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Influence of Adrenergic Agonists on Early Atherosclerotic Events

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INFLUENCE OF ADRENERGIC AGONISTS ON EARLY ATHEROSCLEROTIC
EVENTS

By

Jesica S. Westwright

A THESIS

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EVENTS

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Psychosocial stress is related to cardiovascular disease (CVD) morbidity and mortality, but the mechanisms of this relationship remain incompletely understood. The present *in vitro* study investigated the effect of catecholamines, a subclass of stress hormones, on the early CVD processes of monocyte adhesion and migration. For adhesion, THP-1 monocytes (MO) and human aortic endothelial cells (HAEC) were separately treated with catecholamines prior to static adhesion assay. MO migration was investigated using a Transwell migration protocol. HAEC adrenergic receptor mRNA expression and HAEC adhesion molecule surface expression were evaluated. Adrenergic agonism/antagonism experiments were utilized to determine the role of specific adrenergic receptor subtypes in adhesion and migration. Catecholamine treatment of HAEC, but not MO, increased adhesion of MO to HAEC, which was attenuated by β_2-adrenergic receptor blockade. β-adrenergic activation also increased surface expression of intercellular adhesion molecule-1. Catecholamine treatment did not increase production of pro-inflammatory cytokines known to enhance cellular adhesion, suggesting a direct effect of catecholamines. Catecholamines were also mildly chemotactic for MO. These results provide a mechanism by which psychosocial stress, working through catecholamines, could promote early CVD processes of MO adhesion and migration.

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Chapter 1: Introduction

Cardiovascular diseases (CVD) remain the leading worldwide cause of death

(World Health Organization, 2017). In order to successfully treat CVD, it is critical to

understand how CVD risk factors translate into biological mechanisms of disease,

particularly at early stages of disease. One important behavioral and modifiable risk

factor for CVD is psychosocial stress, which occurs when environmental demands exceed

one's ability to adapt and cope (Cohen, Janicki-Deverts, & Miller, 2007; Yusuf et al.,

2004). A significant body of human research has shown that increased psychosocial

stress is associated with increased CVD morbidity and mortality (Lagraauw, Kuiper, &

Bot, 2015; Linden, Stossel, & Maurice, 1996; Yusuf et al., 2004). Furthermore, data from

animal studies show that psychosocial stress accelerates CVD processes at the level of

the blood vessel wall (Kaplan, Manuck, Clarkson, Lusso, & Taub, 1982; McCabe et al.,

2002). Although psychosocial stress clearly contributes to CVD pathology, the specific

biological mechanisms of this relationship are unknown. Thus, the present study utilized

an *in vitro* model to evaluate how psychosocial stress, through activation of biological

stress systems, might affect early CVD processes.

Overview of Vascular Biology Processes

To understand the role of psychosocial stress in CVD processes, it is necessary to

discuss the biology of the vessel wall and how it is influenced by biological stress

systems.   The following sections provide an overview of the role of the initiation and

progression of atherosclerosis, the underlying cause of many CVDs, as well as the role of

the sympathetic nervous system (SNS) and its outputs within the vasculature.

Development of atherosclerosis. Atherosclerosis is an intricate process involving the interaction of immune cells with cells of the vessel wall to promote localized inflammation within the vasculature (Ruparelia, Chai, Fisher, & Choudhury, 2017). It is the underlying cause of many types of CVD as well as adverse cardiovascular events, including coronary heart disease, coronary artery disease, myocardial infarction ("heart attack"), and ischemic stroke (Ellulu et al., 2016). The atherosclerotic process is initiated by the accumulation of low-density lipoprotein cholesterol (LDL) in the space beneath the endothelium, the innermost layer of the vessel wall that contacts the circulating blood. Accumulation of LDL serves as an inflammatory stimulus for the production of pro-inflammatory cytokines and chemokines (cell signaling molecules) by endothelial cells comprising this layer (Moore & Tabas, 2011). Chemokines attract circulating monocytes, a type of immune cell, to the specific area of inflammation (van der Vorst, Döring, & Weber, 2015), while cytokines stimulate the production of cellular adhesion molecules that allow the monocytes to adhere to the endothelium (Ramji & Davies, 2015). Following adherence, monocytes migrate through the endothelial layer to cause a localized thickening of the wall referred to as an atherosclerotic lesion or plaque (Lagraauw et al., 2015). As monocytes and their metabolic products continue to accumulate the lesion becomes increasingly susceptible to rupture, causing adverse cardiovascular events like heart attack and stroke (Moore & Tabas, 2011). Thus, efforts to reduce CVD morbidity and mortality require an understanding of factors that retard or accelerate monocyte recruitment during early stages of atherosclerotic disease.

Sympathetic nervous system influences on the vasculature. The sympathetic (i.e. "fight or flight) branch of the autonomic nervous system is responsible for preparing

body systems to respond to environmental demands, including psychosocial stress

(Cohen et al., 2007). SNS nerves primarily release norepinephrine (NE) and innervate the

heart and blood vessels to regulate blood flow throughout the body (Kvetnansky, Lu, &

Ziegler, 2013). Furthermore, SNS innervation of the adrenal glands stimulates the release

of epinephrine (EPI) into circulation (Kvetnansky et al., 2013). NE is also present in

circulating blood, but at much lower concentrations than EPI (Kvetnansky et al., 2013).

NE, EPI, and drugs that mimic their actions are collectively referred to as catecholamines

and act on adrenergic receptors expressed on endothelial and immune cells (Kvetnansky

et al., 2013). Given their position at the interface of the blood and outer layers of the

vessel wall, endothelial cells may be exposed to both NE released from SNS nerves and

EPI circulating in the blood (Noller et al., 2017; Queen & Ferro, 2006). Similarly, EPI

and NE regulate responses of circulating immune cells as well as immune cell pools in

the bone marrow (Engler, Dawils, et al., 2004). In this way, catecholamines have the

potential to influence atherosclerosis through their action on endothelial cells, monocytes,

or both cell types.

The consequences of adrenergic receptor activation differ depending on the

adrenergic receptor subtype, broadly categorized into α- (αAR1, αAR2) and β- (βAR1,

βAR2, βAR3) subtypes (Hall, 2004; Minneman, Pittman, & Molinoff, 1981). Both NE

and EPI bind to αAR1s and to βAR1, but EPI has a higher affinity for βAR2 relative to

NE (Minneman et al., 1981). Adrenergic receptors are important for regulation of blood

flow by their action on vessel diameter and heart rate (Reiter, 2004), as well as cellular

immune and inflammatory processes (Bierhaus et al., 2003; Reader et al., 2015). Thus,

catecholamines and adrenergic receptors are intimately related to cardiovascular regulation and may be involved in a variety of pro-atherogenic processes.

Summary. Atherosclerosis is pathophysiological process characterized by chronic inflammation. Interactions between endothelial cells and circulating monocytes are critical to atherosclerotic disease promotion, and targeting these interactions is central to the development of novel CVD treatments. Catecholamines are known to contribute to changes in blood pressure, a well-established CVD risk factor, and may also play a role in pro-inflammatory processes relevant to atherosclerosis. The following sections review findings from animal and human studies linking stress, and catecholamines in particular, to CVD outcomes.

Stress, Catecholamines, and CVD Outcomes

A substantial body of human and animal research shows that psychosocial stress has a negative impact on CVD processes and outcomes. Furthermore, research utilizing β-adrenergic receptor blocking drugs (β-blockers) in CVD treatment implicates that catecholamines may facilitate the relationship between stress and CVD outcomes.

Stress negatively impacts CVD outcomes in humans. Both acute and chronic stress are associated with the incidence of CVD and CV events (e.g. heart attack) (Lagraauw et al., 2015). For example, psychosocial factors (encompassing depression, general stress, financial stress, life events, and perceived control) were significantly related to heart attack risk in an international case control study, accounting for 30% of the population attributable risk after controlling for traditional risk factors (Yusuf et al., 2004). In addition, depression and low social support, common in CVD patients, are

related to cardiac morbidity and mortality (Berkman et al., 2003). Taken together, these findings suggest that many forms of stress contribute to CVD morbidity and mortality in humans.

Stress contributes to atherosclerotic development in animals. In addition to human findings linking stress to CVD outcomes, research utilizing animal models of CVD have shown that exposure to chronic stress accelerates the development of atherosclerosis. Kaplan et al. (1982) reported that socially dominant cynomolgus monkeys fed a high-fat diet and subjected to repeated social stress had significantly greater coronary artery atherosclerosis than socially-stressed subordinate monkeys or unstressed monkeys. McCabe et al. (2002) extended these findings to a rabbit model genetically predisposed to atherosclerotic development, showing that exposure to daily social stress increased atherosclerotic lesion severity relative to rabbits housed alone or in a positive social environment. In addition, repeated stress increases atherosclerosis and inflammatory markers in the *ApoE -/-* mouse model of atherosclerosis (Bernberg, Ulleryd, Johansson, & Bergstrom, 2012; Zhang et al., 2010). Finally, there is evidence that exposure to chronic stress may contribute to the initiation as well as the progression of atherosclerosis. A study in rats fed a normal diet found that stress exposure significantly increased circulating LDL cholesterol and measures of oxidative stress, resulting in the initiation of early atherosclerotic lesions (Devaki, Nirupama, & Yajurvedi, 2013), further indicating an important role for stress during early atherosclerotic disease. In summary, research across animal models of CVD are in agreement that chronic stress accelerates the progression of atherosclerosis.

Catecholamines may underlie the stress-CVD relationship. Blocking the effects of catecholamines using β-blockers has been shown to improve CVD processes and outcomes in both humans and animals. Early animal studies showed that treatment with β-blockers reduced progression and severity of atherosclerotic lesions in rabbits fed a high-fat diet (Spence, Perkins, Kline, Adams, & Haust, 1983; Whittington-Coleman, Carrier, & Douglas, 1972). As a result, β-blockers have been widely used as a component of successful CVD treatment for human patients (Al-Gobari, El Khatib, Pillon, & Gueyffier, 2013; Larochelle, Tobe, & Lacourciere, 2014). Importantly, the best CV outcomes from β-blocker treatment are achieved in patients under 60, suggesting that β-adrenergic antagonism may be most beneficial at early stages of atherosclerotic disease (Poirier & Lacourciere, 2012). With regard to the relationship between psychosocial stress and CVD, β-blockers have been shown to attenuate the development of atherosclerotic lesions in monkeys subjected to psychosocial stress (Kaplan, Manuck, Adams, Weingand, & Clarkson, 1987). Taken together, these results indicate that β-blockade is associated with improved CVD, implicating the SNS as a mediating factor in CVD outcomes.

The primary use of β-blockers in the CVD context is to reduce blood pressure (Larochelle et al., 2014). During stress, activation of adrenergic receptors increases cardiac output and dilates peripheral vessels to increase blood flow to the limbs, which can increase blood pressure and contribute to vascular damage (Charkoudian & Rabbitts, 2009; Valentini & Parati, 2009). Thus, it could be posited that psychological stress impacts CVD outcomes primarily though effects on blood pressure and increased shear stress within the vessel. However, animal data linking social stress to atherosclerotic

progression have shown that this relationship can occur under hyperlipidemic conditions without overt changes in blood pressure (McCabe et al., 2002), which indicates that activation of β-adrenergic receptors may have pro-atherogenic effects beyond increasing shear stress within the vessel.

Summary. Stress is related to worse CVD outcomes in animals and humans. Given the role of the SNS in vascular control and the beneficial effects of β-blockers, it appears that catecholamines may play a role in this relationship. Furthermore, catecholamines may play a role in CVD processes above and beyond their impact on heart rate and vessel constriction/dilation. Critically, additional mechanisms of adrenergic receptor activation may be important for immune and inflammatory processes of early CVD.

Effects of Catecholamines on Immune and Inflammatory Processes

Although the role of catecholamines in early CVD processes has not been directly investigated, there is a growing body of research that catecholamines are involved in a number of pro-inflammatory processes in immune cells and other cells, including pro-inflammatory cytokine production, cell trafficking, cellular migration, and cellular adhesion. The literature in this domain is subsequently reviewed.

Pro-inflammatory signaling. Catecholamines have been shown to increase pro-inflammatory signaling across cell types, including immune and endothelial cells. Macrophages, tissue-resident cells which differentiate from monocytes, exhibit an enhanced pro-inflammatory cytokine response to inflammatory stimuli following catecholamine exposure (Bailey, Kinsey, Padgett, Sheridan, & Leblebicioglu, 2009). Catecholamine treatment also increases pro-inflammatory intracellular signaling and pro-

inflammatory cytokine production in cultured monocytes and vascular endothelial cells (Bierhaus et al., 2003; Jin et al., 2011; Stohl et al., 2013). In monocytes, these effects are attenuated by α1- or β-adrenergic antagonism, but not α2-adrenergic antagonism (Bierhaus et al., 2003). Additionally, immune cells from humans exposed to a social stressor show increases in pro-inflammatory cytokine production (Bierhaus et al., 2003). These data suggest a pathway by which social stress, through activation of the SNS may influence the inflammatory status of monocytes/macrophages and endothelial cells.

Immune cell trafficking. In addition to effects on cellular inflammatory signaling, catecholamines may mediate the trafficking of immune cells to areas of inflammation. Social stress in mice initiates the trafficking of immune cells, including monocytes, from resident pools in the bone marrow into circulation (Engler, Bailey, Engler, & Sheridan, 2004; Heidt et al., 2014). Furthermore, Heidt et al. (2014) found that social-stress induced increases in immune cell trafficking was concurrent with increased recruitment of immune cells to developing plaques in an atherosclerotic mouse model, suggesting that immune cells may have homed specifically to vulnerable regions of the vessel wall. Cells trafficked in response to social stress have been described as "pro-inflammatory," with a characteristic pattern of chemokine receptor expression and the tendency to be recruited specifically to areas of inflammation (Geissmann, Jung, & Littman, 2003; Powell et al., 2013). Stress-induced increases in cell trafficking and macrophage inflammatory status are attenuated in socially stressed mice treated with β-blockers, implicating that β-adrenergic receptor activation is the underlying mechanism (Hanke, Powell, Stiner, Bailey, & Sheridan, 2012). Results from these studies indicate that catecholamines could increase trafficking of cells to areas of inflammation within the

vessel wall, potentially leading to increased cell infiltration and progression of atherosclerotic lesions.

Cellular migration. Migration of monocytes to *specific sites* of the blood vessel wall is a critical event in the initiation and progression of atherosclerosis (van der Vorst et al., 2015). Although a number of chemokines play a key role in this process, some evidence suggests that catecholamines themselves may also be chemoattractive (a substance that attracts cells). Straub et al. (2000) demonstrated that physiological levels (10 pM – 1 nM) of NE or the β-adrenergic agonist isoproterenol (ISO) were chemoattractive for monocytes and macrophages, but α1- and α2-agonists had no chemotactic effect. In addition, (Masur, Niggemann, Zanker, & Entschalden, 2001) reported that treatment of carcinoma cells with 10 μM NE significantly increased spontaneous cell migration. Migration was inhibited by the addition of the nonselective β-adrenergic antagonist propranolol, but not by the β1-adrenergic antagonist atenolol, suggesting that the locomotion effect was due to activation of β2-adrenergic receptors. The presence of SNS nerve fibers in vascular regions prone to atherosclerosis (e.g. the aortic arch) suggest the potential for high localized output of catecholamines in these regions (Noller et al., 2017). Thus, catecholamines may not only increase trafficking of immune cells into circulation, but may promote trafficking of immune cells in to the vessel wall in early stages of atherosclerosis.

Cellular adhesion. In order for monocytes to traffic into the into the vessel wall to form lesions, they must first adhere to the endothelium. This process is mediated by cellular adhesion molecules (CAMs) expressed on the endothelium and on circulating immune cells, including monocytes (Moore & Tabas, 2011). There is evidence that, in

general, catecholamine treatment increases adhesion of immune cells to vascular endothelial cells, which can be attenuated by adrenergic receptor blockade (Chen et al., 2005; Jin et al., 2011; Strell et al., 2012; Sundstrom et al., 2003). Chen et al. (2005) and Strell et al. (2012) found that adhesion was increased following treatment of vascular tissue or cultured endothelial cells with catecholamines, and blocked by βAR antagonism, suggesting that endothelial cells, rather than circulating immune cells, were the primary facilitators of increased adhesion. Indeed, there is some evidence that catecholamine treatment increases CAM expression on endothelial cells (Chen et al., 2005; Jin et al., 2011). However, catecholamines may also increase CAM expression in immune cells. In circulating immune cells, psychosocial speech stressors may increase integrin expression, CAMs responsible for firm adhesion to the endothelium, and increase shedding of L-selectin, a CAM which mediates initial tethering to the endothelium and is shed following firm adhesion (Goebel & Mills, 2000; Redwine, Snow, Mills, & Irwin, 2003). Therefore, it is important to clarify whether it is endothelial cells or circulating immune cells that are influenced by catecholamines to increase cellular adhesion, as this may have implications for CVD treatments targeting the adhesion process.

In addition to lack of certainty regarding which cell type is affected by catecholamines in the context of cellular adhesion, there are other gaps in current knowledge of the role of catecholamines and cellular adhesion as it relates to atherosclerosis specifically. Firstly, cellular response to any biochemical stimulus, including adrenergic receptor activation, is highly dependent on the specific cell type. For example, βAR2 agonists are anti-inflammatory for airway epithelial cells in the context of asthma, reducing pro-inflammatory cytokine production and CAM expression

(Sabatani et al., 2003), in contrast to the pro-inflammatory and pro-adhesive effect of catecholamines in endothelial-immune cell interactions. In atherosclerosis, the most critical adhesion interactions occur between *arterial endothelial cells* and *monocytes* (Moore & Tabas, 2011), but the role of catecholamines in adhesion of these specific cell types has not been previously investigated. In addition, it is not clear which adhesion molecules (expressed on immune cells or on endothelial cells) are influenced by catecholamines to increase adhesion. Finally, it is unknown whether catecholamines increase cellular adhesion directly, or by increasing secretion of pro-inflammatory cytokines/chemokines from either cell type. Strell et al. (2012) reported that NE-induced adhesion of cancer cells to microvascular endothelial cells was dependent upon endothelial secretion of the chemokine GROα, whereas Chen et al. (2005) reported that EPI treatment did not increase TNFα or IL-6 production in cardiac tissue. Further investigation is warranted to better understand how catecholamines influence cellular adhesion, and to determine if this mechanism could link psychosocial stress to adverse CVD outcomes.

Summary. In various contexts, catecholamines appear to promote pro-inflammatory activity in immune and endothelial cells. Although these processes have not been directly investigated in the context of atherosclerosis, these findings suggest that catecholamines have the potential to participate in pro-inflammatory interactions between monocytes and vascular endothelial cells. Investigation of these interrelationships has the potential to yield important insights into the role of stress in CVD.

Study Rationale

Catecholamines have been implicated in immune and inflammatory processes relevant to atherosclerosis, but have not been specifically studied within the context of atherosclerosis to determine if catecholamine effects on cellular inflammatory signaling, trafficking, migration, and adhesion could explain the relationship between catecholamines and atherosclerotic progression. The purpose of the present study was to evaluate whether catecholamines influence two processes relevant to early atherosclerosis: the adhesion of monocytes to arterial endothelial cells and monocyte migration/chemotaxis. We hypothesized that catecholamines would 1) increase adhesion of monocytes to human aortic endothelial cells (HAECs) in an *in vitro* static adhesion assay and 2) act as a chemoattractant for monocytes. Furthermore, we hypothesized that activation of βARs, rather than αARs, would be responsible for effects on monocyte adhesion and migration. To address gaps in the literature regarding adhesion, we evaluated which cell type (arterial endothelial cells or monocytes) facilitated catecholamine-driven increases in adhesion, and whether increases in adhesion were mediated by elevated production of pro-inflammatory cytokines.

Chapter 2: Methods

Cell culture

Materials. Adrenergic agonists and antagonists were obtained from Sigma (St. Louis, MI). CellTracker Green CMFDA dye was obtained from Thermo-Fisher (Waltham, MA). Primary human aortic endothelial cells (HAEC) from Cascade Biologics (Thermo-Fisher, Waltham, MA), and human THP-1 monocytes (MO) were used. Human THP-1 cells are a monocytic cell line derived from a patient with leukemia (Tsuchiya et al., 1980). The cells utilized in the present study were obtained from American Type Culture Collection (Manassas, VA).

Cell Cultures. HAEC were grown in Medium 200 containing low-serum growth supplement (Thermo-Fisher, Waltham, MA) and used between passages 5-12. MO were maintained in RPMI medium containing 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 μM mercaptoethanol, and 10% fetal bovine serum. All cells were cultured at 37°C in a humidified 95% air-5% CO_2 incubator.

Adhesion Assays

HAEC were plated at 5,000-30,000 cells per well in gelatin-coated 48-well plates within one week of the experiment. Experiments were conducted with cells that were >80% confluent. HAEC were incubated in complete media (37˚C, 5% CO_2) with or without adrenergic agonists for 6 hours. For antagonist experiments, HAEC were pre-incubated with adrenergic antagonists for 10 minutes prior to addition of adrenergic agonists. MO were labeled with CellTracker Green CMFDA (Life Technologies, Thermo-Fisher, Waltham, MA) at a concentration of 0.5 μM according to the manufacturer's protocol on the day of the experiment. MO were incubated for 6 hours in

complete RPMI media with or without adrenergic agonists, then placed in complete Medium 200 for the adhesion assay. Sixty thousand MO were added per well, incubated at 37˚C (5% CO_2) for 60 minutes, and washed to remove non-adherent cells. Adherent MO were determined by fluorescent microscopy and quantified as cells/mm^2.

Migration Assays

MO were maintained in serum-free RPMI medium containing HEPES, sodium pyruvate, and 1 mg/ml BSA for 4-18 hours prior to the migration assay. The same medium supplemented with adrenergic agonists was added to the lower chamber of a 24-well plate containing Transwell inserts with a 5 µm pore size (Sigma-Aldrich, St. Louis, MO). One hundred thousand MO were added to each insert and incubated for 4 hours at 37˚C (5% CO_2). Following the assay, insert membranes were fixed and stained as described by Justus, Leffler, Ruiz-Echevarria, and Yang (2014), and the number of migrated cells adhered to the membrane was quantified by light microscopy.

Agonist/antagonist studies

Norepinephrine (NE), isoproterenol (ISO; non-selective βAR agonist), and phenylephrine (PHE; non-selective αAR agonist) were used to evaluate the effect of adrenergic agonists on monocyte adhesion and migration. Atenolol (selective βAR1 antagonist) and ICI 118,511 (selective βAR2) were used in antagonism experiments to determine adrenergic receptor subtypes responsible for adhesion and migration. Catecholamine doses ranging from 10^{-5} M to 10^{-12} M were used to establish dose-response curves.

Cellular Adhesion Molecule Expression

HAECs were grown to ~80% confluence in gelatin-coated 60 mm dishes and treated with ISO (1 - 100 nM) in complete Medium 200 for 6 hours. Cells (~ 1were collected by scraping and evaluated for surface expression of CAMs by flow cytometry according to standard staining protocols. The following anti-human antibodies and isotype controls were used: ICAM-1 (BD Biosciences; Cat. # 564077, isotype control Cat. # 562438), VCAM-1 (BD Biosciences; Cat. # 563525, isotype control Cat. # 552834), E-selectin (BD Biosciences; Cat. # 563359, isotype control Cat. # 562652), P-selectin (eBioscience; Cat # 17-0626-82, isotype control Cat # 17-4714-41), and VE-cadherin (endothelial cell marker; BD Biosciences; Cat. # 565672, isotype control Cat. # 563330). Median florescent intensity was used to quantify expression of each marker.

Adrenergic Receptor Expression

Expression of adrenergic receptor subtypes in HAEC was evaluated using quantitative polymerase chain reaction (qPCR). HAEC were grown to ~80% confluence in gelatin-coated 60mm dishes. RNA was isolated from ~1 million cells using the RNAeasy kit (Qiagen, Valencia, CA) and incubated with DNAse I (Qiagen, Valencia, CA). The reverse transcription reaction was used to prepare cDNA (Applied Biosystems, Life Technologies, Carlsbad, CA) following manufacturer's instructions. The following Thermo Fisher Scientific (Waltham, MA) inventoried human primers were used: βAR1 (Cat #: Hs02330048_s1), βAR2 (Cat #: Hs00240532_s1), βAR3 (Cat #: Hs00609046_m1), αAR1b (Cat #: Hs00171263_m1), αAR1d (Cat #: Hs00169865_m1),

αAR2a (Cat #: Hs01099503_s1), αAR2b (Cat #: Hs00265090_s1), αAR2c (Cat #: Hs03044628_s1), and HPRT-1 (hypoxanthine phosphoribosyltransferase 1; housekeeping gene; Cat #: Hs02800695_m1).

The TaqMan gene expression assay was used to quantify gene expression using real-time PCR (Thermo Fisher Scientific). Amplification of twenty micrograms of cDNA was performed with TaqMan Universal PCR Master Mix. Reactions were run with universal cycling conditions on an Applied Biosystems Step-One Plus RT-PCR system. Samples in triplicate were normalized to the housekeeping gene, HPRT-1. The comparative Ct method ΔΔCT (threshold cycle (Pfaffl, 2001)) was utilized to analyze relative quantification.

Pro-Inflammatory Cytokine Production

HAEC pro-inflammatory cytokine production was investigated as a potential intermediary between catecholamines and MO adhesion to HAEC. HAEC are known to produce pro-inflammatory cytokines in response to inflammatory stimuli, which increase expression of adhesion molecules on HAEC cell surface (Libby, 2012). To determine if MO adhesion to HAEC was indirectly mediated by increased pro-inflammatory cytokine production by HAEC, pro-inflammatory cytokine production in response to adrenergic agonist/antagonist treatment was quantified by enzyme-linked immunosorbent assay (ELISA). Supernatants were collected from HAEC treated with adrenergic agonists/antagonists as described above. For IL-6 assay, samples were diluted 1:5 in complete HAEC media prior to analysis (BD Biosciences, San Jose, CA). TNFα and IL-1β were analyzed undiluted via high-sensitivity ELISA (R&D Systems, Minneapolis, MN).

Statistical Analysis

Data are represented as mean ± SEM. Results are evaluated by paired *t*-tests or ANOVA utilizing post hoc Tukey tests to compare experimental and control conditions. An α-level of 0.05 was required for statistical significance.

Chapter 3: Results

Catecholamines Increase MO Adhesion to HAEC

In order to investigate the general role of catecholamines in adhesion of MO to HAEC, MO and HAEC were separately incubated with 100 nM NE prior to the adhesion assay (Figure 1). Treatment of HAEC with NE increased adhesion of MO to HAEC ($p < 0.05$), whereas there was a trend toward decreased adhesion when MO were treated with NE ($p = 0.07$). Treatment of both HAEC and MO with NE also increased adhesion relative to control ($p < .05$), and resulted in a trend toward decreased adhesion when compared to NE treatment of HAEC alone ($p = .10$)

Given limited published data on HAEC AR expression, we evaluated expression of βAR and αAR mRNA in HAEC by RT-PCR (Figure 2). HAEC expressed the AR subtypes βAR1, βAR2, αAR1d, αAR2a, and αAR2c. αAR2b and βAR3 were not detectable by PCR. Relative expression of βAR2 mRNA was five-fold higher than expression of any of the other ARs.

To elucidate specific effects of βAR and αAR activation on adhesion, HAEC were treated with ISO (βAR2/ βAR1 non-selective agonist) or PHE (αAR1 non-selective agonist) prior to adhesion assay (Figure 3). ISO treatment of cells increased binding across multiple doses, although the differences did not reach statistical significance ($p = .07$ at most effective dose). In contrast, PHE treatment of cells resulted in no change or a slight decrease in adhesion levels, indicating that βAR activation was responsible for increased binding of MO to HAEC.

Adrenergic antagonism experiments were utilized to determine the βAR subtype responsible for the effect of ISO on adhesion (Figure 4). Pre-treatment of HAEC with the

βAR2-selective antagonist ICI 118,551 attenuated ISO-induced increases in MO adhesion ($p < .01$) indicating that βAR2-specific activation is required for adhesion effects.

The role of βAR activation in HAEC CAM surface expression was evaluated using flow cytometry. ISO increased surface expression of ICAM-1 relative to control, $p < .05$ (Figure 5). Surface expression of VCAM-1, E-selectin, and P-selectin did not increase with ISO treatment (data not shown, all $p > .05$).

Catecholamine Effects on Adhesion Are Not Mediated by Pro-Inflammatory Cytokines

As stated above, pro-inflammatory cytokines, especially TNFα and IL-1β, are known to increase adhesion of immune cells to ECs. Therefore, we investigated the role of these cytokines, as well as IL-6, in our adhesion paradigm to determine if pro-inflammatory cytokines indirectly mediated the effect of catecholamines on adhesion. ISO treatment of HAEC did not result in detectable levels of TNFα or IL-1β in the supernatant, suggesting that increased adhesion was not indirectly mediated by pro-inflammatory cytokines (data not shown). In contrast, treatment of HAEC with ISO had a significant effect on IL-6 secretion [$F(3,8) = 43.45$, $p < .001$] such that ISO treatment increased IL-6 secretion at all doses ($p < .05$ for all ISO doses, Figure 6a). However, treatment of HAEC with a high dose of IL-6 (10 ng/ml) prior to adhesion assay had no effect on adhesion of MO to HAEC ($p > .10$), suggesting that increased IL-6 production did not mediate the increase in adhesion (Figure 6b).

NE is Mildly Chemotactic for Monocytes

In addition to evaluating the role of catecholamines in adhesion of MO to HAEC, we also sought to determine if catecholamines can act as chemoattractants for MO. At a

concentration of 10 nM, NE inducted migration of MO into the Transwell membrane,

indicating that NE is mildly chemotactic for monocytes ($p < .02$, Figure 7).

Chapter 4: Discussion

Psychosocial stress has long been known to have a negative impact on CVD outcomes, but the underlying biological mechanisms of this relationship are not well understood. In the present study, we demonstrated that stress hormones of the SNS (catecholamines) promoted atherosclerotic processes in cultured arterial endothelial cells and monocytes. Specifically, catecholamines increased adhesion of MO to HAEC and acted as a mild chemoattractant for MO *in vitro*. These results provide preliminary evidence of the mechanisms by which psychosocial stress, acting through catecholamines, could contribute to CVD morbidity and mortality.

Catecholamines Increase Cellular Adhesion by Acting on HAEC

It was hypothesized that pre-treatment of HAEC or MO with catecholamines would increase adhesion of MO to HAEC in an *in vitro* static adhesion assay. Previous research has indicated that catecholamines may increase cellular adhesion and adhesion molecule expression in both immune cells (Goebel & Mills, 2000; Redwine et al., 2003) and endothelial cells (Jin et al., 2011). However, no prior studies specifically evaluated the separate contributions of catecholamine pre-treated immune and endothelial cells in cellular adhesion. For this reason, we had no *a priori* hypotheses regarding which cell type would be important for mediating MO adhesion to HAEC. In the present study, pre-treatment of HAEC with NE increased adhesion of MO to HAEC, whereas pre-treatment of MO with NE had no effect on adhesion. These findings are consistent with Jin et al. (2011), who reported that EPI treatment increased CAM surface expression in human umbilical cord endothelial cells, and with Chen et al. (2005), who showed that treatment of heart tissue with EPI increased adhesion of immune cells by increasing heart CAM

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expression. Thus, our results suggest that in the context of atherosclerosis, catecholamines act primarily on arterial endothelial cells, rather than monocytes, to mediate cellular adhesion.

Effect of Catecholamines on Adhesion is Mediated by βAR2s

As hypothesized, activation of βARs, but not αARs, mediated catecholamine-induced increases in MO adhesion to HAEC. Treatment of HAEC with the βAR agonist ISO increased MO binding, while treatment with the α1AR agonist PHE did not. Furthermore, we showed that ISO treatment increased surface expression ICAM-1 in HAEC, indicating that ISO may have increased MO binding by enhancing expression of this CAM. These results are consistent with studies showing that β-blockade attenuates EPI-stimulated increases in adhesion and CAM expression in endothelial cells (Chen et al., 2005; Strell et al., 2012).

The present study further showed that ISO-mediated increases in adhesion were blocked by a highly selective βAR2-selective antagonist, demonstrating that βAR2s were the specific AR subtype responsible for increased adhesion of MO to HAEC. βAR2 mediation of adhesion was consistent with our mRNA data, which showed that βAR2s were much more highly expressed in HAEC than any of the other adrenergic receptors. Previous research supports that βAR activation in general increases pro-inflammatory processes, including immune cell trafficking, cellular adhesion, and pro-inflammatory cytokine production (Bailey et al., 2009; Bierhaus et al., 2003; Chen et al., 2005). However, specific activation of βAR2s is often considered to be anti-inflammatory, as this βAR subtype mediates vasodilation to reduce blood pressure (Reiter, 2004) and attenuates inflammatory processes in airway epithelial cells (Sabatani et al., 2003). In

contrast, our findings indicate that βAR2-activation in HAEC may promote recruitment of MO to atherosclerotic lesions, suggesting that in some contexts βAR2-activation may be pro-inflammatory. Taken together, the results of the present study suggest a model by which catecholamines, via activation of βAR2s and increased cellular adhesion, promote CVD pathology.

Catecholamines Influence Adhesion Directly

One of the most well established effects of catecholamines on cellular inflammatory processes is their promotion of pro-inflammatory cytokine production (Bailey et al., 2009; Bierhaus et al., 2003; Jin et al., 2011; Stohl et al., 2013). In turn, pro-inflammatory cytokines increase cellular adhesion by increasing CAM expression on endothelial cells (Libby, 2012). Thus, we sought to determine if increases in pro-inflammatory cytokine production were responsible for effects of catecholamines on adhesion. There were no detectable levels of TNFα and IL-1β in supernatants of HAEC treated with ISO or NE. Catecholamine treatment did increase production of IL-6 in our study, consistent with findings in microvascular endothelial cells (Strell et al., 2012). However, pre-treatment of HAEC with IL-6 did not increase adhesion of MO during the adhesion assay. Thus, it appears that catecholamines may mediate ICAM-1 expression and MO binding directly, rather than by indirectly increasing production of pro-inflammatory cytokines.

Norepinephrine is Mildly Chemoattractive for Monocytes

In addition to the effects of catecholamines on adhesion, we hypothesized that catecholamines would act as chemoattractants for monocytes, which could promote trafficking of monocytes across the vessel wall in the context of atherosclerosis.

Consistent with Straub et al. (2000) and Masur et al. (2001), the present study showed that norepinephrine was mildly chemoattractive for MO, inducing migration of MO into the Transwell membrane in an *in vitro* migration assay. These results indicate that catecholamines may act on monocytes in addition to endothelial cells to promote monocyte recruitment to developing atherosclerotic lesions.

Limitations and Future Directions

The present study utilized an *in vitro* model to investigate specific mechanisms of the relationships between catecholamines and atherosclerotic progression. Our findings that catecholamines promote cellular adhesion and migration *in vitro* warrant further investigation in an *in vivo* model to determine if these mechanisms can explain the relationship between catecholamines and atherosclerosis in a more complex environment. Prior to *in vivo* studies, additional *in vitro* work may be beneficial to confirm the present results. Adhesion assays that mimic conditions of blood flow within the vessel should be conducted to confirm the effect of catecholamines on MO adhesion to HAEC observed under static conditions. Adhesion and migration findings should also be confirmed using primary human monocytes, to ensure that results are similar to those obtained with THP-1 monocytes in this study. Finally, the effect of catecholamines on HAEC CAM expression should be further explored. Future work should utilize antagonism experiments to confirm βAR-facilitated increases in ICAM-1.

Conclusion

This study demonstrated that catecholamines can promote *in vitro* monocyte adhesion and migration, processes that are critical to monocyte recruitment in early CVD.

The results of this study provide evidence for a mechanism by which social stress contributes to negative outcomes in CVD, and supports the use of β-blockers in early CVD.

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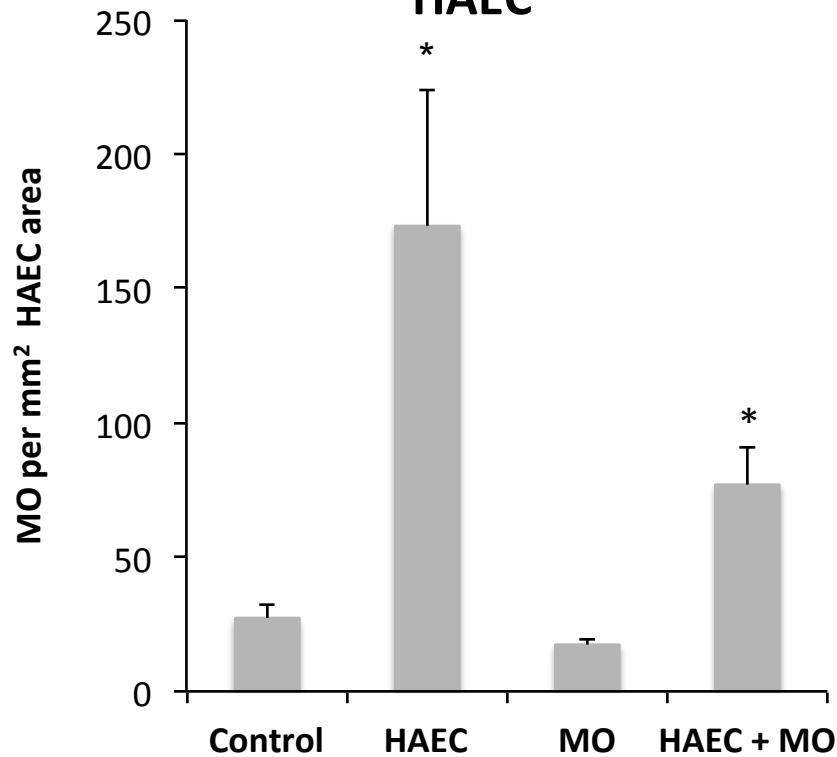
Effect of NE on MO Adhesion to HAEC

Figure 1. Effect of NE on adhesion. Data are expressed as MO counts per mm^2 HAEC area (five to six replicates per group). NE dose was 100 nM. Pre-treatment of HAEC with NE increased adhesion of MO to HAEC (*$p < .05$ relative to control), whereas pre-treatment of MO showed a trend toward decreased adhesion ($p = .07$). Treatment of both HAEC and MO with NE increased adhesion relative to control (*$p < .05$), and showed a trend toward decreased adhesion relative to treatment of HAEC alone ($p = .10$).

HAEC Adrenergic Receptor Expression

	βAR2	βAR1	αAR1D	αAR2A	αAR2C
Mean Relative Quantification	1.05	0.20	0.08	0.04	0.07
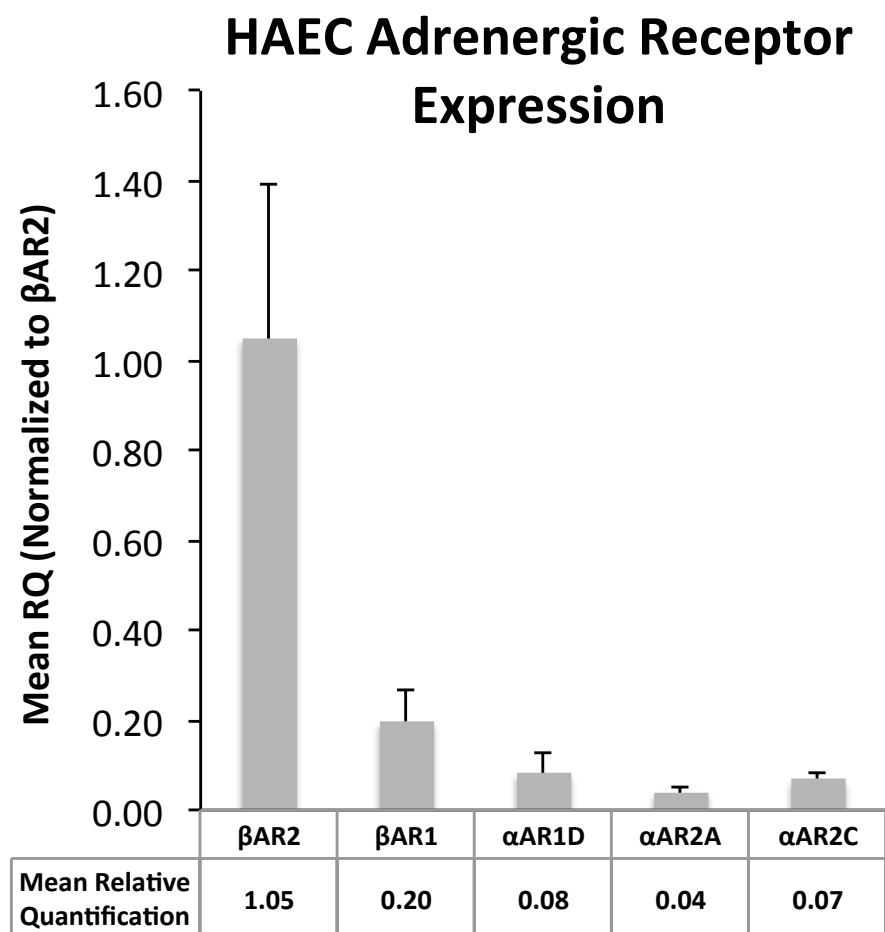
Figure 2. AR subtype mRNA expressed in HAEC. HAEC expression of βAR2 was five-fold higher than expression of any other ARs. Data (three replicates per group) are expressed as fold change relative to βAR2 after normalizing to the housekeeping gene HPRT-1. α2b-adrenergic and β3-adrenergic receptors were not detectable by PCR.

Effect of βAR and αAR Activation on MO Adhesion to HAEC
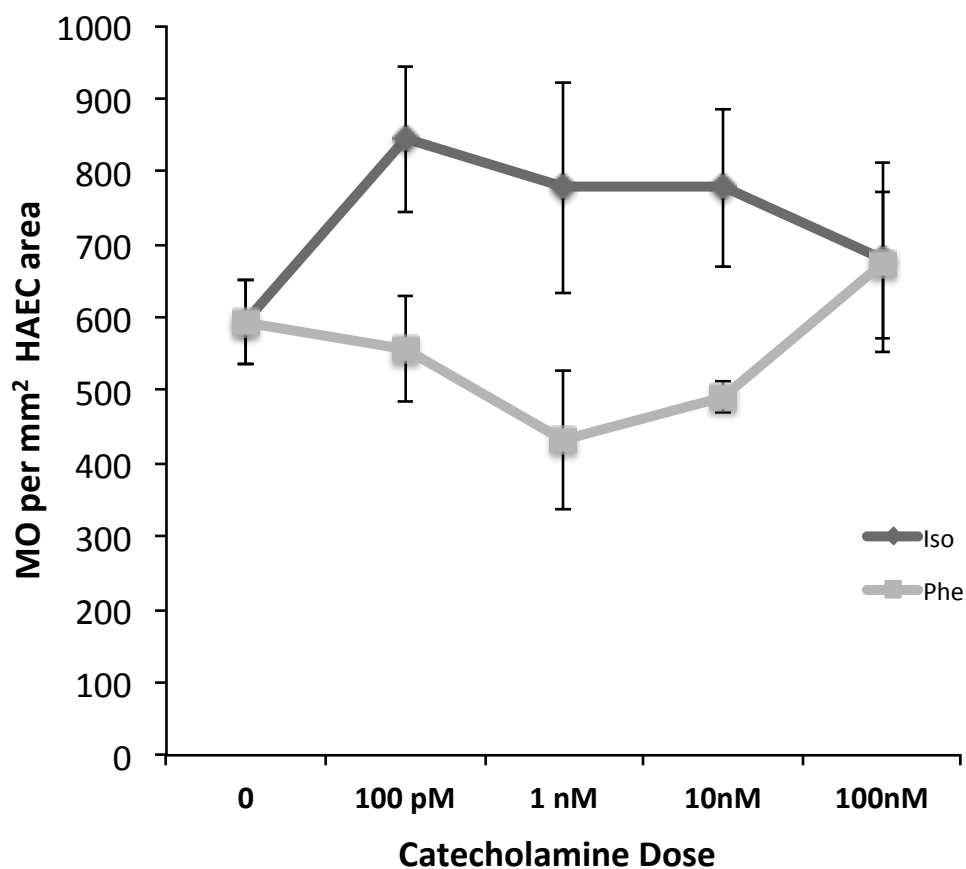
Figure 3. Effect of βAR and αAR activation on adhesion. Data are expressed as MO counts per mm^2 HAEC area (four replicates per group). ISO (βAR1/ βAR2 non-selective agonist) increased adhesion of MO to HAEC, with the most potent effect at the 100 pM dose. In contrast, PHE (αAR1 selective agonist) did not increase MO binding.
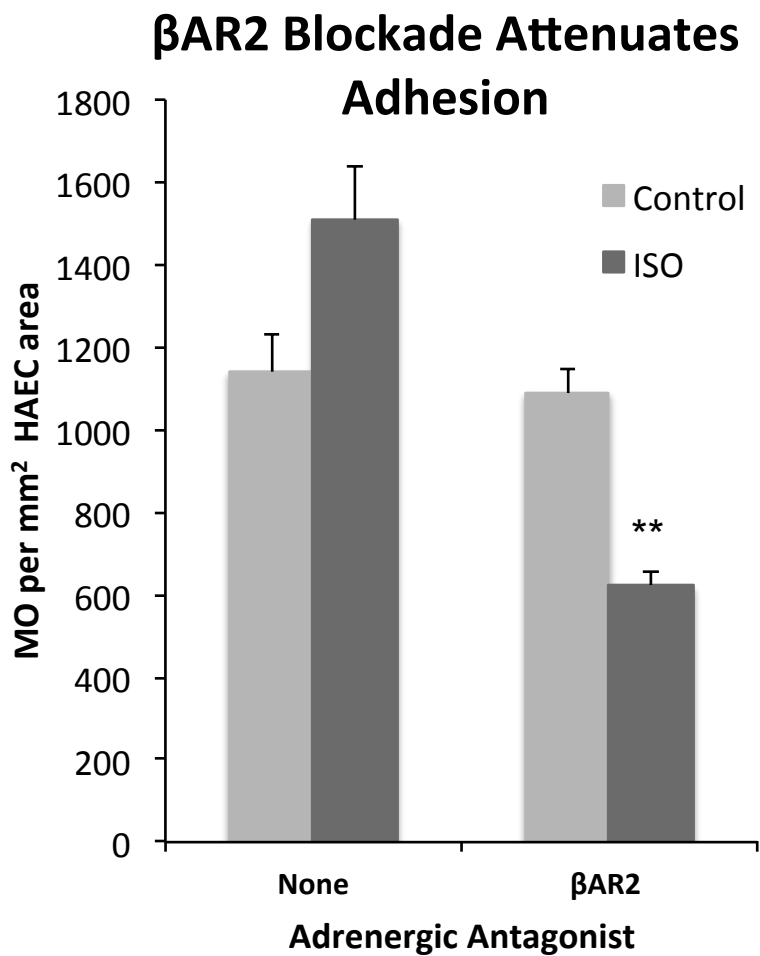
Figure 4. βAR2 blockade attenuates the effect of ISO on MO adhesion to HAEC. Data are expressed as MO counts per mm^2 HAEC area (two to four replicates per group). Pre-treatment of HAEC with 1 μM ICI 118,551 (βAR2 antagonist) blocked ISO-induced increases in adhesion (**p < .01 comparing ISO alone to ISO + ICI 118,551).
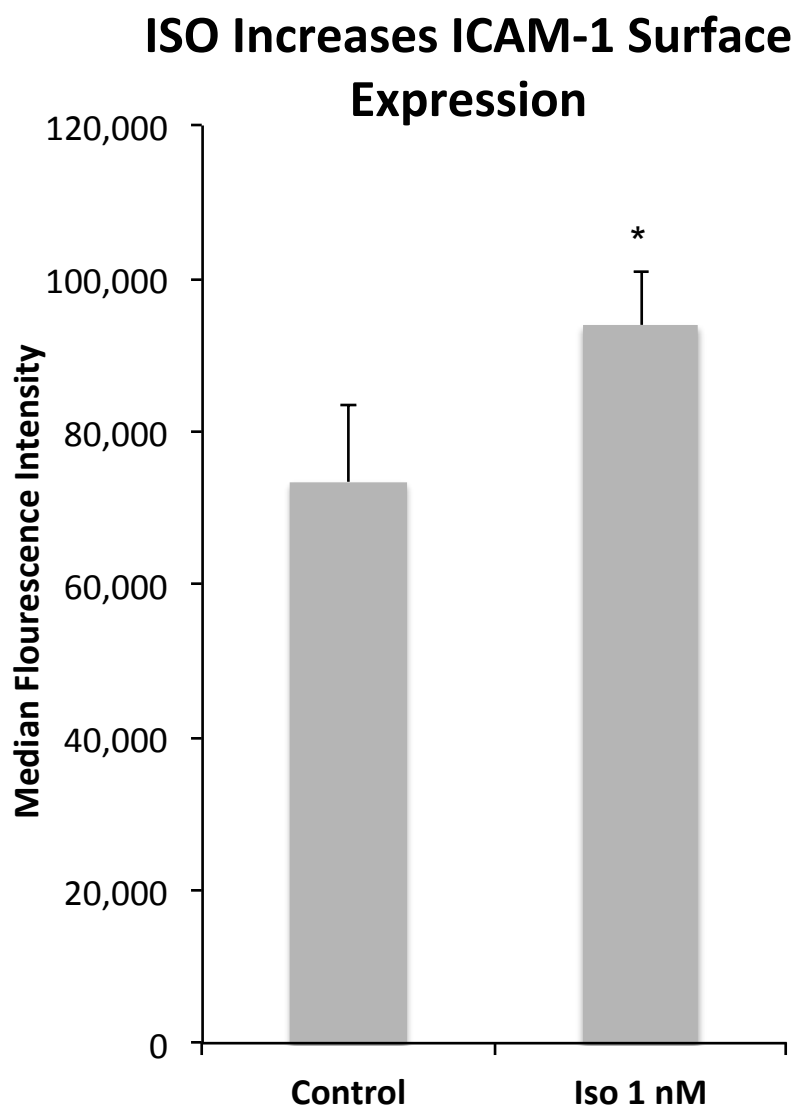
Figure 5. Effect of catecholamines on ICAM-1 expression. Treatment of HAEC with 1 nM ISO increased cell surface expression of the cellular adhesion molecule ICAM-1 (*p < .05, N = 3 replicates per group).
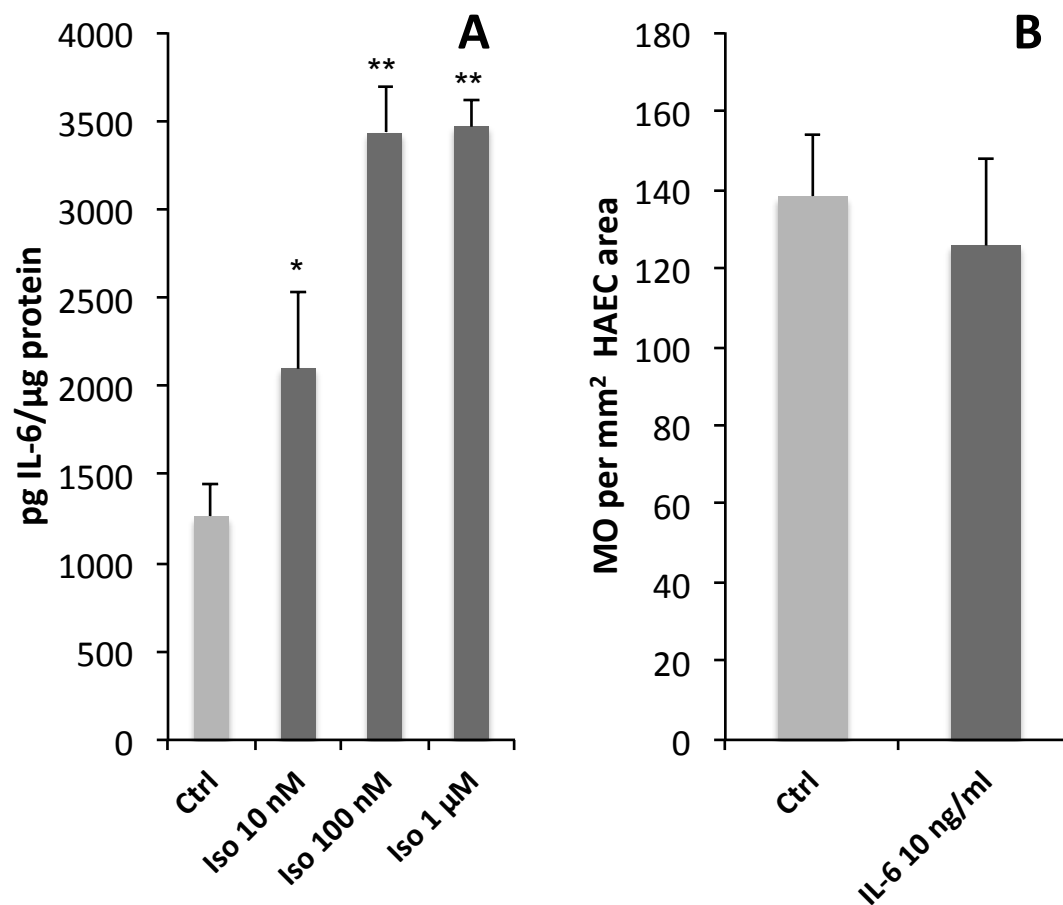
Figure 6. (A) Effect of catecholamine treatment on IL-6 production from HAEC. Treatment of HAEC with ISO increased IL-6 production in a dose-dependent fashion (*p < .05, **p < .01 ISO vs. control; three replicates per condition). (B) Effect of IL-6 on MO binding to HAEC. Pre-treatment of HAEC with 10 ng/ml IL-6 had no effect on MO adhesion to HAEC (four replicates per condition).
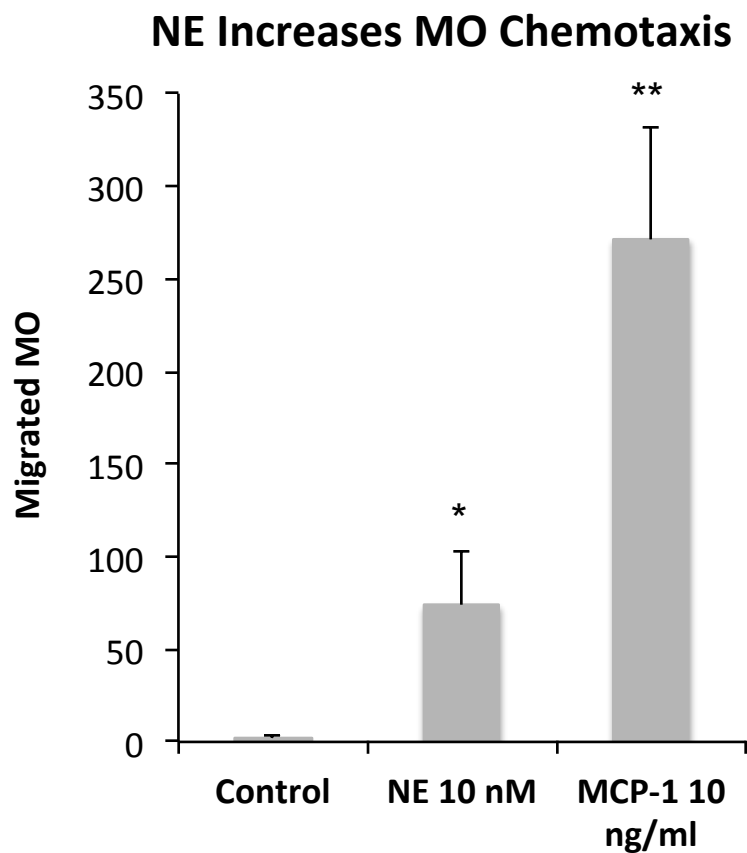
NE Increases MO Chemotaxis

Figure 7. NE is mildly chemotactic for monocytes. Data represent the number of monocytes that migrated into the membrane of a Transwell migration assay toward NE or Monocyte chemoattractive protein-1 (MCP-1) in the lower chamber (two to four replicates per group). Relative to negative control, 10 nM NE induced migration of MO into the membrane (*$p < .05$). MCP-1 served as a positive control for migration. (**$p < .01$).