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# Carotid Endothelial Responses to Proinflammatory Mediators

Susana M. Tente

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By:

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Submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biology with a Neuroscience track from the Department of Biology of Seton Hall University

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**Abstract**

Chronic inflammatory conditions trigger vascular endothelial cell activation and the expression of endothelial proinflammatory molecules, thereby providing a possible immunomodulatory role for the endothelium. The human coronary artery endothelial cell (hCAEC) is the principal site of large blood vessel atherosclerosis, a chronic inflammatory condition that is associated with extensive morbidity and mortality. We examined hCAEC responses to the pro-inflammatory cytokines, tumor necrosis factor α (TNF-α) as well as the inhibitory molecule somatostatin (SRIF-14) and the stimulant methamphetamine (MA). We observed that a 24 hr TNF-α challenge (10 ng $ml^{-1}$) resulted in increased expression of the proinflammatory cytokine, interleukin 6 (IL-6), eight-fold, as determined by enzyme linked immunoabsorbent assay. To establish IL-6 production, hCAEC cells were stimulated with the pro-inflammatory mediators tumor necrosis factor α, (TNF-α; 10 ng $ml^{-1}$). Following stimulation, cell supernatants were collected and assayed for IL-6 by enzyme-linked immunosorbent assay (ELISA).

To establish protein expression, an immobilized array of cytokine and chemokine antibodies was implemented. We observed a TNF-α induced up-regulation of the cytokines including IL-6, IL-8 and IL-12. To determine if the inhibitory peptide SRIF-14 could modulate mediator expression, membrane array experiments were completed. hCAEC cells were treated with 100 nM SRIF-14 alone as well as with 100 nM SRIF-14 + TNF-α , 10 ng $ml^{-1}$ for a period of 24 hours. Additional membrane experiments were executed to investigate the protein expression following treatment with the stimulant MA. hCAEC cells were treated with 25 ng $ml^{-1}$ for a period of 24 hours.

Our results show that TNF-α can up regulate both hCAEC, as well as promoting the secretion of the cytokine IL-6. Treatments of hCAEC cells with SRIF-14 reveal the expression of key mediators IL-6, IL-17 and adhesion molecule VCAM-1. Stimulation of the endothelium with MA results in an expression of IL-8 as well as oncostatin M (OSM). These findings provide evidence that the carotid endothelial cell actively participates in the inflammation process and performs an immunomodulatory role in the cardiac vasculature.

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## Introduction

Inflammation is the process by which the body will react to any noxious stimuli that can threaten normal homeostasis. Chronic inflammation characteristically results in the disruption of normal processes including deterioration of tissues as seen in autoimmune disorders, neurodegenerative diseases and coronary artery disease (CAD) (Zweres, 2002). Atherosclerosis is a chronic inflammatory condition and degenerative process that ultimately affects the endothelial layer of the arterial vessel wall. In the normal vasculature, the endothelium will serve as the first barrier between the blood and surrounding tissues. Given the apparent consequences of heart disease, this investigation will focus on the immunomodulatory role of TNF-α, the indirect stimulatory properties of the addictive drug methamphetamine as well the inhibatory effects somatostatin may have on the heart.

Many well known risk factors including cigarette smoking, hypertension, infection, obesity, insulin resistance and diabetes can further instigate damage to the endothelium and consequently lead to the development of atherosclerosis. The progression of atherosclerosis will often result in the restriction of blood flow to the heart, thickening of the intima and the development of thrombosis (Ross, 1999). The pathological appearance of coronary artery disease and its clinical outcome are well known. Nonetheless, there remains much to be established with regard to the expression of key mediators and their ability to instigate the manifestation of the disease. Tumor necrosis factor alpha (TNF-α), a proinflammatory cytokine, has been shown to play an important role in immunity. There is strong evidence indicating an association between TNF-α and the acceleration of atherosclerosis. This potent cytokine is proven to initiate a signaling cascade that includes additional mediators which will also increases tissue damage during an inflammatory event. This study will focus on the simultaneous detection of any mediators expressed when the endothelium is in contact with TNF-α.

Current data also suggests that the naturally occurring inhibitor somatostatin may also have an effect on the heart (Clemens et al., 1999). Experiments to be discussed later, will also elaborate on the current research that suggests somatostatin's inhibatory role on the coronary vasculature and usefulness as a therapeutic approach to the disease.

Moreover, this investigation also seeks to better understand the effects of the highly addictive drug methamphetamine. Increasing literature suggests that humans who take the drug often suffer severe or fatal cardiac events. The compound methamphetamine is

currently a very popular misused drug. In vitro studies to be discussed later were designed to examine methamphetamine's role as a chemical mediator and any stimulatory effect it possess on the vasculature.

In order to fully understand the effects that chemical mediators have on the heart, it is necessary to also mention the essential role they have in immunity. In response to a given stimuli, cytokines are immunoregulatory proteins secreted by leukocytes, mast cells, macrophages, T helper ($T_H$) cells and other cell types (Goldsby *et al.,* 2000). Pro-inflammatory cytokines such as TNF-α and IL-1β have the ability to trigger and activate the coronary endothelium, leading to the communication between endothelial cells and circulating immune cells. More specifically, cytokines will act as messengers that bind to specific receptors on the membrane of target cells. TNF-α has two receptors TNF-R1 and TNF-R2, which are constitutively expressed in most tissues. The binding actions of the cytokine to the receptor can result in the initiation of a multitude of signaling cascades. The caveats to these actions are that the respective responses can destroy tissues as much as they have the ability for repair. In the case of the carotid vasculature, the cross talk between these cells will prompt endothelium to take on an active form marked by the expression of additional mediators including adhesion molecules, chemokines and cytokines (Zeuke et al., 2002). The role of these mediators is often to recruit and further perpetuate the immune response.

Chemokines are also considered to be potent mediators due to their chemoattractant effects at sites of tissue injury or infection. Chemokines will often be expressed as a response to the release of primary cytokines including IL-1β and TNF-α, respectively. As a superfamily of small polypeptides, chemokines can mediate the migration of leukocytes as well as growth and activation (Gertzen et al., 1999).

As Libby's studies have previously demonstrated, in atherosclerosis, the initiation of such entities can cause complex pathological changes in the middle and large arteries of the human body (2002). The enhanced production of cytokines and chemokines will lead to leukocyte adhesion and transmigration across the vascular endothelium. The disruption of the functional integrity of the tissues by the leukocyte extravasation will initiate the formation of atherosclerotic lesions on the surface of the endothelial cells. These lesions will eventually become lipid-laden plaques which are typically composed of inflammatory cells including macrophages and T-lymphocytes (Von Der Thusen et al., 2003). The accumulation

of the cellular debris will ultimately cause a swelling of the arterial wall. The ability for the intimal lesions to form and lead to plaque formation and destabilization can be potentially lethal. Thus, both chemical and cellular constitituents of the atheromatous plaques are thought to not only have a defining role in the proliferation of inflammation, but also development and stability of the lesion itself (Behrendt, 2002). As Wang et al., has previously documented that both interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) contribute to atherogenesis and the development of plaque formation (1996). Wang's studies further illustrate the importance of chemokines MCP-1 and IL-8 and their function in inflammation. Both molecules can induce the chemoattraction of monocytes and the activation T-cells as well as adhesion molecules. In addition to causing a local immune response, the recruitment of macrophages and T-cells will promote the induction of additional cytokines that will ultimately result in the expression of the enzymatic peptide metalloproteinase (MMP). MMP is capable of advancing the activation of adhesion molecules including VCAM-1 and other bioactive proteins that can lead to plaque rupture (Gertzen et al., 1999).

The widespread effects of proinflammatory molecules have also been reported in both human atherosclerotic lesions and in the ischemic rat heart (Parissis et al., 2002). Reports indicate that there is a substantial increase of chemokine levels found in the plasma of individuals with ischemic heart disease (Filippitos and Kardaras, 2002). More specifically, the chemoattractant protein, granulocyte colony macrophage stimulating factor (GM-CSF) is a classic example of a chemical mediator that regulates monocyte migration and macrophage differentiation (Filippitos and Kardaras, 2002). Additionally, the role of chemokines and their effects on the endothelium are also supported by experiments performed by Gu et al., using gene-knockout mice (1998). Studies designate that the ablation of the gene encoding MCP-1 in apoE$^{-/-}$ mice (low density lipoprotein receptor-deficient mice) resulted in a reduction of the size of atherosclerotic lesions and a significantly lower and delayed mortality (Gu et al., 1998) and (Lucas and Greeves, 2001). MCP-1 is an important immunoregulator in that can modulate the immune response by respectively attenuating Th-1 or Th-2 pathway differentiation. When the chemokine is not present it typically favors the anti-inflammatory Th-2 immune response, which produces cytokines IL-4, IL-5 and IL-13. Conversely, Th1-type cytokines tend to produce the proinflammatory responses responsible for killing intracellular parasites and for perpetuating autoimmune responses.

The ability for the endothelium to become activated by TNF-α or other pro-inflammatory mediators including IL-6 and IL-1β at sites of tissue injury can facilitate the destruction of tissues and further augment the inflammatory response. As demonstrated in experiments detailed in this study, TNF-α can cause the specific expression of potent cytokines, including the marker IL-6, for which there is increasing evidence that IL-6 is specific marker of inflammatory diseases.

TNF-α has long been established as a potent inflammatory cytokine associated with several diseases including Rheumatoid arthritis and Crohn's disease. TNF induced endothelial cell activation has also been reported to promote vascular leakiness and the structural reorganization of the endothelium (Von Der Thusen et al., 2003). The induction of TNF-α has the ability to initiate a cascade of events that will enhance the effects of chronic inflammation.

Elevated plasma levels of inflammatory cytokines including IL-6 have been previously detected in patients with stable or unstable angina and myocardial infarction (Zeuke *et al.*, 2002). Together IL-6, IL-1β and TNF-α cytokines are all proven to exert negative effects in isolated cells of the heart (Zeuke *et al.*, 2002). More specifically, the multipotential cytokine TNF-α, is also known to induce the production of adhesion molecules ICAM-1 and ELAM-1 as well as cytokines and chemokines including IL-1, IL-6, IL-8 and GM-CSF.

Investigations in this study were intended to further understand the modulatory actions of TNF and its ability to ameliorate multiple factors that contribute to inflammatory diseases. Additionally, experiments were designed to investigate the simultaneous expression of inflammatory mediators by stimulating human coronary artery endothelial cell (hCAEC) monolayers with the proinflammatory compound TNF-α.

Increasing evidence supports that somatostatin may also have an important inhibitory role on the heart. SRIF (somatotropin release inhibitory factor) is a naturally occurring neuropeptide primarily produced in tissues of the hypothalamus, brain, pancreas and gut. Somatostatin is composed of peptides that exist in two forms SRIF-14 and SRIF-28 and contain 14 and 28 amino acids, The peptide is also best known for its distinct inhibitory properties on endocrine and exocrine secretion specifically, the inhibition of insulin and growth hormones (Weckbecker et al., 2003). There currently exists a limited amount of information regarding the modulatory role of somatostain on the endothelium. Additionally,

there exist conflicting reports relating SRIFs inhibitory role in the expression of proinflammatory mediators IL-1β and TNF-α and data found in rodent models versus human studies (Weckbecker et al., 2003).

Membrane array experiments in this study were designed to offer a unique profile of the effects SRIF on hCAEC. Should proinflammatory compounds have the ability to instigate the inflammatory response, experiments using SRIF were performed to confirm whether the inhibitor could attenuate inflammatory expression. SRIF based therapies may be useful for inflammatory conditions including the reduction of joint inflammation in patients with rheumatoid arthritis as well as other destructive conditions such as psoriasis, inflammatory bowel disease and Grave's disease.

Previous research also demonstrates that there may be a correlation between methamphetamine (MA) use and deficient cardiovascular function. This suggests a potential cardiotoxic effect of MA (Varner et al., 2002). Methamphetamine is a synthetic derivative of amphetamine, which even in small doses can increase wakefulness, physical activity and increase appetite (Karch, 1996). Methamphetamine is an addictive stimulant that has been a popular drug of abuse for many years in the United States, a majority being due to its easy accessibility. The drug can exist in a powdered form, therefore, it can be ingested, snorted or injected (Varner et al., 2003). Chronic users develop a tolerance of the drug and addicts will likely take higher doses in order to intensify its effects. There is increasing clinical evidence to suggest that frequent use of methamphetamine results in other medical complications and an increasing number of cardiac related deaths (Varner et al., 2003) Additionally, the effects of repeated exposure to the drug can further instigate a number of medical complications including arrhythmias, intracranial bleeding, and congestive heart failure (Treweek et al., 2007). Previous research of methamphetamine has focused on the drugs neuropharmacological actions. There remains a paucity of research with regard to the effects methamphetamine may have on other parts of the body, specifically the coronary endothelium. Since methamphetamine has stimulatory properties, is a highly addictive, and is often misused worldwide, it is advantageous to investigate whether a correlation exists between methamphetamine abuse and coronary artery disease or other cardiovascular maladies. Experiments were thereafter performed to better elucidate the mechanisms of actions and affects of pro-inflammatory mediators as well as to what affects the indirect agonist methamphetamine, may have on the expression of mediators on carotid endothelium.

**Methods:**

*Tissue Culture:*

Adult human coronary artery endothelial cells (hCAEC) (figure 1.) and all hCAEC tissue culture media were obtained from Clonetics, BioWhittaker (Walkersville, MD). The cells were grown in T-75 culture flasks containing Endothelial Cell Basal Medium (EBM) supplemented with 5% fetal calf serum, bovine brain extract (12 $\mu g\ ml^{-1}$), recombinant human epidermal growth factor (10ng $ml^{-1}$), gentamicin (50 $\mu g\ ml^{-1}$), and hydrocortisone (1 $\mu g\ ml^{-1}$) in a humidified atmosphere in 5% $CO_2$ at 37° C. The hCAEC were passaged when the monolayers reached 80% confluence. Cell monolayers were then rinsed twice with 5ml Hepes buffered saline solution (HBSS), and detached using 5ml of 0.05% w/v trypsin in 0.53 EDTA for 3-5 minutes. The cells were neutralized with the addition of 5ml trypsin neutralizing solution. All sterile cell culture materials including plastic dishes and pipettes were obtained from Corning (Corning, NY).

**Figure 1. :** Human Coronary Artery Endothelial Cells in culture, Courtesy of Cell Applications, Inc. (San Diego, California)

*Analyses of IL-6 expression via Human Enzyme-Linked Immunosorbent Assay:*

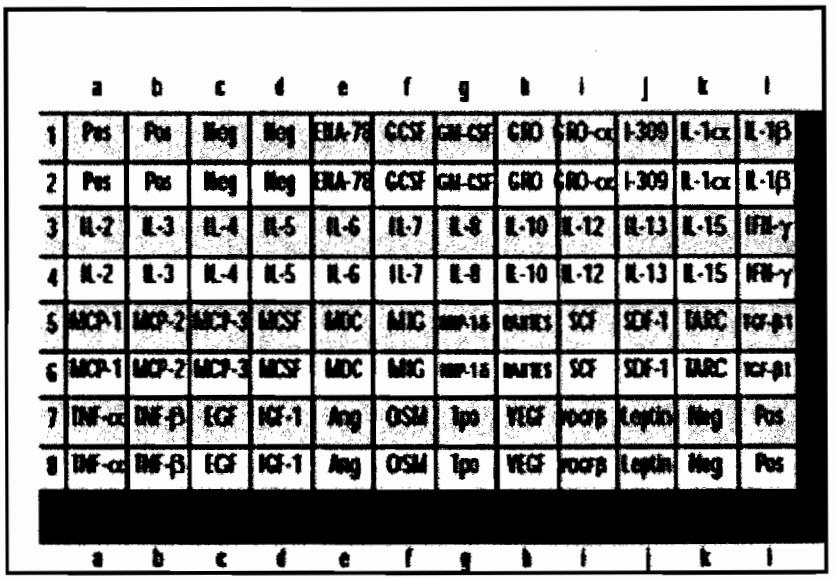
Antibodies to IL-6 were quantitatively determined with a sandwich ELISA kit obtained from eBioscience (San Diego, CA). For experiments, approximately 2 x $10^4$ hCAEC were plated into 2 24-well cultures dishes the presence or absence of 10 ng $ml^{-1}$ TNF-α. purchased from Upstate (Lake Placid, NY). The cells were incubated at 37°C for a period of 24 or 48 hours. Following incubation, cell supernatants were collected and stored at -80°C until use.

For the completion of the ELISA experimental procedure, all methods and reagent were used as directed in the kit provided by eBioscience. Nunc Maxisorb 96 well plates were coated with 100 µl/well of capture antibody in Coating Buffer. Each plate was then sealed and allowed to incubate overnight at 4 C °. Following the overnight incubation, the capture antibody (coating buffer) was aspirated from the wells and washed 3 times with >300 µl/well wash buffer. Each well was blocked with 200 µl/well of a 1X assay diluent and incubated at room temperature for 1 hour, followed by a repeat wash. The amount of 100 µl/well of standard was added to the appropriate wells and 2-fold serial dilutions of the top standards were used to create a standard curve. An experimental concentration of 100µl/well of the hCAEC supernatants were added to the appropriate wells and the plates were sealed. Following 2 hour incubation at room temperature, the samples were aspirated and the plates were washed with wash buffer for a total of 5 washes. A concentration of 100 µl/well of detection antibody diluted in 1X assay diluent was then added and the plates were again sealed and incubated at room temperature for 1 hour. The detection antibody was aspirated and plates were once again washed for a total of 5 washes. Next, 100 µl/well of Avidin-HRP diluted in 1X assay diluent were added to the plates and allowed to incubate at room temperature for 30 minutes, followed again by a wash step as described previously. A concentration of 100 µl/well of substrate solution were added and plates were again incubated at room temperature for 15 minutes and shortly after 50 µl/well of stop solution was added to each well. Plates were read at a wavelength 450 nm and data was analyzed.

*Determination of cytokine expression using TranSignal RayBio Human Cytokine Antibody Arrays:*

In order to determine cytokine expression, hCAEC monolayers were grown in a T-75 culture flask to 75% confluence. For experiments using TNF-α, cells were incubated in the presence or absence of 10 ng ml $^{-1}$ TNF-α for a period of 24 hours in a 37°C humidified incubator at 5% $CO_2$ : 95% air (Table 1.). For experiments using MA, hCAEC were incubated in the presence or absence of 25 ng ml $^{-1}$ MA for a period of 24 hours in a 37°C humidified incubator at 5% $CO_2$ : 95% air (Table 1.). For experiments using somatotropin release inhibitory factor (SRIF-14), cells were incubated in the presence or absence of 100 nM for a period of 24 hours in a 37°C humidified incubator at 5% $CO_2$ : 95% air (Table 2.). Following incubation, array cell supernatants were collected and samples were incubated to permit cytokine binding. Cytokine proteins present in the samples would bind to the anti-cytokine antibody present on the membrane array and create a "sandwich" around the bound cytokines. Captured cytokines were detected with a mixture of biotin-labeled anti-cytokine labeled antibodies.

Detection was completed by the use of HRP-based chemiluminescence (Redwood City, CA). Data was visualized and analyzed using Alpha Imager TM System (San Leandro, CA). The relative expression levels of cytokines were determined by comparing signal intensities between control and treated membranes. The HRP-conjugated antibody present on every membrane serves as a positive control and was used to identify the membrane orientation and to compare relative expression between different membranes. Endothelial Cell Basal Medium (EBM) supplemented with 5% fetal calf serum, bovine brain extract (12 μg ml$^{-1}$ ), recombinant human epidermal growth factor (10ng ml$^{-1}$ ), gentamicin (50 μg ml$^{-1}$ ), and hydrocortisone (1 μg ml$^{-1}$) served as the negative control on each membrane. For best possible results, several different exposures of x-ray film were taken and then compared to each respective schematic diagram of the TranSignal Raybio Human Cytokine Array provided by Panomics (Redwood City, CA).



| | a | b | c | d | e | f | g | h | i | j | k | l |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Pos | Pos | Neg | Neg | ENA-78 | GCSF | GM-CSF | GRO | GRO-α | I-309 | IL-1α | IL-1β |
| 2 | Pos | Pos | Neg | Neg | ENA-78 | GCSF | GM-CSF | GRO | GRO-α | I-309 | IL-1α | IL-1β |
| 3 | IL-2 | IL-3 | IL-4 | IL-5 | IL-6 | IL-7 | IL-8 | IL-10 | IL-12 | IL-13 | IL-15 | IFN-γ |
| 4 | IL-2 | IL-3 | IL-4 | IL-5 | IL-6 | IL-7 | IL-8 | IL-10 | IL-12 | IL-13 | IL-15 | IFN-γ |
| 5 | MCP-1 | MCP-2 | MCP-3 | MCSF | MDC | MIG | [illegible] | RANTES | SCF | SDF-1 | TARC | TGF-β1 |
| 6 | MCP-1 | MCP-2 | MCP-3 | MCSF | MDC | MIG | [illegible] | RANTES | SCF | SDF-1 | TARC | TGF-β1 |
| 7 | TNF-α | TNF-β | EGF | HGF-1 | Ang | OSM | Tpo | VEGF | uPAR | Leptin | Neg | Pos |
| 8 | TNF-α | TNF-β | EGF | HGF-1 | Ang | OSM | Tpo | VEGF | uPAR | Leptin | Neg | Pos |

**Table 1.** TranSignal RayBio Human Cytokine Array 3 (MA6020)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Apo1/Fas | | Leptin | | Rantes | | ICAM-1 | | IL-2 | | IL-7 | | pos | |
| B | CTLA | | MIP1α | | TGFβ | | VCAM-1 | | IL-3 | | IL-8 | | pos | |
| C | Eotaxin | | MIP1β | | IFNγ | | VEGF | | IL-4 | | IL-10 | | neg | |
| D | GM-CSF | | MIP4 | | TNFα | | IL-1α | | IL-5 | | IL-12(p40) | | neg | |
| E | EGF | | MIP-5 | | TNFRI | | IL-1β | | IL-6 | | IL-15 | | pos | |
| F | IP-10 | | MMP3 | | TNFRII | | IL-1Rα | | IL-6R | | IL-17 | | pos | |

**Table 2.** TranSignal RayBio Human Cytokine Array 3.0 (MA6150 & MA6160)

*Source of Methamphetamine:*

Methamphetamine (figure 2.) used in these studies, was kindly supplied by Dr. Sulie Chang, Associate Professor of Biology at Seton Hall University, South Orange, NJ.

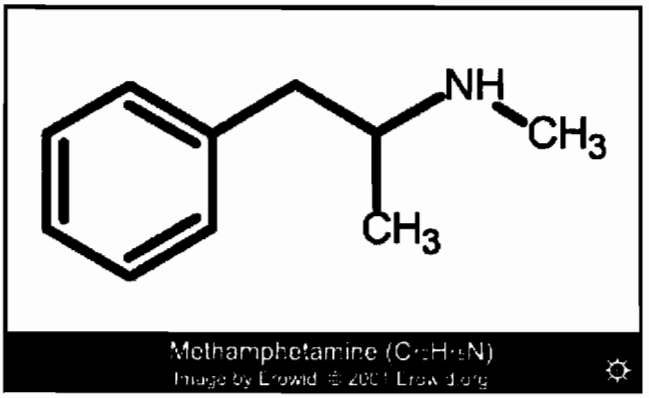



**Figure 2.** Structure of methamphetamine.

**Results:**

*Interleukin-6 as an inflammatory marker:*

Interleukin-6 is a well established multifunctional cytokine produced by several cell types including the endothelium, smooth muscle, macrophages and lymphocytes (Saadeddin et al., 2003). Interleukin-6 is known to regulate both humoral and cellular responses as well as play a pivotal role in inflammation. IL-6 has the ability to stimulate the expression of tissue factor and low density lipoprotein receptors in macrophages, as well as the aggregation of platelets and adhesion molecules (Ikeda, 2003). Most notably, IL-6 has been suggested to regulate the expression of other cytokines in endothelial cells, including both IL-1β and TNF-α which are known to enhance inflammation (Von Der Thusen et al., 2003).

Therefore, in order to confirm whether a well known potent mediator could promote hCAEC expression of a pro-inflammatory molecule, we chose to stimulate hCAEC cells using 10 ng $ml^{-1}$ TNF-α. and assay the results using a Human IL-6 ELISA kit.

The data summarized in figure 3., reveal that TNF-α stimulated endothelial cells up-regulate IL-6 expression. Although TNF- α stimulated cells were shown to only produce a 2 pg $ml^{-1}$ expression level of IL-6, there was however, a significant difference in comparison to control levels thus, exemplifying the pro-inflammatory effects of TNF- α and its correlation to IL-6.

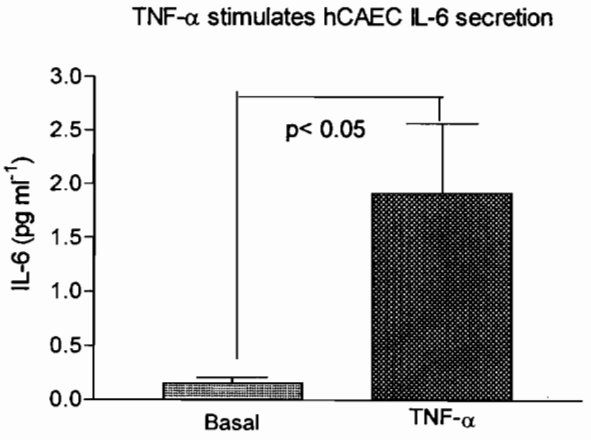



**Figure 3.** Tumor Necrosis Factor-α Induced Expression of Interleukin-6

*Identification of cytokines released from TNF-α treated hCAEC cultures:*

To adequately profile cytokines expressed by human coronary artery endothelial cells, a high-throughput protein based array system was implemented. Human coronary artery endothelial cells were incubated in the presence or absence of 10 ng $ml^{-1}$ TNF-α in T-75 tissue culture flasks, for a period of 24 hours. To determine the relative quantities of cytokine present, signal intensity values were obtained by measuring the pixel density values of each membrane and analyzed and compared using NIH Scion Image. Two different versions of the cytokine membranes were utilized to reflect a greater number of cytokines to be profiled, although there are no variances in sensitivity between each individual version of the membrane. According to the data obtained in figure 4, the TNF- α treated membrane in comparison to the control membrane demonstrates the expression of multiple cytokines identified in figure 5. Most notably, the treated membrane exhibits a distinct cytokine milieu composed of both pro-inflammatory cytokine IL-6 as well as the expression of chemoattractant proteins IL-8, granulocyte colony macrophage stimulating factor (GM-CSF) and monocyte chemoattractant protein (MCP-1).

Chemoattractant cytokines such as IL-8 and GM-CSF are especially important due to their ability to induce chemoattraction of monocytes and activated T-cells (Saadeddin *et al*,. 2002). More specifically, IL-8 is a well established pro-athrogenic factor and also presumed to accelerate athrogenesis by increasing the endothelial adhesiveness for monocytes (Von Der Thusen et al,. 2003). Gerstzen et al, 1999 demonstrates that monocytes contribute to the development of atherosclerotic lesions in mouse models. More interestingly, Gerstzen reveals that mice lacking receptors for these chemokines are less susceptible to atherosclerosis and have fewer monocytes in vascular lesions.Thus, reaffirming the important function of chemoattractants and their actions on the endothelium.

The marked expression of IL-6 directly correlates with previous studies suggesting the multipotential cytokines pro-athrogenic effects. IL-6 is produced by monocytes and macrophages, which are constituents of the atherosclerotic plaque. This particular cytokine also has the abilty to lead to the up-regulation of E-selectin, ICAM-1 and VCAM-1 which increases endothelial cell adhesion. IL-6 can also stimulate the release of inflammatory mediators MCP-1, IL-8 and IL-6 itself (Romano et al., 1997). Therefore, the expression of these markers on the array membrane further suggests the endothelium's ability to become activated and initiate an inflammatory response as well as to lend further support to our

previous ELISA experiments concerning the TNF-α stimulated endothelial cell expression of IL-6. The data shown in figure 6, represents cytokines profiled using the TranSignal Cytokine Antibody array version 3.0 (Table 2.). In comparison to the control membrane, cells treated with TNF-α reveal the expression of multiple cytokines as summarized in figure 7. Most notably, signal densities quantified by desitometry demonstrate significant increases in both IL-6 and IL-8 in comparison to the untreated control membrane as seen in figure 7. With consideration that an enzymatic biotin-streptavidin interaction is used to visualize the bound cytokine, any increase in positive controls from the control membrane to the treated membranes, may be reflective of the high degree of sensitivity of the membrane due to the linkage between the capture antibody, target protein, and detection antibody. Other relevant cytokines expressed in the TNF-α treated membrane include the expression of the proinflammatory cytokine IL-17 and IL-12. Emerging evidence continues to confirm that the production of IL-17 by activated T cells, and the widespread expression of the IL-17 receptor make this interleukin family a strong pro-atherogenic candidate (Von Der Thusen et al., 2003). IL-12 is a heterodimeric interleukin which consists of two components, of which the p40 subunit has been shown to be the major inducible chain (Uyemura et al., 1997). IL-12 is also T cell cytokine which is naturally produced by macrophages and B lymphocytes. Studies using carotid artery specimens obtained from human patients during endarterectomy reveal that IL-12 p40 mRNA and IL-12 p70 protein are present in atherosclerotic plaques (Uyemura et al., 1997). This evidence suggests that IL-12 more than likley has a significant role in chronic inflammatory conditions, including atherosclerosis. Interestingly, there was also an expression of MMP3. MMP3 is a variant in the promoter of stromelysin-1, which belongs to the family of matrix metalloproteinases involved in connective tissue remodeling. This variant has been associated with thickening of the carotid artery and progression coronary artery disease (Rudeck et al., 2002).

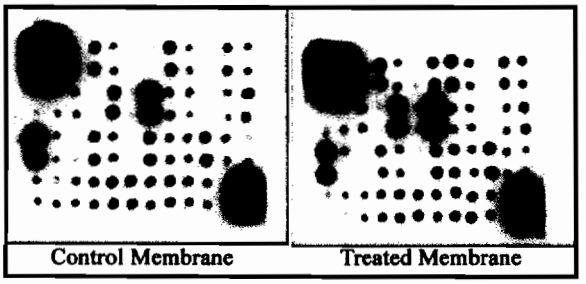



**Figure 4.** TNF-α induced cytokine expression of human coronary artery endothelial cells.

A

TranSignal RayBio Human Cytokine Array 3

| Untreated Control Membrane | | TNF-α Treated Membrane | |
|---|---|---|---|
| *Cytokines Expressed* | *Mean Pixel Density* | *Cytokines Expressed* | *Mean Pixel Density* |
| Positive Control | 130.72 | Positive Control | 64.55 |
| Negative Control | 10.14 | Negative Control | 48.23 |
| MCP-1 | 84.07 | MCP-1 | 87.19 |
| IL-8 | 82.49 | IL-6 | 89.07 |
| | | GM-CSF | 49.7 |
| | | IL-8 | 94.15 |
| | | MIP-1 | 48.83 |
| | | GRO | 56.38 |
| | | SDF-1 | 50.21 |

B

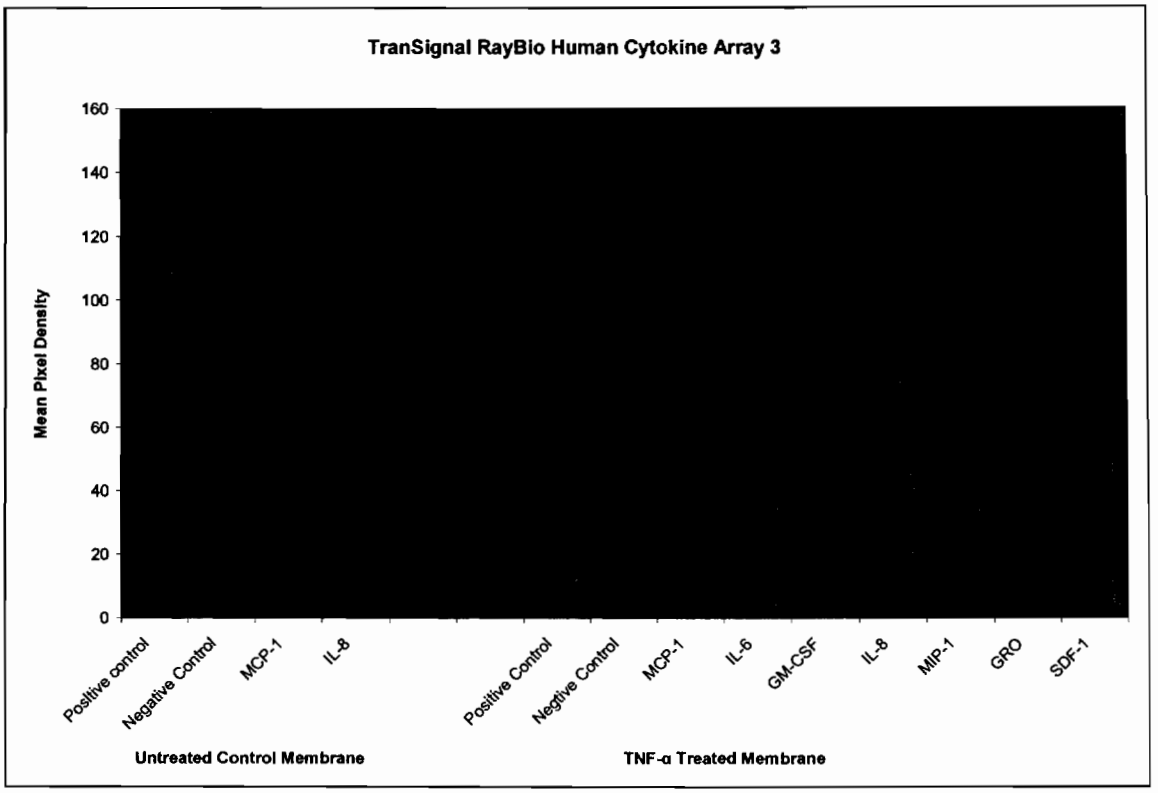



**Figure 5.** (A and B) Quantification by Densitometry of TNF-α induced cytokine expression of hCAEC.

*Cytokine profile of SRIF-14 treated hCAEC*

Results indicate that immunomodulatory properties of somatostatin prove to be advantageous in the development of new therapeutic approaches of drugs for the use in inflammatory diseases. Chowers et al., (2000) investigations are an additional indication that the effects of somatostatin regarding the modulation of inflammatory and anti-inflammatory cytokines secreted from intestinal epithelial cells have shown that somatostatin can inhibit TNF-alpha-induced secretion of IL-8 and IL-1β. The specific role somatostain may have on the carotid endothelium including the progression of atherosclerosis remains unclear. In order, to gain a better perspective on the effects of somatostatin, we chose to profile cytokine expression by treating hCAEC in the presence of 100 nM SRIF-14 alone and hCAEC monolayers in the presence of 100 nM SRIF-14 + 10 ng ml $^{-1}$ TNF-α for a period of 24 hours (figure 6.). A denstiometric analysis of 100 nM SRIF-14 treated cells indicates that there was once again an increased expression of several proinflammatory cytokines including IL-6 and IL-17 as well as the chemokine IL-8 and adhesions molecule VCAM-1 in comparison to the control membrane (figure 7.) Cells treated with both 100 nM SRIF-14 + 10 ng ml $^{-1}$ TNF-α also produced a considerable number of cytokines involved in inflammatory conditions (figure 7).

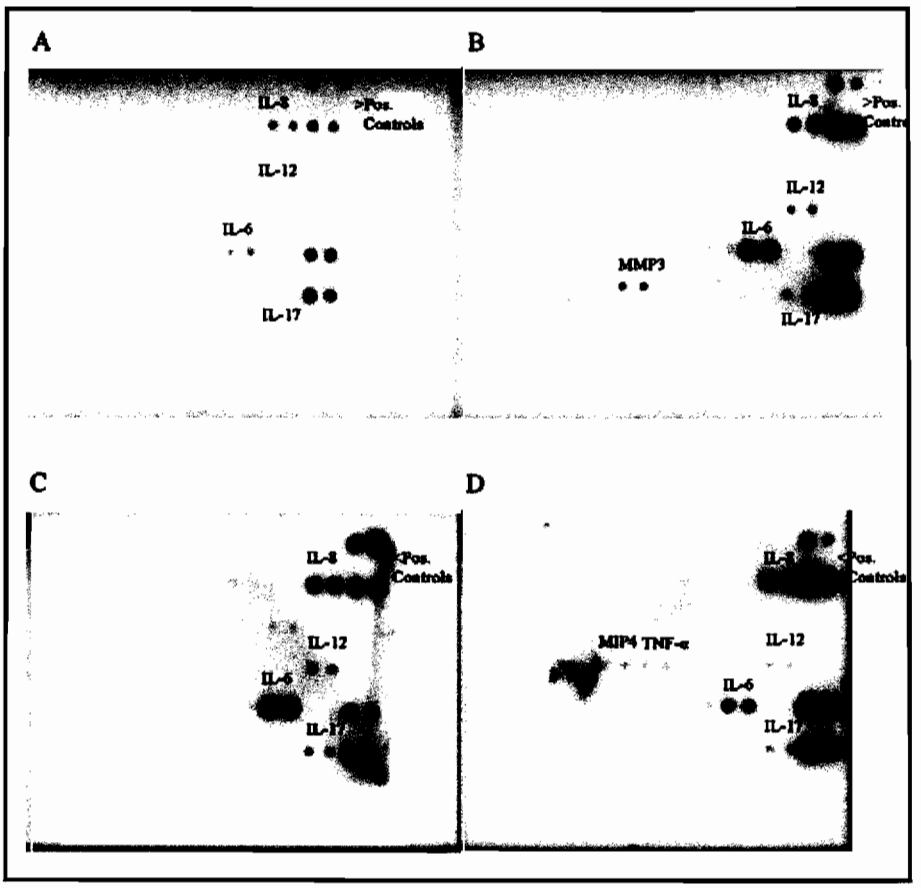


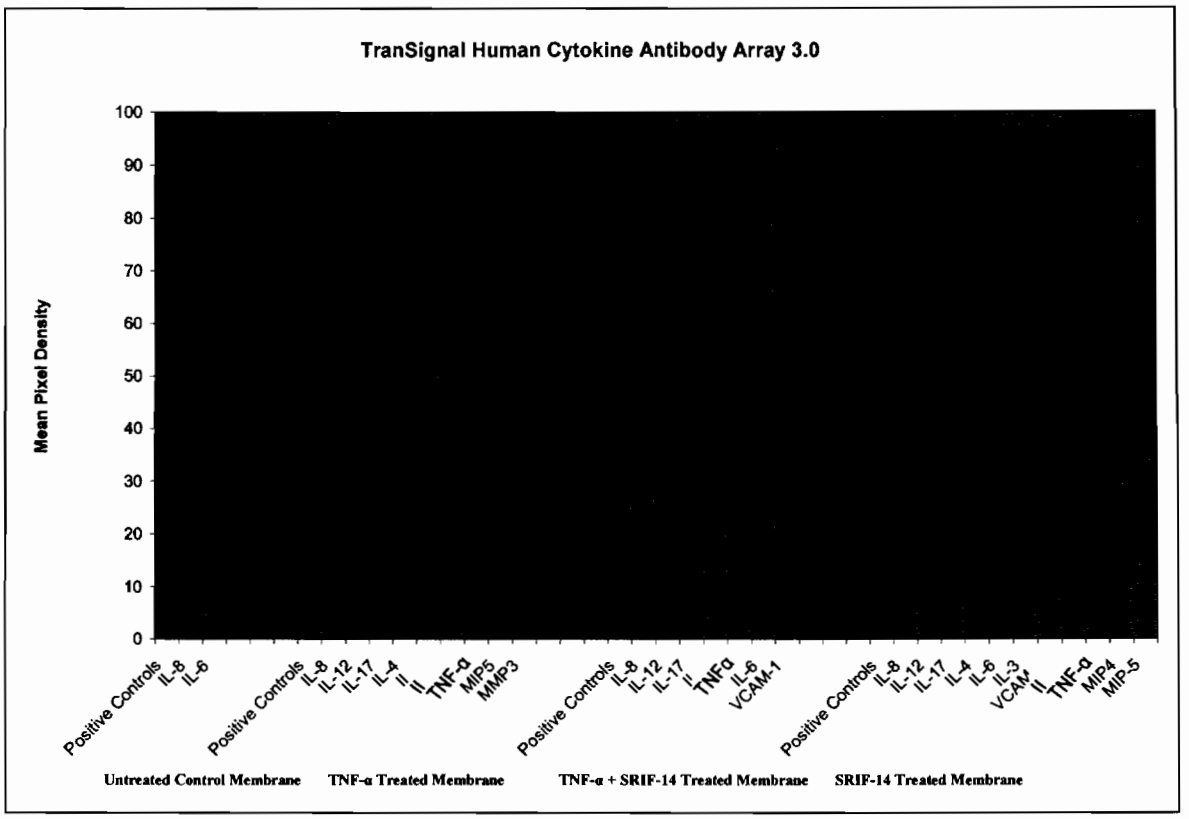

**Figure 6.** *TransSignal Human Cytokine Antibody Array 3.0. (MA6150 & MA6160)* Induced cytokine expression of human coronary artery endothelial cells. (A) Control membrane, hCAEC incubated in untreated EBM. (B) TNF-α treated cells incubated in the presence of 10 ng ml $^{-1}$ TNF-α. (C) hCAEC incubated in the presence of 100 nM SRIF-14. + 10 ng ml $^{-1}$ TNF-α . (D) hCAEC incubated in the presence of 100 nM SRIF-14. All membranes were treated simultaneously for a period of 24 hours.

**Assessment of TNF-α treated Human Coronary Artery Endothelial Cells**

| | Primary Sources | Endothelial Cells | | Monocytes/Macrophages | |
|---|---|---|---|---|---|
| | **Primary Sources, Target and Effects** | | | | |
| | Effects/Expression | **Increase** | **Decrease** | **Increase** | **Decrease** |
| **IL-1α**<br>**IL-1β** | Endothelial Cells, MΦ, SMC dendritic cells, B cell | IL-1, IL-6, MCP-1, M-CSF, ICAM-1, VCAM-1, E-selctin, apoptosis | proliferation | IL-6<br>PDGF-AA, superoxide | apolipoprotien E |
| **IL-4** | Th2 Cells, endothelial cells | P-selectin | | | macrophages |
| **IL-6** | Th2 Cells, Endothelial Cells MΦ, SMC, Monocytes | IL-6, migration, ICAM-1, VCAM-1, MCP-1, IL-8, E-selectin | HGF proliferation | MCP-1 | |
| **GM-CSF** | Hematopoietic cells | | | Monocytes/macrophages | |
| **IL-12** | Endothelial cells, MΦ | VCAM-1 | | | |
| **IL-17** | T Cells | IL-2, MCP-1 | | Macrophages | |
| ***Chemokines*** | | | | | |
| **IL-8** | Endothelial Cells, MΦ, SMC | Monocyte/macrophage Adhesion | | | TIMP-1 |
| **MCP-1** | Endothelial Cells, MΦ, SMC Leukocytes, fibroblasts | | IL-8 | ROS, monocytes | |
| **SDF-1** | Stromal cells, Platelets | Aggregation | | monocytes | |
| **GRO, GROα** | Leukocytes, monocyte arrest | Adhesion | | | |
| **VEG-F** | Endothelial Cells, MΦ, SMC | migration, proliferation | | monocytes | |

**Table 3.** Assessment of cytokine expression as a result of TNF-α stimulation.

**A**




**B**

**TranSignal RayBio Human Cytokine Array 3.0**

| Untreated Control Membrane | | TNF-α Treated Membrane | | TNF-α + SRIF-14 Treated Membrane | | SRIF-14 Treated Membrane | |
|---|---|---|---|---|---|---|---|
| *Cytokines Expressed* | *Mean Pixel Density* | *Cytokines Expressed* | *Mean Pixel Density* | *Cytokines Expressed* | *Mean Pixel Density* | *Cytokines Expressed* | *Mean Pixel Density* |
| Positive Controls | 27.24 | Positive Controls | 81.16 | Positive Controls | 80.35 | Positive Controls | 37.56 |
| IL-8 | 12.47 | IL-8 | 48.7 | IL-8 | 59.23 | IL-8 | 70.09 |
| | | IL-12 | 22.12 | IL-12 | 30.77 | IL-12 | 12.58 |
| | | IL-17 | 44.39 | IL-17 | 18.65 | IL-17 | 19.39 |
| | | IL-4 | 5.09 | IL-4 | 28.11 | IL-4 | 12.32 |
| | | IL-6 | 82.33 | TNF-α | 2.95 | IL-6 | 38.43 |
| | | IL-1β | 16.88 | IL-6 | 83.66 | IL-3 | 18 |
| | | TNF-α | 3.74 | VCAM-1 | 22.13 | VCAM-1 | 15.4 |
| | | MIP5 | 7.61 | | | IL-1β | 8.66 |
| | | MMP3 | 16.75 | | | TNF-α | 12.12 |
| | | | | | | MIP4 | 32.86 |
| | | | | | | MIP5 | 10.31 |

**Figure 7.** (A and B) Quantification by densitometry of hCAEC cytokine expression.

*Cytokine expression of Methamphetamine induced hCAEC cultures*

Current investigations reflect that chronic use of methamphetamine is capable of severe effects to the heart. In order to determine whether MA could initiate a pro-inflammatory event, hCAEC were treated with 25 ng ml $^{-1}$ for a period of 24 hours. According to figure 9, the treated membrane exhibited the expression of growth factor VEGF, OSM and Leptin in addition to the cytokines seen in the control membrane. Oncostatin M (OSM) is a member of the interleukin IL-6 family of cytokines produced by activated monocytes and T-lymphocytes. OSM has been shown to have both pro- and anti-inflammatory properties. OSM is known to modulate the expression of molecules, including tissue inhibitors of metalloproteinases and matrix metalloproteinases (MMPs). As mentioned previously in this study, metalloproteinases have been shown to degrade type IV collagen that supports endothelium (Schonbeck et al., 1998) Therefore, the resulting actions of the MMP enzymes can promote plaque erosion.

OSM's pro-inflammatory properties also include the induction of adhesion molecules (P-selectin, E-selectin, VCAM-1, ICAM-1), and leukocyte migration. Therefore, the expression OSM along with presence of the plasma protein leptin which is associated with obesity and the progression of atherosclerosis can suggests that the MA induction of hCAEC will cause a pro-inflammatory event to occur. There was also a marked expression of chemokines IL-8, SDF-1 and MCP-1 present on both membranes. The data offered a unique profile of chemical mediators and further insight as to the effects that MA may have on the endothelium. Table 4, defines cytokines expressed on the membrane as well as their respective effects on hCAEC.

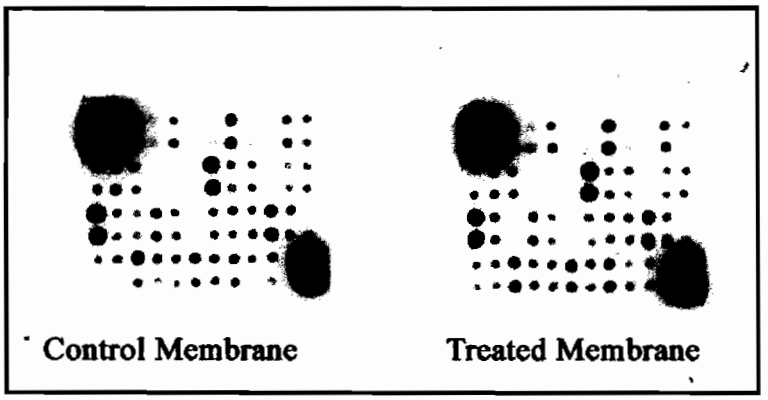
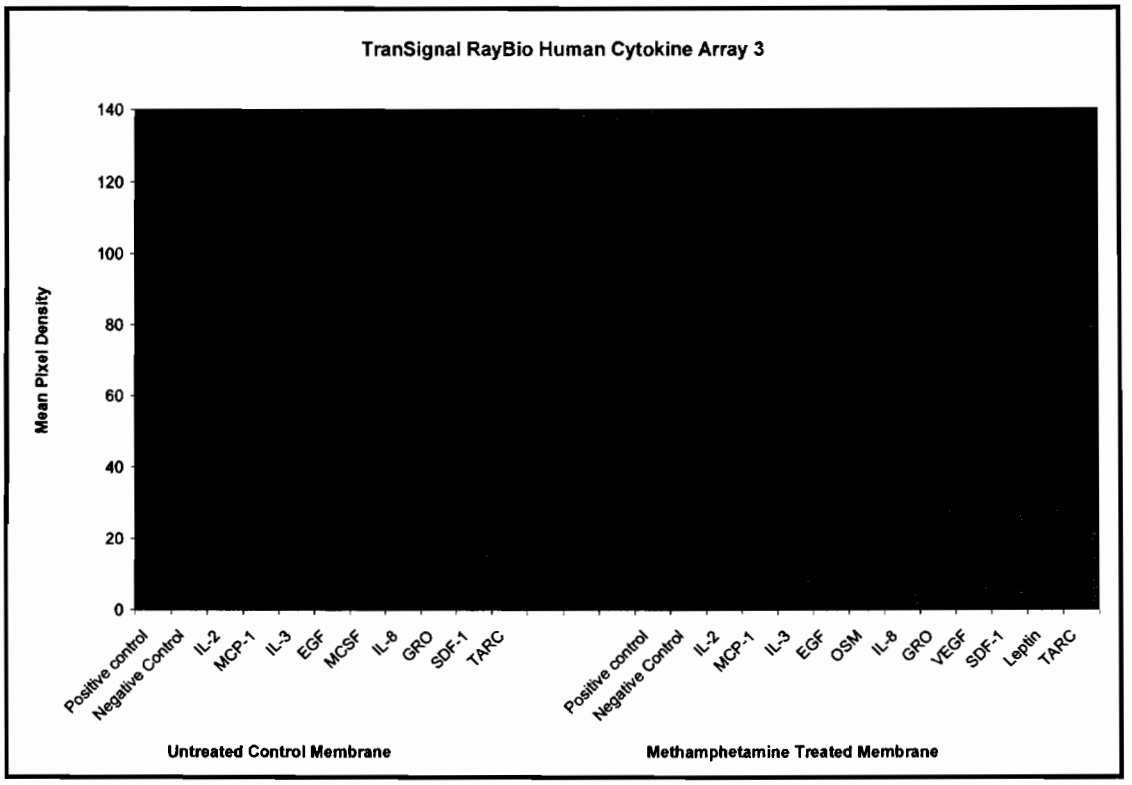



**Figure 9.** Identification of hCAEC cytokines released as a result of methamphetamine exposure.

| Assessment of Methamphetamine treated Human Coronary Artery Endothelial Cells | | | | | |
|---|---|---|---|---|---|
| **Primary Sources, Target and Effects** | | | | | |
| Primary Sources | | Endothelial Cells | | Monocytes/Macrophages | |
| Effects/Expression | | **Increase** | **Decrease** | **Increase** | **Decrease** |
| **IL-8** | Endothelial Cells, MΦ, SMC | Monocyte/macrophage Adhesion, endothelial chemotaxis | | monocytes | TIMP-1 |
| **MCP-1** | Endothelial Cells, MΦ, SMC Leukocytes, fibroblasts | Monocyte/macrophage adhesion migration | | IL-8 | ROS, monocytes |
| **EGF** | Platelets, leukocytes | | | | |
| **SDF-1** | Stromal cells, Leukocytes, | Monocytes, T lymphocyte | | | monocytes, |
| **GRO, GROα** | Leukocytes | proliferation, migration, adhesion | | | monocyte arrest |
| **OSM** | Activated T lymphocytes, Monocytes, endothelial cells | P-selectin, E-selectin leukocyte adhesion, IL-6, GRO, ENA-78 | IL-8, MMP,GM-CSF | | |
| **VEG-F** | Endothelial Cells, MΦ, SMC | migration, proliferation | | monocytes | |

**Table 4.** Assessment of cytokines expressed as a result of MA stimulation of hCAEC.

A




B

TranSignal RayBio Human Cytokine Array 3

| Untreated Control Membrane | | Methamphetamine Treated Membrane | |
|---|---|---|---|
| *Cytokines Expressed* | *Mean Pixel Density* | *Cytokines Expressed* | *Mean Pixel Density* |
| Positive Control | 95.29 | Positive Control | 119.1 |
| Negative Control | 57.57 | Negative Control | 27.81 |
| IL-2 | 40.05 | IL-2 | 36.99 |
| MCP-1 | 94.8 | MCP-1 | 71.06 |
| IL-3 | 45.12 | IL-3 | 47.05 |
| EGF | 37.32 | EGF | 38.46 |
| MCSF | 34.6 | OSM | 32.57 |
| IL-8 | 78.55 | IL-8 | 77.17 |
| GRO | 41.41 | GRO | 49.09 |
| SDF-1 | 47.01 | VEGF | 32.29 |
| TARC | 34.28 | SDF-1 | 54.21 |
| | | Leptin | 44.88 |
| | | TARC | 32.82 |

**Figure 10.** Quantification by densitometry of methamphetamine induced hCAEC.

**Discussion:**

Atherosclerosis is a chronic inflammatory disease involving the interaction between the vascular endothelium, pro-inflammatory cytokines, chemoattractants, macrophages and T-lymphocytes. The expression of pro-inflammatory mediators is not only important due to their characteristics in inflammation but also their potential to further augment an injurious response.

The objective of this study was to examine the protein expression of human carotid endothelial cells following exposure to diverse chemical mediators. More specifically, to examine the expression of the cytokine IL-6 following hCAEC treated with TNF-α. Additionally, to observe any possible inhibatory abilities of somatostatin and also to examine any potential chemical mediators expressed following treatment with the stimulatory compound methamphetamine. To further understand the effects of possible cytokine expression, cells were stimulated with TNF-α, and later treated with the inhibitory molecule SRIF-14 as well as the stimulant MA.

Stimulation of vascular wall cells by the proinflammatory cytokine TNF-α, induced the expression of multiple cytokines and chemokines. Heart endothelial cells stimulated with TNF- α revealed an expression of key cytokines including IL-6 and IL-1β. Previous investigations suggest that it is possible for TNF-α to act in positive feedback loop by inducing IL-6 which leads to further induction of potent proteins IL-1β, IL-17 and IL-4 that are capable of vascular damage. IL-6 and TNF-α are now well known risk indicators of atherosclerosis. As demonstrated in investigations by Lindmark et al., (2001) arterial diseased patients with increased plasma levels of IL-6 were associated with an increased risk for mortality. Additionally, cohorts of patients with acute ischemic stroke have also been known to exhibit increased concentrations of TNF-α, IL-1β and IL-6. Previous research by Lucus and Greeves (2001) explains that recruited macrophages in atherosclerotic lesions contribute to the disease process. Whereby, cells express high levels of scavenger receptors which have the ability to endocytose modified forms of LDL that are found in the foam cells within the coronary lesions. Beyond macrophages ability to clear debris and pathogens, the stimulatory and modulating properties can also instigate tissue damage and prevent new blood vessel formation. TNF-α is primarily produced by macrophages in response to inflammatory stimuli. Macrophages also posses the ability to secrete growth factors,

cytokines IL-1 as well as inflammatory mediators such as cytotoxic inducing IL-12 which influence the growth and development of other cell types within the atherosclerotic lesion (Von Der Thusen et al., 2003). Based on the published research at the time of this study, strong evidence suggested that it would be advantageous to measure the expression of IL-6 following treatment with TNF-α in heart endothelial cells. The expression of IL-6 measured by ELISA, following the administration of TNF-α on hCAEC confirmed that TNF-α could infact elicit an inflammatory response.

Cytokine antibody arrays (figure 4 and figure 6), were then chosen to demonstrate the multitude of cytokines and chemokines that are activated in response to an inflammatory event. Due to the fact that it would be both expensive and time consuming to complete an ELISA experiment for each cytokine in question, it was therefore advantageous to implement a system such as the antibody array membranes. The array membranes are unique in that they are not only based on the sensitive "sandwich" ELISA method for the detection of proteins, but also offer the simultaneous detection of multiple human cytokines. Therefore, we chose to use the membranes to offer a concurrent profile of the cytokines present in the vascular endothelium and implement the ELISA technique specifically towards the effects of TNF-α hCAEC expression of IL-6 only.

The results in this study suggest that all of the cytokines expressed in the array experiments have a significant role in the inflammatory pathway. As mentioned previously, proinflammatory compounds including IL-1β, IL-12, IL-4 as well as the chemoattractant IL-8 are known to have a significant role in inflammation. IL-8 has been documented to have an important role in tumor growth, angiogenesis, and metastasis (Li et al., 2003). IL-6 is known to have widespread effects in the immune system, and MCP-1 is known for its ability to recruit macrophages at sites of tissue injury. As demonstrated in Figure 5. cells treated with TNF-α revealed and increased expression of IL-8 and IL-6 as well as MCP-1.

Increasing literature implies that the naturally occurring inhibitor somatostatin, can have an effect on the heart. Studies by Yan et al., 2005, have recently demonstrated that treatment of hCAEC with somatostatin is known to decrease the expression somatostatin receptors on the heart endothelium and thus resulting in a decrease of TNF-α expression as well as a reduction in cell proliferation. In order to determine whether the inhibitor SRIF could

attenuate inflammatory expression, a membrane array experiment was also implemented to observe any possible expression that SRIF treated hCAEC could exhibit.

The treatment of SRIF alone on the endothelial cells demonstrated an expression of IL-6, IL-8 as well IL-17.

TNF-α and SRIF-14 (figure 7.) treatments of hCAEC were chosen to offer a profile of the expression of relevant mediators which may correlate with an inflammatory endothelium. It was also advantageous to determine whether administration of both compounds simultaneously would attenuate any mediator expression previously noted. Cells induced with TNF-α and SRIF-14 reveal the presence of multiple cytokines including IL-4 and the adhesion molecule VCAM-1 suggesting that an inflammatory event may have taken place. IL-4 has been previously implicated to have pro-atherogenic effects demonstrated by the cytokine's ability to up regulate adhesion molecules. In particular, the presence of adhesion molecules P-selectin and VCAM-1 are known to mediate the adhesion of neutrophils and monocytes to activated platelets and endothelial cells (King et al., 2002). Also, due to the immune response activated lymphocytes and mast cells are typically detected in human atherosclerotic lesions. Studies indicate that IL-4 is released during the activation of both these cell types. IL-4 mRNA has previously been detected in human and mouse atherosclerotic lesions (King et al., 2002).

With consideration that SRIF has predominantly inhibitory effects it was useful to determine if SRIF-14 treatment of hCAEC would induce the expression of any proinflammatory cytokines. As seen in figure 7. cells treated with SRIF-14 offered a profile consisting of the relative expression of proinflammatory molecules IL-6, IL-12 as well as the chemotactic factor IL-8 and adhesion molecule VCAM-1. Due to the qualitative nature of these findings the results may also suggest that the carotid endothelium may actively contribute to the inflammatory process and/or SRIF-14 itself has the ability to induce pro-inflammatory mediators.

In addition, to investigating molecules that may instigate an inhibitory response such as SRIF, the chemical MA was also implemented to determine if the compound could have any stimulatory affect on the endothelium. Increasing literature suggests that humans who take the drug often suffer severe or fatal cardiac events (Karch et al., 1999). Common side

effects which are often potentially harmful, include the chemical's ability to increase heart rate, rapid metabolism muscle breakdown. Since the drug MA is a potent and increasingly popular drug of abuse, it was thought to be advantageous to perform a profile experiment to examine inflammatory protein expression.

Following treatment with MA, the results indicated a presence of growth factor VEGF. VEGF may support the endothelium's ability to induce inflammation. As seen in the profile of the cytokines expressed in response to MA treatment of hCAEC (Figure 10.). Growth factor VEGF is known to play a role in the chemotaxis of monocytes and macrophages (Inoue et al., 1998). VEGF is reported to induce the migration and proliferation of endothelial cells as well as increase vascular permeability. Studies of human coronary artery plaques also reveal that there is a higher expression of the VEGF growth factor mRNA in comparison to normal carotid arteries (Inoue et al., 1998). Densitometric analysis of the membrane treated with methamphetamine revealed a presence of a negative control. Ideally, it would have been advantageous to repeat this experiment to rule out any contamination and reconfirm the presence of all mediators expressed on this membrane. It is also important to note, that the positive controls present in each membrane array were spotted at a fairly high concentration. Thus, signals that the controls generated were expected to be very strong and only act as a qualitative indicator of membrane detection. Therefore, it would be uncommon to have identical signals in the positive control spots from membrane to membrane.

From the results gathered in this study it was confirmed that TNF-$\alpha$ and IL-6 do play a major role in the inflammatory response and have the ability to further propagate tissue injury. Although further experiments would prove useful, this study demonstrates that SRIF can also have an effect on the endothelium. SRIF may not necessarily act as inhibitor with respect to certain cytokine and chemokine expressed (Weckbecker et al., 2003). However, due to previous well established research SRIF based therapies may be useful for inflammatory conditions including the reduction of joint inflammation in patients with rheumatoid arthritis as well as other destructive conditions such as psoriasis, inflammatory bowel disease and Grave's disease.

**Conclusion:**

The primary objective of inflammation is to specifically localize and eliminate the irritant and repair the adjacent tissue. The results of this study indicate that the activation of the endothelium results in the production of cytokine Interleukin-6, which is a known pro-inflammatory marker. Further evidence suggests that the proinflammatory molecule, TNF-α has the ability to increase IL-6 expression. Antibody array membrane experiments also offer a concise profile of the cytokines, chemokines and adhesion molecule that are present when triggered by a proinflammatory event, thus also suggesting that the vascular endothelium can itself be pro-inflammatory. Somatostatin is known as a naturally occurring neuropeptide with widespread effects throughout the human body. Since inflammation plays a critical role in the progression of vascular disease, understanding the interaction between SRIF and the expression of inflammatory mediators can be advantageous in the development of new therapeutic approaches.

With respect to atherosclerosis as a chronic inflammatory condition, further research would be necessary to understand the underlying mechanisms of inflammation and the interactions of inflammatory biomarkers. Future study of proinflammatory mediators would be valuable for the expansion of therapeutic approaches and development of drugs specifically targeted towards proinflammatory mediators such as IL-6.

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