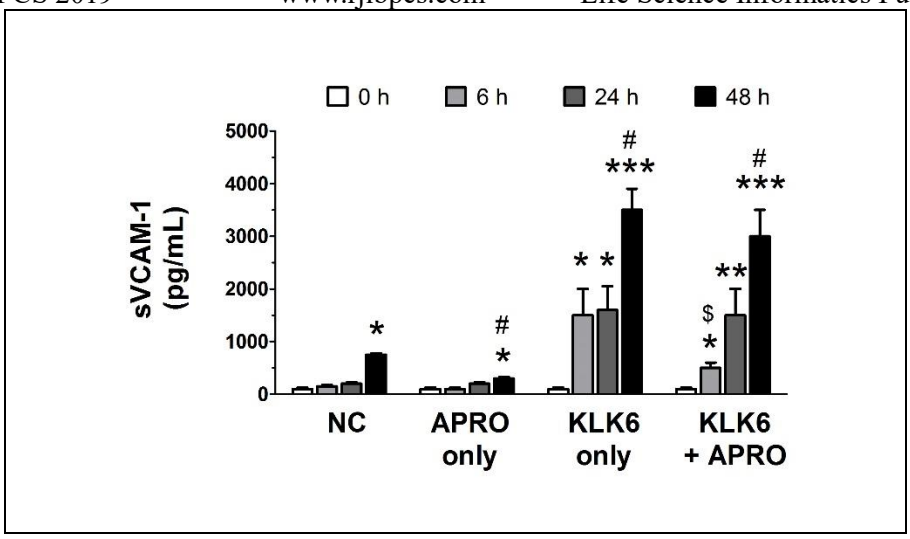

Figure 6: Aprotinin and Kallikrein-6 affect the production of sVCAM-1 over time

HCEC were incubated in medium for indicated times in the absence or presence of KLK6 (5 μg/mL) and aprotinin (100 KIU). sVCAM-1 production in HCEC were measured by ELISA at designated time points. sVCAM-1 concentrations are expressed as pg/mL and represent the mean ± SEM.

*, $P < 0.05$ versus zero minutes for each group. #, $P < 0.05$ versus negative controls at same time point. **, $P < 0.01$ versus negative controls. ***, $P < 0.005$ versus negative controls. $ < 0.05 vs KLK6 alone at same time point. $n = 4$.

DISCUSSION

The vasculature is a complex myriad of vessels that allows blood, nutrients and oxygen to flow from the heart to the organs, and the return of carbon dioxide, hormones, contaminants and waste products to the heart, lungs and kidneys. In order to accomplish this feat, the cardiovascular and pulmonary systems must be efficient and without compromise. Internal inflammatory agents and other deleterious effectors may diminish or interrupt these vital processes. External contaminants and pollutants are independent risk factors for cardiovascular disease (CVD). Cigarette smoke extracts permeate through the alveoli of the lungs into the blood and thereby contaminate the blood with harmful compounds. These contaminants can impinge upon the endothelium of the arteries and affect the overall physiology of the blood vessels and their constituents. While there are many vascular endothelial surface molecules, VCAM-1 and ICAM-1 play important roles in the physiology of the vasculature, especially in their interactions with serum monocytes. Monocytes that are transported by the blood in the normal (non-inflammatory) physiological state roll along the endothelium with minimal interference or adhesion to the endothelium. Increased expression of VCAM-1 and ICAM-1 is a response to pro-inflammatory mediators [20-22]. In the pathophysiological state (during inflammation), accumulation of mCAMs on the surface of vascular endothelial cells increases and in doing so increases the propensity of MO to bind to and transmigrate the endothelium, thereby upregulating the pathophysiology of atherosclerosis [12,23,24]. Thereafter, these MO transform into mature MΦ, setting up a perfect storm for

dysfunctional inflammation and atherosclerosis [25]. Of special interest, long-term smokers are subject to increased risk of atherosclerosis and CVD. VCAM-1 has been implicated as an important player in the development of plaque, and mVCAM-1 expression is increased in the presence of cigarette smoke extract [17]. Monocytes bind mVCAM-1 on the vascular endothelial surface, thus, if mVCAM-1 is increased in smokers, increased binding of monocytes to the vascular endothelium would explain the increased incidence of CVD. However, while cigarette smoke components increase mVCAM-1 expression, the appearance of atherosclerotic plaque often takes years to manifest, suggesting that our understanding of how the increased expression of surface VCAM-1 and monocyte interactions that lead to plaque formation is incomplete. We report here novel factors that regulate the amounts of the soluble form of VCAM-1 produced by human carotid endothelial cells (HCEC). Two endogenous *in vitro* and *in vivo* pro-inflammatory stimuli, bacterial lipopolysaccharide (LPS) and TNFα, increased production of both mVCAM-1 and sVCAM-1 (Fig 1). The mVCAM-1 induction has been observed previously [21], but the concomitant increase in sVCAM-1 levels has not been reported to our knowledge. sVCAM-1 is produced by proteolytic cleavage of mVCAM-1 on the cell surface. Therefore, the increase in sVCAM-1 due to enhanced mVCAM-1 expression was expected. We and others have hypothesized that sVCAM-1 may act as a "decoy" protein by binding to ligands (e.g. VLA-4) present on circulating monocytes, and thereby preventing monocytes from adhering to the vascular endothelium via mVCAM-1. In previous work, we observed that CSE increased mVCAM-1, but not mICAM-1, on the surface of HCEC. We expanded these experiments to evaluate CSE effects on sVCAM-1. Unexpectedly, while CSE increased mVCAM-1, sVCAM-1 levels decreased in both a dose- and time-responsive manner (Fig 2). Since cigarette smoking can increase the risk of plaque formation, in a mechanism likely due to increased mVCAM-1 expression and monocyte binding, these results suggested that reduction of sVCAM-1 may also participate in plaque formation. We postulate that cigarette smoke decreases sVCAM-1 levels and that reduced sVCAM-1 "decoy" proteins in the circulation allow increased monocyte binding to the vascular endothelium (with increased mVCAM-1), eventually resulting in plaque formation. Since sVCAM-1 is synthesized by proteolytic cleavage of mVCAM-1 at the endothelial cell surface, we further reasoned that CSE inhibited this enzymatic cleavage event, likely by reducing the gene expression of the VCAM-1 protease. Others have previously suggested putative VCAM-1 proteases, including Neutrophil Elastase (a serine protease) and ADAM17 or TIMP-3 (metalloproteases). However, these publications did not evaluate human vascular endothelial cells in their experiments [26]. Since HCEC incubated with TNFα or LPS produced increased amounts of sVCAM-1, this implied that vascular endothelial cells produce their own VCAM-1 processing protease. To determine the vascular endothelial cell VCAM-1 protease that was negatively regulated by CSE, we incubated HCEC with or without CSE and evaluated the expression of proteases using RNA microarray analyses. We observed that CSE influenced the

expression of several members of the kallikrein serine protease family. We performed confirmatory experiments using protease inhibitors in LPS-stimulated HCEC (Fig 3). We tested the effects of leupeptin (serine protease inhibitor), EDTA (metalloprotease inhibitor), Bestatin (aminopeptidase inhibitor), and aprotinin (a serine protease inhibitor known to inhibit kallikrein activity). Both leupeptin and aprotinin increased mVCAM-1 and decreased sVCAM-1, further suggesting that the endogenous VCAM-1 processing protease is a serine protease, possibly a member of the kallikrein family. We further evaluated the effects of one member of this family, Kallikrein-6 (KLK6), a representative kallikrein candidate for a VCAM-1 processing protease. KLK6 increased sVCAM-1, but not sICAM-1, production in HCEC cultures in a dose-dependent fashion (Fig 4). KLK6-dependent increases in sVCAM-1 were reduced in HCEC cultures upon addition of aprotinin (Fig 5B), a serine protease inhibitor known to inhibit kallikreins. Furthermore, the addition of aprotinin to unstimulated cultures suppressed sVCAM-1 production (Fig 5A). Finally, HCEC were incubated for various times in the absence or presence of KLK6 and/or aprotinin (Fig 6). In control cultures (no KLK6 or aprotinin), sVCAM-1 steadily accumulated over 48h. Addition of KLK6 significantly increased sVCAM-1 levels compared to negative control cultures, whereas aprotinin reduced these increases. In cultures containing KLK6 plus aprotinin, aprotinin ceased to inhibit KLK6-induced production of sVCAM-1 levels by 24h, possibly due to inactivation of aprotinin by endogenous aprotinin-specific proteases. The regulation of *in vivo* concentrations of mVCAM-1 and sVCAM-1 appears to be tightly controlled by a serine protease, most likely by a member of the kallikrein family. CSE disrupts the expression of this protease, allowing induction of surface mVCAM-1 with concurrent suppression of production of the "decoy" sVCAM-1. We hypothesized that increased incidence of CVD and plaque in smokers is due to the dysregulation of sVCAM-1 production, thereby allowing enhanced monocyte binding and transmigration across the vascular endothelial barrier. Further research is needed to identify the exact kallikrein protease(s) responsible for VCAM-1 processing and identification of the responsible protease may provide additional therapeutic targets for the prevention of atherosclerotic plaque production.

4. CONCLUSION

Lipopolysaccharide (LPS) and tumor necrosis factor-alpha (TNFα) are part of a plethora of serum-based substances that are involved with vascular inflammation. In this study, both LPS and TNFα increased the presence of endothelial cell membrane VCAM-1 (mVCAM-1) and ICAM-1 (mICAM-1) with preliminary, but short-lived concomitant increases in soluble VCAM-1 (sVCAM-1) and ICAM-1 (sICAM-1). Kallikrein-6 (KLK6) (an endothelial protease) increases sVCAM-1, but not sICAM-1, in a dose-dependent manner and is inhibited in the presence of the protease inhibitor aprotinin. The presence of cigarette smoke extract in serum decreases the concentration of soluble VCAM-1 (sVCAM-1), but not soluble ICAM-1 (sICAM-1). Serum monocytes (MO) role along vascular endothelial cells in healthy vessels, but bind to increased numbers of mVCAM-1 in the

beginning stages of inflammation. KLK6 decreases the likelihood of MO-endothelial cell binding by increasing serum sVCAM-1. However, in the state of inflammation, cigarette smoke extract increases mVCAM-1 with concomitant decreases in sVCAM-1 escalating the likelihood of MO binding to and transmigrating vascular endothelial cells, setting up a “perfect storm” of increased inflammatory cytokines and chemokines and the onset of atherosclerosis and vascular disorders.

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CONFLICT OF INTEREST

There are no financial interests or conflict of interests that exist in the science and production of this manuscript.

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