ETHANOL AFFECTS VASCULAR ENDOTHELIAL GROWTH FACTOR AND ITS RECEPTORS IN CORONARY MICROVASCULAR ENDOTHELIAL CELLS

By

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ETHANOL AFFECTS VASCULAR ENDOTHELIAL GROWTH FACTOR AND ITS RECEPTORS IN CORONARY MICROVASCULAR ENDOTHELIAL CELLS

ABSTRACT

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Background and Purpose: Cardiovascular disease is the leading cause of death in the United States. Moderate alcohol consumption has a cardiovascular protective effect, part of which is mediated by positive effects on high density lipoproteins (HDL). However, these effects only account for about 50% of the cardiovascular protective effects of ethanol. The endothelium is a key player in development of atherosclerosis, and many endothelial functions are controlled by vascular endothelial growth factor (VEGF). Ethanol increases VEGF and its receptors in some systems, but the effects of ethanol on the VEGF system in endothelial cells are not known. Accordingly, the purpose of the study is to examine the influence of ethanol on the expression of VEGF and VEGF receptors (VEGFR) in porcine coronary microvascular endothelial cells (PCMEC). Methods: PCMEC were exposed to one of two ethanol concentrations or no ethanol as a control (0, 10, or 25 mM) and four durations (0.5, 2, 4, or 8 h). VEGF and VEGFR proteins were analyzed quantitatively with ELISA and Western Blot techniques, respectively. VEGFR proteins were also analyzed qualitatively with scanning laser confocal microscopy. Results: With increasing ethanol concentrations, protein levels of...
VEGF (p=0.006) and VEGFR-1 (p=0.0007) increased significantly, whereas VEGFR-2 decreased significantly (p=0.0492). Increasing duration of ethanol exposure caused VEGF protein levels to increase (p<0.0001), but had no effect on either VEGFR-1 or VEGFR-2 (p=0.564 and p=0.475, respectively). **Discussion:** We conclude that ethanol at physiologically relevant concentrations can increase VEGF and VEGFR-1 in PCMEC. VEGF and VEGFR-1 are involved in endothelial maintenance and repair, which can attenuate the atherosclerotic process, and may be involved in the cardiovascular protective effects of moderate alcohol consumption. **Keywords:** VEGF, VEGF receptors, ethanol, endothelial cells
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CHAPTÊR ONE
INTRODUCTION

INTRODUCTION

Cardiovascular disease, including coronary artery disease (CAD), cerebrovascular disease, and peripheral vascular disease, is the leading cause of death in the United States (3). Main contributors to cardiovascular disease morbidity and mortality include myocardial infarction and stroke due to atherosclerosis. Many studies have reported that moderate alcohol consumption decreases the risk of cardiovascular disease. Individuals who consume one to two drinks per day are at a lower risk of myocardial infarction (52, 88, 164, 183) and stroke (139) compared to both heavier drinkers (3 or more drinks per day), and those who abstain from alcohol.

Moderate alcohol consumption has been shown to inhibit the progression of atherosclerosis (43, 44), the disease process underlying myocardial infarction and stroke, but the mechanisms are not well established. Increased high-density lipoprotein (HDL) concentration (61, 74, 88, 91) accounts for approximately 50% of the cardiovascular protective effects attributable to moderate alcohol consumption (91, 129). Other known mechanisms that may explain part of the remaining 50% of the cardiovascular protective effects include an increase in fibrinolysis, which reduces fibrinous plaque formation and prevents blood clotting (1, 16, 133, 160), and suppression and inhibition of smooth muscle cell proliferation and migration (73, 102).

Ethanol has been reported to increase vascular endothelial growth factor (VEGF) expression in cultured smooth muscle cells (SMC) (68), and increase the expression of VEGF
and both VEGF receptor types (VEGFR-1 and VEGFR-2) in rat skeletal muscle (60). Because VEGF and the VEGF receptors are involved in many functions of the endothelium, they may be involved in part of the cardiovascular protective effect of ethanol consumption. VEGF promotes endothelial cell proliferation, migration, and differentiation (47, 108, 181). In addition, VEGF plays an important role in regulating other functions of the endothelium, including the control of blood flow, coagulation, fibrinolysis, vascular permeability, and angiogenesis (new blood vessel formation), many of which are important processes in the development and progression of atherosclerosis (19, 49, 55, 92, 120, 146, 181). Although atherosclerotic plaque progression is associated with increased VEGF expression (29, 34, 77), studies involving VEGF administration in vivo have demonstrated conflicting results. Both increased atherosclerotic plaque progression (26, 27) and decreased neointimal thickening of injured blood vessels (6, 166) have been reported with the administration of VEGF in vivo. Similar to VEGF, administration of ethanol to injured blood vessels has reduced neointimal area and decreased neointimal thickening (100, 101, 112).

Although changes in VEGF expression are important, changes in VEGF receptor expression could be equally important in mediating the effects of ethanol and VEGF on endothelial functions. Many endothelial processes are stimulated by VEGF ligand binding to one of two VEGF receptors, VEGFR-1 and VEGFR-2, found primarily on endothelial cells. VEGFR-2 stimulation by VEGF causes receptor auto-phosphorylation and results in endothelial cell proliferation, migration, differentiation, tube formation, increased vascular permeability, angiogenesis, and maintenance of vascular integrity (14, 63, 108). Although the role of VEGFR-1 is not well established, stimulation of VEGFR-1 may counteract the effects of VEGFR-2 as it has been reported to inhibit endothelial cell proliferation (86, 184). VEGFR-1 is up regulated
following endothelial denudation (172), indicating it may play a major role in endothelial repair and maintenance in response to vascular injury.

**STATEMENT OF PROBLEM**

Few studies have examined the effects of moderate ethanol on VEGF and the VEGF receptors (60, 68), and none have been reported in endothelial cells. Gu et al. (68) have reported that following a single exposure of moderate ethanol, VEGF protein increased in coronary artery vascular smooth muscle cells (CAVSMC), and ethanol increased VEGF mRNA expression and angiogenesis in chick chorioallantoic membranes. Gavin and Wagner (60) reported that VEGF, VEGFR-1, and VEGFR-2 expression in rat skeletal muscle were up regulated by moderate ethanol, however, skeletal muscle tissue contains many different cell types, and the effect of ethanol on the individual cell types was not examined. Increased VEGF levels have also been reported in rat gastric mucosa in response to ethanol-induced injury (82, 158).

Although all of the studies previously mentioned involve a single dose at one time point (with the exception of Gu et al. (68)), the cardiovascular protective effects of ethanol are observed in individuals who consume one to two drinks per day over a time course of years. Thus, these studies may have limited application to chronic ethanol exposure. Therefore, the purpose of the current study is to examine durational effects of two ethanol concentrations on VEGF and VEGF receptor proteins in porcine coronary microvascular endothelial cells (PCMEC). Porcine cells were chosen because pigs have many similarities to humans: the cardiovascular anatomy and physiology of pig hearts are similar to human hearts (76), pigs metabolize lipoproteins (39), and they spontaneously develop atherosclerotic plaque (150). Pigs are a good model to study the effects of alcohol as there is a conserved synteny between porcine
and human genes that encode alcohol dehydrogenases, enzymes that oxidize various alcohols (43).

**HYPOTHESES**

**HYPOTHESIS 1**

Ethanol exposure will cause an increase in VEGF protein in PCMEC in a time-dependent manner.

**Rationale:** Ethanol has been shown to upregulate VEGF in coronary artery vascular smooth muscle cells, CAM, and skeletal muscle. The single study on time effects of ethanol exposure on VEGF reported that VEGF protein increased with time (6 to 18 h) in coronary artery vascular smooth muscle cells (68). Based on this limited evidence, we expect VEGF protein to increase with ethanol exposure over time in endothelial cells as well.

**HYPOTHESIS 2**

Ethanol exposure will cause an increase in VEGFR-1 protein in PCMEC in a time-dependent manner.

**Rationale:** In the one published study involving the effects of ethanol on VEGF receptors, VEGFR-1 mRNA increased following 1.5 h of ethanol exposure in rat skeletal muscle (60). Because mRNA upregulation usually precedes protein synthesis, VEGFR-1 protein may have also increased, although it was not measured. Because there are no conflicting studies reported, we are led to hypothesize that VEGFR-1 will increase in the PCMEC to be used in this study. Although no studies have examined the effects of ethanol on VEGFR-1 in endothelial cells, nor have they examined multiple time points to help formulate a time-dependent
hypothesis, it is logical to expect that continued ethanol exposure may lead to an increase in VEGFR-1 protein expression in endothelial cells.

**Hypothesis 3**

Ethanol exposure will cause an increase in VEGFR-2 protein in PCMEC in a time-dependent manner.

**Rationale:** VEGFR-2 mRNA was increased following 1.5 h of ethanol exposure in rat skeletal muscle, in the only published study involving the effect of ethanol on VEGF receptors (60). Because increased protein expression usually follows mRNA upregulation, we hypothesize that ethanol will increase VEGFR-2 protein in our cells. No data exists to help formulate a time-dependent hypothesis or the effects of ethanol on VEGFR-2 in endothelial cells, but since VEGFR-2 protein is up regulated with ethanol in skeletal muscle that contains endothelial cells, it is reasonable to expect that continued ethanol exposure will result in enhanced expression of VEGFR-2 in endothelial cells.
CHAPTER TWO
REVIEW OF LITERATURE

BACKGROUND

Moderate ethanol consumption has positive effects on the cardiovascular system. One to two drinks per day reduces the risk of cardiovascular disease, including myocardial infarction, stroke, and ischemia-reperfusion injury (52, 114, 129, 139, 164, 183). Several known factors and mechanisms are involved in these processes, but this study will focus on the unknown effects of ethanol on endothelial cells, VEGF, and the VEGF receptors. VEGF and its receptors are involved in the control of many physiological and pathological endothelial functions, such as vascular permeability, blood flow, coagulation, fibrinolysis, angiogenesis, and atherosclerosis (19, 27, 49, 120, 146, 181). VEGF mRNA and protein levels are increased by ethanol (60, 68, 82, 83, 158), and these ethanol-induced changes in VEGF expression may play a role in the cardiovascular protective effects of moderate ethanol consumption. In this chapter, the discussion will focus on the individual effects of ethanol, VEGF, VEGF receptors, and endothelial cells in physiological and pathological conditions and the importance of their interactions in endothelial functions.

ETHANOL

Alcohol consumption has many complex physiological effects on the body. Because ethanol is both water-soluble and lipid soluble, it can be distributed into lipid membranes and the cytoplasm of all cells in the body (38). Heavy drinking and binge drinking have harmful effects that include increased risk of mental problems, liver injury, pancreatitis, hypertension,
cardiovascular disease, and many cancers (2, 40, 127, 164), thereby increasing overall morbidity and mortality. However, moderate alcohol consumption has cardiovascular protective effects (52, 88, 168).

**CARDIOVASCULAR PROTECTIVE ROLE OF ETHANOL**

Epidemiological studies suggest a U- or J-shaped association between alcohol consumption and myocardial infarction and ischemic stroke (52, 88, 139, 168). Low to moderate alcohol consumption of 1-2 drinks per day (<50 g/day) has a cardiovascular protective effect of reduced myocardial infarction and stroke that is greater than seen in either abstinence or heavy alcohol consumption (>100 g/day) (88, 139). One standard drink contains approximately 12 g ethanol, which is provided by 360 ml of beer, 150 ml of wine, or 45 ml of liquor (68, 183).

One potential mechanism for the cardiovascular protective effects of ethanol is to inhibit the initiation and progression of atherosclerosis. Emeson et al. (45) examined atherosclerotic lesions in C57BL/6 female mice (hyperlipidemic mice that develop atherosclerotic lesions and willingly drink alcohol) fed high- or low-fat diets containing 0%, 3%, or 6% ethanol. There was a dose-related ethanol inhibition of atherosclerotic lesion development in mice fed high-fat diets when measuring mean total area (\(\mu m^2 \times 10^3\)) of fatty streak formation (0% ethanol, 12.6; 3% ethanol, 5.9; 6% ethanol, 2.0), whereas no atherosclerotic lesions were observed in mice fed low-fat diets. In another study, mice were fed a high-fat liquid diet containing either 0% or 6% alcohol for 24 or 48 weeks, with another group of mice consuming 0% alcohol for the first 24 weeks and 6% alcohol from weeks 24 to 48 (44). Aortas of mice fed 0% ethanol had fatty streak formation (initial formation of atherosclerotic lesions) at 24 weeks, which increased 2.45 fold by 48 weeks. Mice that consumed 6% ethanol diets had smaller lesions, approximately one-fourth
that of controls at 24 weeks, and one-eighth that of controls at 48 weeks. The mice that consumed 0% ethanol for the first 24 weeks and 6% ethanol from weeks 24 to 48 had fatty streaks at 24 weeks, but these lesions stabilized and did not progress once ethanol consumption began. Such cardiovascular protective effects of ethanol to inhibit atherosclerotic plaque progression may involve changes in plasma lipids, especially elevated serum HDL cholesterol concentrations (61, 74, 88, 91), inhibited or suppressed smooth muscle cell (SMC) proliferation and migration (73, 102), or increases in fibrinolysis (1, 16, 133, 160).

**HIGH DENSITY LIPOPROTEIN**

HDL concentration is negatively associated with a risk of coronary heart disease. Gaziano et al. (61) demonstrated a significant inverse relation between alcohol consumption and risk of myocardial infarction, with a positive association between alcohol consumption and HDL and its subfractions (HDL$_2$ and HDL$_3$). In this study, and many others, the increase in total HDL was associated with a reduced risk of myocardial infarction. Similarly, Keichl et al. (88) demonstrated that moderate alcohol consumption reduced the risk of carotid atherosclerosis compared to both non drinkers and heavy drinkers, and the moderate alcohol consumption was associated with elevated HDL cholesterol and a decrease in other lipoproteins. In a study of squirrel monkeys, Hojnacki et al. (74) demonstrated that moderate ethanol consumption (12% of dietary caloric intake) increased HDL, whereas higher doses of ethanol increased both HDL and LDL.
SMOOTH MUSCLE CELL EFFECTS

SMC proliferation and migration from the media to the intima of blood vessels are key events in intimal thickening leading to atherosclerosis, and ethanol has been shown to inhibit these events (73, 102). In one study, human subjects were fasted overnight and given a high fat meal (68% fat) with 0.5 g ethanol/kg body weight (corresponding to approximately 3 cans of beer) on one day, or without ethanol on a different day. Following the meal, blood was drawn every 30 min for 5 h, and hourly thereafter, and SMC proliferation was determined. SMC proliferation following ethanol-containing meals was reduced at each time point compared to control meals (102). Hendrickson et al. (73) examined SMC migration in cultured human umbilical vein SMC, and reported that ethanol pretreatment inhibited SMC migration in a dose-dependent manner in static cultures (maximal inhibition of 60.8% observed at 40-80 mM ethanol). SMC migration was also inhibited in pulsatile-flow SMC cultures, which is a better mimic of in vivo conditions.

CLOTTING MECHANISMS

Increased fibrinolytic activity and decreased platelet aggregation may also explain part of the cardioprotective effects of moderate ethanol consumption (1, 16, 133, 160). Tabengwa et al. (160) reported that human umbilical vein endothelial cells exposed to low ethanol concentrations (0.1% v/v) showed increased expression of urokinase-type plasminogen activator receptors (u-PAR). These receptors are involved in increasing surface-localized fibrinolytic activity, which may be cardioprotective. In addition, ethanol suppresses plasminogen activator inhibitor type-1 (PAI-1), an inhibitor of fibrinolysis, which may also increase fibrinolysis and reduce the risk of cardiovascular disease (65) by decreasing fibrin deposits and the associated thrombotic events.
that may lead to myocardial infarction and stroke. However, heavy alcohol consumption and binge drinking (>50 g alcohol/day) have been reported to inhibit fibrinolysis, which may lead to fibrin deposits on the intima and their incorporation into plaque growth, increasing the risk of cardiovascular disease (168).

Although increased HDL, decreased SMC proliferation and migration, and increased fibrinolysis explain some of the cardiovascular protective mechanisms of ethanol consumption, they do not explain the entire effect. Ethanol is absorbed rapidly through mucosal membranes into the blood stream (117). Consequently, one of the first cells to be affected by ethanol is the endothelial lining of blood vessels. Endothelial cells are involved in many physiological and pathological processes in the body, including atherosclerosis (30, 115). Ethanol exposure may affect endothelial functions, and could potentially be involved in some of the cardiovascular protective effects of ethanol consumption. The next sections will discuss many of the functions of endothelial cells, and the role of VEGF in endothelial function. Interactions of ethanol, VEGF, and endothelial cells will be discussed in later sections.

**Endothelial Cells**

Endothelial cells line all blood vessels. It is estimated that in a normal adult human endothelial cells have a surface area of >1000 m² (106). Both in vivo and in vitro, endothelial cells form a single cell layer often described as "cobblestone" in appearance, which may be a criterion for identification, although it is not a strong identification marker (71, 106, 141). Endothelial cells are the only cells in the body that are exposed to both a moving fluid under pressure (blood) and a solid substrate (blood vessel wall) (71, 106). In vivo, the lumenal side of endothelial cells is exposed to blood, and the ablumenal side is exposed to interstitial fluid and
extracellular matrix (106). In healthy subjects, endothelial cells maintain a flat epithelioid geometry that helps to prevent turbulent blood flow through the blood vessels (15) and generates an antithrombotic surface (30).

**Structure**

The endothelial cell is composed of many different structures, including a round-ellipsoid nucleus that is surrounded by a dense perinuclear cytoplasm. This perinuclear cytoplasm contains many organelles, and consists of thin sheets that "interlock" with neighboring cells to form a barrier between the blood and tissues (71). Endothelial cells are also quite rich in membrane-bound organelles, vesicular and tubular membranous structures, and free ribosomes (79). An organelle that is unique to the endothelial cell is the Weibel-Palade body (177), which has been identified in human, pig, rat, and mouse endothelium (79). Weibel-Palade bodies are specific markers for endothelial cells (111, 173), but may not be present in all endothelial cells (81). They may function as storage and processing organelles for von Willebrand factor (vWF) (173). Pinocytotic vesicles, coated pits, and coated vesicles are also found in endothelial cells (71). The cytoplasm of the endothelial cell contains three types of cytoskeletal filaments: actin-containing microfilaments, intermediate filaments, and microtubules (15). These structures are involved in cell movement, transport of organelles, and maintenance of cell shape and attachment (15). Cultured endothelial cells retain a number of these in vivo structural properties making them suitable for in vitro studies (71).

Endothelial cells also have many membrane receptors that are involved in endothelial cell functions. Some of the membrane receptors found on the endothelium that relate to the present
study include vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), CD31+, CD36+, vWF, VEGFR-1, and VEGFR-2.

**IDENTIFICATION OF ENDOTHELIAL CELLS**

There are three criteria commonly used to positively identify cultured endothelial cells: 1) contact inhibition of growth at confluence resulting in a monolayer of endothelial cells; 2) indirect immunofluorescence with vWF (factor VIII related antibody); and 3) specific uptake of acetylated low-density lipoprotein (106, 141). Other characteristics of cultured endothelial cells may include CD31+ and CD34+ markers, angiotensin converting enzyme (ACE), endothelial nitric oxide synthase (eNOS) secretion, prostacyclin secretion, cobblestone appearance at confluence, and “tube” formation in Matrigel (an artificial extracellular surface containing laminin and collagen type IV to which endothelial cells can adhere) (106, 141).

**ENDOTHELIAL CELL FUNCTION**

For many years endothelial cells were thought to be a passive layer of cells separating blood and tissue. Then in the early 1980’s it was reported that relaxation of isolated arteries induced by acetylcholine depended on the presence of endothelial cells (57). It is now known that endothelial cells are involved in many physiological and pathological processes, some of which include blood vessel barrier function and permeability, coagulation, fibrinolysis, blood flow regulation, and angiogenesis (4, 9, 25, 46, 115, 119, 147, 161). Due to its location in the body, the endothelium is the first component to be exposed to the blood and its many factors, including hormones, growth factors, other components secreted by cells and tissues in the body, as well as ethanol and other substances introduced into the blood (104). VEGF is directly or
indirectly involved in many endothelial functions, and its effects will be included in the following sections.

**Blood Barrier/Vascular Permeability**

Endothelial cells form a barrier between blood and other tissues that is important in regulating movement of fluids and solutes from the blood into the tissue (104). This barrier varies in structure and function depending on the organs in which the cells are found. For example, the cerebral microvasculature endothelium has tight junctions between endothelial cells that form the blood-brain barrier, whereas the hepatic endothelium has large fenestrations that allow solutes to easily pass through (141). Many factors affect vascular permeability and barrier function, including VEGF, which has been implicated as one of the most potent regulators of vascular permeability (42). VEGF was first isolated in 1983 as a factor responsible for the increased vascular permeability of tumors, and consequently was named vascular permeability factor (49). Increased vascular permeability can be beneficial or detrimental. Hyperpermeability can lead to pathological conditions such as edema, atherosclerosis, tumor metastasis, and other diseases, many of which are associated with elevated levels of VEGF. For example, Kawasaki disease is an acute systemic vasculitis that affects children and results in elevated VEGF levels compared to normal children (163). Another condition is POEMS (Crow-Fukase) syndrome, where patients have general edema due to hyperpermeability. This may be due to increased VEGF levels, as Watanabe et al. (176) reported that patients with this syndrome had VEGF levels more than 10-fold higher than the levels found in healthy individuals.

Increased vascular permeability is also important for endothelial cell migration during angiogenesis (9). During this time, the junctions between cells are loosened when contraction
processes occur in the margins of cells (170). Gaps may form allowing substances such as plasma proteins to leak into the extravascular space leading to matrix formation that supports blood vessel growth (171). In tumors, blood vessel growth must occur for the tumor to grow to more than 2-3 mm in diameter (53). The new blood vessels that are formed are “leaky”, and it is believed that this hyperpermeability of the new tumor blood vessels allows tumor cells to pass into the bloodstream, promoting tumor metastasis (42). In atherosclerosis, increased permeability allows enhanced passage of LDL across the endothelium and thus promotes accumulation of LDL in the arterial wall, which are early steps in the pathological process of atherosclerosis (122). This will be discussed in greater detail in future sections.

Vasoregulation, Coagulation, and Fibrinolysis

Endothelial cells play an important role in regulating blood flow. In response to specific stimuli such as hypoxia, growth factors, and cytokines, SMC and endothelial cells produce VEGF and other factors that stimulate endothelial cells to secrete substances affecting blood flow (182). Vasoactive substances released by endothelial cells stimulate SMC that are located in juxtaposition to the endothelial cells, leading to vasodilation or vasoconstriction of the blood vessels (56). Vasodilators secreted from endothelial cells include, but are not limited to, nitric oxide, acetylcholine, prostacyclin, and endothelium-derived hyperpolarizing factors (56, 115, 121). Vasoconstrictors are also released from the endothelium, including prostaglandins, reactive oxygen species, and endothelin (56, 115). Aging and injury to endothelial cells can lead to dysfunction of blood flow regulation. Inability of the blood vessels to fully vasodilate can be caused by reduced secretion of vasodilators, increased secretion of vasoconstrictors, or an abnormal smooth muscle cell response to the vasoactive substances (115). This can lead to
hypertension, which can then cause additional injury to endothelial cells (104), and to a compromised ability to regulate blood flow properly.

The endothelium is also responsible for regulating blood coagulation and fibrinolysis. Resting endothelial cells normally generate an active antithrombotic surface (30), but inflammation or injury to the endothelium increases expression of VEGF. VEGF, and other factors, stimulate endothelial cells to increase adhesion molecules (89), tissue factor, and thrombomodulin (22, 23), activating the coagulation cascade (189). Fibrinogen in blood plasma is acted on by thrombin to produce fibrin, which forms the fibrous meshwork of blood clots to help seal damaged blood vessels, providing an ideal matrix for new microvessel growth during wound healing (169). Although coagulation and fibrin formation is important in wound healing, fibrin formation within blood vessels can lead to blood vessel occlusion. To help prevent this, the endothelium can synthesize, store, and bind fibrinolytic proteins (92). Endothelial cells exposed to fibrin clots increase secretion of tissue plasminogen activator (tPA) and decrease PAI-1 to increase fibrinolytic activity (55). Endothelial cells also secrete urokinase plasminogen activator (uPA), and express both tPA and uPA receptors (tPAR and uPAR) (70, 137). VEGF may be involved in the fibrinolytic process as well, as VEGF stimulates tPA, uPA, PAI-1, and uPAR (107, 130). Obviously, the balance between coagulation and fibrinolysis is an important function of the endothelium.

ANGIOGENESIS AND VASCULOGENESIS

Formation of new blood vessels occurs in many different situations. Vasculogenesis is the process of endothelial cell differentiation and development of a primitive vascular system in developing embryos, whereas angiogenesis is the development of new capillaries from pre-
existing blood vessels (134). VEGF plays a critical role in both vasculogenesis and angiogenesis. For example, if one or both VEGF alleles are absent, embryos will die during gestation due to abnormal blood vessel formation (24, 48). In adults, angiogenesis most commonly occurs in the female reproductive system (148), during wound healing and tissue repair (110), and with exercise training in skeletal muscle (18). Pathological angiogenesis also occurs in ischemic conditions such as those found in tumors, myocardial ischemia, diabetic retinopathy, atherosclerosis, and rheumatoid arthritis (37, 54, 78).

VEGF is involved in several steps required for angiogenesis. Initially, VEGF increases permeability of the endothelium (132), allowing increased flux of plasma proteins into the extravascular space. This leads to clotting of extravasated fibrinogen, which provides a matrix for new blood vessel growth (42). VEGF also enhances endothelial cell proliferation and migration, which are followed by lumen formation and basement membrane production (37). Growth and expansion of tissue masses beyond a diameter of 2-3 mm requires angiogenesis (53), which is important in tumor growth and other pathological conditions. VEGF is involved in regulating many of these processes.

Elevated VEGF expression and greater microvascular density are association with tumor growth and expansion in more advanced tumors (62, 144). Increased vascular density is usually an indication of increased potential for metastasis and decreased survival rates in many cancers including bladder (144), prostate, lung, stomach, ovary, cervix, and squamous cell carcinoma of the head and neck (185). New blood vessels are highly permeable, with little basement membrane and fewer intercellular junctional complexes than normal vessels, which provides a means of passage for tumor cells to enter the bloodstream and metastasize (42). One exception to this was reported by Ogawa et al. (125), where women in early stage (stage I or II) clear cell
ovarian carcinoma with high microvascular density had significantly better progression-free survival. In this case, it was suggested that angiogenesis might augment the response to adjuvant therapy.

The general response to VEGF administration to tissue or cells is increased endothelial cell proliferation, migration, tube formation, and/or blood vessel formation. In rats, administration of 4.8 or 480 pM of recombinant human VEGF (rhVEGF) by intraperitoneal injection twice daily for 5 days significantly increased vascularized area and total microvascular length in the mesentery compared to controls (124). Yue et al. (180) reported that embryonic quail hearts that were incubated with increasing concentrations of VEGF$_{165}$ (5-20 ng/ml) for 48 h had more individual endothelial cells and tube formation compared to control hearts. Total tube length and diameter in the quail hearts also increased with higher VEGF$_{165}$ concentrations (50-100 ng/ml). In a different study, 13-day old chick chorioallantoic membranes (CAM) showed angiogenic effects of 0.5, 1, 2, 3, or 4 µg of VEGF$_{165}$ placed on a Thermonox tissue cover slip and then applied to the CAM (178). Weak angiogenic effects (brush-like capillary formation) of 0.5 µg of VEGF$_{165}$ could be seen with a stereomicroscope 3 days after application, with stronger effects (more brush-like capillary formation) seen with increasing doses of VEGF$_{165}$ (178). It was concluded that the new capillary formation was due to VEGF$_{165}$ and not just normal growth of blood vessels.

VEGF has been proposed as a therapeutic agent due to its beneficial effects (increased capillary or collateral development) in patients with ischemic heart disease who are poor candidates for mechanical revascularization or bypass surgery (72). Hendel et al. (72) administered low-dose (0.005 or 0.017 µg/kg) or high-dose (0.05 or 0.17 µg/kg) rhVEGF via coronary injection in subjects with known severe coronary artery disease. Single Photon
Emission Computed Tomography (SPECT) imaging was performed at rest or during stress (exercise-, dobutamine-, or dipyridamole-induced stress) at baseline, and 30 and 60 days after rhVEGF administration. Dose-related improvements were observed as high doses were associated with improved myocardial perfusion and improvements in collateral count density, whereas no significant change was observed with low doses (72).

VEGF administration may also have detrimental effects. Using a different method of VEGF administration, Lee et al. (95) implanted primary murine myoblasts expressing murine VEGF and β-galactosidase into the ventricular wall of 8-week-old male mice, while control mice were injected with myoblasts that expressed β-galactosidase gene only. All of the surviving mice in the VEGF-treated group had vascular tumors that replaced the myocardium or extended through the endocardium and protruded into the cavity, whereas control mice had normal myocardium (95). This study shows that overexpression of VEGF in non-ischemic hearts can lead to vascular tumors near the implantation site, and supports the idea that VEGF administration may lead to adverse side effects of inappropriate blood vessel growth, especially in individuals at risk of cancer or diabetic retinopathy (175).

Just as VEGF administration can induce angiogenic processes, blocking or preventing VEGF production can impair angiogenesis. Maes et al. (105) compared bone formation of wild-type mice with VEGF\(^{120/120}\) mice. VEGF\(^{120/120}\) mice express exclusively the VEGF\(_{120}\) isoform, and do not express VEGF\(_{164}\) and VEGF\(_{188}\) isoforms (equivalent to human VEGF\(_{121}\), VEGF\(_{165}\), and VEGF\(_{189}\) isoforms, respectively). Long bones of VEGF\(^{120/120}\) mice were shorter and thinner than those in wild-type mice due to disturbed vascularization. Bone blood vessel diameter was larger and blood vessel density was reduced in the VEGF\(^{120/120}\) mice (105). Another study compared collateral vessel development in surgically induced ischemic limbs of C57 wild-type
mice versus apoE<sup>-/-</sup> mice (mice with an inherited deficiency of apoE lipoprotein that leads to hypercholesterolemia) (35). ApoE<sup>-/-</sup> mice had a slowed restoration of blood flow to the ischemic limb and reduced collateral vessel development after surgery compared with C57 mice, effects which were associated with reduced VEGF protein in the ischemic limbs of apoE<sup>-/-</sup> mice compared with C57 control mice (35).

In addition to VEGF, many other factors are involved in initiating angiogenesis in physiological or pathological conditions. Some other endogenous factors that promote physiological and pathological angiogenesis, as reviewed in Rubanyi (138), include, but are not limited to:

- Adenosine
- IGF-1
- Hormones
- Estrogens
- Follistatin
- Proliferin
- Interleukin 8
- Erythropoietin
- Angiogenin
- Scatter factor
- Acidic fibroblast growth factor
- Basic fibroblast growth factor
- Transforming growth factor-alpha and -beta
- Tumor necrosis factor-alpha
- Monocyte chemoattractant protein 1
- Platelet-derived endothelial cell growth factor
- Granulocyte-macrophage colony-stimulating factor
- Prostaglandins E1, E2
- Cytokines and chemokines
- Peptide growth factors
- Hematopoietic growth factors

ATHEROSCLEROSIS

The atherosclerotic process is associated with several VEGF-induced endothelial functions, such as increased cellular adhesion molecules, increased vascular permeability, and angiogenesis. Briefly, atherosclerosis may occur in the following manner, beginning when the endothelium is injured by mechanical stress, carbon monoxide, inflammation and infection, hypertension, cholesterol, high blood glucose, or toxic chemicals, among other factors (104). Endothelial cells express binding sites for monocytes (VCAM-1) and secrete monocyte chemotactic agents. Monocytes attach to the injured endothelial cells, then migrate into the
intima where they are converted into macrophages. Increased blood vessel permeability allows enhanced passage of LDL across the endothelium into the interstitial space of the intima, where the macrophages oxidize LDL to form foam cells (122). Subsequently, SMC migrate into the intima and divide, and these cells secrete collagen and other proteins involved in atherosclerosis (162). Mature plaques, which are composed of foam cells, proteins, SMC, macrophages, lymphocytes, cholesterol and cellular debris, then develop (64). Continued build-up of this plaque can occlude blood vessels completely, and unstable plaque can break off to form an embolus that can also occlude a blood vessel further downstream leading to stroke or myocardial infarction.

Atherosclerotic plaque, like tumors and other tissue, needs a vascular supply in order to increase in size (186). VEGF is thought to be involved in atherosclerotic plaque growth and development, and increased vascularity has been associated with increased VEGF in atherosclerotic plaque (29, 34, 77). Chen et al. (29) studied vascularity and VEGF expression in atherosclerotic lesions in human coronary arteries obtained at autopsy within 16 h of death. Coronary arteries were classified according to atherosclerotic lesion type (normal with diffuse intimal thickening, and progressing from early type I to advanced type VI) as defined by the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association (153-155). Normal coronary arteries showed no intimal neovascularization, whereas in atherosclerotic lesions, the more advanced the atherosclerotic lesion (from type I to type VI) the more often the neointimas contained newly formed blood vessels (29). Although VEGF-positive cells were observed in all coronary artery sections, including normal arteries, greater numbers of VEGF positive cells were observed in the more advanced lesions, and the number of VEGF positive cells correlated well (p<0.0001) with the degree of intimal vascularity. Couffinhal et al. 
examined VEGF expression in normal and atherosclerotic human arteries, including thoracic aorta, saphenous vein, and internal mammary artery segments, as well as in tissue samples from coronary arterial lesions that were retrieved during directional atherectomy.

VEGF was detected in 97% of the atherectomy specimens examined, and the VEGF immunostaining was more extensive in the pathological specimens compared with the normal vessel specimens. Inoue et al. (77) also examined human coronary artery segments harvested during autopsy within 3 hours after death. Arteries with diffuse intimal thickening had virtually no staining for VEGF in endothelial cells or SMC, and only occasional staining for VEGF in macrophages. Early atherosclerotic lesions with hypercellularity showed some VEGF-positive staining in macrophages, endothelial cells, and SMC (77). Advanced atherosclerotic plaques showed extensive VEGF staining in endothelial cells and macrophages of the microvessels, with virtually no staining of SMC. Overall, hypercellular atherosclerotic lesions and atheromatous plaques had significantly higher VEGF-positive cell area (24-29%) than coronary arteries with diffuse intimal thickening (77). These studies suggest that VEGF is important in atherosclerotic plaque development and progression.

Because of the strong correlation of VEGF, microvasculature, and atherosclerotic lesions, many studies suggest important roles for angiogenesis and VEGF in the progression or inhibition of atherosclerotic plaque (6, 7, 26, 27, 116, 166, 167). Moulton et al. (116) reported that subcutaneous injections of the angiogenesis inhibitors endostatin or TNP-470 (a selective inhibitor of endothelial cell proliferation and migration) reduced the amount of neovascularization and atherosclerotic plaque formation in mouse aorta. Intimal neovascularization and increased numbers of capillaries and endothelial cells were observed in advanced atherosclerotic lesions in control mice, and treatment for 16 weeks with endostatin or
TNP-470 inhibited plaque growth by 85% and 70%, respectively. Percentages of plaques containing any intimal vessels was also significantly lower in treated mice compared with control mice (116). This result raises the question: If anti-angiogenic factors reduce or inhibit atherosclerosis, does administration of angiogenic factors increase atherosclerosis progression (78)? Celletti et al. (27) reported that compared to control animals, a single intraperitoneal injection (2 µg/kg) of rhVEGF165 in mice doubly deficient in apolipoprotein E and apolipoprotein B100 resulted in a 14-fold increase in cross-sectional plaque area, a 5-fold increase in maximal plaque thickness, and less uniform (thus, less stable) plaques in thoracic aortas. Increased CD34+/flk-1 cells, and increased bone marrow and circulating macrophages/monocytes, which are potential atherogenic macrophage precursors, were also observed in rhVEGF-treated rats (27). Treatment with rh-VEGF also increased endothelial staining for the endothelial cell markers vWF and CD31+, indicating a higher vascularity of lesions in the rhVEGF group compared to controls. In another study by this group (26), a single intramuscular injection of rhVEGF (2 µg/kg) to New Zealand White rabbits, significantly increased aortic mean plaque area (23-fold increase), circumferential plaque extension (5.2-fold increase), maximal plaque thickness (2.75-fold increase), and total CD31+ positive area compared to control rabbits. Lazarous et al. (94) reported that dogs that underwent unilateral balloon denudation of the iliofemoral artery had greater percent luminal stenosis (50%) following administration of 720 µg VEGF per day for 7 days compared with control dogs (22%), suggesting that VEGF administration exacerbated the neointimal response to vascular injury (94).

In contrast, Asahara et al. (6) found that VEGF administration did not accelerate atherosclerosis in rats. Deendothelialization injury of the left common carotid artery was
performed on rats using an inflated balloon, and immediately after injury, 100 µg VEGF or control saline solution was administered to the artery. Neointimal thickening was significantly reduced with VEGF treatment compared to control, as intima-to-media ratio (I/M) was less at 2 weeks (I/M 1.23 in controls, compared to 0.81 in VEGF-treated rats) and 4 weeks (I/M 1.60 in controls, compared to 0.86 in VEGF-treated rats) (6). Two weeks after injury, the reendothelialization of the injured vessel was accelerated in the VEGF-treated rats compared with the control rats (percentage of reendothelialized area, 80.7% and 44.0%, respectively). Accelerated reendothelialization continued 4 weeks after injury in VEGF-treated compared to control rats (94.9% and 75.6% percentage of reendothelialized area, respectively). Endothelial cell proliferative activity was also significantly higher in control rats compared to VEGF-treated rats. In a different study conducted by this group (7), male New Zealand White rabbits underwent balloon injury and transfection with 400 µg of phVEGF\textsubscript{165} plasmid DNA or plasmid pGSVLacZ control in the femoral artery. LacZ-transfected arteries developed progressive luminal diameter narrowing (55%) and neointimal thickening through 4 weeks post injury. VEGF-transfected arteries, however, had reduced luminal diameter narrowing (approximately 23%) and significantly less intimal thickening compared to controls (7). Thrombotic occlusion was also significantly less in VEGF-transfected rabbits (3.1%) compared with control LacZ-transfected rabbits (21.9%). Reendothelialization of the balloon-injured femoral artery was nearly complete by 7 days in the VEGF-transfected arteries, whereas LacZ-transfected arteries were <50% complete. LacZ-transfected arteries had impaired vasomotor response to endothelium-dependent agents 4 weeks after injury, whereas VEGF-transfected arteries had near-normal endothelium-dependent vasoreactivity within 1 week of injury, indicating greater recovery of physiological functions. These studies suggest that VEGF may be important in
maintenance and repair of injured endothelium, which may be necessary for normal endothelial function and inhibition of neointimal formation.

Effects of VEGF administration on stent endothelialization (167) and intimal formation have also been examined (166). In both studies, New Zealand White rabbits underwent balloon-injury of the external iliac artery followed by metallic stent implantation. Rabbits received nothing (controls) or either purified protein rhVEGF$_{165}$ (100 µg) in PBS or PBS alone administered via local catheter (166, 167). In one study, stent endothelialization was significantly accelerated with VEGF administration compared to PBS alone or controls at day 4 (48% vs. 6.6% vs. <20%) and day 7 (91% vs. 29% vs. <40%). The percentage of stent surface covered with thrombus was also decreased with VEGF delivery compared to PBS delivery (167). To follow up on this study, Van Belle et al. (166) examined intimal formation following stent implantation. At 4 weeks following arterial injury, maximal intimal area within the stent was significantly lower in the VEGF group compared to either the PBS or control groups (0.87 mm$^2$, 1.47 mm$^2$, and 1.44 mm$^2$, respectively). Maximal percent of cross-sectional area narrowing was also significantly lower in the VEGF group compared to PBS or control groups (19%, 34%, and 32%, respectively). These studies contradict the hypothesis that VEGF administration increases atherosclerotic plaque formation, and support a role of VEGF in the maintenance and repair of the endothelium. Many factors may affect the response of VEGF administration, such as the vessel examined, the amount and source of VEGF being administered, vessel status (injured or non-injured), species, and the balance of VEGF with other endothelial or neovascularization growth factors.

Another benefit of VEGF administration may be attenuation of myocardial ischemia-reperfusion injury. Ischemia-reperfusion injury mainly occurs with a clot that subsequently
dissolves, or during cardiac surgery, where ischemia occurs before and during a procedure, and reperfusion following surgery can lead to endothelial or mechanical dysfunction (17). For example, rat hearts rendered ischemic for 120 min, followed by reperfusion for 40 min that were infused with VEGF every 30 min throughout the ischemic period had significantly improved recovery of cardiac output, coronary flow, and stroke work, as well as significantly less creatine kinase release (an indicator of cardiomyocyte damage) compared to control hearts (103).

Studies have also been conducted using similar models (100, 101, 112), but with ethanol administered following vascular injury rather than VEGF, with results similar to those of Asahara et al. (6, 7) and Van Belle et al. (166, 167). Balloon denudation of both iliac arteries was performed in New Zealand white rabbits, and 10% or 15% v/v alcohol solution was administered to one iliac artery, with the other artery serving as an untreated control (101). In arteries treated with 10% and 15% ethanol, mean neointimal areas were reduced by 43% and 55%, respectively, and they showed less neointimal thickening compared to control arteries (101). Liu et al. (103) performed balloon catheter overstretch injury in coronary arteries of domestic pigs, and one coronary artery received local delivery of 15% ethanol solution, whereas the other coronary artery served as a control. Average thrombus thickness and neointimal area at the site of injury were significantly reduced in ethanol treated vessels compared to controls. The number of proliferating SMC was also significantly reduced with ethanol-treatment compared with controls (100). The effect of ethanol consumption on atherosclerotic progression was studied in New Zealand white rabbits (112) given water (control) or an alcohol solution (2.5 ml alcohol in 500 ml water) for six days before and 10 weeks after balloon endothelial denudation of iliac arteries (112). Angioplasty was then performed at the site of greatest atherosclerotic narrowing 14 days after endothelial denudation, and eight weeks after angioplasty, rabbits were
killed and examined for neointimal formation. Neointimal area was significantly reduced by an average of 26% in the alcohol-treated group compared to the control group, and the lumen to neointimal ratio was twofold greater in alcohol-treated group compared to the control group (112). These similarities to the results of VEGF treatment suggest that there may be some relation between ethanol and VEGF in endothelial function.

**Vascular Endothelial Growth Factor**

VEGF is a 45 kDa basic, heparin binding, homodimeric glycoprotein (181) that is related to platelet-derived growth factor (PDGF) and the family of fibroblast growth factors (FGF). However, VEGF is distinct from the other growth factors, as it not only can stimulate endothelial cell growth but also can modify endothelial vascular permeability and has procoagulant activity (31). As discussed above, many physiological and pathological processes depend on VEGF expression.

**Function**

VEGF has several functions. It modulates expression of adhesion molecules on the surface of endothelial cells (87, 89), increases endothelial cell proliferation and migration, and promotes angiogenesis in vivo and in vitro (49, 136, 181). It was once thought that VEGF did not affect other cell types (50), however, this is now known not to be true. VEGF targets retinal epithelial cells (152), trophoblasts (36), and cells of the central nervous system, including astrocytes and neurons (157). It is also important in the differentiation of hypertrophic chondrocytes, osteoblasts, and osteoclasts involved in bone formation (105).
**Structure and Homologues**

Six different isoforms can be generated from alternative splicing of the VEGF gene, with these isoforms in humans containing 121, 145, 165, 183, 189, and 206 amino acids, respectively, (96, 97, 120, 136). VEGF\(_{165}\) is the most common form produced by normal cells (181) and is the most biologically active form, due to its abundance in both physiological and pathological processes (120). The VEGF\(_{165}\) isoform is a heparin-binding, homodimeric glycoprotein (181), whereas the other isoforms have slight differences in structure. VEGF\(_{121}\) and VEGF\(_{165}\) lack exon 6, whereas VEGF\(_{121}\) and VEGF\(_{145}\) lack exon 7 (136, 181). These three VEGF isoforms (121, 145, and 165) are secreted and form dimeric proteins, whereas the other two isoforms (189 and 206) are believed to remain sequestered in the pericellular matrix when they are produced (181). Members of the VEGF/platelet-derived growth factor (PDGF) family have other homologues, including VEGF-A (commonly referred to as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF) (31). These homologues have similar structure and share a significant amino acid sequence homology to VEGF (136).

**Location**

VEGF expression has been identified in healthy blood vessels throughout the body, including vessels in the cerebrum (51, 84, 118, 174), bone (105), heart (99), and skeletal muscle (60). VEGF expression has also been found in atherosclerotic plaques (29, 34, 77), several vascular neoplasms, and many other tumors (125, 144). VEGF is mainly synthesized in SMC (34) and diffuses to the adjacent endothelium to modulate endothelial function. Although it was initially thought that endothelial cells do not express VEGF, subsequent studies have shown that
endothelial cells do express VEGF (51, 84, 118, 174), as do cultured mesangial cells (90) and macrophages (33).

**REGULATION OF VEGF**

VEGF regulation and gene expression are affected by many factors, including hypoxia (109), interleukin-1β (IL-1β), IL-10, IL-13, basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGF-β1) (49), nitric oxide (NO) (13), hyperglycemia and hypoglycemia (90, 131, 140, 156), stretch (99, 187), shear stress (32, 58), and ethanol (60, 68, 82).

Hypoxia is a potent inducer of VEGF expression via an oxygen-responsive transcription factor, hypoxia inducible factor-1 (HIF-1) (98, 118, 158). Tissue hypoxia and subsequent VEGF expression is a common feature in many pathologies such as tumor growth (93, 113), retinopathies, cardiac ischemia (66, 93) and brain ischemia (149), as well as in the physiological condition of exercise (8, 18, 69). Hypoxia can also induce VEGF expression in cultured endothelial cells (10, 98, 118), indicating that there may be an autocrine, as well as paracrine, regulation of VEGF (20, 21). Chronic hypoxia reduces VEGF gene expression in the brain (93) and reduces VEGF and its receptors, VEGFR-1 and VEGFR-2, in skeletal muscle (126).

**VEGF RECEPTORS**

VEGF receptors (VEGFR) are cell surface tyrosine-kinase receptors (31) that have a cytoplasmic region with an insert sequence within the catalytic domain, a single hydrophobic transmembrane domain (181) and seven immunoglobulin-like domains in their extracellular region (120). A main characteristic of these receptors is their ability to undergo autophosphorylation when activated by VEGF ligand (31). There are now three known VEGF
receptors: VEGFR-1/fms-like-tyrosine kinase-1 (Flt-1); VEGFR-2/ fetal liver kinase-1 (Flk-1, human form KDR); and VEGFR-3/fms-like-tyrosine kinase-4 (Flt-4) (31, 120, 181). A main structural difference between VEGFR-3 and the other two VEGF receptors is that VEGFR-3 undergoes a proteolytic process upon VEGF ligand binding that yields disulfide-linked polypeptides of 120- and 75-kDa. These three receptors, along with recently discovered co-receptors neuropilin-1 and –2, bind different isoforms of the VEGF family members (reviewed in Table 1), and are involved in regulation of different physiological processes (31).

VEGF RECEPTOR-1

VEGFR-1 is a 180 kDa glycoprotein (75, 135) that binds VEGF with high affinity, but VEGFR-1 is weakly expressed in endothelial cells (108). Activation of VEGFR-1 in endothelial cells promotes cell migration, but may not efficiently induce cell proliferation (184), and this may negatively regulate blood vessel formation (86). VEGFR-1 plays a role in the development of normal vascularization and embryonic development. Disruption of the VEGFR-1 genes results in abnormalities of blood vessels in homozygous animals (120). VEGFR-1 knock-out mice are able to develop mature, differentiated endothelial cells, but the vessels are large and disorganized (181). VEGFR-1 is up regulated in endothelial cells following endothelial denudation (172), indicating that VEGFR-1 may be involved in vascular repair and maintenance. VEGFR-1 can bind two of the three soluble isoforms of VEGF (VEGF$_{121}$, and VEGF$_{165}$) (120), and also binds VEGF-B and PIGF (31). VEGFR-1 also has a soluble variant, sVEGFR-1, which is secreted from endothelial cells and may sequester the ligand to inhibit VEGF activity (19, 145).
VEGFR-1 has been reported to be expressed on quiescent endothelium of normal human adult vessels (34), and to a greater extent in proliferating vascular endothelium (11). VEGFR-1 is also expressed in renal mesangial cells (165), trophoblast cells (28), and is the only VEGF receptor expressed on monocytes (181).

**VEGF Receptor-2**

VEGFR-2, a 200-235 kDa glycoprotein (75, 135), mediates most of the effects of VEGF (181), and because of this is thought to be the most important receptor for VEGF. VEGFR-2 plays a role in vasculogenesis and angiogenesis (19), where its activation results in cell proliferation, migration, differentiation, tube formation, increased vascular permeability, and maintenance of vascular integrity (188). VEGFR-2 is needed for differentiation of endothelial cells, and for migration of primitive precursors of endothelial cells, which leads to a subsequent formation of blood vessels (142). Without the VEGFR-2 gene, embryos die before birth because differentiation of endothelial cells does not occur and, thus, blood vessels do not form (143). VEGF binds with lower affinity to VEGFR-2 than to VEGFR-1, but VEGFR-2 expression is higher on endothelial cells than VEGFR-1 (108). VEGFR-2 binds all three soluble isoforms of VEGF (VEGF_{121}, VEGF_{145}, and VEGF_{165}) (120), as well as the homologues VEGF-C, VEGF-D, and VEGF-E (31). VEGFR-2 is expressed in all vascular endothelia adult human vessels (34, 181) as well as in renal mesangial cells (165), retinal progenitor cells (179), and hematopoietic stem cells (85).
VEGF Receptor-3 and Neuropilin-1 and -2

The VEGFR-3 has only recently been identified (31, 128) and the function of this receptor is not well known, although it appears to be involved mainly in lymphangiogenesis (181). VEGFR-3 is preferentially expressed on lymphatic endothelium, and is not found on vascular endothelium (181). VEGFR-3 does not bind VEGF itself, but it does bind other members of the VEGF family, VEGF-C and VEGF-D (31). VEGFR-3 may also be involved in the development of the cardiovascular system, as VEGFR-3 deficient embryos die of cardiovascular failure prior to the development of lymphatic blood vessels (41).

Neuropilin receptors differ from VEGF receptors as they belong to a class of collapsin/semaphorin receptors (31), rather than the tyrosine-kinase receptor class. The binding of VEGF to neuropilin receptors does not display direct signaling activities, but neuropilin-1 expression on endothelial cells does enhance VEGF binding to VEGFR-2 and the migration of endothelial cells in response to VEGF (151). One recent study has also reported that neuropilin-1 is important in wound angiogenesis (110).

VEGF Receptors and Angiogenesis

As previously discussed, VEGF is one of the major factors involved in angiogenesis. Because angiogenesis is involved in so many processes, both physiological and pathological, it is important to determine the role of the VEGF receptors on angiogenesis, as targeting VEGF receptors may be a therapeutic approach for many conditions. It has been reported that VEGFR-2 is the main receptor involved in VEGF signaling (181); however, there is other evidence that all VEGF receptors may be involved in angiogenesis. Marchand et al. (108) examined the effects of antisense (AS) oligonucleotides for VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) mRNA on
angiogenesis. In this study, phosphate-buffered saline (PBS), VEGF, or VEGF combined with either AS-Flk-1, AS-Flt-1, or AS-scrambled (i.e., control) oligonucleotides were administered in mouse testis. After 14 days, angiogenesis was determined by measuring new capillary blood vessel growth. VEGF administration increased blood vessel growth by over 200% compared with the control. The combination of VEGF and the AS of both Flk-1 and Flt-1 almost completely prevented this effect of VEGF, indicating that both VEGF receptors Flk-1 and Flt-1 are essential to VEGF-induced angiogenesis in mouse testis (108).

**Ethanol, VEGF and VEGF Receptors**

There are very little published data regarding the effects of ethanol on VEGF and the VEGF receptors. To date, Medline searches through PubMed and BioMedNet have identified a few published articles relating specifically to ethanol and VEGF, and several more involving ethanol-induced gastric injury and VEGF.

**Ethanol, VEGF, and Angiogenesis**

The effects of moderate ethanol on VEGF expression were examined in SMC and chick CAM (68) and rat skeletal muscle (59). In both of these, ethanol increased VEGF. Canine coronary artery vascular smooth muscle cells (CAVSMC) were isolated and cultured with ethanol (10 or 20 mmol/l) for 2, 6, and 18 h (67). Ethanol exposure caused a dose-dependent increase in both VEGF mRNA and protein, measured by Northern blot and ELISA, respectively. CAVSMC exposed to 10 and 20 mmol/l ethanol for 18 h had increased VEGF protein levels of 19% and 68%, respectively, compared to control. No statistically significant time-dependent increases in VEGF protein were observed in the CAVSMC, but 20 mmol/l ethanol for 6 h did
increase VEGF protein levels by 27%. In the same study, Gu et al. (68) also examined the effects of moderate ethanol on angiogenesis in chick CAM. Eggs were incubated for 9 days, and then exposed to normal saline (control) or ethanol at 0.125 or 0.25 g/kg/day for 7 days. Ethanol and saline were administered through a small hole (2-3 mm) in one end of the egg. Ethanol administration of 0.25 g/kg/day for 7 days increased blood vessel formation in CAM, as shown by greater branching of vessels and higher vascular density compared with saline treated CAM. Vessel length density was also increased 53% in 0.25 g/kg/day ethanol treated CAM compared with saline treated CAM, corresponding to a 1.48 fold increase in VEGF mRNA expression in the ethanol treated CAM compared with saline treated CAM. No significant effects were observed at 0.125 g ethanol/kg/day. This is not direct proof that VEGF causes angiogenesis, but the results are consistent with the hypothesis that ethanol-induced VEGF plays a role in angiogenesis.

Gavin and Wagner (60) examined VEGF mRNA expression in rat gastrocnemius skeletal muscle after a single intraperitoneal injection of either saline or 17 mmol/kg ethanol. Approximately 1.5 h after injection, VEGF mRNA was significantly increased (3-fold) in rat gastrocnemius skeletal muscle in ethanol treated animals compared with controls. They did not look at capillary density or angiogenesis in this model.

Jones et al. (83) examined the effects of ethanol on an endothelial-derived cell line (EA hy926). Cells incubated with 1.0% or 2.5% ethanol for 3 hours formed tubelike structures (in vitro angiogenesis) when plated on Matrigel (83), whereas control cells not exposed to ethanol did not form tubelike structures. The greatest tubelike formation occurred when the EA hy926 cells were incubated for 3 hours in 2.5% ethanol. Surprisingly, they also reported that 2.5%
ethanol for only 5 min induced tubelike formations (83). This is one of the first papers to report that ethanol can induce angiogenesis in vitro.

**ETHANOL AND VEGF RECEPTORS**

To date, there is only one published report on the effects of ethanol on VEGF receptor expression. Gavin and Wagner (60) examined VEGF receptor mRNA expression in rat gastrocnemius skeletal muscle after a single intraperitoneal injection of either saline or 17 mmol/kg ethanol. Approximately 1.5 h after injection, both Flt-1 mRNA and Flk-1 mRNA were significantly increased (approximately 2-fold each) in rat gastrocnemius skeletal muscle in ethanol treated animals compared with controls. There have been no studies of the effects of ethanol exposure on VEGF and its receptors in endothelial cells.

**ETHANOL INJURY AND VEGF**

Jones et al. (82) reported that intragastric administration of 1.5 ml 100% ethanol caused severe gastric mucosal injury in rats. This hemorrhagic necrotic injury that was caused 3, 6, and 24 h following ethanol administration was associated with increased VEGF mRNA (629%, 553%, and 385%, respectively) and protein (367%, 318%, and 185%, respectively). Angiogenesis, as shown by numerous tubes of migrating endothelial cells, was also observed in gastric mucosal sections 24 hr after administering 100% ethanol. To assess the relationship between VEGF and angiogenesis, anti-VEGF antibody was administered concurrently with 50% ethanol administration. Again, ethanol-induced injury was observed, but angiogenesis was severely impaired, as shown by a 3-fold reduction in tubes of migrating endothelial cells compared with controls receiving pre-immune IgG concurrent with 50% ethanol.
In a similar study by Szabo et al. (158), rats received intragastric administration of 2 ml of 70% ethanol or saline. Significant increases in VEGF protein were observed by immunohistochemistry 3, 6, and 24 hr after ethanol injury. VEGF expression was localized in regenerating mucosal endothelial cells in microvascular tubes that were undergoing angiogenesis in response to the ethanol injury. It has been suggested that VEGF may be involved in acute gastroprotection and chronic ulcer healing following mucosal injury from ethanol administration (159). These studies involving ethanol-induced injury used doses that are much greater than what is considered moderate ethanol levels, and so may not be relevant to cardiovascular protective effects of moderate ethanol consumption. However, these studies demonstrate the importance of VEGF in the angiogenic response to ethanol-induced gastric mucosal injury.

**SUMMARY**

In summary, VEGF and endothelial cells are very important in the maintenance and function of the cardiovascular system. They are involved in controlling blood flow, vascular permeability, coagulation, fibrinolysis, angiogenesis, and in maintaining blood vessel integrity. Many of these are important steps in the development and progression of atherosclerosis. Ethanol has been shown to increase the expression of VEGF in several models. Previous studies have examined dose/concentration effects of ethanol on VEGF expression in several models and cell types, but so far, only one has examined the effects of ethanol on VEGF receptors. In addition, only one study has examined time-dependent effects of ethanol, and that study was in SMC. Finally, no studies have been conducted in endothelial cells. Accordingly, the purpose of this study is to examine the effects of ethanol duration and concentration on both VEGF and
VEGF receptors in coronary microvascular endothelial cells, because they may be involved in endothelial functions associated with the cardiovascular protective effects of ethanol.
CHAPTER THREE
METHODS AND MATERIALS

RESEARCH DESIGN

Time-dependent effects of ethanol exposure on VEGF and VEGF receptors in PCMEC were examined. PCMEC were grown to confluence in 100 mm plates. PCMEC were exposed in a 3 x 4 factorial design to one of four control (no ethanol for four times; 0.5, 2, 4, and 8 h) and eight experimental conditions consisting of two doses of ethanol (10 and 25 mM) for the four times. Five plates per condition and one plate of coverslips per condition were studied (giving six samples per condition for culture media):

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These ethanol concentrations were chosen because they represent the lower and upper ranges of blood alcohol concentrations that can be achieved with moderate alcohol consumption of 1-2 drinks. In some individuals, ethanol can be detected in the blood for up to 4 h or longer following the ingestion of one standard drink, so the durations that were chosen are well within the ranges that can be achieved with moderate alcohol consumption.

Protein levels of the VEGF receptors VEGFR–1 and VEGFR–2 from cultured cell lysate were detected by Western blot, and VEGF protein in supernatant and cell lysate was detected quantitatively by enzyme linked immunosorbent assay (ELISA). Immunocytochemistry using scanning laser confocal microscopy was used to qualitatively analyze VEGF, VEGFR-1, and VEGFR-2, and to determine the purity of the PCMEC cultures with markers specific for
endothelial cells (vWF, CD31+, and VE Cadherin). Alpha-smooth muscle actin (α-SMA) was also analyzed, as others have shown that porcine coronary microvascular endothelial cells express α-SMA (5, 80).

**CELL CULTURING PROCEDURE**

**CELL ISOLATION AND CULTURE**

First passage slaughterhouse PCMEC that have previously been isolated by our group were used (81). Briefly, under aseptic conditions, small pieces (2 x 1 cm) of left ventricular tissue were removed from the heart of each pig and the inner and outer one-thirds of the ventricle wall, which contain most of the macrovessels (123) were removed from these pieces, leaving the remaining middle portion of the ventricular wall. The vascular tissue contained in the middle portion is primarily microvessels (12). To digest the tissue, it was placed in a petri dish with Type II collagenase, and minced with a razor blade. The petri dish containing the tissue was placed on a warm (37°C) thermal pad on an orbital rocker for 30 min, after which the tissue was aspirated up and down in a transfer pipet to aid cellular dispersion. The petri dish was returned to the orbital rocker for an additional 30 min, and then the tissue was transferred to 15 ml centrifuge tubes. Dulbecco’s Modified Eagle Medium (DMEM) was added to the petri dishes and gently swirled. This wash was added to the centrifuge tubes. After centrifuging (800x g, 30 sec), supernatant was filtered using a 100 µm mesh nylon cell strainer (Falcon). This filtrate was centrifuged (800x g, 5 min). The remaining pellet was suspended in complete DMEM and re-centrifuged to wash the cells (3 times), and the cells were plated into 100-mm tissue culture dishes. The culture dishes were placed in an incubator (37°C, 5% CO₂) for 2 h, then washed with PBS three times to remove unattached cells. Cells were incubated and the media changed.
after the first 24 h and every 48 h thereafter. Cells were allowed to grow until they approached confluency (approximately 7-10 days) and then were harvested and frozen in liquid nitrogen for cryopreservation.

**Thawing and Plating Cells**

Prior to thawing the cells, a 100-mm tissue culture plate (Greiner, ISC Biologicals, Kaysville, UT) was coated with 1 ml of a 5 µg/ml fibronectin (Roche Biochemicals, Indianapolis, IN) solution. This solution was applied for 30 min, and the plate was agitated throughout the 30 min to maintain a uniform layer of coating solution. A 15 ml conical centrifuge tube was prepared with 14 ml of cold DMEM (Gibco BRL, Rockford, MD) containing 20% fetal bovine serum (FBS, Hyclone Labs, Logan, UT), 2 mM sodium pyruvate (Sigma, St Louis, MO), 90 µg/ml heparin (Sigma, St Louis, MO), 100 IU/ml penicillin (Gibco BRL, Rockford, MD), 100 µg/ml streptomycin (Gibco BRL, Rockford, MD), and 4 mM MgSO₄ (JT Baker Chemical, Phillipsburg, NJ). The remaining DMEM was warmed in a 37°C water bath.

Cells were removed from liquid nitrogen, quickly thawed in a warm (37°C) water bath, and were added to a second, empty 15 ml conical centrifuge tube. The 14 ml of cold supplemented DMEM solution previously measured out was slowly added to the cells in 3-4 drop increments, up to a total volume of 5-6 ml, swirling between increments. After 5-6 ml of the cold DMEM was added to the cells, increments of up to 500 µL cold DMEM were added to the cells with continued swirling of the cells between increments until all of the cold DMEM was added. The cells were centrifuged (5 min, 800x g, 26°C) and the media aspirated. Warmed DMEM (6 ml) was added to the cell pellet and the solution aspirated repeatedly to break up the cell clump. Excess fibronectin was aspirated from the previously coated plate and the cells and media were
plated into the fibronectin-coated plate. Ten µg/ml cAMP (Sigma, St Louis, MO) was added to the media in the plate. The plate was allowed to incubate (37°C, 5% CO₂) until cells were approximately 75-80% confluent.

**CELL MAINTENANCE/FEEDING**

The newly plated cells were fed 18-24 h after the initial plating, then every 48 h thereafter. DMEM was aspirated from the plate, and 2 ml of warm TC-PBS was added to the plate to wash the cells. The plate was gently swirled, the TC-PBS aspirated, and 5 ml of new, warm DMEM added to the plate, which was then returned to the incubator.

**PASSAGING CELLS**

Fourth passage cells were used for the experiments in this study. Cells were passaged when they reach approximately 80% confluency and split 1 to 5 for the first split and 1 to 4 for the second and third splits. Plates needed for passaging were coated with fibronectin (see methods used in “Thawing and Plating Cells” section). DMEM was aspirated from the plates and 5 ml of warm TC-PBS was added to each plate and the plate swirled to wash the cells. TC-PBS was aspirated from the plates, and 2 ml of warm 0.25% trypsin/EDTA (Gibco BRL, Rockford, MD) added to the plates. The trypsin was on the plates for 1-2 min while the cells were observed under a microscope. When the cells were observed to begin to lift off of the plate, the plate was tapped gently with quarter turns between taps until all of the cells were dislodged. Once the cells all lifted off the plate, 5 ml of warm DMEM was added to the plate to neutralize the trypsin. The cells were transferred to a conical centrifuge tube and centrifuged (5 min, 800x g, 26°C). The media was aspirated and the cells in the pellet were resuspended in warm DMEM.
(1 ml of DMEM added for each new plate to be incubated). Excess fibronectin was aspirated from the new plates and 1 ml of cell suspension added to each plate containing 5 ml of new, warm DMEM to make a total of 6 ml of media per plate. Plates were placed in an incubator (37°C, 5% CO₂), and maintained according to the methods detailed in the previous section.

**ETHANOL EXPOSURE**

For the 10 and 25 mM ethanol concentrations used in this experiment, the appropriate volumes of 100% ethanol were added to DMEM as follows (evaporation made the ethanol concentration less than 100%, so back calculation was used to give appropriate final concentration):

<table>
<thead>
<tr>
<th>Ethanol Concentration</th>
<th>Ethanol</th>
<th>DMEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM EtOH:</td>
<td>77.8 µl</td>
<td>165 ml</td>
</tr>
<tr>
<td>25 mM EtOH:</td>
<td>194.4 µl</td>
<td>160 ml</td>
</tr>
</tbody>
</table>

Once cells reached confluence in passage four, plates for different experimental conditions were subjected to one of the two ethanol concentrations for each of the four durations or to no ethanol (control). In the experimental plates, DMEM was aspirated from the plates and cells were washed with 2 ml warmed TC-PBS. The TC-PBS was aspirated and 5 ml of either 10 mM or 25 mM ethanol DMEM was added per plate. The plates were then exposed to 0, 10, or 25 mM ethanol for 0.5, 2, 4, or 8 h.

**Harvesting Cells**

Cells and media were harvested immediately after the end of the ethanol exposure in the experimental plates, and a set of control plates were harvested with each set of experimental
plates. The media for each plate was aspirated and saved in separate tubes and stored at -20°C until analyzed. Each plate of cells was then washed with 5 ml warm TC-PBS. The TC-PBS was aspirated and 1.0 ml of mammalian protein extraction reagent (M-PER, Pierce, Rockford, IL) solution was added to each plate, then let stand for approximately 2 min. Cells were scraped from the plate with a plastic cell scraper, and the plate let stand for another 2 min. The M-PER solution from each plate containing the cells was transferred to a 1.5 ml Ependorff tube and centrifuged (5 min, 1000 rpm, 26°C). Cell lysates were stored at -20°C for until analyzed.

**ETHANOL SPECTROPHOTOMETRY**

Ethanol concentrations in the media were measured to determine the beginning and ending ethanol concentrations to which the cells were exposed. Each experimental condition was measured with plates containing PCMEC or plates without PCMEC to compare the ethanol evaporation, and to determine if PCMEC were metabolizing ethanol. Using a spectrophotometer (Beckman DU 640, Fullerton, CA), the absorbance measured at a wavelength of 340 nm is directly proportional to the alcohol concentration in the sample. Following the alcohol kit directions (Diagnostic Chemicals Limited, Oxford, CT), 10 µl deionized water, standard or sample was pipetted into separate ultraviolet (UV) methacrylate cuvettes, and 1.4 ml of ethanol buffer and 0.1 ml of NAD-ADH reagent was added to each cuvette. Samples were incubated for 10 min at 37°C and absorbance was measured. Ethanol concentration in mmol/L was calculated by the following method according to the kit directions:

\[
\text{Ethanol mmol/L (}\%\text{w/v)} = (A/A_s) \times 20 \text{ mmol/L}
\]

where \(A\) is the absorbance of the unknown, \(A_s\) is the absorbance of the standard, and 20 mmol/L is the concentration of the standard.
**PROTEIN QUANTITATION ASSAY**

*CELL LYSATE*

The BCA protein assay kit (Pierce, Rockford, IL) was used to determine sample total protein concentration. Following manufacturer directions, 25 µl of each standard (serially diluted with MPER) or cell lysate sample was pipetted in triplicate into separate microplate wells. Working reagent (200 µl) was added to each well and the plate was shaken thoroughly on a plate shaker for 30 sec. The plate was covered and incubated at 37°C for 30 min, following which the plate was cooled to room temperature and the absorbance measured at 562 nm on a plate reader (Spectra Max Plus, Molecular Devices, Sunnyvale, CA). Unknown protein concentration was calculated from a four-parameter standard curve prepared by plotting each blank-corrected standard versus its concentration.

*CELL CULTURE MEDIA*

Standards for the cell culture media protein assay were prepared with DMEM used in the experimental conditions. A standard curve with M-PER was used to determine protein concentrations of standards containing DMEM with added FBS. DMEM standards and samples for protein assay were diluted 1:3 and 1:4, respectively, and were pretreated with a Compat-Able protein assay preparation reagent kit (Pierce, Rockford, IL) to remove the interfering substance Phenol Red, a component of the DMEM. Briefly, 50 µl of each diluted sample or protein standard and 500 µl of Compat-Able protein assay preparation reagent 1 was added to a 2.0 ml microcentrifuge tube. The tubes were mixed and allowed to stand at room temperature for 10 min, and 500 µl of Compat-Able protein assay preparation reagent 2 was added to each tube.
The tubes were mixed again and centrifuged at 12,000 g for 10 min. Each tube was inverted, the supernatant discarded, and the tube blotted on clean paper towel. The pellet of each tube was then dissolved in 50 µl of BCA working reagent and vortexed vigorously to solubilize the pellet. Each standard and sample was then run in duplicate with the BCA protein assay similar to cell lysate samples. For the cell culture media, unknown protein concentrations were calculated from a linear standard curve prepared by plotting each blank-corrected standard versus its concentration.

**Enzyme Linked Immunosorbent Assays**

A human VEGF DuoSet ELISA development kit (R&D Systems, Minneapolis, MN) was used on PCMEC supernatant and cell lysate to determine levels of secreted and sequestered VEGF protein, respectively. The kit antibodies recognized VEGF\textsubscript{165} and VEGF\textsubscript{121} isoforms. Following manufacturer directions, a 96-well plated was coated with 100 µl of VEGF capture antibody per well, sealed, and incubated overnight at room temperature. The wells were aspirated and washed with 350 µl of wash buffer three times, one minute per wash. Plates were blocked with 300 µl of block buffer in each well and incubated at room temperature for 1 h, then washed (1 min x 3). One hundred µl of sample or standards were added per well and the plates were covered and incubated for 2 h at room temperature. Plates were washed (1 min x 3), 100 µl of detection antibody was added to each well, and plates were covered and incubated for 2 h at room temperature. Washing was repeated (1 min x 3) and 100 µl of Streptavidin-HRP was added to each well. Plates were covered to avoid direct light and incubated for 20 min at room temperature. The plates were washed a final time (1 min x 3) and 100 µl of substrate solution was added to each well, with plates incubated in the dark for 20 min at room temperature. Stop
solution (50 µl) was added to each well and the plate gently tapped to ensure thorough mixing. The optical density of each well was determined immediately using a microplate reader (Molecular Devices, Sunnyvale, CA) set to 450 nm with wavelength correction set to 570 nm. VEGF protein concentrations were determined from a blank-corrected four-parameter standard curve.

**WESTERN BLOT**

VEGFR-1 and –2 were analyzed by Western blot. Cell lysate samples were diluted 1:2 in sample buffer and heated at 97°C for 4 min. Five µl of standard ladder (Bio-Rad Laboratories, Hercules, CA) or 10 µl of each sample were added to wells of a 7.5% bis-acrylamide gel (Bio-Rad Laboratories, Hercules, CA) and electrophoresed for 30 min at 200 volts. Protein was blotted onto a supported nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) at 400 mA for 2 h. Blots were then blocked overnight at 4°C in 5% block buffer (Bio-Rad Laboratories, Hercules, CA) and incubated in VEGFR-1 or –2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Blots were washed (4 x 5 min) and incubated in secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) at room temperature for 45 min. After washing (4 x 5 min) blots were incubated with SuperSignal Pico West Chemiluminescent Substrate (Pierce, Rockford, IL) for at least 5 min and signal was detected and analyzed with a ChemiDoc XRS program (Bio-Rad Laboratories, Hercules, CA). Blots were stripped (Pierce, Rockford, IL) and reprobed with the other VEGFR antibody not used first, following the same protocol as described.
SCANNING LASER CONFOCAL MICROSCOPY

Cells grown on 13-mm coverslips (Nalge Nunc International, Rochester, NY) were fixed in 5 ml of ice-cold absolute methanol for 5 min. The coverslips were airdried and stored at –20°C until they were analyzed.

IMMUNOCYTOCHEMISTRY

Immunocytochemistry was performed on passage four cells to demonstrate characteristics of endothelial cells by immunostaining for platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31), vWF, VE Cadherin, and α-smooth muscle actin (Table 2). The durational effects of ethanol on the expression of VEGF and VEGF receptors in PCMEC were qualitatively analyzed using confocal microscopy (Table 2).

Cells grown for confocal microscopy analyses were rehydrated by placing coverslips cell side down on a 50 μL drop of thawed, filtered PBS on dental wax in a humidified chamber for 5 min. Excess PBS was removed by gently touching the edge of the cover slip to filter paper. Each cover slip was placed cell side down on a 50 μL drop of filtered Tris-buffered saline (TBST-BSA, pH 8.3) containing 10 mM Tris, 250 mM NaCl, 0.3% Tween-20 and 1% bovine serum albumin (BSA) on dental wax in a humidified chamber and incubated at room temperature for 1 h to block non-specific IgG binding sites.

Primary antibody was prepared in TBST-BSA at a 1:50 dilution (see Table 2). The coverslips were removed from the TBST-BSA and each cover slip was placed cell side down on a 50 μL drop of the appropriate primary antibody solution, then incubated in a humidified chamber overnight at room temperature. Coverslips were washed in 50 μL drops of TBST-BSA three times for 10 min, blotting off the excess between washes. Oregon Green secondary
antibody was prepared in TBST-BSA at a 1:100 dilution (See Table 2). Coverslips were removed from the third wash, blotted on filter paper, and placed cell side down on 50 μL drop of the secondary antibody solution. Samples were again placed in a humidified chamber and stored in a dark drawer for 1 h at room temperature. Coverslips were washed 3 x 10 min in 50 μL drops of PBS media, returned to the humidified chamber and placed in a dark drawer between washes. In order to stain nuclei, coverslips were mounted on a drop of Vectashield Mounting Medium for Fluorescence with Propidium Iodide (Vector Laboratories, Burlingame, CA) on a glass cover slide (22 x 60 mm, VWR Scientific, Media, PA). Cells were incubated in the dark at least 30 min before viewing on the scanning laser confocal microscope.

**CONFOCAL MICROSCOPY PROTOCOLS**

The Lasersharp 3.2 program was used on the scanning laser confocal microscope (BioRad Laboratories, Hercules, CA), with red and green triple labeling method and Texas Red and fluorescein isothiocyanate (FITC) settings for red and green channels. The following starting points were used, and adjusted as needed:

- **Texas Red:** Iris: 2.0  Gain: ~1000  B Level: -15
- **FITC Green:** Iris: 3.0  Gain: ~1350  B Level: -20
- **Objective:** 60  Power: 30%  Speed: Normal  Search: Normal

**STATISTICAL ANALYSES**

Data were tested for normality and equal variance. All data were normally distributed with equal variance, with the exception of VEGF protein in the media. Because no VEGF was detectable in media samples without ethanol, 0 mM ethanol data were deleted from the ANOVA.
Data from 10 and 25 mM ethanol concentrations were transformed using a square root transformation, which resulted in normal distribution and borderline equal variance (p=0.047). Transformed data were used in the statistical calculations. The figures all use non-transformed data. For VEGF and VEGFR protein, mean data values from 96-well ELISA plates and Western blot densitometry were evaluated using a two-way analysis of variance (ANOVA) for ethanol concentration and duration. Ethanol concentrations from the ethanol evaporation/metabolism curve were evaluated for two-way ANOVA for time and presence of cells. If there was a significant interaction, multiple comparisons were performed to determine significant differences among cell means using a Bonferroni-corrected t-test. If no significant interaction was detected, multiple comparisons for each level were made using marginal means. Correlations between total protein and VEGF, and total protein and each VEGFR were also examined. Data are reported as mean ± SD, with significance set at p<0.05. Data were analyzed with Number Cruncher Statistical System software (NCSS, Kaysville, Utah).
REFERENCES


ETHANOL AFFECTS VASCULAR ENDOTHELIAL GROWTH FACTOR AND ITS RECEPTORS IN CORONARY MICROVASCULAR ENDOTHELIAL CELLS

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ABSTRACT

**Background and Purpose:** Cardiovascular disease is the leading cause of death in the United States. Moderate alcohol consumption has a cardiovascular protective effect, part of which is mediated by positive effects on high density lipoproteins (HDL). However, these effects only account for about 50% of the cardiovascular protective effects of ethanol. The endothelium is a key player in development of atherosclerosis, and many endothelial functions are controlled by vascular endothelial growth factor (VEGF). Ethanol increases VEGF and its receptors in some systems, but the effects of ethanol on the VEGF system in endothelial cells are not known. Accordingly, the purpose of the study is to examine the influence of ethanol on the expression of VEGF and VEGF receptors (VEGFR) in porcine coronary microvascular endothelial cells (PCMEC). **Methods:** PCMEC were exposed to one of two ethanol concentrations or no ethanol as a control (0, 10, or 25 mM) and four durations (0.5, 2, 4, or 8 h). VEGF and VEGFR proteins were analyzed quantitatively with ELISA and Western Blot techniques, respectively. VEGFR proteins were also analyzed qualitatively with scanning laser confocal microscopy. **Results:** With increasing ethanol concentrations, protein levels of VEGF (p=0.006) and VEGFR-1 (p=0.0007) increased significantly, whereas VEGFR-2 decreased significantly (p=0.0492). Increasing duration of ethanol exposure caused VEGF protein levels to increase (p<0.0001), but had no effect on either VEGFR-1 or VEGFR-2 (p=0.564 and p=0.475, respectively). **Discussion:** We conclude that ethanol at physiologically relevant concentrations can increase VEGF and VEGFR-1 in PCMEC. VEGF and VEGFR-1 are involved in endothelial maintenance and repair, which can attenuate the atherosclerotic process, and may be involved in the cardiovascular protective effects of moderate alcohol consumption.
INTRODUCTION

Cardiovascular disease is the leading cause of death in the United States (3). Main contributors to cardiovascular disease morbidity and mortality include myocardial infarction and stroke due to atherosclerosis. Epidemiologic studies consistently show that moderate alcohol consumption (one to two drinks per day) decreases the risk of negative cardiovascular events such as stroke and myocardial infarction (33, 49, 56) compared to heavy drinking (3 or more drinks per day) or abstention. Moderate alcohol consumption inhibits the progression of atherosclerosis (21, 22), the disease process leading to myocardial infarction and stroke, but the mechanisms are not well established. Increased HDL accounts for approximately 50% of the cardiovascular protective effects attributable to moderate alcohol consumption (35, 47). Other mechanisms to explain the remaining 50% may, in part, include an increase in fibrinolysis (1, 10), inhibition of smooth muscle cell proliferation and migration (39), and other unknown mechanisms.

Endothelial cell injury and dysfunction, early events in atherosclerosis, are characterized by increases in blood vessel permeability, coagulation, and inflammation, each favoring atherosclerotic plaque development (23). Vascular endothelial growth factor (VEGF) influences many of these endothelial functions and has been shown to increase atherosclerotic plaque development (12, 13, 36). VEGF stimulates endothelial cells to increase adhesion molecules (34) and attract monocytes, which are known to increase procoagulant activity and contribute to fibrin deposition (16), both of which are involved in atherosclerosis. However, VEGF may also have cardiovascular protective effects. It is a survival factor for endothelial cells and promotes endothelial repair after injury (6, 25), and stimulates the plasminogen activator system to
increase fibrinolysis (48). Because an intact, healthy endothelium inhibits atherosclerosis, VEGF system activation may also inhibit atherosclerosis.

Interactions between VEGF and its two main receptors VEGFR-1 and VEGFR-2, determine the effects of VEGF. VEGF receptors are cell surface receptors that are members of the tyrosine-kinase receptor family (15). VEGF binding to VEGFR-2 promotes increased vascular permeability, endothelial cell proliferation, migration, differentiation and tube formation, all involved in angiogenesis (8, 26). In atherosclerosis, angiogenesis may be beneficial by increasing oxygen to ischemic or hypoxic areas of the heart, thus providing a protective effect, but it may also be detrimental by providing a blood supply to atherosclerotic plaque and supporting continued plaque growth and development (14). Activation of VEGFR-1 by VEGF in endothelial cells inhibits VEGFR-2 induced endothelial cell proliferation (57), which may negatively regulate blood vessel formation. VEGFR-1 also enhances vascular repair and maintenance (50, 52). VEGF binds to VEGFR-1 with higher affinity than VEGFR-2 (41), so VEGFR-1 may sequester the VEGF ligand and thereby inhibit activation of VEGFR-2.

Ethanol has been shown in some systems to affect VEGF and its receptors. It increases VEGF expression in cultured smooth muscle cells (SMC) (27) and increases the expression of VEGF, VEGFR-1 and VEGFR-2 in rat skeletal muscle (24). Blocking VEGF abolishes ethanol-stimulated angiogenesis in vitro (11). However, the effects of ethanol on VEGF and VEGFR in endothelial cells are unknown. Accordingly, the purpose of this study was to examine whether exposure to moderate ethanol concentrations affects VEGF and VEGFR proteins in porcine coronary microvascular endothelial cells (PCMEC). Porcine cells were chosen because pigs metabolize lipoproteins (19) and spontaneously develop atherosclerotic plaque (51) similar to humans.
METHODS AND MATERIALS

RESEARCH DESIGN

Duration and concentration effects of ethanol exposure on VEGF and VEGF receptors in PCMEC were examined. Confluent PCMEC (n=6 plates per experimental condition) were exposed to one of three ethanol concentrations (0 (control), 10, or 25 mM) for one of four durations (0.5, 2, 4, or 8 h) in a 3 x 4 factorial design.

CELL CULTURE

PCMEC that had been isolated as previously described (30) were cultured in 100-mm tissue culture plates (Greiner, ISC Biologicals, Kaysville, UT) pre-coated for 30 min with a 5 µg/ml human, plasma-derived fibronectin solution (Roche Biochemicals, Indianapolis, IN) in phosphate buffered saline (PBS). Cryopreserved cells were quickly thawed in a warm (37°C) water bath and washed with 14 ml of cold complete DMEM (Gibco BRL, Rockford, MD) containing 20% fetal bovine serum (FBS, Hyclone Labs, Logan, UT), 2 mM sodium pyruvate, 90 µg/ml heparin, 100 IU/ml penicillin, 100 µg/ml streptomycin, and a total concentration of 4 mM MgSO₄ (complete DMEM). Cells were centrifuged (800x g, 5 min) and the media aspirated. Complete DMEM (6 ml) was added to the cell pellet and the solution aspirated repeatedly to break up the cell clump. This cell suspension solution was added to the fibronectin-coated plate. Dibutyryl cAMP was added to media to achieve a 500 µM concentration, and the cells were incubated (37°C, 5% CO₂). Complete DMEM was changed 18-24 h after the initial plating, and every 48 h thereafter. Cells were passaged when they reached 75-80% confluence using 0.25% trypsin/EDTA (Gibco BRL), and were then centrifuged (800x
g, 5 min), resuspended in complete DMEM, and plated on fibronectin-coated plates. Fourth passage cells were used for the experiments in this study.

**Ethanol Exposure**

Once cells reached confluence in passage four, plates of cells and coverslips were subjected to the experimental conditions. Each plate was incubated with 5 ml of complete DMEM with an ethanol concentration of 0, 10, or 25 mM for 0.5, 2, 4, or 8 h. No adjustments in pH were required, as ethanol did not affect the pH of the media at these concentrations. Cells and media were harvested immediately after the end of the ethanol exposure. Media samples were collected and cells were washed with 5 ml of PBS. Cell lysates were obtained using 1.0 ml of mammalian protein extract reagent (M-PER, Pierce Biotechnology, Rockford, IL). Samples were stored at –20°C until analyzed.

**Ethanol Spectrophotometry**

Ethanol concentration in the media was measured in each experimental condition with an ethanol assay kit (Diagnostic Chemicals Limited, Oxford, CT) before and immediately after ethanol exposure to detect beginning and ending ethanol concentrations. Measurements were made in plates with and without PCMEC to determine ethanol evaporation versus possible cellular metabolism of ethanol. Following manufacturer directions, 10 µL deionized water, standard or sample were pipetted into separate ultraviolet methacrylate cuvettes, and 1.4 ml of ethanol buffer and 0.1 ml of NAD-ADH reagent were added to each cuvette. Samples were incubated for 10 min at 37°C and absorbance was measured by spectrophotometry (Beckman DU640, Fullerton, CA) at a wavelength of 340 nm. At this wavelength, measured absorbance is
directly proportional to the alcohol concentration in the sample. Ethanol concentration in mmol/L was calculated by the following method according to the kit directions:

\[
\text{Ethanol mmol/L (\%w/v)} = (A/A_s) \times 20 \text{ mmol/L}
\]

where \( A \) is the absorbance of the unknown, \( A_s \) is the absorbance of the standard, and 20 mmol/L is the concentration of the standard.

**Protein Analyses**

**ELISA.** VEGF protein in conditioned media was measured using a human VEGF DuoSet capture ELISA kit (R&D Systems, Minneapolis, MN), following manufacturer directions. Antibodies in the kit recognize the VEGF\(_{165}\) and VEGF\(_{121}\) isoforms. Following manufacturer directions, each well of a clear, 96-well plate (Corning Inc., Acton, MA) was coated with 100 µl of VEGF capture antibody and plates were sealed and incubated overnight at room temperature. The wells were washed with 350 µl of wash buffer (3 x 1 min). Wells were blocked with 300 µl of block buffer, incubated at room temperature for 1 h, and washed (3 x 1 min). Triplicate wells were loaded with 100 µl of sample or standards and the plates were covered and incubated for 2 h at room temperature. Plates were washed again (3 x 1 min), 100 µl of detection antibody were added to each well. Plates were covered and incubated for 2 h at room temperature. Washing was repeated (3 x 1 min) and 100 µl of Streptavidin-horseradish peroxidase were added to each well. Plates were incubated in the dark for 20 min at room temperature. After washing a final time (3 x 1 min), 100 µl of substrate solution was added to each well, and plates were incubated 20 min at room temperature. Stop solution (50 µl) was added to each well and the plate gently tapped to ensure thorough mixing. The optical density of each well was determined immediately using a microplate reader (Molecular Devices, Sunnyvale, CA) set to 450 nm with wavelength
correction set to 570 nm. VEGF protein samples were measured in triplicate, and VEGF concentrations were determined by comparing samples to a blank-corrected, four-parameter standard curve. Total protein concentrations in the media were measured in duplicate with the BCA protein assay kit (Pierce Biotechnology) after the media was pretreated with a Compat-Able protein assay preparation reagent kit (Pierce Biotechnology) to remove the interfering substance Phenol Red. Results were meaned, VEGF protein was normalized to total protein, and values were expressed as pg of VEGF protein per mg total protein.

**Western blot.** VEGFR-1 and –2 proteins were analyzed by Western blotting. Cell lysate samples were diluted 1:2 in sample buffer and heated at 97°C for 4 min. Five µl of standard ladder (Bio-Rad Laboratories, Hercules, CA) or 10 µl of each sample were added to wells of a 7.5% bis-acrylamide gel (Bio-Rad Laboratories) and electrophoresed (Mini Protean III, Bio-Rad Laboratories) for 35 min at 200 volts. Protein was blotted onto a supported nitrocellulose membrane (Bio-Rad Laboratories) at 400 mA for 2 h. Blots were then blocked overnight at 4°C in 5% block buffer (Bio-Rad Laboratories) and incubated in VEGFR-1 antibody (Table 1) for 1 h at room temperature. Blots were washed (4 x 5 min) and incubated in secondary antibody conjugated to horseradish peroxidase (Table 1) at room temperature for 45 min. After washing (4 x 5 min) blots were incubated with SuperSignal Pico West Chemiluminescent Substrate (Pierce Biotechnology) and signal was detected and analyzed with a ChemiDoc XRS system and Quantity One software (Bio-Rad Laboratories). Blots were stripped with Restore Western Blot Stripping Buffer (Pierce Biotechnology) and reprobed with the VEGFR-2 antibody, following the same protocol described above.
**SCANNING LASER CONFOCAL MICROSCOPY**

Scanning laser confocal microscopy was used to determine the purity of the endothelial cell cultures by immunostaining for the endothelial cell markers platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31+), von Willebrand factor-related antigen (vWF), vascular endothelial (VE)-cadherin and α-smooth muscle actin (Table 2). Effects of ethanol concentration and duration on cell-associated VEGF receptors in PCMEC were qualitatively analyzed using confocal microscopy, as follows.

Cells grown on coverslips were fixed in 5 ml of ice-cold absolute methanol for 5 min to permeabilize the membrane. The coverslips were airdried and stored at −20°C until analyzed. Cells were rehydrated in filtered PBS for 5 min, and then were incubated in filtered Tris-buffered saline (TBST-BSA, pH 8.3) containing 10 mM Tris, 250 mM NaCl, 0.3% Tween-20 and 1% bovine serum albumin (BSA) in a humidified chamber at room temperature for 1 h to block non-specific binding sites. Cells were incubated in a humidified chamber overnight at room temperature in primary antibody (Table 2) prepared in TBST-BSA at a 1:50 dilution. Coverslips were washed (3 x 10 min) by placing each cover slip on a 50 μL drop of TBST-BSA. Secondary antibody (Table 2) was prepared in TBST-BSA at a 1:100 dilution and the coverslips were incubated in secondary antibody in a dark drawer for 1 h at room temperature. Coverslips were washed (3 x 10 min) in PBS and mounted on a drop of Vectashield Mounting Medium for Fluorescence with Propidium Iodide (Vector Laboratories, Burlingame, CA) on a glass cover slide (22 x 60 mm, VWR Scientific, Media, PA) to stain the nuclei. Cells were incubated in the dark at least 30 min and visualized using a Bio-Rad 1024 scanning laser confocal microscope at 60x (BioRad Laboratories).
**Statistical Analyses**

Data were tested for normality and equal variance. All data were normally distributed with equal variance, with the exception of VEGF protein in the media. Because no VEGF was detectable in media samples without ethanol, 0 mM ethanol data were deleted from the ANOVA. Data from 10 and 25 mM ethanol concentrations were transformed using a square root transformation, which resulted in normal distribution and borderline equal variance (p=0.047). Transformed data were used in the statistical calculations. The figures all use non-transformed data. For VEGF and VEGFR protein, mean data values from 96-well ELISA plates and Western blot densitometry were evaluated using a two-way analysis of variance (ANOVA) for ethanol concentration and duration. Ethanol concentrations from the ethanol evaporation/metabolism curve were evaluated for two-way ANOVA for time and presence of cells. If there was a significant interaction, multiple comparisons were performed to determine significant differences among cell means using a Bonferroni-corrected t-test. If no significant interaction was detected, multiple comparisons for each level were made using marginal means. Correlations between total protein and VEGF, and total protein and each VEGFR were also examined. Data are reported as mean ± SD, with significance set at p<0.05. Data were analyzed with Number Cruncher Statistical System software (NCSS, Kaysville, Utah).

**Results**

**VEGF protein.** The effects of different ethanol concentrations and durations on VEGF protein in supernatant are shown in Figure 1. VEGF protein was not detectable in supernatant of control cells (no ethanol). When comparing data from cells exposed to 10 and 25 mM ethanol,
there was no significant interaction between ethanol concentration and duration for the amount of VEGF protein in the media \((p=0.679)\). Both increasing ethanol concentration and duration increased VEGF protein levels in the media \((p=0.006, \text{ and } p<0.0001, \text{ respectively})\). There was no correlation between VEGF protein and total protein \((R^2=0.0306, p=0.1537)\).

**VEGF receptors.** Figures 2 shows a representative Western blot and quantitative densitometry normalized to total protein for VEGFR-1. Bands for VEGFR-1 \((180 \text{ kDa})\) appeared just below the 200 kDa standard ladder band (Fig. 2b). For VEGFR-1 there was no significant interaction \((p=0.978)\) between ethanol concentration and time on VEGFR-1. There was a significant main effect of ethanol concentration as the amount of VEGFR-1 increased in cell lysate with 25 mM ethanol concentration \((p=0.0007)\). No significant main effect of time was detected \((p=0.564)\). There was no correlation between VEGFR-1 protein and total protein \((R^2=0.016, p=0.3356)\). VEGFR-1 signal intensity appears to increase with increasing ethanol concentrations compared to control (no ethanol) in confocal microscopy images (Fig. 3).

VEGFR-2 \((235 \text{ kDa})\) bands were just above the 200 kDa standard ladder band in Western blots, and densitometric analyses showed a significant decrease in signal at 25 mM ethanol concentration \((p=0.0492, \text{ Fig. 4})\). There was no time effect on VEGFR-2 \((p=0.475)\), or interaction between ethanol concentration and time \((p=0.978)\). There was no correlation between amounts of VEGFR-2 protein and total protein \((R^2=0.0405, p=0.1750)\). Although there was a significant decrease in VEGFR-2 at 25 mM ethanol compared to 0 or 10 mM ethanol with quantitative analysis, VEGFR-2 does not appear to change much with 10 and 25 mM ethanol exposure in the qualitatively analyzed confocal microscope images (Figure 5).

**Ethanol concentration.** Ethanol concentration of the media measured in plates with and without PCMEC significantly decreased over time for both 10 mM and 25 mM ethanol
(p<0.0001 for both concentrations, Figure 6). After 8 h, approximately 62% of the ethanol was lost in the 25 mM ethanol media (25.1 mM to 9.5 mM ethanol), whereas approximately 56% of the ethanol was lost in the 10 mM ethanol media (10.1 mM to 4.4 mM ethanol). There was no significant difference in ethanol concentration between plates with and without cells at 25 mM ethanol (p=0.097). At 10 mM ethanol, there was a significant difference in ethanol concentration between plates with and without cells (p=0.009), as plates containing cells had a lower ethanol concentration at each time point. However, differences were not large enough to be detected by a Bonferroni post hoc test. There was no significant interaction between time and the absence or presence of cells for 10 or 25 mM ethanol media (p=0.405 and p=0.441, respectively).

Cell characterization. All of the endothelial cell markers tested were present in 99-100% of the PCMEC examined, as seen in Figure 7. In addition, PCMEC express α-SMA (Fig. 7).

DISCUSSION

This study was designed to determine the effects of moderate ethanol exposure on VEGF and its receptors in coronary microvascular endothelial cells. The main findings of this study are that 25 mM ethanol concentrations increase levels of VEGF and VEGFR-1 protein, and decrease those of VEGFR-2 protein in PCMEC. Moderate alcohol consumption reduces the risk of cardiovascular disease by inhibiting the progression of atherosclerosis (21, 22). The ethanol concentrations used in this study (10 and 25 mM) are equivalent to blood alcohol concentrations of approximately 0.046 and 0.115%, respectively, where 0.08% (approximately 17.5 mM) is the legal limit of blood alcohol concentration for operating a vehicle in most states. Ingestion of one to two standard drinks can induce blood alcohol concentrations within these ranges, and ethanol
can be detected in the blood for up to 4 hours or longer (42). Thus, the concentrations and durations used are physiologically relevant.

We chose to study endothelial cells in vitro because of the importance of endothelium in atherosclerosis. The purity of our cultures was established by the presence of several endothelial-specific markers. PECAM-1/CD31+, one of the most reliable endothelial cell markers (4), VE-cadherin (9), a cell adhesion molecule, and vWF (43, 53) are all considered to be specific endothelial cell markers that would not be expressed by other potentially contaminating cells (e.g., fibroblasts, smooth muscle cells, mesothelial cells). The uniformly positive expression of each of these endothelial specific markers in our cell cultures establishes that the cultures were homogenous and that the endothelial phenotype was preserved in the cells used for experiments. Although α-smooth muscle actin is typically associated with smooth muscle cells, others have shown, as we have in this study, that porcine coronary microvascular endothelial cells also express α-smooth muscle actin (4, 30).

There is debate about whether VEGF is beneficial or detrimental in atherosclerosis. VEGF is involved in most of the functions of the endothelium. It is important in repairing and maintaining the endothelium, and is a pro-survival factor for endothelial cells (37), as it induces expression of antiapoptotic proteins (25). But, VEGF also may stimulate atherosclerotic plaque development. VEGF expression is increased in atherosclerotic arteries compared to normal arteries (18, 28). In addition, exogenous VEGF increases in atherosclerotic plaque development in various models (12, 13, 36), although the physiological relevance is unclear because VEGF concentrations used in these studies are much higher than those found in vivo. VEGF stimulates angiogenesis and arteriogenesis (46, 55), which have been proposed to be involved in the cardiovascular protective effect of ethanol because new blood vessels may increase blood flow to
ischemic or hypoxic areas of the heart that are blocked by atherosclerotic plaques (29). However, neovascularization may also support atherosclerotic plaque development as blood vessels in the vaso vasorum provide oxygen for cells in the developing plaque. Advanced atherosclerotic plaques have greater blood vessel density compared to less advanced plaques (14, 28, 45). Clearly, the positive and negative effects of VEGF on processes involved in atherosclerosis complicate interpretation of increased levels of this growth factor.

Normal endothelial function is important in maintaining vascular homeostasis by maintaining vascular tone, balancing coagulation and fibrinolysis, and regulating the inflammatory process (54). Endothelial dysfunction occurs early in the atherosclerotic process, and maintaining a healthy endothelium attenuates the atherosclerotic process (7, 54). Endothelial injury can occur by several mechanisms, including, for example, hypertension, high cholesterol, hyperglycemia, inflammation and infection, mechanical stress, carbon monoxide, and toxic chemicals (40). Smooth muscle cell proliferation increases following endothelial injury leading to intimal hyperplasia (17). Inhibition of smooth muscle cell proliferation has been proposed as a cardiovascular protective mechanism of ethanol, as ethanol administration following vascular injury inhibits intimal formation and atherosclerotic plaque progression (38, 44). Likewise, VEGF administration accelerated reendothelialization and return of endothelial integrity and normal function following vascular injury, which attenuated atherosclerotic plaque progression (5, 6). Dorafshar et al. (20) reported that VEGF inhibits SMC proliferation in the absence of endothelial cells, to give an additional possible cardiovascular protective mechanism of VEGF to reduce smooth muscle cell proliferation and neointimal hyperplasia.

Ethanol and VEGF. Increasing ethanol concentrations and durations increased VEGF production and secretion in PCMECs. The effects of moderate ethanol on VEGF expression in
vitro have been examined in various models, including smooth muscle cells, chick chorioallantoic membranes (CAM) (27), and rat skeletal muscle (24). Gavin and Wagner (24) reported that VEGF mRNA was significantly increased (3-fold) in rat gastrocnemius skeletal muscle one hour following a single intraperitoneal injection of 17 mmol/kg of ethanol. Gu et al. (27) reported that ethanol exposure of 10 and 20 mmol/l ethanol for 18 h caused a dose-dependent increase in VEGF protein (19 and 68%, respectively) in coronary artery vascular smooth muscle cells. In the same study, they also reported that CAM exposed to ethanol for 7 days demonstrated increased VEGF mRNA expression (1.48-fold) and increased blood vessel formation. Ethanol stimulates angiogenesis, an effect that might be due to VEGF. Jones et al. (31) reported that 1.0% or 2.5% ethanol caused the formation of tube-like structures in vitro in an endothelial-derived cell line (EA hy926) when cells were plated on Matrigel. Surprisingly, they reported that an exposure duration of as little as 5 min at 2.5% ethanol was sufficient to induce in vitro angiogenesis (31). Burns and Wilson (11) showed that VEGF was required for ethanol-mediated in vitro angiogenesis. In that study, either ethanol exposure or VEGF administration produced similar angiogenic responses in human umbilical vein endothelial cell (HUVEC) cultures. When anti-VEGF antibodies were added, the angiogenic response was abolished for both VEGF and ethanol, suggesting that ethanol increases the angiogenic response by increasing levels of VEGF (11).

**Ethanol and VEGF receptors.** Because VEGF receptors mediate the effects of VEGF, understanding potential effects of experimental treatments on VEGF receptors is important in interpreting findings. Only one study to date has been published on the effects of ethanol on VEGF receptors. Gavin and Wagner (24) reported that a single injection of 17 mmol/kg ethanol increased both VEGFR-1 and VEGFR-2 mRNA in rat gastrocnemius muscle. Our results in
PCMEC support the finding that ethanol increases VEGFR-1 levels. One of the main functions of VEGFR-1 is its role in endothelial maintenance and repair after injury. An increase in VEGF and VEGFR-1 stimulated by ethanol could help maintain endothelial homeostasis to decrease the incidence and severity of atherosclerotic diseases. VEGFR-1 activation may negatively regulate endogenous levels of VEGF, by binding VEGF and removing it from circulation (50). This would decrease the amount of VEGF available to activate VEGFR-2 and thus may reduce cell proliferation and migration, vascular permeability, fibrosis, and other events that lead to atherosclerosis.

In contrast to the study by Gavin and Wagner (24), we found that increased ethanol concentration at 25 mM decreased VEGFR-2 in PCMEC. The difference in VEGFR-2 expression between these two studies could be due to numerous factors, such as differences in the ethanol concentrations, species studied, cell type, site of action, in vivo versus in vitro conditions, or various other undefined factors. VEGFR-2 activation stimulates cell proliferation and migration, processes involved in angiogenesis (46). VEGFR-1 may oppose angiogenesis stimulated by VEGFR-2 as it inhibits the cell proliferation mediated by VEGFR-2 (57). Kearney et al. (32) reported VEGFR-1 deficient (flt-1^{-/-}) embryonic stem cell cultures and VEGFR-1 mutant (flt-1^{+/+}) embryos had increased endothelial cell division and vascularization compared to controls. Assuming that changes in receptor levels represents changes in functional receptor activity, our finding of decreased VEGFR-2 coupled with increased VEGFR-1 suggest that cell proliferation, and thus angiogenesis could be impaired by moderate ethanol exposure. If, indeed, plaque development requires angiogenesis as suggested by others (14, 58), inhibition of the angiogenic process might inhibit growth of atherosclerotic plaque. Because VEGFR-1 mediates endothelial repair and VEGFR-2 is the primary receptor controlling VEGF-stimulated vascular
permeability, cell proliferation and migration (both endothelial cells and smooth muscle cells), differentiation, and tube formation, the increased levels of VEGF and VEGFR-1 coupled with decreased VEGFR-2 levels suggest that endothelial repair processes could be enhanced and processes involved in atherosclerosis could be impaired with ethanol exposure. However, since we did not measure VEGF receptor function or phosphorylation, this is speculative.

_**Ethanol evaporation and metabolism.**_ Because evaporation would expected in a non-sealed culture dish, the linear decrease in ethanol concentrations in media during the 8 h incubation period is not surprising. We chose this method to mimic in vivo conditions following ethanol consumption, where the body metabolizes ethanol, resulting in a steady decrease in ethanol concentration in the blood over time. At 25 mM ethanol, there was no significant difference in concentrations of media incubated with or without PCMEC. Although ethanol concentrations were slightly lower in plates containing PCMEC than plates without cells in all samples of both ethanol concentrations measured, only the 10 mM ethanol concentration was significantly different. Handling time for media from the plates with PCMEC was longer because in these plates, media was collected and cells harvested prior to determination of ethanol concentration, whereas in the plates without cells, ethanol concentration was determined immediately at the end of the exposure time. Blood vessels metabolize ethanol, but most of the metabolism is thought to occur in the tunica media that contains smooth muscle and connective tissue (2), rather than in the endothelium. With our conflicting results, we are unable to determine if the PCMEC were metabolizing ethanol.

**Summary.** In summary, this study demonstrates in PCMEC that 25 mM ethanol concentration: 1) increases VEGF protein levels; 2) increases VEGFR-1 protein levels; and 3) decreases VEGFR-2 protein levels. To our knowledge, this is the first study to examine the
effects of ethanol on VEGF and VEGF receptors in endothelial cells. Possible enhanced endothelial repair and maintenance, from increased VEGFR-1 and reduced vascular permeability, cell proliferation, and migration from decreased VEGFR-2 can attenuate the process of atherosclerosis. Thus, the cardiovascular protective effects of ethanol may be due in part to inhibition of atherosclerotic plaque progression itself, rather than an increase in collateral vessel development to compensate for advanced atherosclerotic plaque-induced tissue ischemia. Because changes in VEGF receptors may be as important in mediating atherosclerotic plaque formation and progression as VEGF itself, understanding the effects of VEGF requires an understanding of each of the components of the VEGF system. To better understand how ethanol inhibits atherosclerosis, additional studies using microvascular cells, particularly endothelial cells, are needed.

Acknowledgements. Research support was provided by the Alcoholic Beverage Medical Research Foundation and the Washington State University Alcohol and Drug Abuse Program. We thank Rick Meek, Sheryl Cooney, and Robert Anderberg from the research lab at The Heart Institute of Spokane for their support and assistance. We thank Chris Davitt from the Electron Microscopy Center at Washington State University for her help in obtaining confocal microscope images, Bryan Slinker for his assistance with statistical analyses, and Sally Blank for her helpful discussions and technical assistance.
REFERENCES


Table 1: Western blot antibodies and concentrations.

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Table 2: Antibody information for scanning laser confocal microscopy.

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Figure 1: VEGF Protein vs. Ethanol Concentration

PCMEC were exposed to 10 mM or 25 mM ethanol for 0.5, 2, 4, or 8 h. Exposure to moderate ethanol concentrations and durations induces the expression of VEGF protein in conditioned media. Data is expressed as means ± SD (n=6). VEGF protein was not detectable at any time point at 0 mM ethanol, or at 0.5 or 2 h at 10 mM ethanol.

*Significantly different from 0 mM ethanol, p<0.05.
#Significantly different from all other durations, p<0.05.
‡Significantly different from 2 h, p<0.05.
Figure 2: VEGFR-1 in cell lysate

Quantitative densitometry for the ratio of VEGFR-1 protein to total protein (A) and a representative Western blot of VEGFR-1 protein (MW 180 kDa) (B) in cell lysate from PCMEC exposed to 10 or 25 mM ethanol for 0.5, 2, 4, or 8 h. Data at 0 mM ethanol at 0.5 h were normalized to 1.0. All other data were normalized to 0 mM ethanol at 0.5 h to allow for comparisons. Data is expressed as means ± SD (n=5).

*Significantly different from 0 mM ethanol, p<0.05.
Figure 3: VEGFR-1 protein confocal microscope images

Nuclei are stained red with propidium iodide for cellular localization. Expression of VEGFR-1 protein is expressed by green fluorescent signal. A) 0 mM EtOH, 0.5 h; B) 0 mM EtOH, 2 h; C) 0 mM EtOH, 4 h; D) 0 mM EtOH, 8 h; E) 10 mM EtOH, 0.5 h; F) 10 mM EtOH, 2 h; G) 10 mM EtOH, 4 h; H) 10 mM EtOH, 8 h; I) 25 mM EtOH, 0.5 h; J) 25 mM EtOH, 2 h; K) 25 mM EtOH, 4 h; L) 25 mM EtOH, 8 h; M) Representative sample of secondary antibody only.
Figure 4: VEGFR-2 in cell lysate

Quantitative densitometry for the ratio of VEGFR-2 protein to total protein (A) and a representative Western blot of VEGFR-2 protein (MW 200-230 kDa) (B) in cell lysate from PCMEC exposed to 10 or 25 mM ethanol for 0.5, 2, 4, or 8 h. Data at 0 mM ethanol at 0.5 h were normalized to 1.0. All other data were normalized to 0 mM ethanol at 0.5 h to allow for comparisons. Data is expressed as means ± SD (n=5).

*Significantly different from 0 mM ethanol, p<0.05.
Figure 5: VEGFR-2 protein confocal microscope images

Nuclei are stained red with propidium iodide for cellular localization. Expression of VEGFR-2 protein is expressed by green fluorescent signal. A) 0 mM EtOH, 0.5 h; B) 0 mM EtOH, 2 h; C) 0 mM EtOH, 4 h; D) 0 mM EtOH, 8 h; E) 10 mM EtOH, 0.5 h; F) 10 mM EtOH, 2 h; G) 10 mM EtOH, 4 h; H) 10 mM EtOH, 8 h; I) 25 mM EtOH, 0.5 h; J) 25 mM EtOH, 2 h; K) 25 mM EtOH, 4 h; L) 25 mM EtOH, 8 h; M) Representative sample of secondary antibody only.
Figure 6: *Ethanol evaporation curve for conditioned media with or without PCMEC*

Within each concentration, all time points are significantly different from every other time point. The 25 mM conditioned media with cells is not significantly different from conditioned media without cells. The 10 mM conditioned media with cells is statistically significantly different from conditioned media without cells (p<0.05). See discussion.
Figure 7: Endothelial cell characterization markers

Nuclei are stained red with propidium iodide for cellular localization. Expression of endothelial cell characterization markers are expressed by green fluorescent signal. A) PECAM/CD31+; B) Vascular endothelial-cadherin; C) vWF; D) α-smooth muscle actin; E) Representative sample of secondary antibody only.
SERUM STARVATION AND NORMAL GLUCOSE TESTING

PCMEC used in this experiment are sensitive to the conditions to which they are exposed. Previous work in our lab has shown that the PCMEC grow best in DMEM containing 25 mM glucose and 20% FBS. These concentrations of glucose and FBS are higher than those used to culture many cell types, and could be considered additional factors to which the cells are exposed. High glucose concentrations may damage endothelial cells, leading to the production of cytokines, growth factors, and other substances. Also, many cell cultures are grown are serum starved for 24-48 h prior to experimental conditions being applied. To determine if PCMEC could tolerate normal glucose concentrations or serum starvation, cells were exposed to either 5 mM glucose DMEM or FBS-free DMEM and monitored for 48 hours, both visually to detect morphological changes, and by measuring lactate dehydrogenase (LDH) to detect possible cell damage. Cells were examined at nine time points (0, 2, 4, 8, 12, 18, 24, 36, and 48 h) visually with a digital camera (Nikon Coolpix 995, Nikon Inc., Melville, NY) mounted on a microscope (Nicon Eclipse TS100, Nikon Inc., Melville, NY), and with a lactate dehydrogenase kit (Diagnostic Chemicals Limited, Oxford, CT). Plates of cells to be exposed to normal glucose and serum starvation were washed with 2 ml warm TC-PBS, and 5 ml of warm 5 mM glucose DMEM or FBS-free DMEM was added to each plate. Normal glucose and FBS-free media was measured with the LDH kit immediately before it was added to the cells to get an initial LDH concentration reading. Media in plates to be used in the experiment was also measured to get an initial LDH concentration of cells before they were exposed to the new conditions. LDH was measured by adding 10 µl of media and 1.0 ml of LDH-L reagent to a UV methacrylate cuvette and incubating for 2 min at 37°C. The increase in absorbance at 340 nm was recorded at one-
minute intervals with a spectrophotometer (Genesys2, ThermoSpectronic, Rochester, NY) until the absorbance change was constant. LDH activity was calculated by the following equation:

$$\text{LDH U/L} = \frac{\Delta A/\text{min} \times \text{assay volume (ml)} \times 1000}{6.22 \times \text{light path (cm)} \times \text{sample volume (ml)}}$$

$$= \Delta A/\text{min} \times 16238$$

where
- $\Delta A/\text{min}$ = change in absorbance per minute
- Assay volume = total assay volume expressed in ml
- 1000 = converts U/m to U/L
- 6.22 = absorbance coefficient of NADH at 340 nm
- Light path = length of the light path expressed in cm (usually 1.0)
- Sample volume = sample volume expressed in ml
- 16238 = factor derived from the constants in the equation

**SERUM STARVATION**

At confluence, PCMEC normally maintain a monolayer of cells that are contact inhibited, and have an elongated morphology. Cells exposed to serum starved conditions displayed morphological changes that were observable after 2 h. Instead of a flat, full appearance, these cells appeared to draw away from each other. This continued at all time points through 12-18 h, at which time cells began to lift off of the plates. At later time points, the cells that remained attached to the plates began to take on a flatter, more normal appearance. LDH concentrations did not change much through 12 h, but from 18 h on the LDH concentrations increased dramatically. It is unknown whether the increase in LDH was from cells still attached to the plates, or only those that had lifted off of the plates.

**NORMAL GLUCOSE**

Cells exposed to normal glucose concentrations for up to 48 h showed no visible changes in morphology or LDH concentrations. However, when PCMEC grown in high glucose media
were passed and cultured in normal glucose media, cell proliferation was noticeably slower after one week in normal glucose media. PCMEC did not approach confluency after 7-10 d, when they would normally become confluent. After 2 wk the cells were still not near confluency, and cells began to lift off of the plates. Within a few more days, more than 95% of the cells had lifted off of the plates, and the cells were determined to be unusable.
Figure 8. PCMEC following exposure to normal glucose conditions

PCMEC morphology following exposure to normal glucose conditions for different durations. Pictures are representative of all samples examined. A) 0 h, immediately before addition of normal glucose media. B) 2 h of normal glucose exposure. C) 4 h normal glucose exposure. D) 8 h normal glucose exposure. E) 12 h normal glucose exposure. F) 18 h normal glucose exposure. G) 24 h normal glucose exposure. H) 36 h normal glucose exposure. I) 48 h normal glucose exposure.
Figure 9. PCMEC following serum starvation

PCMEC morphology following exposure to serum starvation for different durations. Pictures are representative of all samples examined. A) 0 h, immediately before serum starvation. B) 2 h of serum starvation. C) 4 h serum starvation. D) 8 h serum starvation. E) 12 h serum starvation. F) 18 h serum starvation. G) 24 h serum starvation. H) 36 h serum starvation. I) 48 h serum starvation.
Lactate Dehydrogenase Assay for Low Glucose Media and Serum Starvation in PCMECs

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APPENDIX D

ADDITIONAL WORK
Total Protein vs VEGF Protein

\[ y = 0.0023x - 6.5292 \]

\[ R^2 = 0.0306 \]
Total Protein vs Relative Density of VEGFR-1

\[ y = -0.0004x + 1.8286 \]

\[ R^2 = 0.016 \]
Total Protein vs Relative Density of VEGFR-2

$y = -0.0003x + 1.2049$

$R^2 = 0.0405$
PCMEC were analyzed with confocal microscopy to determine cell culture purity. Cell nuclei were stained red with propidium iodide for cellular localization. Confocal images for each cell marker were examined and nuclei of the cells were counted. These are presented as “Number of Cells”. Each nuclei was examined for expression of each marker indicated by green fluorescence. In the above chart, this is noted as “Cells Expression Marker”. The percent of cells expressing each marker is given in the last column.

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