Studies on the Heme Oxygenase-1 Pathway and Anti-Angiogenic Factors in Preeclampsia and Endothelial Protection

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DECLARATION

I hereby declare that I have composed this thesis and the work described herein is my own unless otherwise stated in the text.

I also declare that the content of this thesis has not been submitted for any other degree or professional qualification.

Wenda Ramma

April 2011.

ABSTRACT

The endothelium plays a pivotal role in the maintenance of vascular homeostasis and its dysregulation promotes vascular complications. This thesis proposes that heme oxygenase-1 (HO-1), an anti-inflammatory enzyme with antioxidant properties, is endothelial protective factor that prevents endothelial injury induced by cisplatin or activated neutrophils. Specifically, this thesis aimed to test (i) that overexpression of HO-1 prevents cisplatin-induced endothelial injury and suppresses caspase activity; (ii) whether neutrophil-endothelial cell activation resulted in the release of soluble Flt-1 (sFlt-1) and soluble endoglin (sEng), the two anti-angiogenic factors known to induce the clinical signs of preeclampsia; (iii) whether HO-1 prevented activated neutrophils from stimulating the release of these factors from the endothelium; (iv) the relative contribution and the co-dependency of neutrophil activation and anti-angiogenic growth factors in preeclampsia where systemic endothelial dysfunction is known to occur. This thesis shows that cisplatin inhibited human umbilical vein endothelial cells (HUVEC) metabolism as measured by MTT assay and resulted in the release of placenta growth factor (PlGF). Immunoblotting confirmed that cisplatin increased cleaved caspase-3 expression in HUVEC. These effects of cisplatin were attenuated in HUVEC infected with adenovirus encoding HO-1 and the effects were exacerbated when HO-1 was silenced by siRNA. Furthermore, cisplatin stimulated PlGF release was suppressed by the overexpression of HO-1. In addition, HO-1 overexpression inhibited angiogenesis as determined by vascular endothelial growth factor-induced capillary tube

formation on Matrigel coated plates. Thus these studies indicate that agents which upregulate HO-1 could increase the effectiveness and tolerability to cisplatin in cancer treatment. Although neutrophils are early contributors to endothelial cell activation, no studies have determined their contribution to the release of sFlt-1 and sEng. We therefore investigated the effect of activated neutrophils on the release of sFlt-1 and sEng in endothelial/neutrophil co-cultures and in the circulation of women with normal pregnancy and preeclampsia. LPS-mediated neutrophil activation stimulated the release of sEng but not sFlt-1 from endothelial cells in culture. In the absence of neutrophils, overexpression of HO-1 in HUVEC down-regulated the release of sEng. In contrast, HO-1 overexpression failed to inhibit the release of sEng in the presence of activated neutrophils. The release of sEng by activated neutrophils-endothelial cell cocultures appears to be mediated by metalloproteinases (MMP) as the broad-spectrum MMP inhibitor (GM6001) attenuated sEng release. Clinical studies demonstrated that sEng, pro-inflammatory interleukin-6 (IL-6) and the soluble markers of neutrophil activation (α-defensins and calprotectin) were all elevated in women with preeclampsia. We identified a direct correlation between neutrophil activation and IL-6 release. However, no correlation could be established between these factors and sEng release in preeclampsia. Hence, these results provide compelling clinical evidence to show that the increase in neutrophil activation and IL-6 release during preeclampsia are unlikely to significantly contribute to the clinical signs of preeclampsia as they fail to correlate directly with the anti-angiogenic factors, which form the final

common pathway to the clinical signs of preeclampsia and systemic endothelial dysfunction.

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ABBREVIATIONS

Ad – Adenovirus

ADMA - Asymmetric dimethylarginine

ALK - activin receptor-like kinase

Ang-1 – angiopoietin-1

Ang-2 – angiopoietin-2

AP-1 - activator protein 1

APS - ammonium persulphate

bFGF – Basic fibroblast growth factor

BVR – Biliverdin reductase

cAMP – cyclic adenonine monophosphate

CEC – circulating endothelial cells

cGMP – cyclic guanosine monophosphate

CO – Carbon monoxide

CRP – C-reactive protein

CYP - cytochrome

DMSO – DiMethyl Sulfoxide

DNA - Deoxyribonucleic acid

EDHF – Endothelial derived hyperpolarizing factor

EDTA - ethylenediaminetetraacetic

ELISA – Enzyme-Linked ImmunoSorbent Assay

eNOS – endothelial nitric oxide synthase

ESAM – Endothelial cell specific adhesion molecule

Fe – free iron

HELLP – Hemolysis, Elevated liver enzymes, Low platelet count

HMEC – Human microvascular endothelial cell

HMGB1 - high mobility group box 1

HNP – Human neutrophil peptides

HO-1 – Heme oxygenase-1

HO-2 – Heme oxygenase-2

HUVEC – Human umbilical vein endothelial cell

ICAM-1 – Inter-Cellular Adhesion Molecule 1

IFN-γ - Interferon-γ

IFU – Infective units

Ig - Immunoglobulin

IL- - Interleukin-

iNOS – inducible nitric oxide synthase

IP - Intraperitoneally

IV – Intravenously

JAM – Junctional adhesion molecule

KPBS – potassium phosphate buffered saline

LDL – low-density lipoprotein

LFA1 - lymphocyte function-associated antigen 1

LPS – Lipopolysaccharide

MAC 1 - macrophage antigen 1

MadCAM-1 - Mucosal addressin cell adhesion molecule-1

MAPK - mitogen-activated protein kinase

MCP-1 monocyte chemoattractant protein-1

MMP - Matrix metalloproteinase

MTT - 3-(4, 5-Dimethylthiazol–2-yl)-2, 5-diphenyltetrazolium bromide thizolyl blue

NADPH - nicotinamide adenine dinucleotide phosphate-oxidase

NFkB - nuclear factor-κB

NGAL – neutrophil gelatinase-associated lipocalin

NO – Nitric oxide

NOS – Nitric oxide synthase

NRP – Neuropillin receptor

OCT-2 – Organic cation transporter -2

PAI-1 - plasminogen activator inhibitor-1

PBS – phosphate buffered saline

PDGF – platelet derived growth factor

PECAM1 - platelet/endothelial-cell adhesion molecule 1

PGF2α - Prostaglandins

PGH2 - Prostaglandins

PGI2 – Prostacyclin

PI3K – PhosphatidylInositol 3-kinase

PIP3 – PhosphatidylInositol (3, 4, 5)-trisphosphate

PKC – Protein Kinase C

PlGF – Placenta growth factor

PMN – polymorphonuclear leukocytes

PMSF - phenylmethylsulfonylfluoride

PSGL-1 - P-selectin glycoprotein ligand 1

RBC – Red blood cells

RIPA - Radio immunoprecipitation buffer

RNA – Ribonucleic acid

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

ROS – reactive oxygen species

SDS-PAGE – Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

sEng – soluble Endoglin

sFlt-1 – soluble Fms-like tyrosine kinase-1

sGC – soluble guanylate cyclase

siRNA – small interfering RNA

SnPP – Tin protoporphyrin

TEMED - Tetramethylethylenediamine

TGF-β - transforming growth factor

TLR – Toll-like receptor

TNF-α – Tumour necrosis factor- α

tPA - tissue plasminogen activator

VCAM-1 – Vascular Cell Adhesion Molecule 1

VEGF - vascular endothelial growth factor

VEGFR- - Vascular endothelial growth factor receptor

VLA4 - very late antigen 4

vWF - von Willebrand's factor

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CHAPTER 1

INTRODUCTION

1.1 THE ENDOTHELIUM

The vascular endothelium is a metabolically active organ, which plays a pivotal role in tissue homeostasis (Molitoris et al., 2002). The endothelium forms the inner surface of all blood vessels and taken together it is an organ of about 1kg of weight with an estimated total surface area in human of about 350m^2 (Pries et al., 2000). This inner layer of the blood vessel is also refered to as the intima. The endothelium is a single-layer of cells, followed by the media consisting of smooth muscle cells and the outer layer, the adventitia (Figure 1.1). The endothelial cells align with the blood stream as indicated in Figure 1.1 by the arrow for the direction of the blood flow.

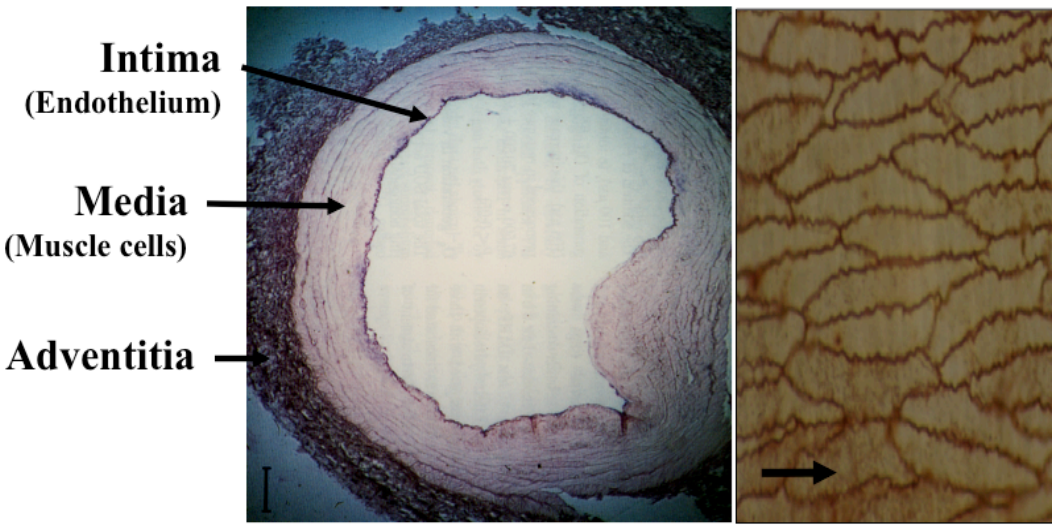


Figure 1.1. **Location and structure of the endothelium.** The left panel represents a porcine coronary artery with the inner layer (intima or endothelium) as a single-layer of cells, followed by the media consisting of smooth muscle cells and the outer layer, the adventitia. The right panel illustrates the alignment of the endothelial cells with the direction of blood flow, indicated by the arrow.

The endothelium acts as an anatomical barrier that prevents the influx of circulating blood into the vessel wall and also controls vascular tone, regulates local cellular growth and the deposition of extracellular matrix. Equally important is its ability to protect the vessel from the potentially harmful consequences of toxic substances and cells that circulate in the blood by mediating hemostatic, inflammatory, and reparative responses to local injury. It also regulates leukocyte trafficking from blood to tissues and maintains the anti-thrombotic and anti-coagulant balance in flowing blood (Pearson, 2000).

1.1.1 Endothelial cell barrier

The endothelial cell lining the wall of blood vessels form a selective barrier for the transport of molecules between blood and tissues. The continuous monolayer of endothelial cells are linked to each other by various types of adhesive structures or cell-to-cell junctions. Three types of junctions have been identified: tight junctions, adherens junctions, and gap junctions (Dejana et al., 1995, Schnittler, 1998). Tight junctions seal the endothelial cell layer and are formed by closely apposed neighboring plasma membranes, which appear to be partially fused. The main transmembrane constituent of tight junctions is occludins (Vestweber, 2000). Adherens junctions are formed by cadherins, VE-cadherin located at the endothelial cell surface. Cadherins are cell adhesion molecules, which are anchored with their cytoplasmic tail to a network of intracellular cytoplasmic proteins, catenins, that are connected to the actin-based microfilament system (Dejana, 1996). Adhesive

structures regulate the vascular permeability to circulating cells. They are controlled according to the need of the irrigated organ. Redistribution of surface cadherins and occludins, stabilization of focal adhesion bonds and progressive activation of matrix metalloproteinases (MMP) are associated with the regulation of endothelial cell permeability (Alexander, 2002).

1.1.2 Haemostatic balance

The endothelium maintains haemostatic balances via the production of procoagulant factors (von Willebrand factor, Tissue factor) and the fibrinolytic plasminogen activator inhibitor-1, coagulation inhibitors thrombomodulin and tissue factor pathway inhibitor, and tissue type and urokinase-type plasminogen activator (John Vane, 1995).

Inflammation, trauma and exposure to various cytokines (such as tumour necrosis factor-α or lipopolysaccharide) shift the endothelium from its normal anti-coagulant state to a procoagulant and prothrombotic state (Rapaport, 1993, Pearson, 1999). Activated endothelial cells secrete platelet-activating factor, which causes aggregation of platelets. The endothelium also synthesizes von Willebrand's factor (vWF), a platelet adhesion protein that is mainly found in the subendothelium and extracellular matrix, areas that do not interact with the platelets unless exposed by injury. Additionally, fibronectin and collagen in the subendothelium also contribute to platelet adherence (Nievelstein and de Groot, 1988). von Willebrand's factor and fibronectin are used as markers of endothelial cell activation (Meyer, 1982).

Activated endothelial cells also express tissue factor. Factor VII binds tissue factor and is activated on the endothelial cell surface. VIIa-tissue factor complex activates Factor X to form prothrombinase complex that cleaves prothrombin to generate thrombin. VIIa-tissue factor complex can also activate factor IX, which forms a complex with VIIIa and phospholipid thereby activating Factor X. Once thrombin is formed, it diffuses into the surrounding plasma or interstitial fluid and produce fibrin strands by cleaving fibrinogen (Rapaport, 1993).

1.1.3 Control of vascular tone

The endothelial cells regulate the vascular tone and blood pressure via the secretion of vasodilators: prostacyclin (PGI_2), nitric oxide (NO) and the endothelium derived hyperpolarizing factor (EDHF). These vasodilators also act to inhibit platelet aggregation and inflammation (Figure 1.2 & Figure 1.3). However, under pathological conditions such as hypoxia or high blood pressure, vasoconstrictive factors such as endothelin, superoxide anion, vasoconstrictor prostaglandins (PGF2α, PGH2) and thromboxane A2 are released from the endothelium (Michiels, 2003).

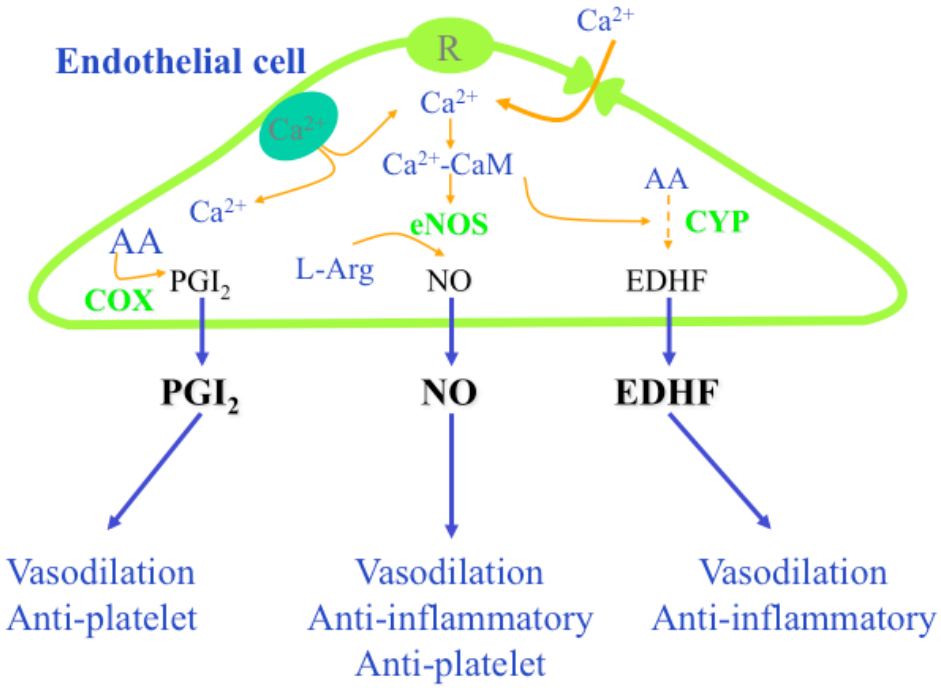


Figure 1.2 **Functions of PGI_2, NO and EDHF in endothelial cells.** PGI_2 is produced from arachidonic acid (AA) metabolism on cyclooxygenase (COX); NO is produced by the action of eNOS on L-arginine and EDHF is derived from arachidonic acid's (AA) action of CYP.

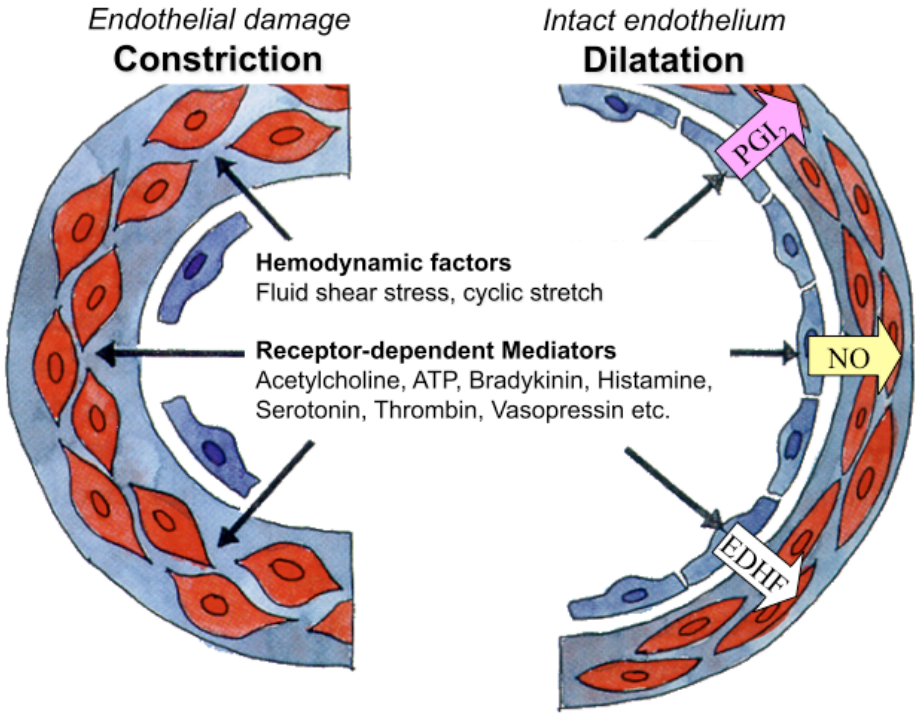


Figure 1.3 **Factors involved in the regulation of vascular tone in blood vessels.** PGI_2, NO and EDHF from intact endothelium (blue single-nuclear layer of cells) cause relaxation of underlying smooth muscle cells (red). In contrast, constriction of smooth muscle cells occurs when the endothelium is damaged.

1.1.3.1 Prostacyclin

Prostacyclin (PGI_2) is derived from arachidonic acid metabolism and is released constitutively by endothelial cells (Moncada et al., 1976). It activates adenylate cyclase to generate cAMP and consequently causes relaxation of the underlying smooth muscle cells. PGI_2 synthesis can also be triggered by a variety of agents such as thrombin, arachidonic acid, histamine or serotonin (Figure 1.3). These agents increase the cytoplasmic concentrations of calcium, via the G-protein-coupled receptors, causing nitric oxide synthase (NOS) and phospholipase A_2 (the initial rate limiting enzyme in the PGI_2 synthetic pathway) to be activated by calcium (Ibe et al., 1989).

1.1.3.2 Nitric Oxide

Nitric Oxide (NO) has a wide range of physiological and pathological activities, including the regulation of vessel tone and angiogenesis in wound healing, inflammation, ischemic cardiovascular and malignant diseases (Ignarro, 1989, Flammer and Luscher, 2010). In 1980, Furchgott and Zawadzki showed for the first time that the integrity of the endothelium was crucial for the relaxation of vascular smooth muscle cells induced by acetylcholine (Furchgott and Zawadzki, 1980). Seven years later, they identified that the endothelium-derived relaxing factor (EDRF) was NO (Palmer et al., 1987, Furchgott, 1988). The physiological concentration of NO ranges from 5 nM to 4 μM (Palmer et al., 1987). NO is synthesized in response to physiologic shear stress or vasopeptides such as acetylcholine, bradykinin, thrombin (Figure 1.3) and polypeptide growth factors like vascular endothelial growth factor (VEGF) (Ahmed et al., 1997).

The endothelial NO is generated by the membrane-bound endothelial NOS (eNOS). The normal function of eNOS requires the dimerisation of the enzyme, the presence of its substrate L-arginine and the essential co-factor (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4), a potent naturally occurring reducing agent (Forstermann and Munzel, 2006). NO-dependent vasodilation is initiated when the phosphoinositol pathway is activated in the endothelial cells, leading to the increase in cytosolic calcium level. The binding of calcium to calmodulin activates eNOS, which then catalyses the

$NADPH^-$- and O_2-dependent five-electron oxidation of L-arginine to form NO and citrulline (Cooke and Dzau, 1997). NO diffuses from the endothelial cells into the vascular smooth muscle cell to activate soluble guanylate cyclase (sGC) to increase cyclic guanosine monophosphate (cGMP) (Figure 1.2). This causes inhibition of the phosphoinositol pathway and a decrease in intracellular calcium accompanied by muscle cell relaxation and vasodilation (Henderson, 1991).

NO is rapidly inactivated in the blood by hemoglobin, superoxide radicals in the vascular walls or oxygen in free solution (Marshall and Kontos, 1990). Failure of endothelium-dependent vasodilatation due to lack of NO synthesis (and/or increased NO destruction by reactive oxygen species) is an early feature of hypercholesterolemia and atherogenesis (Cooke et al., 1991), and chronic inhibition of eNOS exacerbates atherogenesis in animal models (Maxwell et al., 1998). Furthermore, lack of eNOS in mice leads to reduction in endothelium dependent vasodilation and increase in systemic blood pressure (Huang et al., 1995). Apart from its vasodilatory function, NO also inhibits platelet activation, smooth muscle cell proliferation, leukocyte adhesion and is antioxidative (Figure 1.4).

Although eNOS is the predominant form of NOS in the vasculature, an inducible form (iNOS), which can produce approximately 1000-fold greater amounts of NO than does the constitutive form, is also present in the endothelial cells (Star, 1993). iNOS is also present in macrophages,

neutrophils, hepatocytes, cardiac myocytes, chondrocytes, and many other cell types (Lala and Orucevic, 1998). iNOS is induced during inflammation by pro-inflammatory cytokines and bacterial endotoxins. It can locally generate high amount of NO for prolonged period of time, which can cause cellular injury (Wink et al., 1996, Ricciardolo et al., 2004). The other form of NOS, neuronal NOS (nNOS) produces NO in nervous tissue of both the central and peripheral nervous system (Southan and Szabo, 1996). Both iNOS and nNOS are soluble and located predominantly in the cytosol.

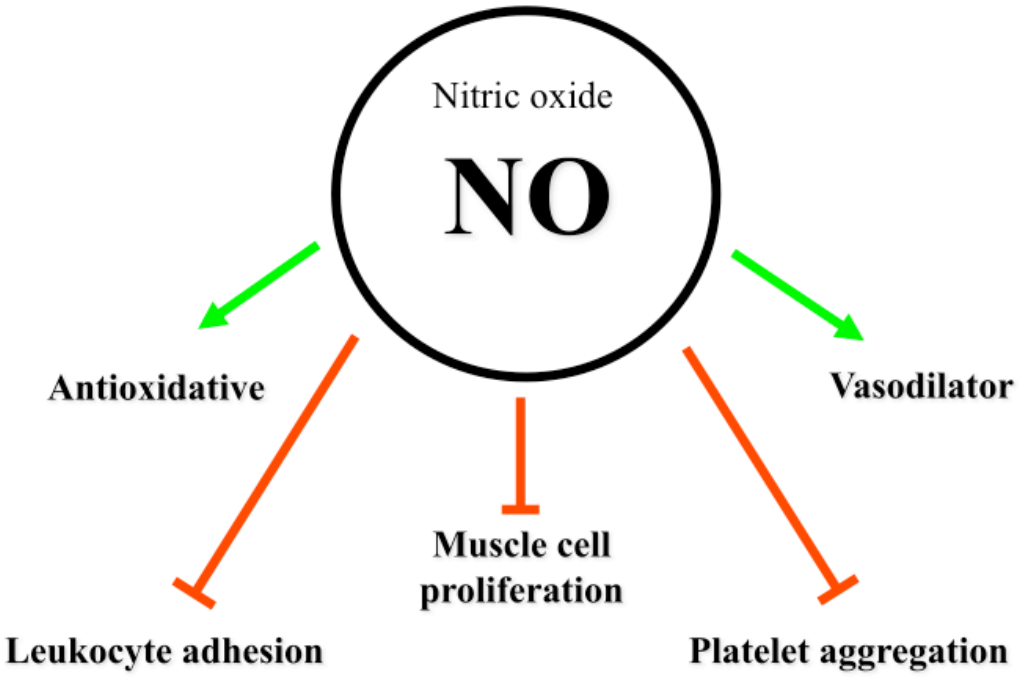


Figure 1.4. **Diagrammatic representation of the physiological properties of nitric oxide (NO).** NO functions as a vasodilator and has antioxidative properties in endothelial cells. It also inhibits leukocyte adhesion, muscle proliferation and platelet aggregation.

1.1.4 Endothelial cell dysfunction

Endothelial dysfunction plays a key role in the development of vascular diseases. It refers to the disruption of any of the processes that is required to maintain healthy endothelial cells and is commonly linked to the abnormal endothelial-dependent smooth muscle relaxation (vasodilation) due to the impairment of the NO-cGMP pathway and decrease in NO bioavailability (Cooke et al., 1991, Bivalacqua et al., 2003) (Figure 1.5). Cardiovascular risk factors such as hypertension, hypercholesterolemia, diabetes mellitus, or chronic smoking stimulate the production of reactive oxygen species and superoxide from NADPH oxidases in the vasculature (Figure 1.5). Superoxide reacts readily with vascular NO to form peroxynitrite, a reactive nitrogen species. The co-factor for eNOS, BH_4, is highly sensitive to oxidation by peroxinitrite. Oxidation of BH_4 leads to its depletion that consequently promotes superoxide production by eNOS (referred to as eNOS uncoupling). This transformation of eNOS from a protective enzyme to a contributor to oxidative stress has been observed in several in vitro models, in animal models of cardiovascular diseases, and in patients with cardiovascular risk factors (Forstermann and Munzel, 2006). Together, these contribute to increase vasoconstriction of the vessel leading to vascular disorders such as atherosclerosis, myocardial ischemia and angina (Henderson, 1991, Schwartz et al., 2010). In healthy endothelium, intravenous injection of acetylcholine causes vasodilation through the NO-cGMP pathway. However, in absence of the normal endothelium as in patients with hypertension, diabetes, hypercholesterolemia, congestive heart failure or atherosclerosis, acetylcholine has a paradoxical vasoconstrictive effect (Henderson, 1991,

Quyyumi, 1998), indicating that the vasodilatory response of acetylcholine is reduced or abolished as a result of endothelial damage (Schwartz et al., 2010).

Physiological NO has multiple effects on the vasculature. In the absence of NO, platelets adhere to the endothelium, aggregate and release platelet-derived factors, while leukocytes also adhere and promote inflammatory responses. Furthermore, low bioavailability of NO leads to an increase in superoxide production, which promotes low-density lipoprotein (LDL) oxidation. It was recently shown than NO *per se* inhibits LDL oxidation (Ahmed et al., 2009). Thus, NO keeps the endothelium healthy not just by its vasodilatory and anti-adhesive properties but also by inhibiting lipid peroxidation. Moreover, in pregnancy, insufficient generation of NO can predispose women to preeclampsia and fetal growth restriction (Ahmed et al., 1997) as well as compromise angiogenesis (Murohara et al., 1998, Ahmad et al., 2006).

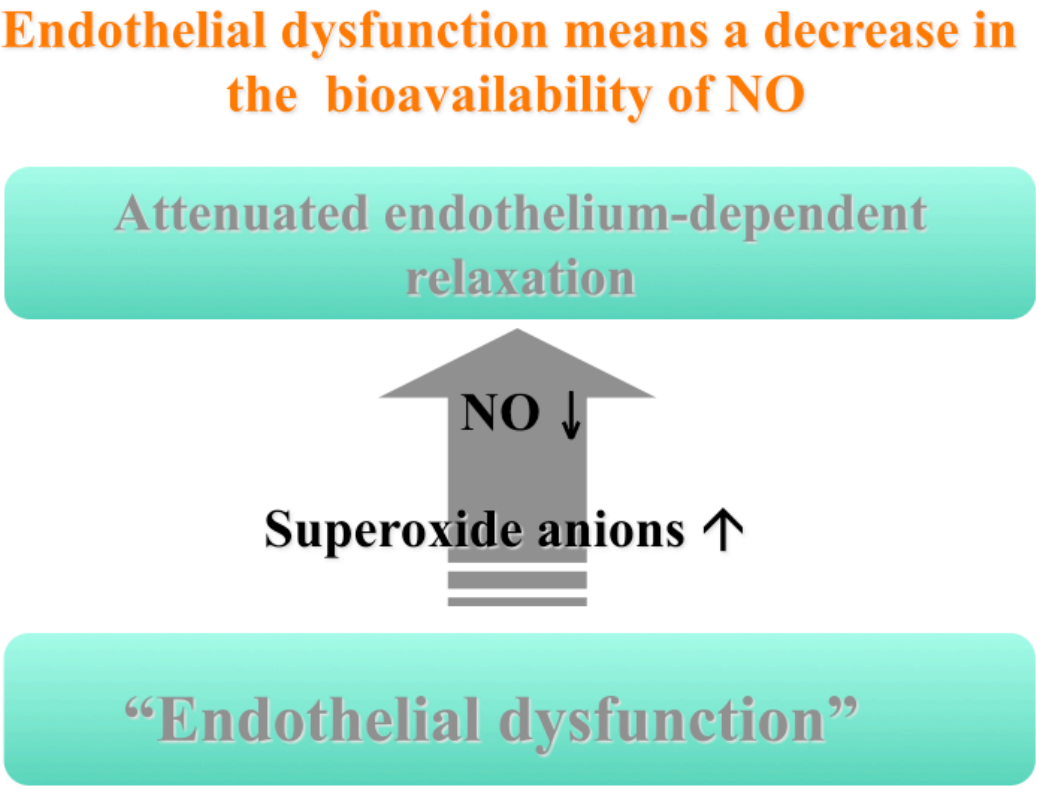


Figure 1.5 **Schematic representation of endothelial dysfunction.** Endothelial dysfunction is a consequence of a decrease in NO bioavailability as a consequence of superoxide anions production from NADPH oxidases, hence impairing the relaxation of the underlying vascular smooth muscles.

1.1.5 Endothelial cell activation

Endothelial cell activation and injury plays an important role in both drug-induced and inflammatory-mediated injury to the endothelium. Both endothelial cell activation and endothelial cell injury are two distinct phenomenons, yet the two are likely to overlap during the activation process (Pober and Cotran, 1990). Endothelial cell activation is a reversible process, which involves the change in morphological structure (increase in cell size and cytoplasmic organelles) of the endothelial cells, without loss of

endothelial integrity. The endothelial cell can return to the quiescent, non-activated state upon withdrawal of cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Pober, 1988, Ballermann, 1998, Blann, 2000). However, uncontrolled endothelial cell activation can progress to injury and apoptosis, which is irreversible and causes endothelial fragmentation and detachment of endothelial cells from the intima (Bach et al., 1997). The terms "endothelial cell activation," "endothelial cell injury/endothelial cell damage," and "endothelial cell dysfunction" are not interchangeable and should be used with a clear definition of each (Blann, 2000). "Endothelial cell activation" is distinct from sublethal injury with consequent endothelial cell dysfunction. Endothelial cell activation may also lead to endothelial cell dysfunction without evidence of vascular injury, as seen in the vascular leak syndrome induced by interleukin-2 (IL-2) (Pober, 1988).

As previously described, endothelial cell dysfunction can manifest itself as an imbalance between relaxing and contracting factors, for example, NO and endothelin; between procoagulant and anticoagulant mediators; or between growth-inhibiting and growth-promoting substances (De Meyer et al., 1997). Hence, the process of endothelial cell activation leading to endothelial cell dysfunction and endothelial cell injury involves a series of immediate and delayed events. The first event may be a very early, immediate immunological activation of the endothelial cells (type I endothelial cell activation) involving the release of stored proteins independent of protein

synthesis, followed by an early delayed activation (type II endothelial cell activation) that involves de novo protein synthesis and secretion of the proteins. Endothelial cell dysfunction with irreversible endothelial cell injury can be produced by uncontrolled and persistent endothelial cell activation and can result in critical local levels of endothelial adhesion molecules, procoagulant molecules, vasodilators, cytokines, chemokines, and endothelial cell necrosis (Zhang et al., 2010).

1.1.6 Markers of endothelial cell injury

The expression of endothelial cell adhesion molecules and other soluble factors released by the endothelium are good markers of endothelial cell activation. Adhesion molecules play a critical role in the recruitment of leukocytes in many forms of vascular injury. Several endothelial cell adhesion molecules belonging to the immunoglobulin (Ig) superfamily play mediate the interaction between endothelial cell and leukocytes. These include intercellular adhesion molecule ICAM-1, ICAM-2, ICAM-3, vascular cell adhesion molecule-1 (VCAM-1), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). Other biomarkers include E-selectin, P-selectin and endothelin-1. Among the numerous biomarkers commonly used, only E-selectin, vWF, MadCAM-1, Asymmetric dimethylarginine (ADMA), and circulating endothelial cells (CEC) that are detached from the endothelium, are considered to be endothelial-specific markers of activated endothelial cells (Zhang et al., 2010). The other markers, although reliable and sensitive for vascular inflammation and injury, are not endothelial-specific and may

derive from multiple types of activated cells, such as neutrophils, platelets, mast cells, macrophages, antigen representing cells or T lymphocytes. Activation of neutrophils and endothelial cells is an early critical event in drug-induced vascular injury and in major pathologies. Increased expression of ICAM-1 has been reported in vasculitic lesions involving nerve and muscle (Panegyres et al., 1992). The levels of soluble ICAM-1 have been shown to be higher in individuals who develop atherosclerosis thereby suggesting that soluble ICAM-1 may serve as a biomarker for such vascular lesions (Lu et al., 2010). E-selectin expressed on endothelial cells binds to the carbohydrate ligands on leukocytes. In patients with diffuse vasculitis, the skin vessels have increased expression of both E-selectin and ICAM-1 (Johnson et al., 2006).

1.2 THE HAEMOXYGENASE SYSTEM

Heme oxygenases are microsomal enzymes responsible for the rate-limiting breakdown of heme to produce equimolar amount of carbon monoxide (CO), free iron (Fe) and biliverdin (Jozkowicz et al., 2007) (Figure 1.6). There are three major isoforms of hemeoxygenases, which are products of different genes (Cruse and Maines, 1988). HO-1, a 32 kDa protein is the inducible form, which is present at very low levels in most quiescent tissues. The spleen and the liver are the only exception where HO-1 is highly expressed, probably due to its role in the recycling of erythrocyte and heme degradation (Braggins et al., 1986). HO-2 is an approximately 36 kDa protein which is catalytically active and shares with HO-1 similar substrate specificity and co-

factor requirements (Trakshel et al., 1986). It is constitutively expressed in the brain, endothelium and testis, supporting a role for this enzyme in both the nervous nd male reproductive systems (Trakshel and Maines, 1988, Bainbridge and Smith, 2005). However, HO-2 is present at lower levels in most tissues including the liver, kidney, spleen the cardiovascular system and the vasculature comprising the endothelial and smooth muscle cell lining of blood vessels (Maines, 1988). HO-2 and HO-1 share less than 50% homology in both amino acid and nucleotide sequence (Rotenberg and Maines, 1990). HO-3 is a newly characterised 33 kDa protein. It is a poor catalyst of heme and its transcript can be found in a variety of organs including the spleen, liver, thymus, prostate, heart, kidney, brain and testes (McCoubrey et al., 1997).

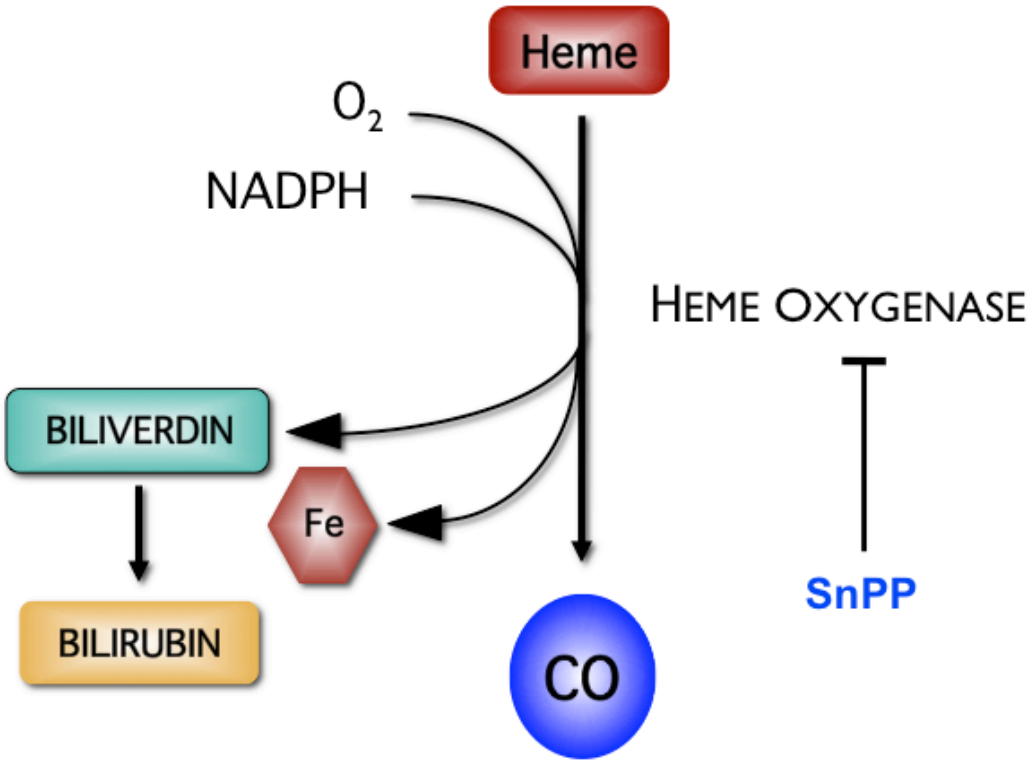


Figure 1.6. **Diagrammatic representation of the heme catabolic pathway.** Degradation of heme catalysed by the enzyme heme oxygenase leads to the production of biliverdin, carbon monoxide (CO) and iron (Fe). Biliverdin is subsequently converted to bilirubin. Tin protoporphyrin (SnPP) is an inhibitor of heme oxygenase.

Free heme is produced from hemoglobin upon lysis of red blood cells. Heme is a pro-oxidant and pro-inflammatory agent, which can be toxic to several cellular components including lipid bilayers, mitochondria, the cytoskeleton and the components of the nucleus (Maines, 1997). As a result, its elimination from the body is essential to prevent excessive oxidative stress, inflammation and also to maintain cell survival. HO acts by degrading heme into biliverdin the gaseous CO and free iron. Biliverdin is subsequently converted to bilirubin by biliverdin reductase (BVR). HO and its catalytic products have important roles in ischemia/reperfusion injury, inflammation, immune

dysfunction, organ transplant, the control of vascular tone and apoptosis (Elbirt and Bonkovsky, 1999, Bainbridge and Smith, 2005). Bilirubin is an antioxidant, which can scavenge free radicals and prevent lipid peroxidation. CO has vasodilatory effects as well as antiapoptotic functions and can protect cells under "stress" conditions (Li Volti et al., 2002, Agarwal and Nick, 2000).

1.2.1 Heme oxygenase-1

Heme oxygenase-1 (HO-1) plays an important role in the body and lack of HO-1 has detrimental consequences. Poss and Tonegawa reported embryonic loss and death within one year of birth in HO-1 knockout mice. In HO-1 knockout adult mice, normochromic, microcytic anemia and progressive chronic inflammation in the kidney and the liver were observed (Fang et al., 2004). HO-1 is induced by stress stimulus such as as heme, heavy metals, platelet-derived factors, peroxynitrite, endotoxin, hypoxia, hyperoxia and cytokines (Dulak et al., 2008, Agarwal and Nick, 2000). A common feature among these inducers is that they are all involved in the imbalance of the redox state of the cell and cause cellular and tissue injury, indicating that increased in HO-1 level under stress condition could be a cellular defense mechanism. Indeed, induction of HO-1 with metalloporphyrins (Amersi et al., 1999) and HO-1 gene transfer (Amersi et al., 1999, Coito et al., 2002) confers protection against various insults and has also been associated to increased xenograft survival in mice models. Figure 1.7 illustrates the multifunctional role of the HO-1/CO system.

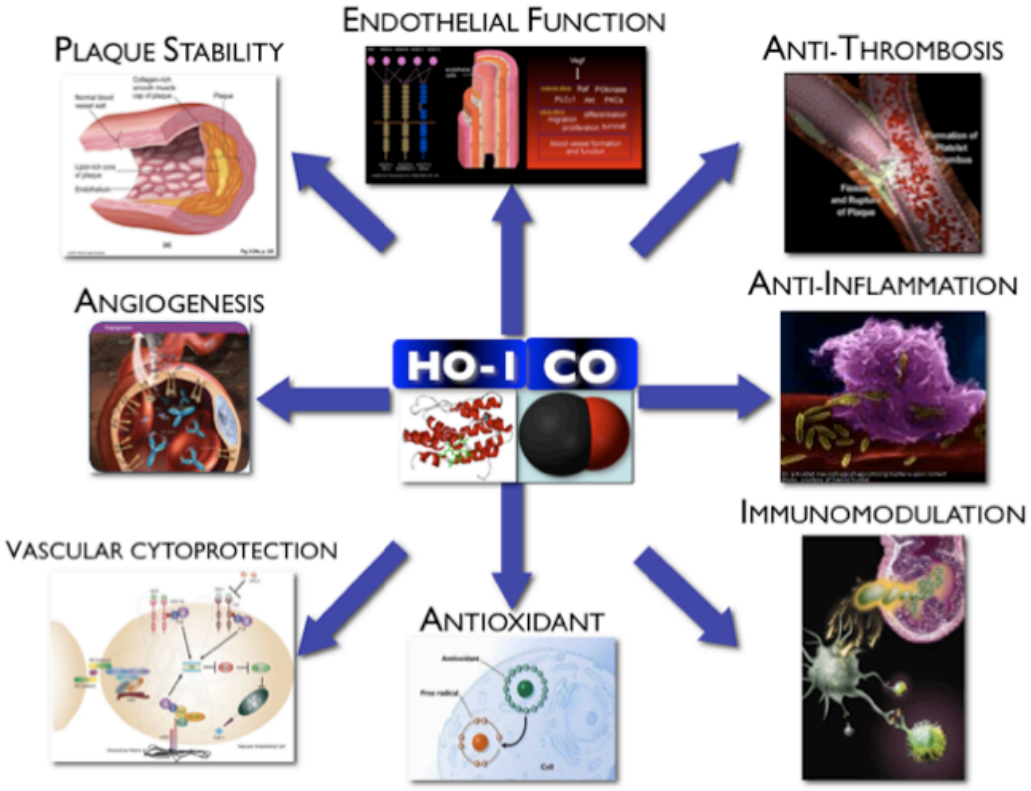


Figure 1.7 **Multifunctional actions of heme oxygenase pathway.** The HO-1/CO system increases plaque stability, ameliorates endothelial function, confer cytoprotection, acts as antioxidant, anti-thrombosis, anti-inflammatory and controls the immune systems.

1.2.1.1 HO-1 and oxidative stress

Stocker et al. first proposed that HO-1 induction might provide cytoprotection against oxidative stress (Stocker, 1990). Indeed, studies in HO-1 deficient mice *in vivo* and their derived cells *in vitro* revealed that these mice are more prone to oxidative challenge than their wild type counterparts

(Poss and Tonegawa, 1997). Several other studies have shown that the induction of HO-1 serves as an adaptive and protective mechanism in various stress conditions (Otterbein and Choi, 2000, Morse and Choi, 2002). Furthermore, pharmacological induction of HO-1 with cobalt protoporphyrin or gene transfer using an adenoviral vector containing HO-1 reduces ischemia-reperfusion injury and prolonged survival after cold ischemia/isotransplantation of fatty livers in the rat (Agarwal and Nick, 2000). Nonetheless, despite the extensive literature on the cytoprotective role of HO-1, few studies have reported the detrimental effects resulting from HO-1 overexpression as a result of the accumulation of reactive iron released during the degradation of heme by HO-1 (Suttner and Dennery, 1999),(Lamb et al., 1999). This suggests that a beneficial threshold for HO-1 overexpression exists and that the exaggerated expression of HO-1 is unlikely to confer cytoprotection

1.2.1.2 HO-1 and inflammation

Induction of HO-1 is associated with reduced inflammation while its inhibition is pro-inflammatory (Willis et al., 1996, Willoughby et al., 2000). The anti-inflammatory property of HO-1 is supported by the fact that the only human who lacked the HO-1 enzymatic activity died of an inflammatory condition (Yachie et al., 1999). Furthermore, HO-1 deficient mice exhibited strong increased in proinflammatory cytokines, including IL-1β, IFN-γ, TNF-α, and interleukin-6 (IL-6) and developed a chronic inflammatory state characterized by splenomegaly, lymphadenopathy,

leukocytosis, as well as hepatic and renal inflammation that progressed with age (Poss and Tonegawa, 1997). Studies have shown that the expression of HO-1 in inflammatory conditions, including pulmonary inflammation, cardiac ischemia and reperfusion injury and hypertension, is potently cytoprotective (Minamino et al., 2001, Yet et al., 2001, Fujita et al., 2001, Otterbein et al., 1999). Furthermore, elevated level of HO-1 mRNA and protein was observed in human atherosclerotic plaques (Wang et al., 1998a) and in the vascular endothelial and smooth muscle cells exposed to oxidised low-density lipoprotein (Ishikawa et al., 1997). In addition, increase in HO-1 gene expression has also been shown to inhibit vascular smooth muscle cell proliferation, hence preventing atherosclerotic lesions (Duckers et al., 2001). Many studies have suggested that CO is largely responsible for the anti-inflammatory nature of HO-1 (Otterbein et al., 2000, Lee and Chau, 2002). However, ferritin, biliverdin and bilirubin may also mediate this effect (Vile et al., 1994, Gray et al., 2002, Nakagami et al., 1993). For example, they can reduce the interaction of leukocytes with the vascular endothelium by altering the expression of various adhesion molecules, such as E-selectin, ICAM-1, and VCAM-1 (Hayashi et al., 1999).

1.2.1.3 HO-1 and apoptosis

HO-1 has cytoprotective effects in cultured cells and in animal models of various diseases in brain, heart, kidney, lung and liver (Fang et al., 2004, Brouard et al., 2000). Apoptosis, or programmed cell death, is an active and tightly regulated process that occurs during normal development, but can be

induced in response to drugs or diseases. Apoptosis is known to commonly follow oxidative stress. The induction of HO-1 following oxidative stress has prompted researchers to investigate the anti-apoptotic properties of HO-1. Indeed, many studies have now confirmed such a role for HO-1. Overexpression of HO-1 in the endothelial cells of coronary microvessel has been shown to protect against the toxicity of heme (Abraham et al., 1995). Furthermore, *in vitro* pharmacological induction of HO-1 using hemin showed a significant decrease in apoptosis induced by potent oxidising agents such as peroxynitrite or cytotoxic agents such as cisplatin (Foresti et al., 1999),(Shiraishi et al., 2000). Other *in vitro* studies have shown that upregulation of HO-1 confers protection to endothelial cells against hydrogen peroxide-mediated cell death (Motterlini et al., 1996a, Motterlini et al., 1996b) and from the cytotoxic effect of tumour necrosis factor α (TNF-α) (Polte et al., 1997). In constrast, inhibition of HO-1 by specific inhibitors enhances the nephtoxic side effect of cisplatin and promotes endotoxin-induced septic shock (Fang et al., 2004).

1.2.1.4 HO-1 in cell proliferation

A number of studies have shown that overexpression of HO-1 in various cell types affects cell growth. In smooth muscle cells, renal and pulmonary epithelial cells, HO-1 overexpression has been shown to inhibit cell proliferation by causing cell arrest (Zhang et al., 2002b, Inguaggiato et al., 2001, Lee et al., 1996b). In the presence of HO-1 inhibitors, the anti-proliferative effect of HO-1 was abolished (Zhang et al., 2002b),(Lee et al.,

1996b). Furthermore, vascular smooth muscle cells lacking HO-1 displayed an increase in proliferation rate compared to those expressing HO-1 (Duckers et al., 2001). Studies investigating the mechanism by which HO-1 may affect cell proliferation have demonstrated that gene-induced or pharmacological overexpression of HO-1 causes up-regulation of p21 in smooth muscle cells (Inguaggiato et al., 2001, Duckers et al., 2001). In contrast, the retroviral overexpression of HO-1 in endothelial cells downregulates p21 and p27, leaving the expression of p53 unaffected (Abraham et al., 2003b). p21 is a cyclin dependent kinase inhibitor acting as a down-stream target of p53 tumour suppressor gene and is involved in promoting cell cycle arrest in the G1 phase of the cell cycle. The mechanism by which HO-1 overexpression regulates p21 is currently unclear. However, these findings strongly suggest that HO-1 can influence the cell cycle and its regulators depending on the cell types.

1.2.1.5 HO-1 in angiogenesis

Some studies have shown that HO-1 has proliferative functions and promotes angiogenesis. For instance, Li Volti et al. showed that NO donors can upregulate the expression of HO-1 and increase the proliferation of vascular endothelium (Li Volti et al., 2002), while the inhibition of HO-1 with zinc protoporphirin (SnPP) or antisense strategies (siRNA) abolished the proliferative effect of HO-1 (Dulak et al., 2008). The involvement of HO-1 in angiogenesis was demonstrated through the transfection of HO-1 gene into coronary endothelial cells, which resulted in the formation of capillary-like

tubular structures (Li Volti et al., 2002). Furthermore, the upregulation of HO-1 in endothelial cells has been linked to increase in VEGF synthesis in those cells, hence suggesting the contribution of HO-1 in angiogenesis (Jozkowicz et al., 2003). However, as human umbilical vein endothelial cells do not produce VEGF, these studies have come into question in recent years.

1.2.1.6 HO-1 in cancer

The expression and proliferative effect of HO-1 in cancer is controversial. Many studies have shown that HO-1 is highly expressed in a variety of tumours including adenocarcinoma, sarcoma, glioblastoma, and melanoma, and squamous carcinoma cells, prostate cancers, pancreatic cancers (Jozkowicz et al., 2007). However, the effect of HO-1 on tumour growth differs depending on the type of cancerous cells. In certain type of cancers, as in murine and human melanoma cell lines, the upregulation of HO-1 has been shown to promote their proliferation (Was et al., 2006), whereas its knockdown, as in pancreatic tumour cell lines, caused a significant reduction in tumour growth (Berberat et al., 2005). In contrast, pharmacological inhibition of HO-1 caused a small but significant increase in proliferation of the rat and human breast cancer cell lines, whereas its pharmacological induction significantly inhibited cell cycle progression in the same cells (Hill et al., 2005). Hence the ability of HO-1 to regulate tumour growth is variable and tumour specific.

1.2.2 Carbon monoxide

Carbon monoxide (CO) is a by-product of HO catabolism. The basal level of CO is generated from HO-2 as a result of the degradation of heme and the physiological turnover of heme (Kajimura et al., 2003, Suematsu et al., 1994) and under physiological conditions, the basal level of CO in the human body is about 20 μM/hr (Johnson et al., 2003). This basal level is responsible for many biological processes, including activation of cGMP when NOS is inhibited (Kaide et al., 2001, Kajimura et al., 2003). During stress conditions, CO is produced from the upregulation of HO-1 (Abraham et al., 2003a).

Like NO, CO activates soluble guanylate cyclase and increases the intracellular concentration of cGMP (Furchgott and Jothianandan, 1991), hence inhibiting platelet aggregation (Brune and Ullrich, 1987) and causing smooth muscle relaxation (Morita and Kourembanas, 1995). CO also inhibits endothelin-1 and platelet-derived growth factor-B to promote vasodilation (Morita and Kourembanas, 1995). Furthermore, CO has anti-inflammatory properties and has been shown to inhibit the production of pro-inflammatory cytokines such as TNF-α, IL-1β or IL-6 from lipopolysaccharide-stimulated macrophages (Otterbein et al., 2000, Sawle et al., 2005). Brouard et al. reported that the anti-apoptotic effect of HO-1 was due to the production of CO in endothelial cells expressing HO-1 and that the release of CO may serve as an intercellular signaling molecule to protect those endothelial cells which do not express HO-1. They suggested that p38 mitogen-activated protein kinase (MAPK) activation might be responsible for

the anti-apoptotic effect of HO-1/CO system in the endothelial cells as observed in other cell type including the kidney epithelial cell line HeLa, cardiac muscle cells and lymphoid Jurkat T cells (Brouard et al., 2000, Silva et al., 2006, Soares et al., 2002).

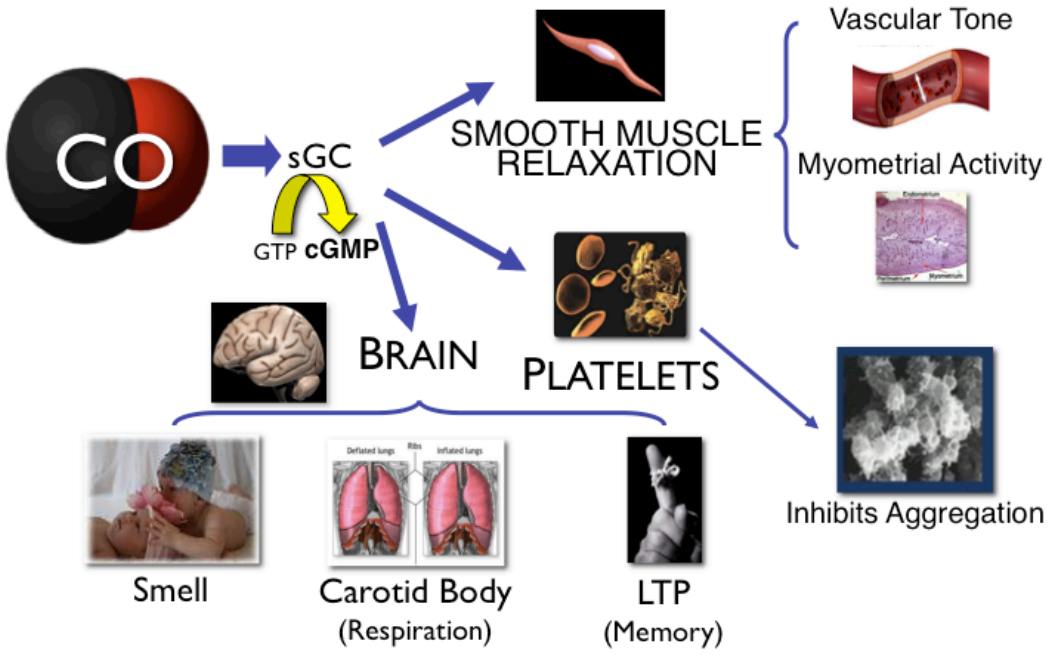


Figure 1.8. **Physiological role of carbon monoxide (CO).** CO is involved in smooth muscle relaxation and inhibits platelets aggregation. It also controls neurological processes involved in sensory function and respiration.

1.2.3 Biliverdin reductase and bilirubin

Human biliverdin reductase (BVR) is a water-soluble enzyme that reduces biliverdin (the product of heme oxygenase HO-1 and HO-2 activity) to bilirubin. It is unique among biological catalysts due to its dual pH/cofactor dependent activity profile (Kutty and Maines, 1981); either NADPH or NADH are used, with pH optima of 8.7 and 7.0, respectively. It also functions as a serine/threonine (Lerner-Marmarosh et al., 2005) and tyrosine kinase (Hunter and Cooper, 1985) and acts as a transcription factor in the MAPK signaling cascade (Salim et al., 2001). A rare feature of BVR is that its biological activity depends on its pH dependent autophosphorylation (optimum pH 8.5), which is reversible (Salim et al., 2001).

Singleton et al. were the first to uncover the enzymatic action of BVR in 1965 (Singleton and Laster, 1965). BVR is expressed in all tissues in two forms: BVR-A (dominant in adults) and BVR-B (dominant in fetal state) (Maines et al., 2007, Florczyk et al., 2008). The kidney has the highest level of BVR (McCoubrey et al., 1995). Unlike bilirubin, biliverdin cannot cross the cell membrane lipid bilayer (Maines, 2005). Experiments in rodents and human cell lines have shown that environmental agents, such as bromobenzene, renal toxin and lipopolysaccharide can activate BVR in the kidney and induce its translocation to the cell nucleus (Maines et al., 2001).

The resulting product of the enzyme activity of BVR is bilirubin, a biologically active and multifunctional protein. Bilirubin is an important cytoprotectant and antioxidant, which protects nuclear components against free radical damage. It also inhibits the production of superoxide-producing NADPH oxidase. The BVR/bilirubin system can act as an important survival factor. For instance, under hemolytic conditions or when HO-1 is induced, a large amount of heme is degraded. Therefore, high level of BVR is required to prevent biliverdin levels from rising above normal levels. The accumulation of biliverdin due to the absence of BVR can cause the fatal "green jaundice". Small interfering RNA (siRNA) knock down of BVR can also cause depletion of bilirubin, hence causing apoptotic cell death via the increase in tissue level of reactive oxygen species (Baranano et al., 2002).

1.3 ANGIOGENESIS

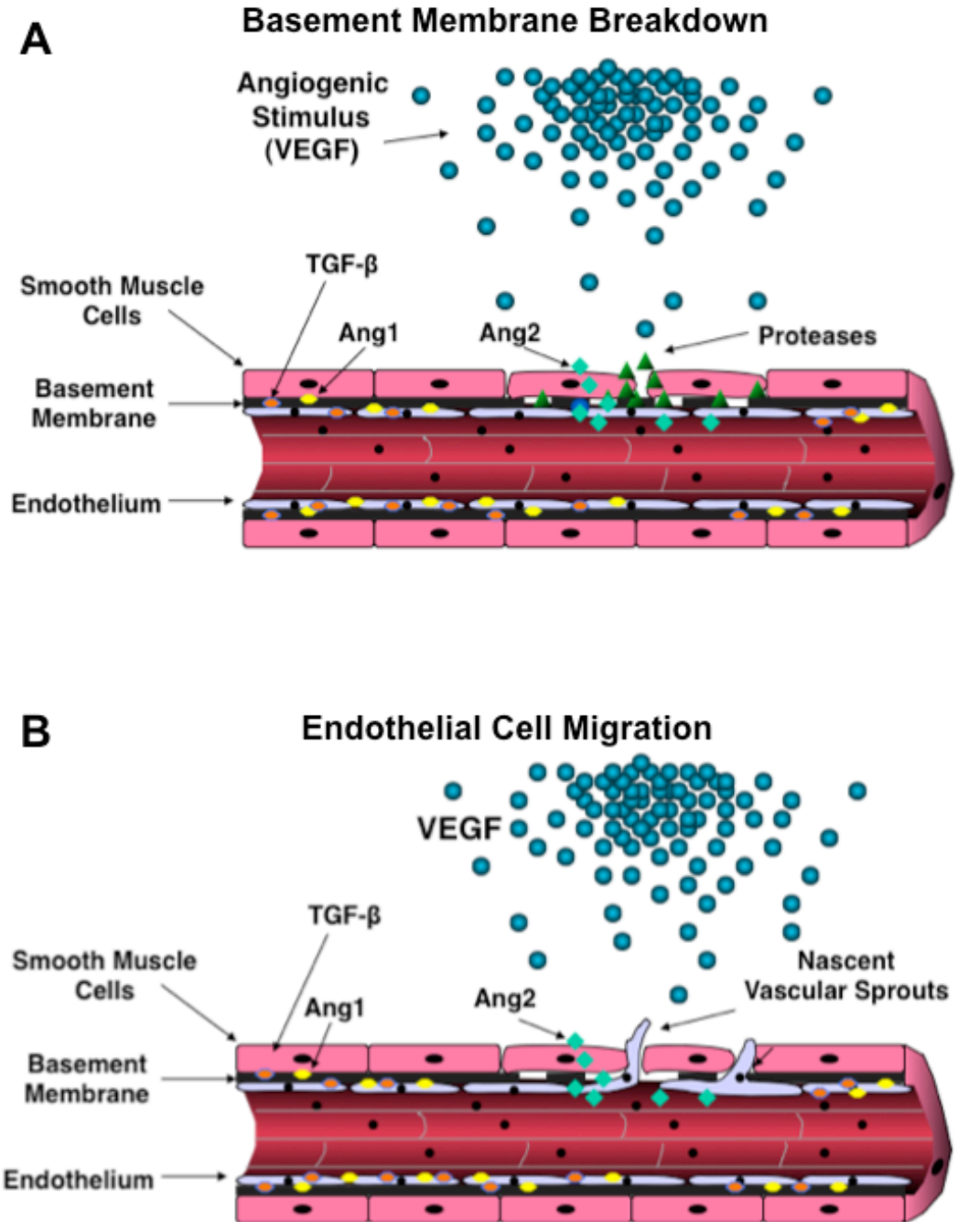
Vasculogenesis and angiogenesis are the two processes involved in the formation of new blood vessels during embryogenesis. Vasculogenesis is unique to embryonic development whereas angiogenesis occurs throughout life. During vasculogenesis, angioblasts proliferate and coalesce into a primitive network of vessels known as the primary capillary plexus. The endothelial cell lattice created by vasculogenesis serves as a scaffold for angiogenesis. Angiogenesis, also refered as neovascularisation, is the formation of new capillaries from pre-existing microvasculature (Ausprunk and Folkman, 1977, Folkman, 1992 #1889, Carmeliet, 2005). It involves the expansion of the endothelium through proliferation, migration and

remodeling where capillaries are formed by spouting or by non-sprouting angiogenesis (Risau, 1997).

Angiogenesis is a dynamic multistep phenomenon, which is regulated by a number of pro-angiogenic and anti-angiogenic (angiostatic) factors (Papetti and Herman, 2002). It sequentially involves the degradation of vascular basement, and interstitial matrix by endothelial cells, the coordinated migration and proliferation of endothelial cells and tubulogenesis and the formation of capillary loops. Angiogenesis is completed by a process known as vascular maturation, whereby pericytes are recruited to stabilize the new vessels by inhibiting endothelial cell proliferation and migration, and by stimulating the production of extracullular matrix (Figure 1.9). In addition, large vessels may become covered with smooth muscle cells providing them with elasticity and vasomotor properties (Carmeliet and Jain, 2000).

In healthy adults, angiogenesis is a tightly regulated process. Under physiological conditions, the rate of proliferation of endothelial cells is very low (Denekamp, 1982). The maintenance of quiescent endothelial cells is thought to be due to negative regulators of angiogeneis, since pro-angiogenic factors have been detected in adult tissues in which there is no angiogenesis. The growth of new vessels only occurs in ovarian cycle, wound healing or bone fracture and in muscles adaptations to exercise (Klagsbrun and D'Amore, 1991, Brown and Hudlicka, 2003). Angiogenesis is a crucial phenomenon during pregnancy and is required for the development of the vascular structures involved in transplacental exchange and for the growth

of the placental vessels. In contrast, in pathological conditions such as atherosclerosis, diabetic retinopathy, rheumatoid arthritis, psoriasis, endometriosis and cancer unrestrained angiogenesis occurs, while in the heart, brain and peripheral ischemia, pre-eclampsia, and nephropathy are characterized by insufficient angiogenesis (Carmeliet, 2005).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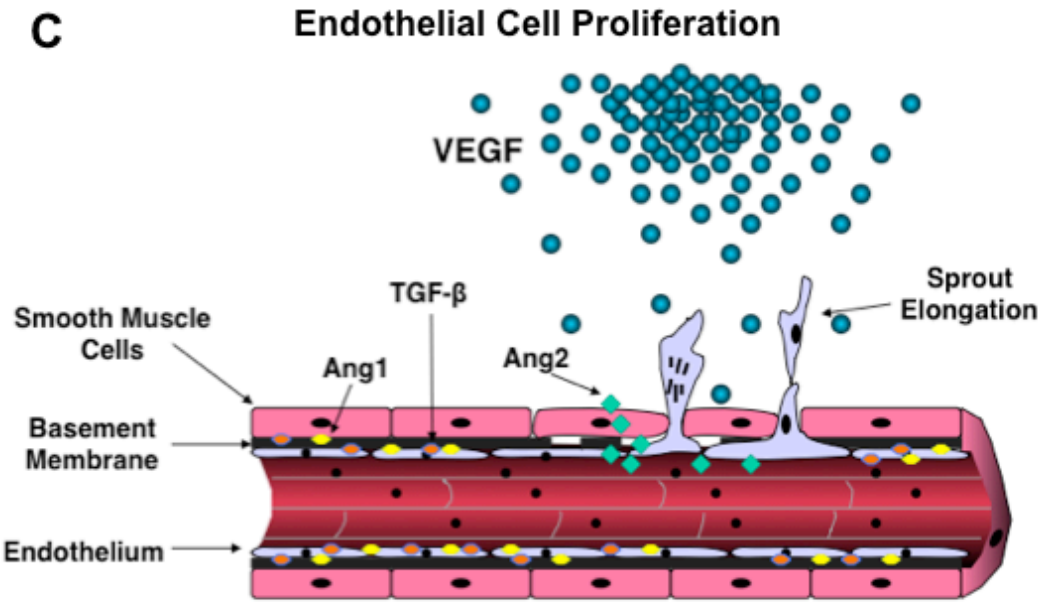
Basement Membrane Breakdown
Angiogenic Stimulus (VEGF)
TGF-β
Smooth Muscle Cells
Ang1
Ang2
Proteases
Basement Membrane
Endothelium
B
Endothelial Cell Migration
VEGF
TGF-β
Smooth Muscle Cells
Ang1
Ang2
Nascent Vascular Sprouts
Basement Membrane
Endothelium


C
Endothelial Cell Proliferation
VEGF
TGF-β
Sprout
Elongation
Smooth Muscle
Cells
Ang1
Ang2
Basement
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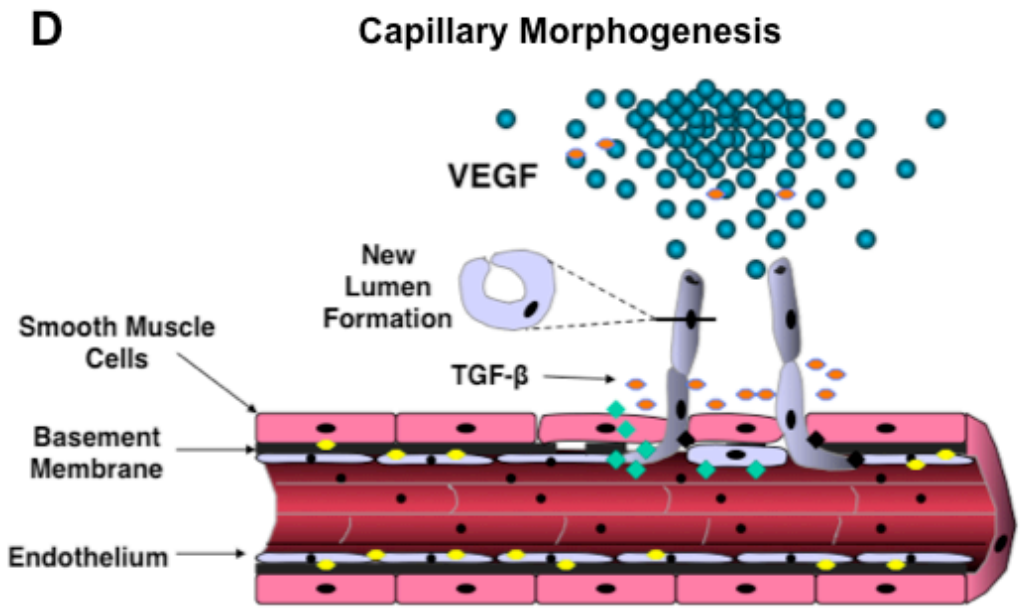
D
Capillary Morphogenesis
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New
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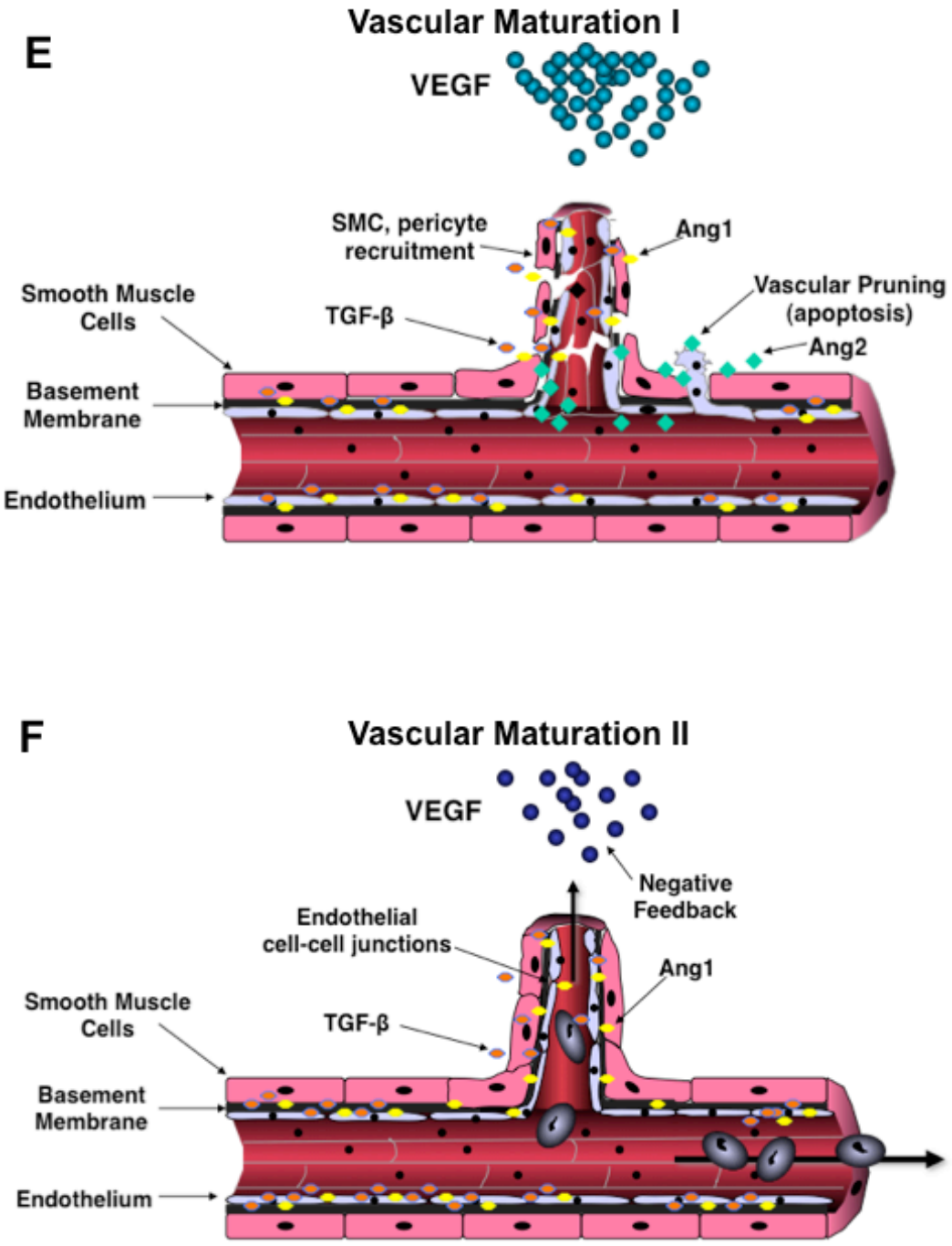

Figure 1.9. **Sequential steps involved in angiogenesis.** VEGF acts as a chemoattractant for endothelial cells migration. Proteases cause basement membrane degradation. Ang-1 binds to its receptor Tie2 and promotes survival and stabilisation of quiescent endothelial cells. The Tie2 antagonist, Ang-2, increases vascular permeability and promotes cell migration and formation of capillary sprout. TGF-β plays a role in vessel maturation, ideally resulting in functional vessels that can conduct blood, oxygen, and nutrients to the site of the angiogenic stimulus and decrease in VEGF.

1.3.1 Physiological angiogenesis

During adult life, physiological angiogenesis is involved in the development of the ovarian follicle and corpus luteum, and in the endometrium in each menstrual cycle (Demir et al., 2010). It is also involved in the wound repair process, where it provides a supply of nutrients and promotes granulation, tissue formation and the clearance of debris. Neovascularisation in wounds depends on cell-cell interactions, cell extracellular matrix interactions and also the balance between angiogenic agonists and antagonists. Tissue injury is followed by exudation of plasma constituents including fibrinogen. Fibrinogen provides the substrate for the generation of a fibrin-containing matrix, which is subsequently replaced by granulation tissue (Calvete, 1994). Replacement involved proteolysis by plasmin, which is generated from plasminogen present in plasma and the interstitial fluid (Miyashita et al., 1988). Plasminogen activators convert plasminogen into plasmin by limited proteolysis (Dano et al., 1985). Granulocytes, monocytes, fibroblasts and capillary endothelial cells are involved in the organization of the primary wound matrix (Lanir et al., 1988). During the later stages of wound repair, macrophages stimulated by hypoxia, cytokines or inflammatory mediators synthesize new factors like transforming growth factor (TGF-β) and VEGF (Knighton et al., 1983). During the terminal stages of healing, the production of angiogenic factors is decreased when granulation tissue is formed and the area is less hypoxic.

The development and endocrine function of the ovarian corpus luteum are dependent on the growth new capillary vessels. Progesterone release by the corpeus luteum is crucial for implantation and maintenance of pregnancy. After ovulation, the vessels invade and ruptured follicle and form a microvascular plexus that nourishes the developing corpus luteum. Multiple mediators are involved in corpus luteum angiogenesis including VEGF, basic FGF (bFGF) (Ferrara et al., 1998), angiopoietin-1 and angiopoietin-2 (Maisonpierre et al., 1997).

1.3.2 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) was first described as a protein able to induce vascular permeability in tumours (Senger et al., 1983). It was later described as a stimulator of endothelial cell proliferation and as a major inducer of angiogenesis and vasculogenesis (Ferrara, 2004). VEGF belongs to the platelet derived growth factor (PDGF)/VEGF family of growth factors which also includes placenta growth factor (PlGF) (Maglione et al., 1991), VEGF-B (Olofsson et al., 1996), VEGF-C (Joukov et al., 1996) (Lee et al., 1996a), VEGF-D (Achen et al., 1998) and VEGF-E (Ogawa et al., 1998, Meyer et al., 1999) (see Figure 1.10).

Vascular endothelial growth factor-A (VEGF-A) is widely known for its critical role in vascular development and angiogenesis. It is an endothelial specific mitogen, which promotes endothelial cell survival and causes endothelium-dependent vasodilation (Schrijvers et al., 2004). Increasing

evidence point to VEGF-A being an important factor that maintains vascular homeostasis. Lee and colleagues showed that the absence of VEGF-A from endothelial cells of mice was associated with organ failure, haemorrhages, intestinal perforations and signs of multiple infarcts. Furthermore, the presence of cleaved caspase-3 in those mice lacking endothelial-VEGF and morphological alterations of the endothelial cells such as membrane blebbing, cell shrinkage, cell rupture, nuclear condensation and exposure to cytosolic component provided support for the importance of VEGF in inhibiting endothelial cell apoptosis. Accumulation of vWF and fibrinogen deposits in the same mice signified endothelial cell damage. Eventually the endothelial specific VEGF deficient mice died from thromboembolism and cardiac ischemic event (Lee et al., 2007).

VEGF also stimulates the release of NO from HUVEC (Ahmed et al., 1997, Papapetropoulos et al., 1997) and NO plays a role in sprouting angiogenesis by mediating vasodilation (Duda et al., 2004). VEGF-A acts via two high-affinity tyrosine kinase receptors (VEGFR-1 and VEGFR-2) and also binds with lower affinity to co-receptors such as neuropilins (NRP) and heparin sulphate containing proteoglycans (Neufeld et al., 1999, Neufeld et al., 2002) (Figure 1.12). VEGF mediated cell proliferation and migration are mediated by VEGFR-2, while VEGFR-1 plays a role in the endothelial cell differentiation and release of nitric oxide (Bussolati et al., 2001). Activation of VEGFR-1 (Bussolati et al., 2001) and VEGFR-2 (Feng et al., 1999, Wu et al., 1999) causes upregulation and phosphorylation of eNOS and induces the release of NO from endothelial cells.

VEGFR-1 is located on the vascular endothelial cells, smooth muscle cells, monocytes and osteoblasts. Alternative splicing of this receptor generates its soluble form, soluble VEGFR-1 (sVEGFR-1), also known as soluble Fms-like tyrosine kinase receptor-1 (sFlt-1). Although sVEGFR-1 lacks the cytosolic region, it can still bind its ligands VEGF and acts as an antagonist (Luttun et al., 2004). In vivo studies in mice have suggested that VEGFR-1 does not play an important role in vascular development. However, mice lacking VEGFR-1 die prematurely (embryonic day 8.5-9.0) because of vascular overgrowth and disorganisation (Fong et al., 1995), whereas mice deficient in VEGFR-2 die in utero at embryonic day 8.5 due to a defect in vasculogenesis resulting from a failure in migration and proliferation of hematopoietic/endothelial progenitor cells (Shalaby et al., 1995, Hidaka et al., 1999, Schuh et al., 1999). In contrast mice without the tyrosine kinase domain of VEGFR-1 survived (Hiratsuka et al., 1998) indicating that VEGFR-1 acts as a decoy receptor to regulate the bioavailability of VEGF for VEGFR-2 activation and development (Hiratsuka et al., 1998, Kearney et al., 2004). However, using chimeric receptor system in endothelial cells, Ahmad and colleagues demonstrated the involvement of VEGFR-1 in adult angiogenesis. They showed that the independent activation of VEGFR-1 and VEGFR-2 causes capillary-like tube formation on Matrigel in an NO-dependent manner (Ahmad et al., 2006).

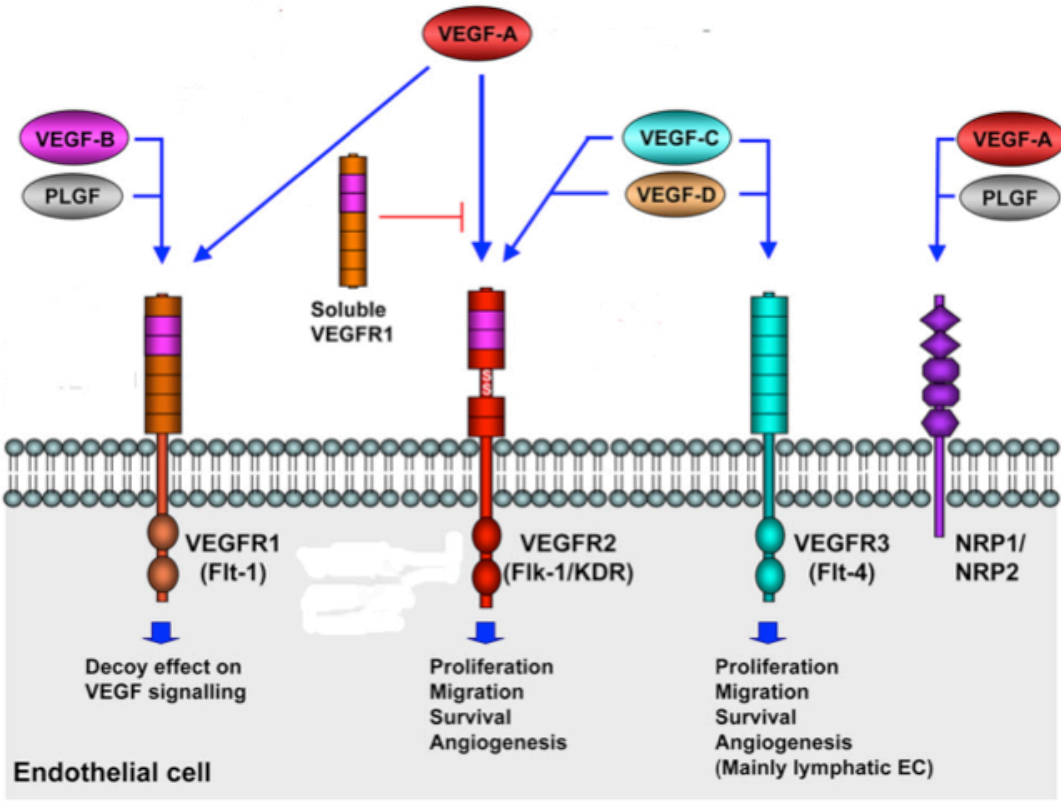

Figure 1.10. **VEGF signaling pathway in endothelial cell.** There are five ligands (VEGF-A, -B, -C and PlGF) and five receptors (VEGFR-1, -2, -3 and NRP1 and NRP2). VEGFR-1 and VEGFR-2 are expressed in the cell surface of most blood endothelial cells. In contrast, VEGFR-3 is largely restricted to lymphatic endothelial cells. VEGF-A binds VEGFR1, VEGFR2, NRP1 and NRP2; VEGF-B interacts with VEGFR1 and NRP1; VEGF-C binds VEGFR2, VEGFR3 and NRP2; VEGF-D interacts with VEGFR2 and VEGFR3; and PlGF interacts with VEGFR1, NRP1 and NRP2 (Adapted from Li, 2009 #3123)

1.3.3 Placenta growth factor

Placenta growth factor (PlGF) was first discovered in the human placenta (Maglione et al., 1991). It is a member of the VEGF family of growth factors (Ribatti, 2008). Alternative splicing of the human PlGF mRNA produces 4

isoforms: PlGF-1 ($PlGF_{131}$), PlGF-2 ($PlGF_{152}$), PlGF-3 ($PlGF_{203}$) and PlGF-4 ($PlGF_{224}$). PlGF-1 and PlGF-3 bind exclusively to VEGFR-1, which is highly expressed in vascular endothelial cells. PlGF-2 binds to the neuropilin-1 (NRP1) and neuropilin-2 (NRP1), which are also expressed in endothelial cells (Figure 1.10). Unlike VEGF, binding of PlGF to VEGFR-1 causes phosphorylation of alternative tyrosine residues and gene expression in endothelium (Autiero et al., 2003).

PlGF is not required for vascular development and its level is low in healthy adult tissues (Carmeliet et al., 2001). However, in normal endothelial cells, PlGF stimulate angiogenesis by amplifying the effect of VEGF. It also promotes monocyte migration (Clauss et al., 1996), stimulates NO release (Bussolati et al., 2001) and prolongs survival and stability of capillary-networks (Cai et al., 2003). In vivo studies have shown that during new blood vessels formation in the adult, endothelial cells become more responsive to VEGF through the upregulation of PlGF and VEGFR-1. PlGF affects endothelial cells directly by binding to VEGFR-1 and inducing its own signalling as well as amplifying VEGF-mediated angiogenesis (Carmeliet et al., 2001). Enhancement in angiogenesis can occur due to the intermolecular crosstalk between VEGFR-1 and VEGFR-2 as a result of activation of VEGFR-1 by PlGF (Autiero et al., 2003).

In pathological situations such as cancer and diabetes there is an increase in PlGF expression, which makes it an attractive target for therapy (Carmeliet et al., 2001). Recent evidence has shown that PlGF/VEGFR-1 signalling is

confined to pathologies characterized by aberrant angiogenesis such as cancer in the adult. The significance of PlGF in tumour development is highlighted by the fact that tumour angiogenesis and growth is inhibited in PlGF null mice, or transgenic mice bearing truncated/signaling-inactive VEGFR-1. PlGF deficient mice show significantly reduced growth and neovascularisation (Carmeliet et al., 2001). Consistent with these findings, PlGF appears to be a survival factor for the endothelium and prolongs stability of capillary-networks (Cai et al., 2003). However, unlike VEGF, which is overexpressed in most tumours, PlGF up-regulation occurs in only a few cancers such as melanomas (Lacal et al., 2000), hypervascular renal cell carcinomas and a subset of meningiomas (Donnini et al., 1999).

Cardiac expression of PlGF promotes wound healing (Iwama et al., 2006) and improves cardiac performance (Roncal et al., 2010) after acute myocardial infarction. PlGF has also been shown to stimulate collateral growth in the ischemic heart and limb (Pipp et al., 2003, Luttun et al., 2002, Kolakowski et al., 2006). On the other side, PlGF is involved in inflammatory angiogenic disorders such as atherosclerosis (Pilarczyk et al., 2008) or arthritis (Yoo et al., 2009). Since monocytes and macrophages express VEGFR-1 receptors, PlGF acts as a chemoattractant and promotes inflammation by increasing monocyte migration, cytokine production (Fu et al., 2009) and tissue factor expression (Carmeliet et al., 2001).

1.3.4 Transforming growth factor-β

Transforming growth factor-β (TGF-β) is part of a superfamily of dimeric growth factors and similar to VEGF, it has the ability to regulate blood vessel formation. It is also involved in many biological processes, including cell proliferation, migration and vessel maturation (Figure 1.9). It also regulates cellular functions involved in development, wound healing, cancer, fibrosis, vascular, and immune diseases. There are three TGF-β isoforms: TGF-β1, -β2 and -β3 (ten Dijke and Arthur, 2007, Blobe et al., 2000). TGF-β1 is expressed in endothelial cells, vascular smooth muscle cells, myofibroblasts, macrophages and other hematopoietic cells. TGF-β2 is localised in epithelial and neuronal cells while TGF-β3 is predominantly expressed in mesenchymal cells, vascular smooth muscle cells, macrophages and endothelial cells (Letterio and Roberts, 1996, Taya et al., 1999, Molin et al., 2002). TGF-β modulates cellular processes by binding to three high affinity cell surface receptors. The type III receptor is the most abundant receptor. However, it is a non-signaling receptor and functions to transfer TGF-β to its signaling receptors, type I and type II. Another type III-related TGF-β receptor that is expressed in endothelial cells is Endoglin, which is made up of an extracellular domain and a cytoplasmic tail homologous to the type III receptor (Duff et al., 2003).

Knockdown studies of TGF-β, its receptors and its downstream signaling proteins, activin receptor-like kinase-1 (ALK1) and ALK5, have revealed the

importance of TGF-β signaling in vascular development. TGF-β1 deficient mice die in utero due to vascular defects (Dickson et al., 1995), while TGF-β1 null embryos display developmental defects and cardiac malformations (Bartram et al., 2001). Mice lacking TGF-β type II receptor (TGF-β RII) or ALK5 die at embryonic day 10.5 due to vascular defects of the yolk sac (Oshima et al., 1996, Larsson et al., 2001). ALK1 knockout mice die at embryonic day 10.5–11.5, whereas mutation in ALK1 caused defective angiogenesis and impaired vascular smooth muscle cell development (Oh et al., 2000). Smad1-deficient mice demonstrate a failure in establishing chorion–allontoic circulation (Tremblay et al., 2001, Lechleider et al., 2001), whereas Smad5-deficient embryos had defects in yolk sac vasculature with enlarged blood vessels (Chang et al., 1999, Yang et al., 1999). Absence of the accessory receptor, endoglin, caused embryonic lethality at embryonic day 10.5–11.5 accompanied by cardiovascular and angiogenic defects due to abnormal vascular smooth muscle cell development (Li et al., 1999, Arthur et al., 2000, Bourdeau et al., 1999). Mice deficient in the accessory TGF-β type III receptor, betaglycan, exhibited lethal proliferative defects in the heart and apoptosis of the liver (Stenvers et al., 2003).

TGF-β exerts bifunctional effects on angiogenesis. It can stimulate and inhibit proliferation of endothelial cells in a dose-dependent manner. Low doses of TGF-β stimulate proliferation and migration, while high doses of TGF-β inhibit these responses. Furthermore, TGF-β regulates the activation state of the endothelium via a fine balance between ALK5 and ALK1 signaling

(Goumans et al., 2002). The TGF-β/ALK5 pathway leads to the inhibition of endothelial cell migration and proliferation, while the TGF-β/ALK1 pathway induces endothelial cell migration and proliferation (Ota et al., 2002). Endoglin, the TGF-β coreceptor, has been shown to regulate the balance between ALK1 and ALK5 signalling (Figure 1.11) (Goumans et al., 2002).

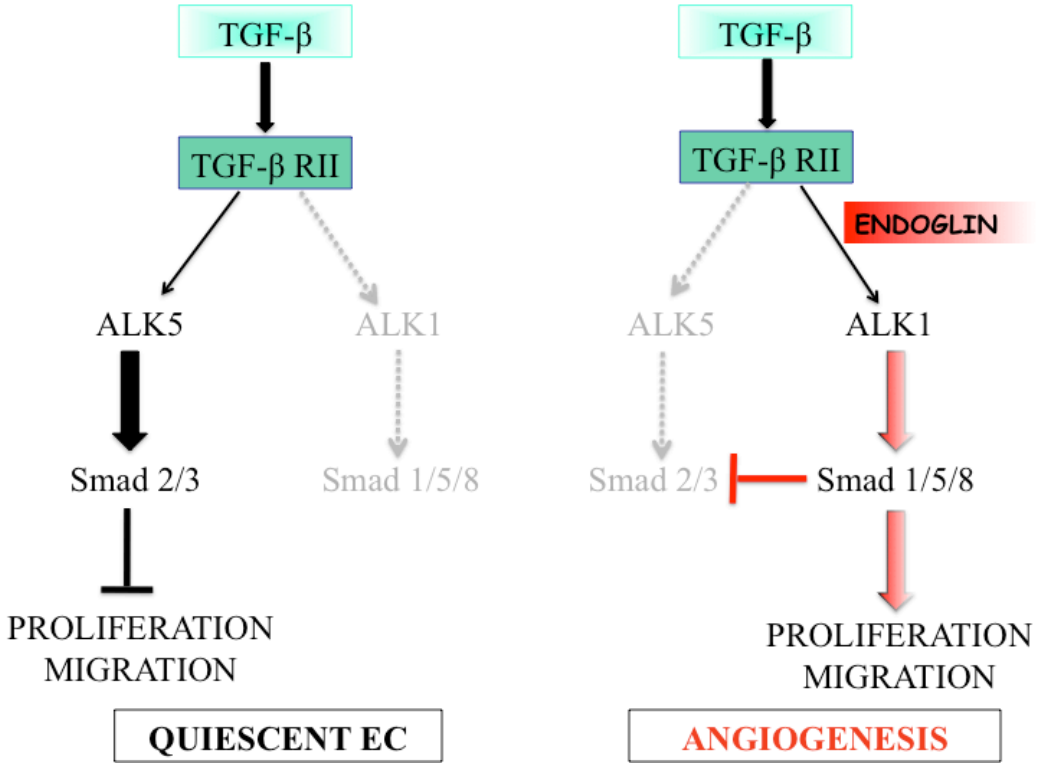

Figure 1.11. **Regulation of angiogenesis by TGF-β/Endoglin**. TGF-β /ALK5 signalling pathway inhibits cell proliferation and migration, whereas the TGF-β/ALK1 pathway promotes proliferation and migration. Endoglin, a coreceptor of TGF-β RII is essential for ALK1 signalling. In the absence of endoglin, theTGF-β /ALK5 signalling is predominant and maintains a quiescent the endothelium (EC). High endoglin expression stimulates the ALK1 pathway and indirectly inhibits ALK5 signalling, thus promoting angiogenesis (Adapted from (Lebrin et al., 2005).

1.3.4.1 Endoglin

Endoglin (also known as CD105) is a type of type I integral membrane glycoprotein that belongs to the zona pellucida family of proteins (Gougos et al., 1992, Llorca et al., 2007). It consists of a large extracellular domain (561 amino acids), a single hydrophobic transmembrane domain, and a short cytosolic domain (Gougos and Letarte, 1990). Endoglin acts as a TGF-β coreceptor and modulates TGF-β1 and TGF-β3 dependent responses, but not TGF-β2 (Cheifetz et al., 1992). Endoglin binds to TGF-β1 or TGF-β3 only when it is associated to TGF-β type II receptor (TGF-β RII) (Barbara et al., 1999). Both the extracellular and intracellular domain of Endoglin interacts with TGFβ- RII. The cytoplasmic domain of Endoglin, rich in serine and threonine residues, is phosphorylated by ALK5 or TGF-β RII (Guerrero-Esteo et al., 2002). The expression of Endoglin in endothelial cell is upregulated by hypoxia (Sanchez-Elsner et al., 2002) and TGF-β1, while TNF-α suppresses its expression (Li et al., 2003). In the absence of endoglin, the growth of endothelial cells is impaired and TGF-β/ALK1 signaling is abrogated whereas the TGF-β/ALK5 signaling is stimulated, indicating that Endoglin may function as a modulator of the balance between TGF-β/ALK1 and TGF-β/ALK5 signaling pathways. Hence Endoglin stimulates TGF-β/ALK1 signaling and indirectly inhibits TGF-β/ALK5 signaling, thus promoting the activation phase of angiogenesis (Figure 1.14) (Lebrin et al., 2004). Moreover, Endoglin can modulate the endothelial cell function independent of TGF-β by regulating cytoskeleton organization, protecting endothelial cells from hypoxia-induced apoptosis, stimulating JNK1 phosphorylation and

regulating the expression of eNOS (Lebrin et al., 2005). Endoglin plays an important role in tumoral and non-tumoral adult angiogenesis (Figure 1.11) (Bernabeu et al., 2009, Jerkic, 2006 #2660). In resting endothelial cells, the level of endoglin is undetectable. However, it is highly expressed in vascular endothelial cells in sites of active angiogenesis during embryogenesis, in inflamed tissues and within tumours (Burrows et al., 1995, Miller et al., 1999, Fonsatti et al., 2000). Mice deficient in endoglin die during embryonic development due to defective angiogenesis indicating the importance of endoglin in vascular development (Arthur et al., 2000).

In ischemia-reperfusion injury and myocardial infarction, the level of endoglin is increased in the ischemic area and border zone (van Laake et al., 2006). Furthermore, in atherosclerosis, the expression of endoglin is elevated in the vascular smooth muscle cells (Conley et al., 2000). In cancer, it is highly expressed in certain tumours, including primary and metastatic lesions of melanoma (Altomonte et al., 1996), in ovarian cancer (Henriksen et al., 1995) and in prostate cancer cells (Jovanovic et al., 2001). Endoglin is also expressed in syncytiotrophoblasts of term placenta (St-Jacques et al., 1994) and its expression is elevated in preeclampsia (Venkatesha et al., 2006).

As previously described, NO produced from eNOS is a major regulator of vascular tone and angiogenesis. In endothelial cells, endoglin is necessary for TGF-β_1-dependent transcription of eNOS (Santibanez et al., 2007). Endoglin induces the expression of eNOS at the transcriptional level in the presence or

the absence of TGF-β in endothelial cells via the TGF-β type I receptor/ALK5 and its downstream substrate Smad2 (Santibanez et al., 2007). In the presence of TGF-β, it increases the expression of eNOS by increasing Smad2 protein levels thereby enhancing TGF-β induction of eNOS and increasing the synthesis of NO. Jerkic and colleagues demonstrated that the concentrations of nitrites, a NO metabolite, in the plasma and urine were lower in $Eng^{+/-}$ than in $Eng^{+/+}$ mice. Moreover, the levels of eNOS is the kidneys and femoral arteries were about half in $Eng^{+/-}$ than in $Eng^{+/+}$ mice and were also reduced in primary cultures of aortic endothelial cells from $Eng^{+/-}$ compared with those from $Eng^{+/+}$ mice. In addition, they showed that overexpression or suppression of endoglin in cultured cells induced a marked increase or decrease in the protein levels of eNOS, respectively (Jerkic et al., 2004). Hence endoglin signaling via TGF-β can have vascular protective effect through the generation of NO and hence confer anti-inflammatory and atheroprotective effect.

1.4 INFLAMMATION

Inflammation is a complex biological response of vascular tissues to harmful injury caused by pathogens, damaged cells or irritants (Ferrero-Miliani et al., 2007). Leukocytes (white blood cells) are important mediators of inflammation. They can be divided into two categories, 1) granulocytes consisting of neutrophils, basophils and eosinophils and 2) agranulocytes consisting of lymphocytes, monocytes and macrophages. Neutrophils are the first cells to be recruited to the site of infection or inflammation. The number

of neutrophils adhering to the endothelium increases with inflammation (Harlan et al., 1981). Neutrophils are important for the successful elimination of pathogens. Activated neutrophils and monocytes/macrophages respond to septic stimuli by producing ROS (e.g., superoxide, hydrogen peroxide), RNS (e.g. peroxynitrite), the myeloperoxidase-derived oxidant, hypochlorous acid and proteases. Together, these events contribute to the neutrophil/macrophage-mediated killing of pathogens. However, the excessive production of ROS and proteolytic enzymes can also cause endothelial cell dysfunction and damage leading to organ dysfunction (Mochida et al., 2007).

Inflammation can be classified as either acute or chronic and systemic inflammation includes both acute and chronic changes. Acute inflammation is the initial response of the body to harmful stimuli and it involves increased movement of plasma and leukocytes (especially neutrophils) from the blood into the injured tissues. The purpose of this response is to eliminate the microbes and remove the cellular debris. If successful, acute inflammation is resolved, restoring the normal tissue architecture or forming a connective tissue scar. If the stimulus is not eliminated then the inflammatory process persists and evolves (Pober and Sessa, 2007). This prolongation of inflammation is known as chronic inflammation. Chronic systemic inflammation can cause damage to the cardiovascular, nervous, endocrine and other systems. For instance chronic inflammation has been associated to the development of insulin resistance and hence the metabolic syndrome and diabetes. In addition, chronic inflammation can also induce damage to the

arterial lining leading to the development of atherosclerosis and cardiovascular disease.

During both acute and chronic inflammation, a variety of soluble factors are involved in the recruitment of leukocyte through the expression of cellular adhesion molecules and chemoattractants. These soluble mediators modulate the activation of the resident cells (such as fibroblasts, endothelial cells, tissue macrophages, and mast cells) and the newly recruited inflammatory cells (such as monocytes, lymphocytes, neutrophils, and eosinophils) (Feghali and Wright, 1997). The soluble mediators include the inflammatory lipid metabolites such as platelet activating factor and the derivatives of arachidonic acid (prostaglandins, leukotrienes, lipoxins), which are generated from cellular phospholipids; the endogeneous vasodilator NO; and a group of cell-derived polypeptides, known as cytokines, which are involved in the orchestration of the inflammatory response by regulating the state of cellular activation and the systemic responses to inflammation (J.I. Gallin, 1992). Furthermore, systemic inflammation consists of changes in circulating acute-phase proteins with or without fever, anemia, leukocytosis, or metabolic adaptations, especially involving liver and adipose tissue (Gabay and Kushner, 1999). There are two types of acute-phase proteins: the positive ones that are increased during systemic inflammation such as C-reactive protein (CRP) or IL-6, or the negative ones that are reduced during systemic inflammation (e.g. albumin). Figure 1.12 summarises the cytokines involved during acute and chronic inflammation.

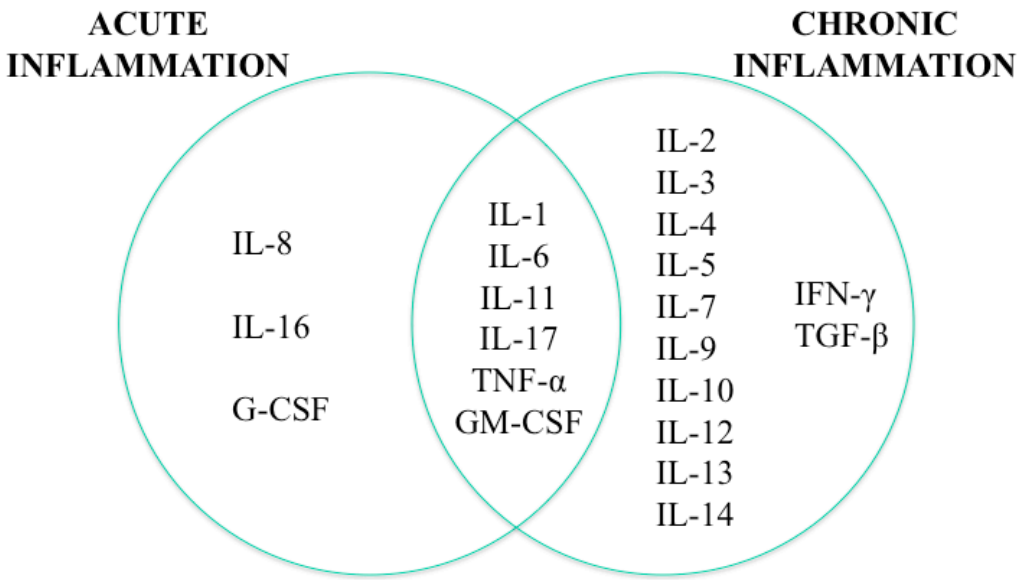

Figure 1.12. **Cytokines involved during acute and chronic inflammation.**

1.4.1 Neutrophil and the endothelium

Neutrophils are the most abundant subclass of leukocytes (60-70%) in the peripheral blood and they are the first line of defense against invading bacteria. The bone marrow produces and releases more than 5–10 × 10^{10} neutrophils in the circulation every day. In human, the average peripheral blood neutrophil count amounts to 2.5–7.5 × 10^{9}/L with a circulating half-life of 6 to 8 h (Sasmono et al., 2007). Defective neutrophil recruitment can have lethal consequences during bacterial infections (Anderson and Springer, 1987). In contrast, inhibition of neutrophil recruitment can have positive effect on ischemia-reperfusion injury (Singbartl et al., 2000), non-bacterial inflammation (Zarbock et al., 2006) and in autoimmune disease (Chiriac et al., 2007).

During acute inflammation, neutrophils are the first to be rapidly recruited to the endothelium followed by monocytes. They can cause "endothelial cell activation" through the release of cytokines, which then upregulate adhesion molecules on the endothelial cell surface, hence amplifying the neutrophil-endothelial interaction and the inflammatory process (Lush and Kvietys, 2000, Ley and Reutershan, 2006). Septic stimuli, such as LPS or TNF- α, activate transcription factors, including NFκB and activator protein 1 (AP-1), resulting in the transcriptional activation of multiple genes that leads to the formation of the pro-inflammatory cytokines (e.g. TNF- α, IFN-γ, pro-inflammatory interleukins, IL-6, IL-1β), and also increases the expression of adhesion molecules such as selectins, ICAM-1, VCAM-1 on endothelial cells and the release of chemokines (e.g. MCP-1, IL-8, fractalkine) by endothelial cells to recruit leukocytes from the blood to the site of infection or tissue damage (Ley and Reutershan, 2006, Liu and Malik, 2006, Abraham, 2003, Rao et al., 2007). E-selectin is upregulated by cytokine stimulation of endothelial cells; P-selectin is upregulated by stimulation of endothelial cells with agents such as thrombin and histamine; and L-selectin is resident on the surface of unstimulated neutrophils (Smith, 1993). E-selectin is primarily important for neutrophil recruitment, whereas ICAM-1 participates in the transmigration of all leukocytes by binding to β_2-integrins (also known as LFA1). VCAM-1 is involved in the recruitment of T cells, monocytes, eosinophils, and basophils, but not neutrophils, which lack the integrin counterreceptor VLA-4 (Langer and Chavakis, 2009). Transendothelial migration is directed by chemokines including IL-8, which attracts neutrophils, and MCP-1, the main attractant

for mononuclear cells (Braunersreuther et al., 2007). Endothelial cells express adhesion molecules and release chemokines at different rate depending on the stimuli to control the extent of leukocytes transmigration across them.

During inflammation, neutrophils roll along the endothelium, respond to inflammatory signals, arrest, and transmigrate (Figure 1.13). The adhesion molecules ICAM-1, VCAM-1 and MADCAM-1 are located on endothelial cells. Proinflammatory cytokines, such as TNF-α or LPS, stimulate endothelial cells to upregulate the expression of these adhesion molecules, which is responsible for the adhesion of neutrophils to the endothelium and their subsequent transmigration through the endothelial cell (Fassbender et al., 1995). The first contact between neutrophils and the endothelium is known as capture or tethering and is mediated by selectins (L-, P- and E-selectins) and their counter-receptors (Kansas, 1996). Selectin binding and the presentation of chemokines by endothelial cells leads to the activation of signaling pathways in neutrophils that cause changes in integrin conformation (inside-out signaling). Depending on the resulting integrin conformation, binding of activated integrins to their counter-receptors in the endothelial cell surface causes either slow rolling or arrest of neutrophils. Slow rolling of neutrophils along the endothelial cells is mediated by β_2-integrins (CD11a/CD18, CD11b/CD18, CD11c/CD18) binding to ICAM-1, ICAM-2 and ICAM-3 (Smith, 1993),(Dunne et al., 2002). The firm adhesion of neutrophils to endothelium is dependent on the time a neutrophil spends in close contact with the endothelium (Jung et al., 1998). Upon arrest, integrins bound to their ligands can signal into the neutrophil (outside-in signaling),

stabilize and strenghten the adhesion (Ley and Zarbock, 2006). They also activate different signaling pathways, and initiate transmigration. During the neutrophil-endothelium interaction, neutrophils receive signals from P-selectin glycoprotein ligand-1 (PSGL1), L-selectin, G-protein-coupled receptors, and integrins, which can activate different pathways and subsequently leading to the activation of neutrophils with actin polymerization, crawling, transmigration through the endothelium, respiratory burst, and degranulation. Neutrophils activation also involves the rapid shedding of L-selectin (Gearing and Newman, 1993) and upregulation of CD11b/CD18 on the neutrophil surface (Smith, 1993). Endothelial cell activation results in the shedding of ICAM-1 from the cell surface into the circulation.

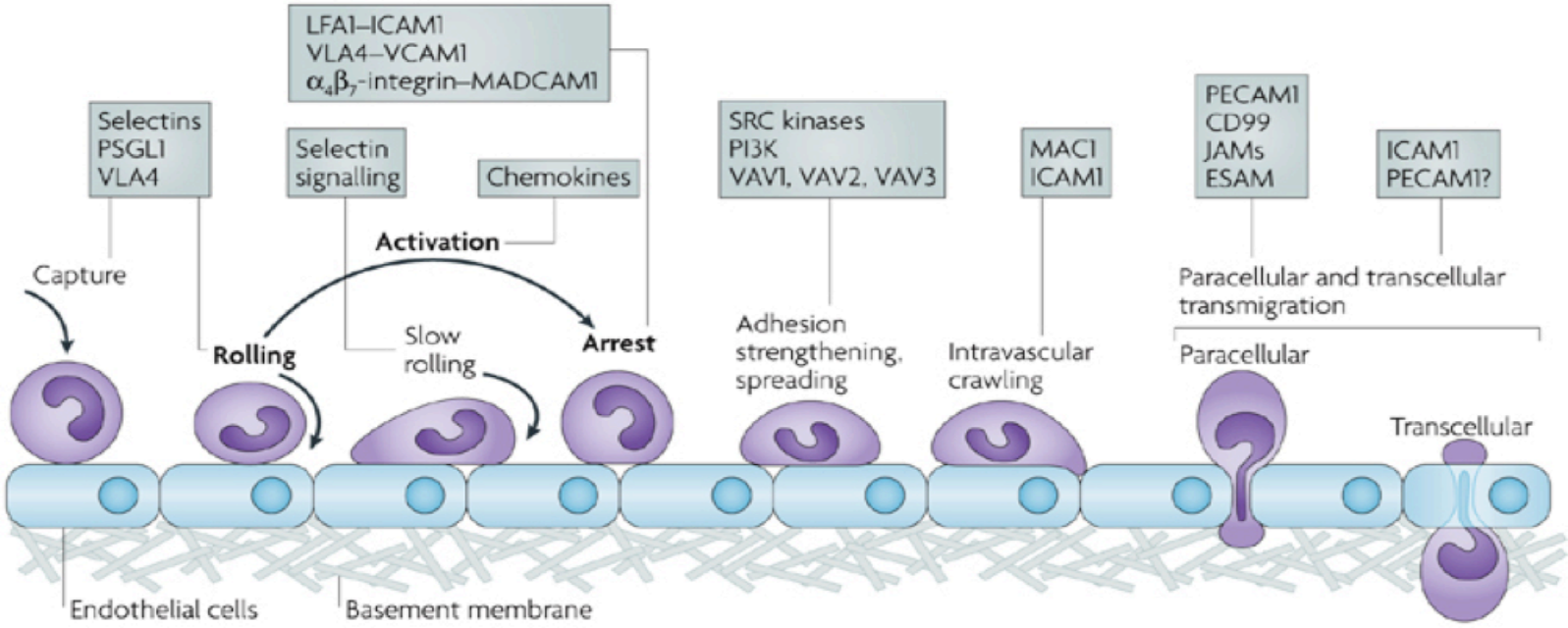

Figure 1.13. **Leukocyte-endothelial cell interaction.** Capture and slow rolling is mediated by selectins, activation is mediated by chemokines, arrest is mediated by integrins on neutrophils binding to adhesion molecules on the endothelial cell surface. Following arrest, neutrophils transmigrate either paracellularly or transcellularly (Ley et al., 2007).

1.4.2 Neutrophil degranulation

Once neutrophils migrate to the site of injury, they function as a secretory cell. The mobilization and release of cytoplasmic granules and secretory vesicles from neutrophils is an important part of the defense mechanism (Faurschou and Borregaard, 2003). Neutrophil degranulation occurs in a two-stage process involving an initial prerequisite "priming" step and a second "activation" step. Priming is essential to prevent unregulated neutrophil activation. Contact with activated endothelium, foreign surfaces, or agents such as LPS, primed neutrophils. Priming also inhibits chemotaxis and apoptosis suggesting that this event may serve to "fix" neutrophils at the inflamed site and increase the lifespan of these cells (Sengelov et al., 1995). Upon stimulation of neutrophils, there is a complete mobilization of secretory vesicles that contain the receptors necessary for the earliest phases of the inflammatory response such as β_2-integrin, CD11b/CD18 and CD35 (Sengelov et al., 1993, Sengelov et al., 1994). Continuous stimulation results in the release of secondary and then primary (azurophil) granules from neutrophils, a process known as sequential degranulation (Bainton, 1973, Bentwood and Henson, 1980). The extra cellular release of the secondary and primary granules is under separate controls. Chemoattractants selectively induce the release of secondary granules under conditions when azurophilic granules are not released (Table 1.1).

Neutrophils release four major types of granules, which include secretory vesicles that are endocytic in origin, tertiary, secondary, and azurophilic primary granules. Tertiary granules rich in proteases, such as MMP-9 and gelatinases, are released during the transmigration of neutrophils across the endothelium to facilitate the penetration of neutrophils through the endothelium. The release of the primary and secondary granules in the extravascular space is dependent on the increases in intracellular calcium. They contain the antibacterial peptides, including myeloperoxidase, α-defensins, elastases, lactoferrin, which are important in the clearance of bacteria and contribute to the degradation of engulfed microorganisms (Sengelov et al., 1993). Table 1.1 summarises the contents of neutrophil degranulation.

Table 1.1. **Contents of neutrophil degranulation**

	PRIMARY GRANULES	SECONDARY GRANULES	TERTIARY GRANULES	SECRETORY VESICLES
Membrane	CD63, CD68	Laminin-R, Vitronektin-R, TNF-R, FPR	CD11b, FPR	CD11, CD14, CD16, FPR, CD10, CD13
Matrix	Azurocidin Cathepsin G Elastase Proteinase-3 Defensins MPO	Lactoferrin, hCAP18 (LL-37), NGAL	MMP-9, lysozyme, gelatinases	Azurocidin, proteinase-3, albumin

1.4.3 Toll-like receptor mediated neutrophil activation

Toll-like receptors (TLR) function as pattern recognition receptors for a broad range of microbial stimuli. Until now, ten human TLR have been identified (Sabroe et al., 2003). Neutrophils express all of these TLRs, except TLR3 (Hayashi et al., 2003). The binding of TLR agonists on neutrophil causes activation of neutrophils (Kutsuna et al., 2004, Suzuki et al., 1999, Yuo et al., 1989). TLR are also expressed in different parts of a cell, such as the plasma membrane (TLR1, TLR2, TLR4, TLR5, and TLR6) or in endo(lyso)somes (TLR3, TLR7, TLR8, and TLR9) (Barton and Kagan, 2009). Table 1.2 summarises microbial TLR agonists and Table 1.3 summarizes the endogeneous TLR agonists.

LPS produced from Gram-negative bacteria is often used as a model of inflammation for *in vitro* and *in vivo* studies and TLR4 is its major receptor for binding (Poltorak et al., 1998, Qureshi et al., 1999). Mice lacking TLR4 are not able to display an inflammatory response to LPS and are highly susceptible to Gram-negative infections such as *Escherichia coli* peritonitis or pneumonia caused by *Klebsiella pneumoniae* or *Acinetobacter baumannii* (Malcolm et al., 2003).

Cellular responses to components of gram-positive bacteria are mainly mediated via TLR2 (Beutler et al., 2003). TLR2 agonists signal via TLR2 heterodimers, TLR2 in combination with TLR1 and TLR2 in combination with TLR6 (Hirschfeld et al., 2000, Aliprantis et al., 2000, Hajjar et al., 2001).

TLR2 ligands induce cytokine and chemokine production, with involvement of phosphoinositide 3-kinase and the Akt signaling pathway (Strassheim et al., 2004).

Table 1.2 **Microbial ligands reported to activate cells via TLR.**

	MICROBIAL LIGANDS	PATHOGENS
TLR 1/2/6	Lipopeptides Zymosan Lipoarabinomannan	Various bacteria, Mycoplasma Yeast Mycobacteria
TLR 3	ds RNA	Viruses
TLR 4	LPS Pneumolysin F-protein	Gram-negative bacteria Strep. Pneumonia Respiratory syncitial virus
TLR 5	Flagellin	Flagellated bacteria
TLR 7/8	Single stranded RNA	viruses
TLR 9	Unmethylated CpG DNA	Viral and bacterial DNA

Table 1.3 **Endogeneous TLR ligands**

	ENDOGENEOUS TLR LIGANDS	DISEASE
TLR 2	Hyaluronan fragments High-mobility group box 1 protein (HMGB1) Biglycan	Lung inflammation Systemic inflammation
TLR 3	RNA (from dead cells) RNA (dead cells)	Skin healing/injury Rheumatoid arthritis
TLR 4	Myeloid-related protein 8 and 14 (Mrp8/14) Type III repeat extra domain of fibronectin Hyaluronan fragments High-mobility group box 1 protein (HMGB1) Oxidized phospholipids Biglycan	Inflammation/sepsis Lung inflammation Inflammation/sepsis Lung injury Systemic inflammation
TLR 7/8	Small nuclear RNA	SLE
TLR 9	HMGB1 Immune complexes/dsDNA	Autoimmune disease (SLE) Autoimmune disease

1.5 DRUG-INDUCED ENDOTHELIAL DYSFUNCTION

Pharmacological agent and cytotoxic drugs can modulate the endothelial cells directly to trigger endothelial dysfunction and tissue toxicity. Indeed, drug-induced vascular injury is an important toxicological concern in the development of new drugs and in existing drug therapy, such as cisplatin chemotherapy. Various clinical studies have shown that one of the important drawbacks of cisplatin-based chemotherapy is the development of vascular injury during and post treatment (Nuver et al., 2010).

1.5.1 Cisplatin – A model of vascular injury

Cisplatin has been used for over 30 years as an anti-cancer drug. It has been the first line treatment for metastatic ovarian and testicular cancers combined with bleomycin or etoposide (Go and Adjei, 1999, Rabik and Dolan, 2007). It is also used against epidermoid carcinomas of the head and neck, refractory non-hodgkins lymphomas, and cancers of the bladder, lung, breast, uterus and cervix (Go and Adjei, 1999). Despite being one of the most effective chemotherapeutic agents, its administration has been hindered by its side effects including ototoxicity, neurotoxicity, nephrotoxicity and vascular toxicity.

The most common mode of administration of cisplatin is a single slow intravenous injection or infusion every 3-4 weeks. It can also be administered in close proximity to the site of the tumour. In ovarian tumour, the most

common method of administration is the intraperitoneal (IP) administration, where the concentration of the drug in the peritoneal cavity is about 50 times higher compared to intravenous (IV) administration. In the treatment of osteosarcoma and osteogenic sarcoma, repeated doses of intra-arterial cisplatin were found to be more effective than intravenous administration of the drug (Hugate et al., 2008). Intra-arterial delivery is also commonly used for hepatic tumours, melanoma and glioblastoma (Liu et al., 1998).

1.5.1.1 Cisplatin-induced apoptosis

Apoptosis is a mechanism through which cells with damaged genetic contents undergo programmed cell death to prevent the proliferation of cells with abnormal genes. It involves cell shrinkage, chromatin condensation, membrane budding, phosphatidylserine externalization and activation of a family of cysteine proteases called caspases (Cummings and Schnellmann, 2002).

Cisplatin is an inorganic molecule which contains two ammonia groups in the cis position and two chlorine molecules. Upon its entry in cells, cisplatin is hydrated and loses its chloride ions and gains two water molecules (Rabik and Dolan, 2007). The resultant highly reactive positively charged molecule has been shown to interact with intracellular nucleophilic molecules such as glutathione, methionine, metallothionein, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA is the major target of cisplatin. Cisplatin interacts with DNA to form DNA-protein and DNA-DNA interstrand and

intrastrand crosslinks (Siddik, 2003). Reports have shown that the cytotoxic effect of cisplatin against neoplastic cells is mostly due to the formation of intrastrand bifunctional adducts, which induces a conformational change in the DNA structure and initiate cell death by apoptosis (Go and Adjei, 1999, Siddik, 2003).

Caspases, a family of aspartic-acid directed proteases, is involved in the signalling pathways leading to apoptosis. Among the caspases, the proteolytic cleavage of caspase-3 plays a critical role in apoptosis (Polverino and Patterson, 1997). Indeed cisplatin exerts its potent chemotherapeutic effect specifically by triggering apoptosis in cancer cells via the activation of caspase-8, -3 and -6 (Seki et al., 2000). Activation of caspase-8 is the earliest event in the caspase cascade, followed by the activation of the intracellular protein Bid, which induces the release of cytochrome c from mitochondria and sequentially activating Apaf-1, caspase-9 and caspase-3 (Li et al., 1998, Luo et al., 1998, Zou et al., 1997, Hakem et al., 1998). Cisplatin-induced apoptosis can also be mediated by the activation of death-receptor pathway involving the FasL or the mitochondrial pathway, which also involves the caspase family of proteins (Rabik and Dolan, 2007, Sheikh-Hamad, 2008) (Figure 1.14). In culture cell lines, cisplatin may cause cell death through the inhibition of sodium-potassium adenosine triphosphatase, calcium channel function and mitochondrial function (Go and Adjei, 1999).

Low concentration of cisplatin given for a prolonged period can also cause apoptosis of cells. Studies on non-cancer cells, for example mouse tubular cells, have shown that high concentrations of cisplatin (800 μM) cause

necrosis whereas low concentrations (8 μM) leads to apoptosis (Lau, 1999). *In vitro* studies have shown that treatment with cisplatin activates caspase-3 in the kidney epithelial cells (Cummings and Schnellmann, 2002). Although caspases have been associated with apoptosis, they have recently been shown to play a role in necrotic cell death. For instance, high doses of cisplatin (50 μM), which causes cell death by necrosis, also induced caspase-3 activation in murine microvascular pancreatic endothelial cells indicating that apoptosis and necrosis may share common pathways (Dursun et al., 2006).

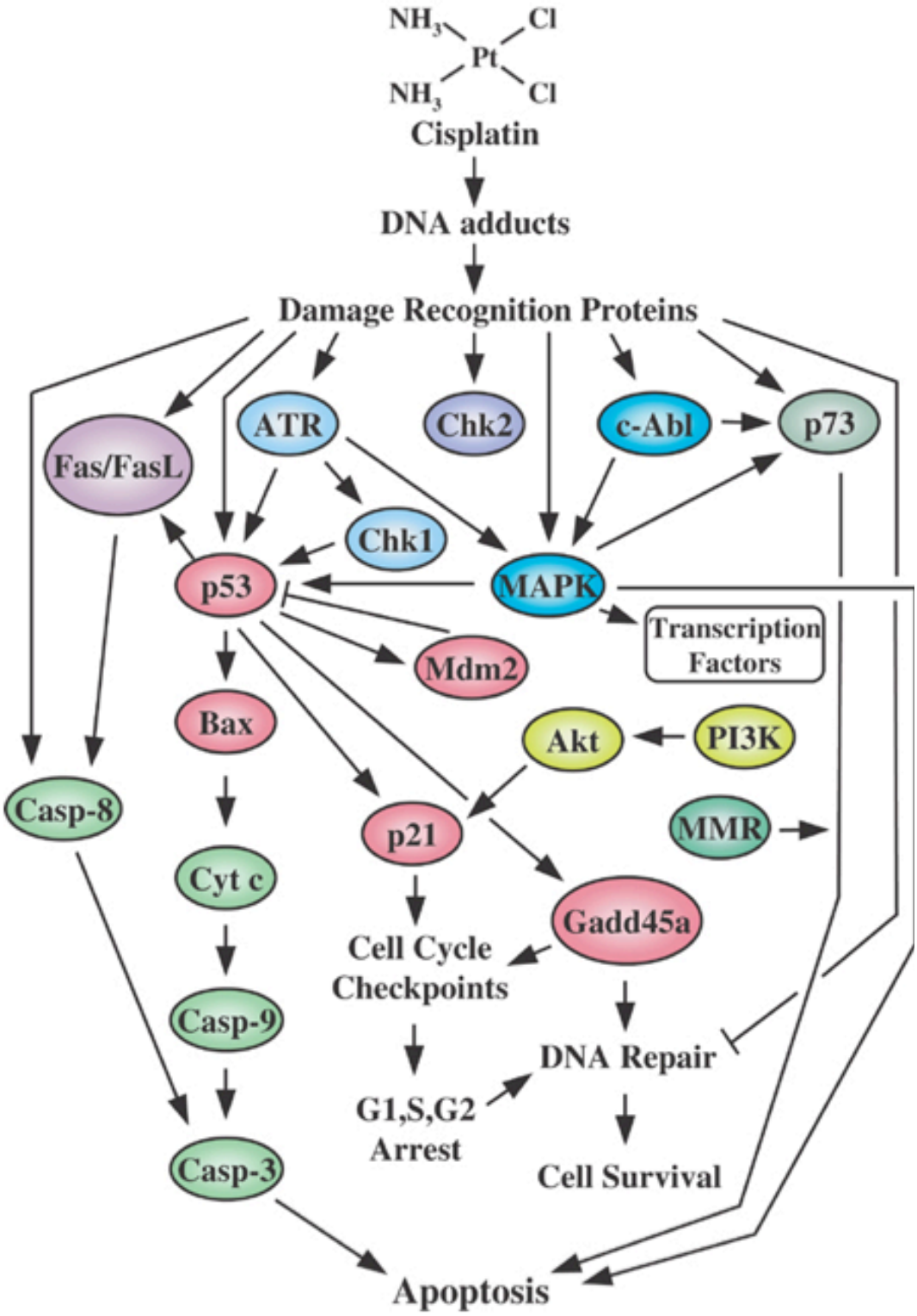

Figure 1.14. **Apoptotic pathways involved in cisplatin treatment**. Cisplatin binds to DNA to form DNA adducts, thereby inhibiting cell proliferation and DNA synthesis. The DNA damage response is transduced via the activation of several death pathways. Activation of caspase-3 is the final pathway leading to apoptosis (Adapted from (Siddik, 2003).

1.5.2 Cisplatin-mediated side effects

Although the use of cisplatin has produced high cure rates, it has also been associated with acute and chronic toxicity in normal tissues.

1.5.2.1 Ototoxicity

Ototoxicity is a common side effect of high doses of cisplatin. It occurs in about 23-54% of patients receiving cisplatin treatment and the functional deficits are observed as hearing loss and/or tinnitus in the frequency range beyond 4 kHz (Rabik and Dolan, 2007). Cisplatin causes damage to the outer hair cells of the cochlea (inner ear) (Rabik and Dolan, 2007) and the organ of corti (Rademaker-Lakhai et al., 2006). It activates specific enzyme in the cochlea to induce the release of reactive oxygen species (ROS) in the tissues of the inner ear (Rybak et al., 2007). The toxic effect of cisplatin is further exacerbated by the reduction of antioxidants level in the cochlea such as glutathione (Rademaker-Lakhai et al., 2006). Apoptosis is responsible for the outer hair cell death of the cochlea via the release of cytochrome c and the activation of caspases-9 and -3 (Rabik and Dolan, 2007).

Cisplatin accumulates in vascular areas of the cochlea to cause vascular damage, which resulted in a dose-related hair cell loss. Miettinen et al. have shown that administration of 0.1% of cisplatin in guinea pigs induced a decrease in blood flow in the cochlea (Miettinen et al., 1997). Detailed electron microscopic study of blood vessels of the stria vascularis of guinea

pigs have shown that endothelial cells were damaged after treatment with cisplatin alone or in combination therapy with Gemtamicin. This included mitochondrial damage, depleted organelles, intracytoplasmic vacuole formation, lipid bodies and cytoplasmic extrusions located on the surface of the lumen. These observations suggested that endothelial cell damage of the strial capillaries in the cochlea contribute to cisplatin-induced ototoxicity (Kohn et al., 1997).

1.5.2.2 Neurotoxicity

Acute and delayed CNS toxicity are observed in about 30% of patients treated with cisplatin in a dose-dependent manner (Dietrich et al., 2004). Cisplatin-DNA complex binds to and causes apoptosis of the dorsal root ganglion neurones. This produces primary sensory neuropathy, which is characterised by a decrease in sensory nerve conduction velocity and selective sensory loss in the extremities (Ta et al., 2006).

1.5.2.3 Nephrotoxicity

Above a concentration of 90 mg/m^2 IV or 270 mg/m^2 IP, cisplatin produces dose-dependent renal toxicity (Deng et al., 2001). Cisplatin is excreted mainly through the kidney. Its concentration is about 5 times higher in the proximal tubular epithelial cells compared to the serum concentration. This difference in concentration is associated to cisplatin-induced nephrotoxicity. Rodent studies have shown that cisplatin is mainly excreted through glomerular filtration and only a very small amount is secreted in the renal tubules.

Tubular reabsorption has not been observed. Cisplatin is taken up via the kidney specific-organic cation transporter-2 (OCT2) in both the proximal and the distal nephrons (Yao et al., 2007). In the proximal tubular cells, cisplatin accumulates in the cytosol, mitochondria, nuclei and microsomes. It is conjugated to glutathione and metabolized to a reactive thiol through a γ-glutamyl transpeptidase and a cysteine S-conjugate β-lyase-dependent pathway. The reactive thiol is responsible for the nephrotoxic effect of cisplatin (Yao et al., 2007).

Cisplatin-induced nephrotoxicity involves oxidative stress, apoptosis, inflammation and fibrinogenesis. Oxidative stress is one of the factors that cause acute renal injury. ROS damage cells by acting on the lipids (lipid peroxidation), proteins (denaturation) and DNA. In the presence of cisplatin, ROS is produced through various pathways including the xanthine-xanthine oxidase system, mitochondria and NADPH oxidase in cells. Cisplatin causes increase in free radical and decrease in antioxidant production by inducing glucose-6-phosphate dehydrogenase and hexokinase activity, which affect the mitochondrial dysfunction. Intracellular calcium level is increased and this activates the NADPH oxidase and stimulates ROS production by mitochondria. The level of superoxide anion, hydrogen peroxide and hydroxyl radical in the kidneys are higher after cisplatin treatment. Reactive nitrogen species (RNS) may also be involved in cisplatin-induced nephrotoxicity. In rats treated with cisplatin, the level of peroxynitrite and nitric oxide was higher compared to the control rats. Peroxynitrite can alter the protein structure and function, cause lipid peroxidation, DNA cleavage

and reduction in cellular defenses by oxidation of thiol pools (Yao et al., 2007).

Cisplatin induces inflammatory reactions in the kidney, which are also associated to renal injury. Degradation of IκB in a time-dependent manner and increase in nuclear factor-κB (NF-κB) binding activity by cisplatin is known to induce the expression of pro-inflammatory cytokines such as TNF-α, which induces apoptosis via the production of ROS. Furthermore, cisplatin increases the expression of cytokines and chemokines such as monocyte chemoattractant protein-1 (MCP-1), ICAM, HO-1, TNF receptor 1 and TNF receptor 2 in the kidney (Yao et al., 2007).

Impairment of renal blood flow is also a consequence of cisplatin treatment. Clinical studies in patients with non-seminomatous testicular carcinoma have shown that those patients exhibit a reduction in effective renal plasma flow and an increase in filtration fraction during cisplatin-based therapy (Offerman et al., 1984). Furthermore, *in vivo* data have demonstrated that the decrease in glomerular filtration rate in early cisplatin-induced acute renal failure was in part due to alteration in renal blood flow and renal vascular resistance (Winston and Safirstein, 1985). Micropuncture studies also confirmed that a single dose of cisplatin caused a decrease in single nephron glomerular filtration rate and an increase in the afferent arteriolar resistance as a consequence of a reduction in both glomerular plasma flow and transcapillary hydraulic pressure difference (Barros et al., 1989). As a consequence, severe decrease in renal blood flow can cause regions of

hypoxia in the kidney. The proximal tubules are very sensitive to ischemic insult and can undergo cell death through both apoptosis and necrosis (Yao et al., 2007).

1.5.3 Cisplatin-induced vascular damage

Until recently, most studies on cisplatin were focussed on the mechanism of anti-tumour activity of cisplatin and on its toxic effect in the ear and the kidney. However, it is now increasingly becoming evident that cisplatin-induced vascular toxicity may contribute to vascular injury not only during chemotherapy but can persist post-chemotherapy.

The vascular toxicities associated with cisplatin include hepatic veno-occlusive disease, Raynaud's phenomenon, myocardial ischemia and infarction, cerebrovascular attacks, venous thrombosis and thromboembolic events and hypertension (de Vos et al., 2004). Raynaud's syndrome and acute vascular ischemic events develops in 37-50% of patients treated with cisplatin-containing chemotherapy (Vogelzang et al., 1981, Hansen et al., 1990, Bokemeyer et al., 1996). Thrombolic events may occur shortly after administration of cisplatin (Weijl et al., 2000), while cumulative doses of cisplatin of more than 400 mg/m^2 have been associated to renovascular hypertension, cardiac ischemia, infarction and cerebrovascular attacks. Moreover, long-term survivors of testicular cancer often develop cardiovascular complications (Raghavan et al., 1992, Nord et al., 2003). In addition, several years after chemotherapy, the long-term survivors of testicular cancer were found to have elevated level of plasma vWf, micro-

albuminuria, inflammation, and an imbalance in the plasma levels of the fibrinolytic proteins tissue-type plasminogen activator and plasminogen activator inhibitor type 1 (Nuver et al., 2004), which indicate endothelial dysfunction.

Cisplatin can directly damage the endothelial cells or indirectly induce endothelial cell damage through inflammation or fibrinolysis. A recent study demonstrated that cisplatin attenuated the production of NO via the inhibition of Akt-eNOS cascades in human umbilical vein endothelial cells (HUVEC) and can hence contribute to the development of atherosclerosis (Sekijima et al., 2011). Cisplatin has also been reported to induce the release of pro-inflammatory cytokines, IL-1 and IL-6 from HUVEC (Shi et al., 1998). Furthermore, increased expression of ICAM-1 has been observed on HUVEC (Yu et al., 2008) and in human microvascular endothelial cells (HMEC) (Nuver et al., 2010) treated with cisplatin. In addition, in a rat model of cisplatin-induced nephrotoxicity, ICAM-1 mRNA level was found to be elevated and antibodies against ICAM-1 abrogated the renal dysfunction (Kelly et al., 1999). These evidence indicate that endothelial dysfunction is associated with cisplatin therapy and support the hypothesis that cisplatin can initiate or promote inflammation leading to the development of cardiovascular complications and atherosclerosis in cancer patients.

Neuropathy associated with cisplatin treatment appears to occur as a result of the destruction of the nerve blood supply of the spine (vasa nervorum). In their *in vitro* studies, Kirchmair et al. showed that cisplatin directly causes

apoptosis of endothelial cells, leading to the degeneration of the vasa nervorum, the destruction of the nerve architecture and the loss of nerve function. Furthermore, their *in vivo* data confirmed that the neuropathy induced by cisplatin was due to the profound endothelial cell apoptosis within the vasa nervorum in the rats. Laser Doppler perfusion imaging revealed decrease in the nerve blood perfusion and the endothelial cell-specific BS1 lectin staining demonstrated reduction in the density of the vasa nervorum (Kirchmair et al., 2005). Together, these observations indicated that damage to the endothelial cells could be the initiating event of the neurotoxic side effect of cisplatin.

1.5.4 HO-1 in cisplatin-induced injury

The role of HO-1 in cisplatin-induced nephrotoxicity and acute kidney injury has been extensively evaluated. Indeed, the expression of HO-1 was found to be induced by cisplatin in the kidney (Agarwal and Nick, 2000). In cisplatin-induced nephrotoxicity, upregulation of HO-1 expression occurs as early as 6 hr after cisplatin administration in both the proximal and distal tubules of the kidney of a rat model (Shiraishi et al., 2000), like reflecting the increase in oxidative stress in the kidney. Indeed, both *in vivo* and *in vitro* experiments have shown that the expression of HO-1 in the kidney can determine the extent of renal injury caused by cisplatin. Inhibition of HO-1 by tin protoporphyrin in cisplatin-induced toxic nephropathy exacerbated the damage in terms of both structural and functional aspects of renal injury (Agarwal and Nick, 2000, Fang et al., 2004). Furthermore, HO-1 null mice were found to exhibit more severe renal failure and greater damage

(increased in apoptosis in both proximal and distal tubules of HO-1 deficient mice and necrosis) compared to wild-type animals treated with cisplatin. In the HO-1 null mice, tubular necrosis, degeneration, loss of brush border and red blood cell extravasation were observed while the damage in the wild-type counterparts was significantly mild. Pharmacological overexpression of HO-1 in human renal epithelial cells using hemin attenuated cisplatin-induced cytotoxicity and inflammation to a large extent (Agarwal and Nick, 2000, Shiraishi et al., 2000). In addition, the pharmacological inhibition of HO-1 expression in the kidney by tin protoporphyrin has been shown to negatively affect renal hemodynamics and caused a reduction in renal blood flow, an increase in renal vascular resistance and an increase in the fractional excretion of sodium in rats treated with cisplatin (Agarwal et al., 1995).

1.6 PREECLAMPSIA

The only definitive therapy for preeclampsia is delivery of the baby and the placenta. Early delivery often has serious consequences for the health of the baby, especially before 32 weeks gestation, whereas watchful waiting often employed to allow for fetal lung maturity, *in utero*, increases maternal risks (Ahmed, 2011b). In developing countries, preeclampsia claims the life of 60,000 mothers every year due to lack of appropriate diagnosis and treatment (WHO, 2005).

1.6.1 Epidemiology and risk factors

Seven percent of healthy women, with no family history of preeclampsia, who start their pregnancy as healthy nulliparas eventually develop preeclampsia (Levine et al., 1997). Studies have shown that a genetic link can be attributed to preeclampsia; the presence of preeclampsia in a first-degree relative increases a woman's risk of severe preeclampsia by 2- to 4-fold (Esplin et al., 2001). The placenta is a product of both mother and father and a history of preeclampsia in the father's mother can also increase the risk (Esplin et al., 2001).

The risk of preeclampsia can be increased by medical conditions such as chronic hypertension, diabetes mellitus, renal complications, obesity and hypercoagulable states (Duckitt and Harrington, 2005). Furthermore, the probability of developing preeclampsia is greater in women who had previously developed the condition. Preeclampsia only develops in the presence of the placenta and disappears quickly after the delivery of the placenta. Hence, conditions, which increase the mass of the placenta, such as multifetal gestations and hydatidiform mole, are known to promote the occurrence of the disorder. Contrary to common-sense expectations, smoking during pregnancy reduces the incidence of preeclampsia (England et al., 2002).

1.6.2 Clinical characteristics

Preeclampsia is defined as the *de novo* onset of hypertension (systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg) and proteinuria (≥ 300 mg/24hr) after 20 weeks' gestation. In extreme but rare cases, serious complications of preeclampsia can include acute renal failure, seizures (eclampsia), pulmonary edema, acute liver injury, hemolysis, and/or thrombocytopenia. The last three signs occur together as part of the hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, a severe variant of preeclampsia. Apart from the hypertension and proteinuria, the central nervous system also plays a role, such as headache and hyperrelexia.

It has recently been suggested that preeclampsia be divided into those cases with early onset (<34 weeks of gestation) and those with late onset (>34 weeks of gestation) (von Dadelszen et al., 2003). In general, cases with early-onset preeclampsia are clinically more severe and usually result in the delivery of a very immature, growth-retarded fetus. Late-onset preeclampsia frequently does not exhibit the placental changes, such an unmodified spiral arteries, characteristic of this disorder (Sebire et al., 2005).

1.6.3 Pathogenesis of preeclampsia

1.6.3.1 Placental vascular remodeling

In normal placental development, extravillous cytotrophoblasts invade the uterine spiral arteries of the decidua and myometrium. These fetal cells replace the endothelial layer of the uterine vessels, transforming them from high resistance vessels to flaccid, low resistance cells. This allows the increase in uterine blood flow needed to sustain the fetus during pregnancy (Meekins et al., 1994).

It is believed that in preeclampsia, this transformation is incomplete, where the cytotrophoblast invasion of the arteries is limited to the superficial decidua and the myometrial segments remain narrow and undilated (Meekins et al., 1994). Furthermore, whereas in normal pregnancy, the invasive cytotrophoblast down-regulate the epithelial-type adhesion molecules and adopt an endothelial cell-surface adhesion phenotype (pseudovasculogenesis); in preeclampsia, cytotrophoblasts do not undergo this switch of cell surface integrins and adhesion molecules, and fail to adequately invade the myometrial spiral arteries (Zhou et al., 2002) (Figure 1.15). However, failure to remodel the uterine arteries is also associated with intrauterine growth restriction, where no signs of hypertension and proteinuria are observed. Hence this effect cannot be seen as preeclampsia-effect.

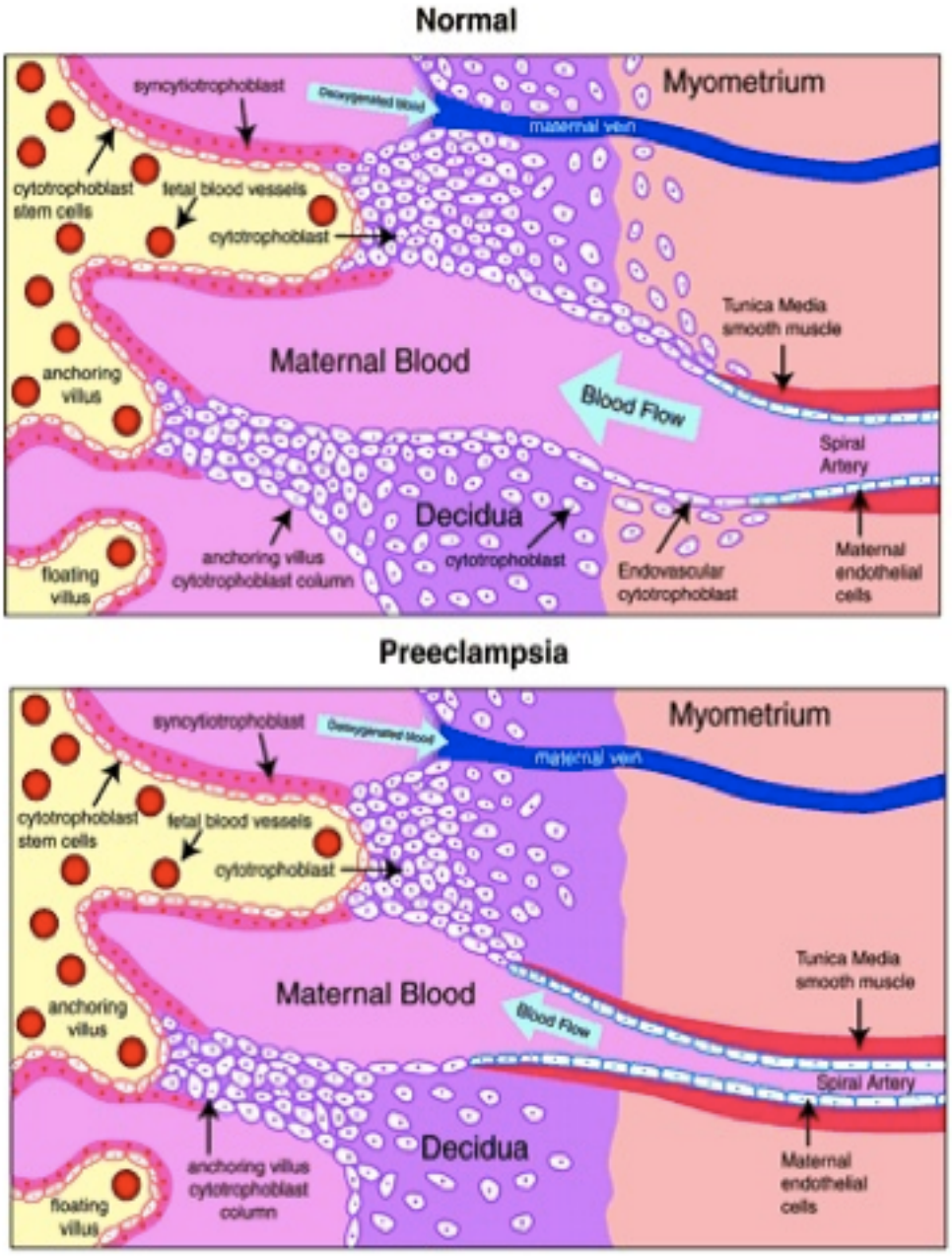

Figure 1.15. **Abnormal placentation in preeclampsia**. In normal placental development, invasive cytotrophoblasts of fetal origin invade the maternal spiral arteries, transforming them from small-caliber resistance vessels to high-caliber capacitance vessels capable of providing placental perfusion adequate to sustain the growing fetus. During the process of vascular invasion, the cytotrophoblasts differentiate from an epithelial phenotype to an endothelial phenotype, a process referred to as "pseudovasculogenesis" or "vascular mimicry" (upper panel). In preeclampsia, cytotrophoblasts fail to adopt an invasive endothelial phenotype. Instead, invasion of the spiral arteries is shallow, and they remain small-caliber, resistance vessels (lower panel) (Lam et al., 2005).

1.6.3.2 Endothelial dysfunction and anti-angiogenic factors in preeclampsia

Although preeclampsia is of placental origin, the tissue affected most is the maternal endothelium. Preeclampsia is characterised by widespread endothelial damage, which leads to proteinurea and hypertension. It has been proposed that circulating factors, originating from the placenta, contribute to the endothelial dysfunction and to the manifestations of the disease (Roberts et al., 1989). Many studies have shown that serum markers of endothelial cell activation are altered in preeclamptic women, including cellular fibronectin, vWF, soluble E-selectin, VCAM-1, platelet-derived growth factor and endothelin (Szarka et al., 2010, Aggarwal et al., 2011, Lok et al., 2008, Strijbos et al., 2010).

VEGF plays a crucial role in the health of fenestrated and sinusoidal endothelium found in the renal glomerulus, brain and liver (Esser et al., 1998) – organs that are severely compromised in preeclampsia. It is also critical for homeostasis (Ferrara, 2004) and activates both VEGFR-1 and VEGFR-2 to stimulate NO required for angiogenesis (Ahmad et al., 2006). Ahmed et al. were the first to propose that preeclampsia may arise due to loss of VEGF activity and by the possible elevation of sFlt-1 (Ahmed, 1997). This hypothesis was later confirmed when it was shown that adenoviral overexpression of sFlt-1 to pregnant rats mimicked the clinical manifestations of preeclampsia (Maynard et al., 2003). Furthermore, cancer patients receiving anti-VEGF therapy (e.g. Avastin) exhibit preeclampsia-like

symptoms (Kabbinavar et al., 2003), further supporting a role for VEGF in preeclampsia. Compelling clinical studies showed that serum levels of sFlt-1 and PlGF gave the highest strength of association with the clinical manifestation of preeclampsia (Levine et al., 2004, Levine et al., 2006, Noori et al., 2010). Together these studies strengthen the proposal that loss of VEGF activity may be responsible for the clinical signs of preeclampsia.

Dysfunction in eNOS activity promotes endothelial dysfunction due to the "uncoupling" of eNOS resulting in the production of superoxide instead of NO (Heitzer et al., 2001) and also leads to increase in endothelial cell permeability in preeclampsia (Wang et al., 2004). It was recently discovered that the anti-angiogenic factor soluble Endoglin inhibits TGF-β signaling in preeclampsia, hence preventing the activation of eNOS (Venkatesha et al., 2006). Neutralisation of TGF-β leads to endothelial dysfunction characterised by impaired endothelium-mediated vasodilatation and elevated expression of surface adhesion molecules, resulting in increased leukocyte adhesion (Walshe et al., 2009).

The physiologic role of PlGF in preeclampsia is less well understood. However, PlGF was shown to stimulate NO release (Khaliq et al., 1999, Bussolati et al.), hence loss of PlGF may also lead to a reduction in NO production. In pregnant rats, inhibition of both VEGF and PlGF is required to produce preeclampsia-like symptoms (Maynard et al., 2003), indicating that decrease in PlGF may be important in the pathogenesis of sFlt-1-induced endothelial dysfunction.

1.6.3.3 sFlt-1: A circulating antagonist to VEGF and PlGF

Soluble Fms-like tyrosine kinase-1 (sFlt-1) or soluble VEGF receptor-1 (sVEGFR-1) is formed from the alternative splicing of the VEGFR-1 (Flt-1) receptor, the endothelial receptor for VEGF and PlGF. sFlt-1 contains the extracellular ligand-binding domain of VEGFR-1 but lacks the transmembrane and intracellular signaling domain. Circulating sFlt-1 acts as a potent antagonist of VEGF- and PlGF-mediated biological activities by binding these ligands and by preventing ligand-receptor dimerisation with full-length VEGF receptors (Kendall and Thomas, 1993) (Fig. 1.9).

In pregnancy, sFlt-1 mRNA is highly expressed in villous and extravillous trophoblast and sFlt-1 protein is present in the supernatant from villous cultures, indicating that vascular growth in the placenta may be locally regulated by this soluble factor produced by the placenta (Clark et al., 1998a). Although it was previously thought that sFlt-1 was restricted to the maternal circulation of pregnant women, recent studies have shown that detectable levels of sFlt-1, of monocytes and endothelial origin, were also present in non-pregnant women (Barleon et al., 2001). This indicated that sFlt-1 might act as a regulator of VEGF bioavailability, which is of importance as continuous low levels of VEGF are essential for endothelial cell proliferation and survival (Luttun and Carmeliet, 2003). It has been proposed that sFlt-1 may regulate the bioavailability of VEGF through its heterodimerisation with VEGF receptors, hence abolishing VEGF-mediated signal transduction

(Barleon et al., 2001).

In preeclampsia, placental expression of sFlt-1 is significantly elevated, hence increasing circulating sFlt-1 (Maynard et al., 2003). Numerous studies in rodents have attributed the preeclampsia-like symptoms observed as hypertension and proteinuria to be a direct consequence of the increased in sFlt-1 expression or circulating levels (Lu et al., 2007, Gilbert et al., 2007, Li et al., 2007, Bytautiene et al., 2010, Suzuki et al., 2009, Costantine et al., 2010). A recent study however showed that sFlt-1 can only induce hypertension and proteinuria in mice above a certain critical threshold (Bergmann et al., 2010). In support of this theory, women with fetal growth-restriction, despite having elevated levels of sFlt-1 compared to controls, do not exhibit signs of hypertension or proteinurea (Wallner et al., 2007).

1.6.3.4 sEng: A circulating antagonist to TGF-β

Dysregulation of TGF-β signaling and elevated levels of soluble Endoglin (sEng) have been reported in preeclampsia. TGF-β is an anti-inflammatory growth factor (Robertson et al., 2003) that activates eNOS (Venkatesha et al., 2006) and endoglin is a transmembrane co-receptor for TGF-β1 and TGF-β3. sEng is the product of the proteolytic cleavage of the NH_2- extracellular domain of the full-length membrane-bound endoglin. It binds to TGF-β1 and reduce its bioavailability. As a consequence, it limits the activity of TGF-β1 signalling and eNOS (Jerkic et al., 2004, Toporsian et al., 2005) and hence promotes vascular dysfunction. Furthermore, sEng also inhibits angiogenesis

to the same extent as sFlt-1 (Venkatesha et al., 2006).

Soluble Endoglin is increased in the circulating level of pregnant women many weeks prior to the clinical onset of the preeclampsia in these women (Levine et al., 2006). Increase in circulating sEng affects endothelial cell integrity *in vivo* (Walshe et al., 2009) and reduces the number of regulatory T cells observed in the systemic circulation of preeclamptic women (Santner-Nanan et al., 2009). The molecular mechanisms regulating sEng release has not been fully elucidated. However, cytokines (TNF-α, IFN-γ) (Cudmore et al., 2007) and angiotensin II type 1-receptor autoantibodies (Zhou et al., 2010) increase both sFlt-1 and sEng and the stress-responsive HO-1 inhibits their release (Cudmore et al., 2007). In pregnant rats, sEng acts synergistically with sFlt-1 to induce endothelial dysfunction, hypertension, severe proteinuria, and HELLP syndrome (hemolysis, elevated liver enzymes and low platelets) (Venkatesha et al., 2006).

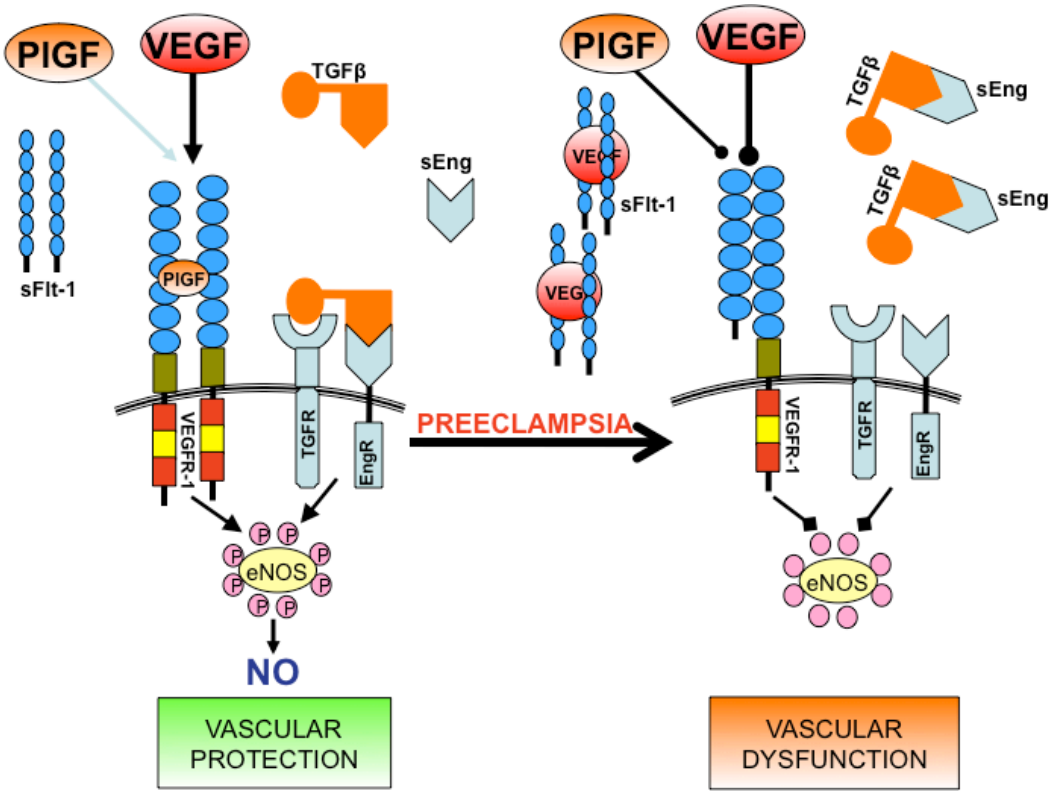

Figure 1.16. **Diagrammatic representation of the role of anti-angiogenic factors in preeclampsia.** A functional endothelial monolayer requires VEGF, PLGF and TGF-β for normal endothelial function via activation of nitric oxide (NO). In preeclampsia VEGF protective signal is compromised due to an excess of soluble Flt-1 (sFlt-1), which is compounded by a decrease in the expression of PlGF and a rise in circulating soluble Eng (sEng).

1.6.4 Inflammation and neutrophil activation in preeclampsia

A generalized systemic inflammation is common to all pregnancies (Willis et al., 2003). Redman and colleagues have proposed that preeclampsia arises as a result of an excessive maternal intravascular inflammatory response to

pregnancy, which may occur because either the stimulus or the maternal response is too strong and involving both the innate and the adaptive immune system (Redman et al., 1999). They also specified that preeclampsia is not intrinsically different from normal pregnancy but it is the extreme end of a continuous spectrum of inflammatory responses that are a feature of pregnancy itself (Redman and Sargent, 2010). It has also been proposed that as a consequence of poor placentation and reduction in uterine blood flow, the increase in oxidative and endoplasmic reticulum stress, both potent pro-inflammatory mediators, may be the cause of preeclampsia (Burton et al., 2009, Redman and Sargent, 2010).

Many studies have stipulated that the contribution of endothelial dysfunction in preeclampsia can be viewed in a larger context as part of the inflammatory network. Activated leukocytes activate the endothelium and vice versa (Zimmerman et al., 1992, Mantovani and Dejana, 1989). More than a decade ago, Greer et al. reported that neutrophils were the major immune cell type, which were activated and were involved in the pathophysiology of preeclampsia (Clark et al., 1998b). However the direct consequence of neutrophil activation in preeclampsia has not been shown, although it is highly probably that the vascular dysfunction observed in preeclampsia is partly due to neutrophil activation and the release of pro-inflammatory cytokines [Sabatier, 2000 #2153;Halim, 1996 #1246;Haller, 1996 #1620.

As previously described, the human neutrophil is a major component of leukocytes and its arsenal includes the plasma membrane-associated electron transporting NADPH-oxidase enzyme system and the intracellular primary, secondary and tertiary cytoplasmic granules. Neutrophils bind and transmigrate through the endothelium via the interaction of endothelial adhesion molecules and surface receptor on neutrophils. Activated neutrophils release granules, ROS and RNS, which are able to mediate vascular damage. Several studies have confirmed that chronic inflammation in the decidua and placenta during preeclampsia may lead to local neutrophil activation in that compartment. Activation of neutrophils has been demonstrated through the marked upregulation of cell surface adhesion molecules including, CD11a, CD11b and CD11c, and the complement related markers CD35 and CD59 in neutrophils of preeclamptic women [Sacks, 1997 #2154; Mellembakken, 2002 #2152; Sabatier, 2000 #2153]. Other studies have shown that soluble markers of neutrophil activation, which is released in the circulation from the degranulation of activated neutrophils, are increased in preeclampsia. These markers include elastases and lactoferrin, which are both significantly elevated in preeclampsia (Halim et al., 1996, Sacks et al., 1998). Other markers of neutrophil activation include α-defensins and calprotectin that have been shown to be elevated in preeclampsia (Greenwald and Ganz, 1987, Braekke et al., 2005).

α-defensins are highly concentrated in the granules of neutrophils. They are highly water soluble, but can also interact with the lipid environment of a

pathogen membrane. Human α-defensins, human neutrophil peptide (HNP) 1–3 is primarily expressed in neutrophils (Greenwald and Ganz, 1987). However, they can also be produced by non-granulocytes including monocytes and lymphocytes (Agerberth et al., 2000), as well as natural killer cells (Chalifour et al., 2004). Defensins have anti-microbial properties against a variety of bacteria, viruses, fungi and parasites. They can modulate the production of inflammatory cytokines and chemokines by inducing the release of TNF-α and IFN-γ from macrophages (Soehnlein et al., 2008). In addition, Miles et al. reported that α-defensins released by apoptotic human neutrophils could inhibit the production of NO in chronic inflammation (Miles et al., 2009). In preeclampsia, recent reports have shown that α-defensins is significantly increased compared to control patients (Prieto et al., 1997).

Calprotectin, also called S100A8/S100A9, is a calcium-binding protein, closely correlated with inflammation (Johne et al., 1997). It originates from neutrophils and macrophages and has various biological functions, including suppression of cell proliferation, apoptosis induction, immune regulation and participation in inflammatory reactions (Passey et al., 1999, Striz and Trebichavsky, 2004). In infectious or inflammatory conditions, the levels of calprotectin in serum, secretion and specific tissues can be detected to reflect severity of diseases to some degree as it is specifically expressed in inflammatory cells (neutrophils and macrophages), implying that it can be regarded as a clinically relevant marker of inflammation (Striz and Trebichavsky, 2004). In preeclampsia, calprotectin is also significantly

elevated (Braekke et al., 2005, Holthe et al., 2005). Hence, both the vascular and the inflammation theory as a cause of preeclampsia have persisted although their causational link has not been established.

CHAPTER 2

HYPOTHESIS AND AIMS

2.1 HYPOTHESIS

Endothelial dysfunction is the hallmark of vascular diseases, including atherosclerosis and preeclampsia. The vascular endothelium plays a vital role in the prevention of vascular dysfunction (Gibbons, 1997). It acts as a barrier, controls the vascular tone, inflammatory, and haemostatic responses as well as angiogenesis (Molitoris et al., 2002). Heme oxygenase-1 (HO-1) is an inducible, cytoprotective, anti-inflammatory enzyme that is widely acknowledged to provide defense against oxidant damage (Keyse and Tyrrell, 1989, Duckers et al., 2001) and to be protective against ischemia-reperfusion injury (Melo et al., 2002, Katori et al., 2002, Zhang et al., 2004, Tsuchihashi et al., 2004). Furthermore, human biliverdin reductase (BVR), a water-soluble enzyme that reduces biliverdin (the product of heme oxygenase HO-1 and HO-2 activity) to the antioxidant bilirubin, also possesses cytoprotective properties (Baranano et al., 2002). The first part of this thesis will test whether HO-1 or BVR protect the endothelium from toxicological-induced injury by inhibiting caspase activation, pro-inflammatory placenta growth factor (PlGF), soluble Endoglin (sEng) and soluble fms-like tyrosine receptor-1 (sFlt-1) release from the endothelium.

Upregulation of the anti-angiogenic factors, sFlt-1 and sEng, was reported to cause endothelial dysfunction and play a key role in the pathogenesis of preeclampsia (Maynard et al., 2003, Ahmad and Ahmed, 2004, Venkatesha et al., 2006). The mechanisms responsible for the release of these factors have

not been fully elucidated. Ahmed and colleagues have challenged the theory that the increase in sFlt-1 and sEng is due to hypoxia or oxidative stress as a consequence of poor placentation and reduction in uteroplacental blood flow {Ahmed and Cudmore, 2009]. This is partly because preeclamptic placenta continues to generate substantially higher levels of sFlt-1 into conditioned media even 24 h *ex vivo* when cultured under atmospheric conditions as compared with normal pregnancy placental explants (Ahmad and Ahmed, 2004). The role of inflammation as the main stimulus for the release of sFlt-1 and sEng has also been proposed (Redman and Sargent, 2009). Hence this thesis aims at determining whether neutrophil activation/inflammation play a causal role in the release of these anti-angiogenic factors in both in vitro experimental cell culture setups and by observational clinical studies.

2.2 AIMS

2.2.1 Cytotoxic drug-induced studies

Cisplatin is a potent anticancer drug. Its benefit is limited due to its long-term cytotoxic effect on normal cells (Pliarchopoulou and Pectasides, 2010). The vascular endothelium is the first point of contact for systemic cytotoxic drug such as cisplatin. Hence, damage to the vascular endothelium is likely to be one of the initial events, which potentially contribute to, if not worsen the side effects associated with cisplatin chemotherapy. Patients treated with cisplatin for testicular cancer often develop vascular complications such as myocardial infarction, stroke and thromboembolic disease and have

increased levels of microalbuminuria and pro-inflammatory mediators which increases their risk of developing atherosclerosis (Nuver et al., 2005).

Numerous studies have shown that HO-1 protect against cisplatin-induced nephrotoxicity and autophagy of kidney cells (Agarwal et al., 1995, Agarwal and Nick, 2000, Bolisetty et al., 2010) and inhibition of HO-1 by tin protoporphyrin (SnPP) prior to cisplatin enhances the nephrotoxic side effect of cisplatin (Shiraishi et al., 2000, Fang et al., 2004). Few studies have investigated the effect of cisplatin on endothelial cells (Yu et al., 2008, Nuver et al., 2010), however, none have directly addressed whether cisplatin-induced endothelial cell damage can be prevented by the induction of HO-1 in the endothelium. As the endothelium is the first contact point for cisplatin, we hypothesized that cisplatin-induced endothelial damage could be minimized by over-expression of the stress responsive enzyme, HO-1, which has cytoprotective, anti-inflammatory and anti-apoptotic properties. Furthermore, we postulate that BVR may also be protective but via the upregulation of HO-1.

Pro-inflammatory cytokine, PlGF is required for macrophage infiltration in early atherosclerotic lesions in atherosclerosis-prone apolipoprotein-E-deficient ($ApoE^{-/-}$) mice (Khurana et al., 2005) and PlGF neutralization by a murine anti-PlGF antibody (αPlGF mAb) reduced inflammatory cell infiltration and atherosclerotic lesion size in these mice (Roncal et al., 2010). We hypothesised that cisplatin may stimulate PlGF release from the

endothelium but the upregulation of HO-1 will inhibit both cisplatin-induced endothelial cell damage and PlGF release from endothelial cells.

The objectives of cytotoxic drug studies were:

(i) To determine whether cisplatin induces cell death in human umbilical endothelial cell (HUVEC)

(ii) To measure whether cisplatin stimulates the release of the pro-inflammatory cytokine, PlGF, or the anti-angiogenic growth factors, sFlt-1 and sEng from HUVEC.

(iii) To test if adenoviral over-expression of HO-1 or BVR suppresses endothelial cell damage as measured by cleaved caspase-3 expression, PlGF, sFlt-1 and sEng release from HUVEC

(iv) To determine whether siRNA-mediated silencing of endothelial HO-1 causes increase expression of cleaved caspase-3 and endothelial cell death induced by cisplatin.

(v) To assess the effect of over-expression of BVR up-regulated HO-1 and hence confer cytoprotection via HO-1

(vi) To examine the angiogenic properties of HO-1 and BVR over-expression in endothelial cells

2.2.2 Role of activated neutrophils in soluble endoglin release in preeclampsia

Preeclampsia, a maternal hypertensive disorder, is associated with increased production of pro-inflammatory cytokines and reportedly marked

neutrophil activation (Greer et al., 1991). Endoglin (CD105), a transmembrane co-receptor for TGF-β1 and TGF-β3, is predominantly expressed by activated, proliferating endothelium during angiogenesis (Jerkic et al., 2006, Duwel et al., 2007) and regulates the activity of endothelial nitric oxide synthase (eNOS) (Jerkic et al., 2004, Toporsian et al., 2005). Proteolytic cleavage of the extracellular domain of endoglin gives rise to sEng, which functions to neutralise TGF-β signaling (Venkatesha et al., 2006) hence causing vascular dysfunction (Walshe et al., 2009). High plasma sEng level is associated with preeclampsia (Levine et al., 2006). *In vivo* studies have shown that sEng acts synergistically with sFlt-1, the natural antagonist of vascular endothelial growth factor (VEGF), to induce maternal endothelial dysfunction and severe preeclampsia (Venkatesha et al., 2006). Pro-inflammatory cytokines (Cudmore et al., 2007) and angiotensin-II receptor type-1 autoantibodies (Zhou et al., 2010) stimulate sEng release while HO-1 inhibits its release (Cudmore et al., 2007). The neutrophil is an important component of the innate immune system. Its production is increased in response to inflammation. Activated neutrophils have the ability to adhere to endothelial cells, to generate high levels of reactive oxygen species and proteases, which can cause vascular injury or inappropriate endothelial activation (Wang et al., 1998b, Lee et al., 2003). As activated neutrophils release large quantities of proteinases, which can cleave cell surface receptors, we hypothesized that activated neutrophils may contribute to the release of sEng from endothelial cells.

The objectives of activated neutrophil studies were:

(i) To investigate whether neutrophils activated by LPS induce sEng release from endothelial cells

(ii) To determine whether endothelial overexpression of HO-1 prevents LPS-activated neutrophils from stimulating sEng release.

(iii) To determine the role of protease and metalloproteinase in the release of endothelial sEng by activated neutrophils.

(iv) To test whether in vitro studies in HUVECs and trophoblasts correlate with clinical in vivo measurements of sEng and markers of inflammation and neutrophil activation.

(v) To measure markers of neutrophil activation and inflammation in normal and preeclamptic plasma and urine.

(vi) To determine the magnitude of neutrophil activation and elevation of anti-angiogenic factors in preeclampsia.

(vii) To investigate using the Hill causational criteria to determine whether neutrophil activation/inflammation is causational for preeclampsia.

(viii) To determine causational relationship between anti-angiogenic factors and preeclampsia.

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

The sources of all purchased materials and gifts are outlined in Appendix I. Apparatus and equipment utilised during the studies are detailed in Appendix II together with corresponding suppliers. Formulations of all solutions and buffers are listed in Appendix III.

Human Umbilical Vascular Endothelial Cells (HUVEC) was obtained from umbilical cords, of normal term deliveries at the Birmingham Women's Hospital (BWH), BWH NHS Trust (Ethics number 09/H1211/83). Umbilical cords remained connected to the placenta and were immediately refrigerated at 4°C post-partum, following standard clamping and cutting procedures. The method used for HUVEC isolation is described in 1.2.2.1.

3.2 METHODS

3.2.1 General maintenance

All tissue culture work, and other work requiring aseptic conditions, was carried out in a class II microbiological safety cabinet. Tissue culture media, plastics and supplements were purchased as sterile. Solutions required to be sterile were passed through a syringe-driven sterilizing filter (0.22μm pore). Glassware was washed and autoclaved prior to use. Cell culture media was

stored in accordance with manufacturer guidelines (i.e. at 4°C) and used before the expiry date.

3.2.2 Cell types

3.2.2.1 Isolation of Human Umbilical Vein Endothelial Cells

Human Umbilical Vein Endothelial cells (HUVEC) were isolated from the veins of umbilical cords through digestion with collagenase as described previously (Jaffe et al., 1973). Detached endothelial cells were collected by flushing the collagenase solution through the umbilical vein with Medium 199 (M199), buffered with Earle's salts and supplemented with 2 mM L-Glutamine, 100 U/ml penicillin, 10 mg/ml streptomycin, 2.5 ng/ml basic Fibroblast Growth Factor, 20 ng/ml Epidermal Growth Factor, and 20% FCS into a sterile 50ml tube. The cell suspension was spun at 80 g for 5 minutes and the resulting cell pellet re-suspended in 10 ml supplemented M199, 20% FCS. Cells were seeded in 0.2% gelatin-coated tissue culture flasks and allowed to attach overnight at 37°C in a humidified incubator with an atmosphere of 95% air, 5% CO_2. The medium was replaced the following day to remove erythrocytes and the cells were grown to confluence. Immunofluorescence for the human von-Willebrand factor (vWf) was employed to ensure the purity of cultures.

3.2.2.2 Isolation of human neutrophils from whole blood

Peripheral blood neutrophils were isolated from whole human blood for the neutrophil-endothelial co-cultures by dextran sedimentation of erythrocytes and density gradient centrifugation of leukocytes. All the procedures were conducted at room temperature.

Healthy donors were consented prior to blood collection. About 40 ml of blood was taken from the median cubital vein of the anterior forearm of a healthy donor by venipuncture. The blood was collected in a 50 ml tube containing 100 μL of 0.5M EDTA blood. The tube was inverted gently several times to mix and either left at 4°C for no longer than 2 hrs or processed immediately. Isolation of neutrophils was performed in two steps:

1) Dextran sedimentation – To remove most of the red blood cells (RBC). The RBC sedimented at the bottom of the tube, while the leukocytes and lymphocytes remain suspended in the solution. 4% dextran solution was added to the whole blood in a 1:6 ratio (1 ml of dextran for 6 ml of blood). The tube was inverted several times to ensure adequate mixing and was let to stand at room temperature for 45 mins to 1 hr until the separation was complete.

2) Percoll gradient – Percoll gradients was prepared from a stock solution of isotonic Percoll. The isotonic stock solution of 90% Percoll was made up by

mixing 9 ml Percoll and 1 ml of 9% NaCl. By further dilutions with NaCl, 79% Percoll and 56% Percoll were prepared. For every 40 ml of whole blood, three 15 ml tubes were prepared, each containing 15 ml of 56% Percoll. Then, very carefully, to avoid mixing, 5 ml of 79% Percoll was layered below the 56% Percoll solution. Equal volumes of the clear mixture of leukocytes and lymphocytes were carefully overlaid onto the gradients in each tube and centrifuged at 100 g, with breaks set to zero for 25 minutes at room temperature. A lower band (white ring) between the 56 and 79% Percoll denotes the layer that contains the neutrophils. Taking care not to disturb the layer of erythrocytes beneath, this layer was carefully aspirated with a pipette to a fresh 50 ml tube. PBS was added to the 50 ml mark of the tube to wash the neutrophils and the neutrophils were spun at 100 g for 10 minutes. The pellet of neutrophils at the bottom of the tube was then resuspended in the appropriate tissue culture medium. The neutrophils were counted using a haemcytometer and resuspended at a concentration of 1.0 X 10^6 cells/ml in M199 containing 5% FBS. The purity of neutrophil isolation was verified at regular intervals by cytospin.

3.2.3 Cell culture

3.2.3.1 Human Umbilical vein Endothelial cell culture

HUVEC were maintained in a humidified incubator in an atmosphere of 95% air, 5% CO_2 at 37°C. Cells were seeded in 80 cm^2 tissue culture flasks in 20% FCS (for HUVEC). 2 mM L-glutamine, 10 U/ml penicillin and 0.1 μg/ml

streptomycin were added to the media. Confluent cells were sub-cultured by aspiration of the media from the cell monolayer and the cells were washed with 15 ml of PBS to remove the serum. Cells were detached by 2-minute incubation with 1ml of Trypsin/ethylenediaminetetraacetic (EDTA). When the cells were detached, 9 ml of media was added to inactivate the digestion of the extra-cellular matrix and the cell suspension was transferred to a sterile 15 ml conical tube. The cells were centrifuged at 80g (for HUVEC) for 5 minutes to pellet the cells. The supernatant was aspirated before the cell pellet was re-suspended in 10 ml of growth medium. The cells were either split between new culture flasks or counted by haemcytometer in preparation for experiments requiring a certain number of cells per well prior to seeding in T-25 flasks or 6, 12, 24 or 96 well tissue culture plates. The cells were then seeded onto gelatin coated culture plates in the following manner:

Table 3.1. **Cell density according to different plates**

Tissue Culture Plate Type (No. Wells/Plate)	Seeding density (No. Cells/Well)	Volume/Well (ml)
6	250,000	1
12	150,000	0.5
24	100,000	0.4
96	10,000	0.1

3.2.3.2 Neutrophil-endothelial cell coculture

As described previously, HUVEC were seeded in 24-well plates and left to attach overnight in M199 complete medium containing 20% FCS. Prior to their addition onto HUVEC, neutrophils were stimulated with LPS for 10 minutes, whereas control neutrophils were left untreated. 500 μl of

neutrophils suspended in M199 complete medium containing 20% FCS were seeded onto the HUVEC at a concentration of 1.0 X 10^6 neutrophils/ml with or without LPS. In separate experiments, neutrophils and HUVEC were also cocultured with various inhibitors or stimulators and incubated at 37°C for 24 hours. After 24 hours, the supernatants were collected in 1 ml eppendorf tubes, centrifuged at 10,000 rpm for 5 minutes to remove endothelial cells and neutrophils debris. Following centrifugation, supernatant were transferred to fresh 1 ml eppendorf tubes and stored at -80°C until analysis.

3.2.3.3 Cell Cryopreservation

A stock of HUVEC was maintained through cryopreservation. HUVEC were trypsinised and pelleted before resuspension in 1 ml of pre-cooled culture medium containing 10% FCS and 10% Dimethyl Sulfoxide (DMSO). The cell suspension was then transferred to sterile cryovials labelled with cell type, passage number, and date of storage. The cryovials were cooled in "Mr Frosty" at a rate of -1°C/minute down to -80°C. Once frozen, the cryovials were transferred to racks and stored in a liquid nitrogen cryostat. When the cryopreserved cells were needed, cells were thawed at 37°C in a water bath for 10 minutes and immediately transferred to tissue culture flasks containing the respective serum containing growth medium. HUVEC were incubated for 16 hours to allow cell attachment followed by a wash with PBS to remove all traces of DMSO and replacement of the growth medium.

3.2.4 Adenoviral infection of cells

Adenoviruses were amplified in HEK-293 cells and purified in house via a double caesium chloride gradient. Unless stated otherwise, viruses were administered to cells at a concentration of 50 Infective units (IFU) per cell. The transduction was carried out in the same culture medium that was used to grow the cells (complete M199 for HUVEC) and the cells were incubated with the virus for 16-20 hrs before the stimulation of the cells.

To minimise the spread of viruses across the lab environment and to avoid cross-contamination of cells, filter tips were always used when handling viruses. This reduced the risk of generating viral aerosols. All vials and pipette tips that had been in contact with virus were treated with 1% TriGene solution prior to disposal. Moreover, all viral work was carried out in a designated hood whilst non-viral work was done in a different hood.

The recombinant, replication-deficient adenovirus-encoding rat HO-1 (AdHO-1) and the Escherichia coli β-galactosidase adenovirus gene (control) were used as described previously (Otterbein et al., 1999). Recombinant and replication-deficient adenoviruses-encoding BVR (AdBVR) was commercially made (Vector BioLabs, Philadelphia, USA). The titres of the viruses are shown in Table 3.2.

Table 3.2. **Titres of adenoviruses**

Virus	Titre (IFU/ml)
Adβ-Gal	1.38×10^{11}
AdBVR	5.25×10^{9} / 6.14×10^{9} / 1.67×10^{11}
AdHO-1	4.32×10^{11}

3.2.5 siRNA gene knockdown

HUVEC were trypsinised as described previously and the cells were electroporated with either ~3 μg HO-1 (sense, 5' GGCAGAGGGUGAUAGAAGAUU-3'; antisense, 5'-UCUUCUAUCACCCUCUGCCUU-3') (Kweon et al., 2006), BVR sense, GCACGAGGAGCAUGUUGAACUCUUG; antisense, CAAGAGUUCAACAUGCUCCUCGUGC) (Zeng et al., 2008) or a universal control siRNA (Dharmacon, USA) using the HUVEC kit II and Nucleofector (Amaxa GmbH, Cologne, Germany) according to the manufacturer's protocol. Equal volumes of cell suspension were then put to gelatine-coated 6-well or 96-well plates in the appropriate volume of MCDB 131 medium and incubated overnight. The following day, the medium was replaced with HUVEC complete medium and the cells were stimulated.

3.2.6 Cisplatin treatment

Confluent HUVEC in 6-, 24- or 96-well plates were treated with different concentrations of cisplatin for the indicated period of time. For each experiment, a fresh stock solution of cisplatin was prepared in Dimethyl sulfoxide (DMSO). A stock solution of 200 mM was prepared by diluting 3 mg of cisplatin in 50 μl of DMSO and further dilutions was carried out in

HUVEC complete media. HUVEC was incubated for 12 and 24 hrs with cisplatin and the cells and supernatants were collected and stored at -80ºC until required.

3.2.7 MTT assay

HUVEC were seeded at a density of 1.0 x 10^5cells/well in 96-well plates, incubated to attach for overnight in complete medium containing 20% FCS. The medium was aspirated from the cells and replaced with 100 μl of fresh medium containing the cisplatin at appropriate concentrations. After 24-hour incubation at 37°C, cisplatin was removed and 80 μl of fresh serum-free medium added and 20 μl of MTT (3-(4, 5-Dimethylthiazol–2-yl)-2, 5-diphenyltetrazolium bromide thizolyl blue) at a concentration of 5 mg/ml was added to each well and the plates incubated in the dark at 37°C for 4 hours. After 4 hours, the medium was carefully aspirated and 150 μl of 99.5% DMSO was added to each well to enable the optical absorbance to be read at 540 nm and 690 nm with the Thermo LabSystems MultiScan Ascent microplate photometer.

3.2.8 Quantitative real-time PCR

Sample preparation and real-time polymerase chain reaction were performed as described previously (Ahmad et al., 2006). Briefly, mRNA was prepared with TRIzol and DNase-1 digestion/purification on RNAeasy columns (Qiagen, West Sussex, UK) and reverse transcribed with the cDNA Synthesis

Kit (Promega, Madison, Wis). Triplicate cDNA samples and standards were amplified in SensiMix containing SYBR green (Quantace, London, UK) with primers specific for HO-1 (sense, 5'-GGG TGA TAG AAG AGG CCA AGA CT-3'; antisense, 5'-GCA GAA TCT TGC ACT TTG TTG CT-3') (Smith et al., 2003) or β-actin. The mean threshold cycle for each HO-1 was normalized to β-actin and expressed relative to control.

3.2.9 Protein chemistry techniques

3.2.9.1 Preparation of samples for protein analysis

Extraction of protein from cell monolayers – After stimulation, the confluent monolayers of cells were washed with ice cold PBS and lysed with ice-cold radio immunoprecipitation buffer (RIPA buffer). The plates containing the cells were left at room temperature for 5 minutes. Cell lysates were collected using a cell scraper and transferred to clean Eppendorf tubes, which were kept on ice. The tubes were centrifuged at 13 000 rpm for 4 minutes and the supernatants containing the extracted proteins were transferred to clean eppendorf tubes. The protein concentration was assessed prior to storage at -80ºC.

3.2.9.2 Estimation of protein concentration

The amount of total protein present in the culture medium was measured before the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

using the Bio-Rad protein assay (Bradford assay) to ensure equal loading of the protein for Western blotting. Protein standards were prepared from a stock solution of 20 mg/ml Bovine Serum Albumin (BSA), diluted in distilled water to give a range of concentrations of 0.2 – 1.4 mg/ml. Thawed proteins were diluted 1:5 in RIPA buffer and 5 μl of each sample and the BSA standards aliquoted in duplicate to each well (using a 96-well plate). 20 μl of Reagent 'A' and 150 μl of Reagent 'B' were added to each well. Reagent 'A' was made up mixing 20 μl of Reagent S to 1 ml of Reagent A. The colour was allowed to develop for 15 minutes and the absorbance was read within 60 minutes in a Multiskan Ascent 96 well plate reader at 690 nm. Results were obtained by plotting of the protein BSA standard concentration against the optical density (OD) to give a straight-line graph. Samples were aliquoted to 30 μg of total cell protein prior to the SDS-PAGE and Western blot analysis.

3.2.10 SDS-polyacrylamide gel electrophoresis

3.2.10.1 Gel preparation

The gels were prepared taking care to ensure the glass plates and spacers were level in the casting chamber and that the plate assembly did not leak. Separating gels were prepared at a range of different acrylamide percentage concentrations dependent on the size of protein being assessed. The gels were prepared in advance taking care to ensure the glass plates and spacers were level in the casting chamber and that the plate assembly did not leak. Separating gels were prepared at a range of % acrylamide concentrations dependent on the size of protein being assessed. Most commonly, 10%

acrylamide gels for separation of bands between 40-120 kDa were prepared and polymerisation initiated by the addition of Tetramethylethylenediamine (TEMED) and 10% ammonium persulphate (APS). Gels were poured into the plate assembly, taking care to prevent the formation of air bubbles. The gel was then overlaid with 70% ethanol, to prevent gel shrinkage and formation of a meniscus, and allowed to polymerise. The 70% ethanol layer was then removed and a 5% acrylamide stacking gel prepared as above poured carefully onto the polymerised separating gel. Sample loading combs were inserted into the stacking gel and the levels topped up during polymerisation to ensure against gel shrinkage. The combs were then removed, the polymerised gels transferred to the running apparatus and the upper and lower chambers of the running apparatus filled with electrophoresis running buffer.

3.2.10.2 Loading of protein samples

Either 30 or 50 µg of total cell protein samples, dependent on the amount of the protein of interest present in the sample, were diluted to a final volume of 25 µl in either 2x, reducing or non-reducing sample buffer containing bromophenol blue as a tracking dye. Immunoprecipitates were routinely resuspended in 50 µl of 2x reducing sample buffer. Reducing buffer contains 200 mM mercaptoethanol and DTT, which chemically reduces disulphide chains and allows separation of individual peptide chains, while non-reducing buffer allows detection of full weight proteins. All samples were boiled for 5 minutes to reduce disulphide bonds or dissociate protein

Antibody binding, prior to loading of equal 25 µl volumes onto the SDS-PAGE gel against a positive control and pre-stained Kaleidoscope molecular weight markers ranging from 17 to 208 kDa. Gels were electrophoresed at 60 V until samples had passed through the 5% stacking gel and the voltage then increased to 120 V. Electrophoresis was stopped when the bromophenol blue tracking dye reached the bottom of the gel.

3.2.10.3 Western Blotting protocol

(i) Assembly of the semi-dry electrophoresis transfer cell unit - The semi-dry electrophoresis transfer cell, composed of transfer cell, gel holder, safety lid with power cables and removable electrode cards, was used to transfer protein from acrylamide gels to nitrocellulose membranes. Following protein electrophoresis, the gels were rinsed in transfer buffer prior to transfer to facilitate the removal of electrophoresis buffer salts and detergents. Hybond ECL nitrocellulose membrane was cut to the dimensions of the gel and wetted in transfer buffer for 5 minutes. In addition, two pieces of thick filter paper were cut to the dimensions of the gel and completely saturated in transfer buffer. The blot transfer cell unit was assembled as shown below; care was taken to ensure no air bubbles were between gel and membrane affecting the efficiency of the transfer. For transfer of protein to occur, a current is passed from the cathode to the anode that is proportional to 0.8 mA x (the area of the nitrocellulose membrane in cm^2).

(ii) Membrane blocking - Following transfer of the protein samples onto the nitrocellulose membrane, non-specific binding sites were blocked by immersing the membrane in blocking solution composed of 5% skimmed milk and 0.1% BSA in Tween Tris buffered saline. Membranes were blocked for 1 hour at room temperature on an orbital shaker. After blocking, membranes were washed in TBST for 15 minutes at room temperature followed by two further 10-minute washes in fresh TBST.

(iii) Primary antibody dilution - Following washing, the membrane was incubated with a primary antibody. Dilution of the primary antibody required to give optimum results for each experiment varied and was determined for each antibody used. Furthermore, the time and temperature of incubation of the membrane with the primary antibody was optimised for each antibody. The dilution of antibody used in this study are summarised in the Table below.

Table 3.3. **Dilutions of primary antibodies used.**

Antibodies	**Species raised in**	**Optimum dilution**	**Source**
β-actin	Mouse monoclonal	1:15 000	Sigma Aldrich, UK
HO-1	Rabbit polyclonal	1:5000	Abcam
Cleaved caspase-3	Mouse polyclonal	1:500	Cell Signaling Tech, Herts, UK
BVR	Rabbit polyclonal	1:5000	Abcam

(iv) Secondary antibody dilution - Before incubation of the membrane with secondary antibody, the membrane was washed as described previously.

Secondary anti-mouse; IgG:horse radish peroxidase (HRP) labelled antibodies were made depending on the species in which primary antibody was raised. They were diluted at 1:5000 (v/v) in 5% milk and incubated with the membrane for 1 hour at room temperature.

(v) ECL detection - Following incubation of the membrane with the appropriate secondary antibody, the membrane was further washed as detailed described previously. For the detection process, the membrane was transferred in the dark room and drained of TBST. ECL detection solution 1 was mixed 1:1 with ECL detection solution 2, added to the membrane and incubated for 1 minute at room temperature. Thereafter, the detection mixture was drained off; the membrane wrapped in Saran Wrap, and exposed protein side up to a sheet of autoradiography film (Kodak, XOMAT AR) in an autoradiographic film cassette with intensifying screens. Various exposure times were used for each membrane to achieve the best results for each blot.

3.2.11 *In vitro* angiogenesis - Tube formation

Assessment of *in vitro* capillary formation used growth factor-reduced Matrigel (Becton Dickinson, Oxford, UK). Matrigel is a basement membrane matrix composed primarily of collagen IV, laminin, entactin, and heparan sulfate proteoglycans. The Matrigel matrix was gently thawed overnight on iceand 50 ul was placed into each well of a 96-well culture plate at 4°C and allowed to polymerize by incubation at 37°C for 30 minutes. After

polymerization of the Matrigel suspension 10,000 cells were plated to each well in culture medium containing 10% FCS. After cells had attached to the Matrigel (90 minutes at 37°C), the FCS containing media was removed and agonist or the vehicle alone in media containing 0.2% BSA was added and incubated for 4-6 hours at 37°C. The tubular network growth area was compared in control and effector-treated Matrigel matrix. In parallel experiments, Trypan Blue exclusion showed that cell viability was >90%. For visualisation of cells, Calcein AM Fluorescent Dye (BD Biosciences, Bedford, MA) was added to the cells at a concentration of 4 μM 30 minutes before imaging. The cells were observed with a Nikon inverted microscope and image analysis for tube length was assessed using Image Pro Plus (Media Cybernetics Inc, Bethesda, MD, USA).

3.2.12 Enzyme-linked immunosorbent assay (ELISA)

3.2.12.1 General ELISA Protocol (DuoSet; R&D, UK)

EIA/RIA PLATES (Corning, UK) were coated with 100 µl/well of capture antibody in PBS pH 7.4, covered with a plastic film and left in the dark at RT overnight. The following day the plates were washed four times with PBS containing 0.05 % Tween (PBS-T) and then blocked for 1 hour using 200 µl/well 1 % BSA in PBS. After three PBS-T washes to remove residual BSA, samples were added at 100 µl/well and plates incubated for 2 hours with agitation at RT. After washing, 100 µl of biotinylated detection antibody in PBS-T was added to each well and incubated for a further two hours at RT with agitation. After washing streptavidin-HRP (1:200) in PBS-T was added

for 20 minutes then washed off. The hydrogen peroxide-colour substrate solution (R&D systems, UK) was added to the plate (see manufacturers instructions), incubated until sufficient colour change was observed and the reaction stopped using 2N H_2SO_4. Presence or absence of the protein of interest was determined by reading the optical density (OD) at 450 nm (adjusted at 540 nm) with a Multiskan Acsent 96 well plate reader and subtracting the blank value (sample diluent only) from the sample absorbance values. The ELISA used for the *in vitro* experiments are shown in the table below.

Table 3.4. **ELISA kit used for *in vitro* experiments**

Protein of interest	Concentration of capture antibody	Concentration of detection antibody	Source
sFlt-1	2 μg/ml	0.5 μg/ml	R&D systems, UK
sEng	0.2 μg/ml	0.4 μg/ml	R&D systems, UK
PlGF	0.2 μg/ml	0.4 μg/ml	R&D systems, UK

3.2.13 Immunohistochemical procedures

Paraffin sections were deparaffinised in xylene and rehydrated with graded ethanol to potassium-phosphate-buffered saline solution (KPBS), pH 7.2. Following antigen retrieval with citrate buffer, the sections were pretreated with 1% hydrogen peroxide for 15 minutes followed by one-hour incubation in 5% donkey serum. The sections were then incubated overnight at 4°C with a primary antibody. Detection was performed with biotinylated donkey anti-rabbit or goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA)

as appropriate followed by avidin-biotin staining (Vectastain Elite ABC, Vector Laboratories, Burlingam, CA) and incubated with 3,3-diaminobenzidine/nickel sulphate as chromogen solution. Specificity of staining was confirmed by omitting the primary antibodies. Specific staining was evaluated semi-quantitatively in a blinded fashion by examining six fields per slide and subjectively scoring on a scale from 0 (no staining) to 5 (intense blue-black staining) the intensity of the chromogen deposited in the placental villous trophoblast, stromal and endothelial cells. Slides stained immunohistochemically purposefully were not counterstained so that morphological changes were hidden to the examiner. For illustration purposes sections were developed using Vector NovaRed (Vector) chromogen and were counterstained with hematoxylin.

Table 3.5. **Primary antibodies used for immunohistochemistry.**

Antibodies	Species raised in/Recognition	Optimum dilution	Source
Human Endoglin (extracellular domain)	Goat/Human	1:500	R&D systems, USA
MMP-14 (catalytic domain)	Mouse monoclonal/Human	1:500	R&D systems, USA

3.3 CLINICAL STUDIES

3.3.1 Patient population, biological samples, clinical definition and clinical laboratory analyses

Maternal blood was collected from women recruited at Yale-New Haven Hospital following admission to Labor and Birth or assessment in high- or low-risk antepartum units. Patient enrolment was performed depending on

clinical presentation in two prospective cohorts, "rule-out preeclampsia" or "rule-out preterm birth", pursuant to protocols approved by the Human Investigation Committee of Yale University. For both enrolment cohorts patients were followed until delivery or discharge, and medical record data was entered prospectively in a de-identified perinatal research database that links the medical record information (including laboratory results and neonatal assessments for the newborns admitted to Newborn Special Care Unit) with the codes on the stored biological samples. This database is continuously updated by specific research staff. The decision to recommend admission, any clinically indicated laboratory tests or procedures, including amniocentesis or delivery of foetus, was made by the primary physician, independent of the research protocols. Patient recruitment, sample processing and abstraction of data from medical records were performed by medical or research staff as appropriate and were independent of me.

After collection, samples for research were transported to the laboratory where they were spun at 3000 g and kept at 4ºC for 20 minutes, then aliquoted in polypropylene cryotubes and stored at -80ºC until analysis.

Maternal serums were collected contemporaneously as previously described (Oliver et al., 2011). Blood samples were collected by venipuncture prior to intravenous administration of fluids and allowed to clot. Blood for plasma collection was collected in a tube containing citrate anti-coagulant. The tube was spun down at 1500 g for 15 mins. The top yellowish supernatant

(plasma) was collected carefully so as to avoid disturbing the white cells in the buffy coat. The plasma was stored at -80ºC until analysis.

Gestational age was established based on last menstrual period and/or early ultrasound evaluations (less than 20 weeks of gestation) in all cases. Severe preeclampsia was defined based on the American College of Obstetricians and Gynecologists criteria (2002) as gestational age greater than 20 weeks, blood pressure of 160 mm Hg systolic or higher or 110 mm Hg diastolic or higher on two occasions at least 6 hours apart, and/or proteinuria of at least 5 g in a 24-hour urine specimen or 3+ or greater on two random urine samples collected at least 4 hours apart. Other elements of the diagnosis included: intrauterine growth restriction (less than 10^{th} percentile), cerebral or visual disturbances (headache, visual change), epigastric or right upper-quadrant pain, pulmonary edema or cyanosis, oliguria (urinary output less than 500 ml in 24 hours) or elements of HELLP syndrome, such as impaired liver function tests (greater than two times normal) and/or thrombocytopenia (less than 100 000 cells/μl). Chronic hypertension was defined as a sustained elevation in blood pressure greater than 140/90 mmHg before pregnancy or before 20 weeks of gestation. Since preeclampsia is a progressive disease and by definition a clinical diagnosis for which no acceptable gold standard is yet available, our data was also analysed based on an outcome measure (the need for delivery for preeclampsia) rather that solely by clinical classification at enrolment.

Selection of samples from the biological repository and assignment to either control or study groups for the purpose of the analyses included herein was based on clinical diagnoses at sample collection and/or outcome and was done not by me and prior to my evaluation of analytes of interest or statistical analysis.

3.3.2 Urine, creatinine quantification and calculations of fractional excretion indicators

For total protein measurements, urine or maternal serum samples were diluted 1:12 or 1:60 respectively, with deionised water and 10 μl of the diluted sample plated in duplicate. 200 μl of 1:50 bicinchoninic acid reagent mixture (BCA protein assay, Thermo Scientific, Rockford, IL) was added and incubated 20 minutes at 37^{o}C then read at 500 nm using a microplate reader against human serum albumin standards.

For creatinine measurement, urine samples were diluted 1:100 and serum samples 1:8 with picric acid (Stanbio Laboratory, http://www.stanbio.com) and spun at 12,000 rpm for 30 minutes. 1:4 dilutions of 3% NaOH and sample supernatant were plated in duplicate and incubated at room temperature for 10 minutes then read at 500 nm using a microplate reader against standard curves derived from known creatinine concentrations.

Fractional excretion indicators (clearance ratios) were calculated using the formula: (urine/plasma analyte concentration)÷(urine/plasma creatinine concentration) X 100. The fractional excretion of a substance represents the proportion of the substance excreted in the urine compared to that filtered by the glomeruli and is generally reported relative to creatinine clearance since creatinine is neither reabsorbed nor significantly secreted.

3.3.3 Enzyme-linked immunosorbent assay (ELISA)

3.3.3.1 General ELISA protocol (Quantikine kit, R&D, USA)

Assay diluent was added at 100 µl/well to pre-coated plates. Samples and standards were then added at 50 µl/well and plates were incubated for 2 hours with agitation at room temperature. After washing, 100 µl of horseradish peroxidate-conjugated detection antibody was added to each well and incubated for a further two hours at room temperature with agitation. After washing, 200 µl of hydrogen peroxide-colour substrate solution (R&D systems, US) was added to the plate (see manufacturers instructions), incubated until sufficient colour change was observed and the reaction stopped using 50 µl of 2 NH_2SO_4. Plates were read at 450 nm with 570-nm wavelength correction using a VERSAmax microplate reader with Softmax Pro 3.1.1 software (Molecular Devices, Sunnyvale, CA) and the blank value (sample diluent only) was subtracted from the sample absorbance values.

3.3.3.1.1 ELISA for sEng

96-well polystyrene microplate pre-coated with mouse monoclonal anti-human Endoglin capture antibody was used. 50 µl of recombinant human sEng standard in assay buffer or serum samples (1:30 dilution) were added to each well and incubated at room temperature for 2 hours. sEng was detected with 200 µL/well of mouse monoclonal anti-human Endoglin detection antibody conjugated to horseradish peroxidase for 2 hours and detected as described above.

3.3.3.1.2 ELISA for PlGF

96-well polystyrene microplate pre-coated with mouse monoclonal anti-human PlGF capture antibody was used. 100 µl of recombinant human PlGF standard in assay buffer or undiluted serum/urine samples were added to each well and incubated at room temperature for 2 hours. PlGF was detected with 200 µL/well of mouse polyclonal anti-human PlGF detection antibody conjugated to horseradish peroxidase for 2 hours and detected as described above.

3.3.3.1.3 ELISA for sFlt-1

96-well polystyrene microplate pre-coated with mouse monoclonal anti-human sFlt-1 capture antibody was used. 100 µl of recombinant human sFlt-1 standard in assay buffer or undiluted urine samples were added to each well

and incubated at room temperature for 2 hours. sFlt-1 was detected with 200 μL/well of mouse polyclonal anti-human sFlt-1 detection antibody conjugated to horseradish peroxidase for 2 hours and detected as described above.

3.3.3.2 ELISA for α-defensins

Plasma α-defensins (HNP 1-3) were assayed according to the manufacturer's protocol (Hycult biotech, Uden, Netherlands). Human HNP 1-3 is released from neutrophils into serum in the process of blood coagulation. This will lead to false positive results. Hence, citrated plasma was used for the detection of α-defensins (HNP 1-3). Briefly, plasma samples were diluted with the dilution buffer provided at 1:1000 in polypropylene tubes. As previously suggested (Craddock et al., 2008), the diluted plasma samples were left to incubate for 1 hr at room temperature to reduce interactions between α-defensins and immunoglobulins present in plasma prior to their addition to the pre-coated plates. Following this, plasma samples and standards were added at 100 μl/well and plates incubated for 1 hour with agitation at room temperature. After washing, 100 μl of diluted tracer was added to each well and incubated for a further 1 hour at room temperature with agitation. After washing, 100 μl of diluted streptavidin-peroxidase was added to each well for 1 hour. After washing, 100 μl of TMB substrate solution was added to the plate and incubated for 20 – 30 minutes, until sufficient colour change was observed and the reaction stopped using 100 μl of stop solution to each well. Plates were read at 450 nm with 570-nm

wavelength correction using a VERSAmax microplate reader with Softmax Pro 3.1.1 software (Molecular Devices, Sunnyvale, CA) and the blank value (sample diluent only) was subtracted from the sample absorbance values. The measurable concentration range was 0.156 – 10 ng/ml.

3.3.3.3 ELISA for calprotectin

Plasma calprotectin (MRP8/14) was assayed according to the manufacturer's protocol (BMA Biomedicals, Rheinstrasse, Switzerland). Citrated plasma samples were diluted to 1:1000 in the assay buffer provided. The samples and standards were added at 100 μl/well in the pre-coated microtiter plate. The plate was incubated for 1 hour with agitation at room temperature. 100 μl of diluted detection reagent (1:400) was added to each well and the plate was incubated overnight (15-17 hours) at 4-8ºC with shaking. The following day, the plate was washed with purified water. After washing, 200 μl of substrate solution was added to each well and incubated for 6-8 minutes at room temperature with agitation until sufficient colour change was observed and the reaction stopped using 100 μl of stop solution to each well. Plates were read at 450 nm with 570-nm wavelength correction using a VERSAmax microplate reader with Softmax Pro 3.1.1 software (Molecular Devices, Sunnyvale, CA) and the blank value (sample diluent only) was subtracted from the sample absorbance values. The measurable concentration range was 0.02 – 0.2 μg/ml.

3.3.3.4 ELISA for Fibronectin

Plasma fibronectin was assayed according to the manufacturer's protocol (American diagnostica Inc., Stamford, CT). Citrated plasma was diluted to 1:300 in the dilution buffer. Plasma samples and standards were added at 100 µl/well and plates incubated for 1 hour with agitation at room temperature. After washing, 100 µl of diluted tracer was added to each well and incubated for a further 1 hour at room temperature with agitation. After washing, 100 µl of detection antibody was added to each well for 30 minutes. After washing, 100 µl of TMB substrate solution was added to the plate and incubated for 15 minutes, until sufficient colour change was observed and the reaction stopped using 100 µl of stop solution to each well. Plates were read at 450 nm with 570 nm wavelength correction using a VERSAmax microplate reader with Softmax Pro 3.1.1 software (Molecular Devices, Sunnyvale, CA) and the blank value (sample diluent only) was subtracted from the sample absorbance

3.4 STATISTICAL ANALYSIS

In vitro studies

All data are expressed as mean ± Standard deviations. Statistical analysis was performed using two-tailed Student's t-test or Mann-Whitney rank-sum tests, as appropriate.

Clinical studies

Comparisons between two groups were performed using Student *t* tests or Mann-Whitney rank-sum tests, as appropriate. Multiple comparison procedures were performed using one way or Kruskal-Wallis analysis of variance (ANOVA) followed by Student-Newman-Keuls or Dunn's post-hoc comparisons, as appropriate. Relationships between variables (correlations) were explored using Spearman rank-order correlations.

SigmaStat 3.0 (Systat Inc. Chicago, IL) or GraphPad Prism (GraphPad Inc.) statistical softwares were used as aids for analysis. A $p<0.05$ was judged to denote statistical significance.

CHAPTER 4

HEME OXYGENASE-1 AND BILIVERDIN REDUCTASE PROTECT AGAINST CISPLATIN-INDUCED ENDOTHELIAL CELL INJURY AND INHIBIT ANGIOGENESIS

4.1 INTRODUCTION

Endothelial cell injury due to exposure to cytotoxic agent, like cisplatin, disrupts normal endothelial cells function. This results in the loss of constitutive protective mechanisms and increase in inflammatory, pro-coagulant, vasoactive, and fibroproliferative responses to injury, which eventually lead to vascular complications. During and post cisplatin therapy, evidence of endothelial dysfunction and damage was observed clinically as hypertension (Valentova and Mladosievicova, 2010), microalbuminuria, atherosclerosis, myocardial infarction, stroke and thromboembolic disease (Nuver et al., 2005). Furthermore, the endothelium being the first point of contact for the potent chemotherapeutic drug, cisplatin, it is plausible that damage to the normal vascular endothelium may be one of the initial events that could contribute or aggravate the well-known toxicities, which include ototoxicity, neurotoxicity, and nephrotoxicity, associated to it. Hence, we hypothesized that the upregulation of the cytoprotective enzymes, heme oxygenase (HO-1) and biliverdin reductase (BVR), would protect the endothelium against the cytotoxic effect of cisplatin.

The protective effect of HO-1 stems from its products, carbon monoxide (CO), free iron (Fe) and biliverdin, generated from the degradation of heme (Jozkowicz et al., 2003). These molecules are associated with anti-apoptotic, anti-inflammatory, antioxidant and anti-mutagenic properties (Ollinger et al., 2007). Apart from reducing biliverdin to bilirubin, BVR can also modulate

cell-signaling pathways by virtue of its ability to act as a transcription factor (McCoubrey et al., 1995) and to phosphorylate and autophosphorylate serine, threonine (Lerner-Marmarosh et al., 2005) and tyrosine residues (Hunter and Cooper, 1985).

HO-1 is upregulated by stress stimulus such as heme, heavy metals, peroxynitrite, endotoxin, hypoxia, hyperoxia, nitric oxide, and various cytokines (Dulak et al., 2008). However, no known inducer of BVR has yet been identified. Although, extensive researches have focused on the protective role of HO-1 in cisplatin-induced kidney injury (Shiraishi et al., 2000, Bolisetty et al., 2010) and few studies have demonstrated the damaging effect of cisplatin on endothelial cells (Nuver et al., 2010, Yu et al., 2008), none have directly addressed whether cisplatin-induced endothelial cell injury can be prevented by the induction of HO-1 or BVR in the endothelium.

Cisplatin treatment is also associated with increased circulating levels of pro-inflammatory mediators including IL-6, IL-1, IL-8 and TNF-α (Toubi et al., 2003). Indeed, as mentioned earlier, cancer patients treated with cisplatin have an increased risk of developing atherosclerosis (Nuver et al., 2004), a disease characterized by inflammation. Atherosclerosis can lead to a number of complications, including ischemia, acute coronary syndromes (unstable angina pectoris and myocardial infarction), and stroke, all of which have been observed in patients treated with cisplatin (Nuver et al., 2005). Hence, targeting the inflammatory processes may be an attractive target to prevent

or treat atherosclerosis. Placenta growth factor (PlGF), a member of the vascular endothelial growth factor (VEGF) family, has been implicated in vascular remodelling, atherosclerosis, and adverse ischemic events in animal models and in humans. It promotes vascular inflammation by binding specifically to VEGFR-1 receptor on monocytes (Luttun et al., 2002, Kodama et al., 2006, Khurana et al., 2005, Pilarczyk et al., 2008). Furthermore, in the atherosclerosis-prone apolipoprotein-E-deficient ($ApoE^{-/-}$) mice, PlGF was required for macrophage infiltration in the early atherosclerotic lesions (Khurana et al., 2005) and neutralization of PlGF by an anti-PlGF antibody reduced the inflammatory cell infiltration and atherosclerotic lesion size in these mice (Roncal et al., 2010). Moreover, endothelial cells treated with PlGF have increased level of monocyte chemoattractant protein-1 (MCP-1) and increased expression of P-selectin (Fu et al., 2009), hence promoting monocyte recruitment and inflammation. Based, on the fact that treatment with cisplatin has been associated with increase risk of atherosclerosis, we investigated whether the release of PlGF from HUVEC was affected by cisplatin treatment.

Angiogenesis is the formation of new blood vessels from pre-existing vessels. Tumors rely on a supply of blood flow in order to grow and proliferate, and hence induce blood vessel growth by secreting various growth factors, including VEGF. Hence, excessive angiogenesis is a characteristic of tumour growth. The tumour endothelial cell is an ideal target for anti-cancer therapy due to their genomic stability compared to cancer cells, which rapidly mutate and acquire drug resistance (Folkman and Klagsbrun, 1987). Recently, HO-1

has also been shown to play a role in angiogenesis (Dulak et al., 2004). Hence, if we are to propose that upregulation of HO-1 confers protection to endothelial cells during chemotherapy, it is important to determine the effect of HO-1 over-expression on VEGF-induced formation of new blood vessels. While a few studies have demonstrated the pro-angiogenic effect of HO-1 and CO in endothelial cells (Deramaudt et al., 1998, Jozkowicz et al., 2003), the outcome of these reports merits further investigations based on a number of discrepancies in relation to the origin of endothelial cell types, the culture conditions, the method of induction of HO-1 or the concentration of HO-1/CO used. Thus, using an adenovirus to over-express HO-1, we investigated whether HO-1 and BVR produce any effect on VEGF-induced capillary-like tube formation in primary cultured endothelial cells.

In summary, the results of this study demonstrated that the over-expression of HO-1 in the endothelium decreases cisplatin-induced damage as assessed by cell viability and cleaved caspase-3 expression. Furthermore, over-expression of BVR upregulates HO-1 expression in endothelial cells and together, they protect the endothelium against cisplatin-mediated cytotoxicity. Interestingly we also found that cisplatin was associated with an increase in PlGF release from the endothelium and that the over-expression of HO-1 inhibits this release. Finally we demonstrated that the overexpression of HO-1 and BVR inhibits VEGF-induced capillary-like tube formation, while the gene silencing of HO-1 and BVR restores the pro-angiogenic effect of VEGF on capillary-like tube formation.

4.2 RESULTS

4.2.1 Toxicity of cisplatin in endothelial cells

To investigate the cytotoxic effect of cisplatin, confluent human umbilical vein endothelial cells (HUVEC) were exposed to cisplatin for 24 hours in 96-wells tissue culture plates and the cell viability was measured by MTT assay. Cisplatin caused a decrease in endothelial cell viability at 24 hours at a threshold concentration of 25 μM (Figure 4.1C). This is consistent with earlier reports, which showed that cisplatin causes cell death in HUVEC from a concentration of 25 μM (L'Azou et al., 2005, Lau, 1999, Cummings and Schnellmann, 2002). The morphological analysis of the HUVEC monolayer using an inverted D-300 Nikon microscope revealed that exposure of cells to 50 μM of cisplatin for 24 hours caused a considerable reduction in the number of cells attached compared to the untreated wells. (Figure 4.1A and 4.1B).

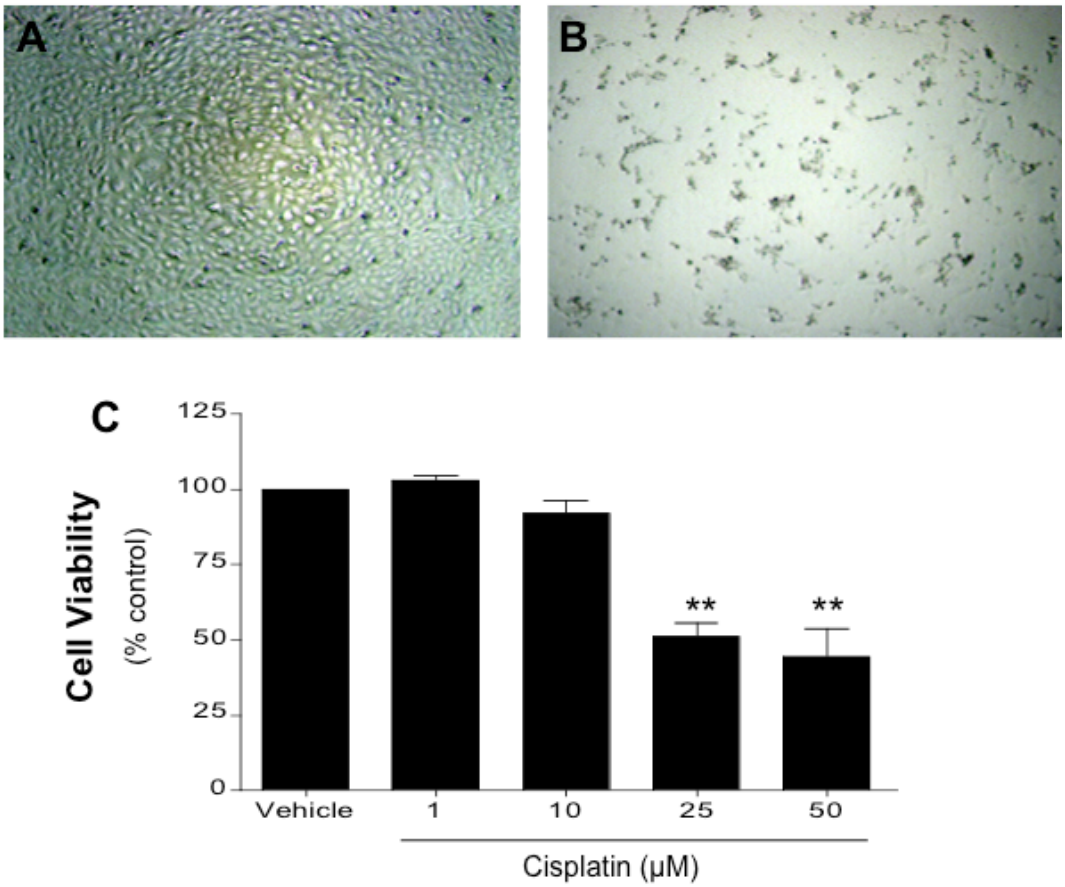

Figure 4.1 **Cisplatin affects endothelial cell viability.** Endothelial cells were treated with increasing dose of cisplatin and the cytotoxic effect was assessed by MTT assay. (A) and (B) are representations of the morphological structure of the untreated cells and the cisplatin-treated cells (25 μM). (C) Cisplatin decreases the cell viability significantly at 25 and 50 μM (** $P<0.01$) compared to untreated cells. Data are expressed as mean ± SEM of three independent experiments performed in triplicate.

4.2.2 Overexpression of HO-1 protects endothelial cells from the toxic effects of cisplatin

HO-1 inhibits apoptosis in endothelial cells (Brouard et al., 2000). To determine the anti-apoptotic effect of HO-1 against cisplatin-induced endothelial damage, HUVEC was infected with an adenovirus encoding HO-1 or β-galactosidase (β-gal) prior to treatment with cisplatin. The results showed that the ability of cisplatin to reduce cell viability was significantly diminished in HO-1 over-expressing HUVEC compared to Adβ-gal expressing cells at 25, 50 and 100 μM of cisplatin ($P<0.01$) (Figure 4.2A).

Cisplatin was reported to cause cell death through the caspase-dependent pathway in the kidney (Dursun et al., 2006) and microarray study in human microvessel endothelial cells (HMEC) showed that the gene expression of the caspase family was decreased in HO-1 overexpressed cells, hence decreasing apoptosis (Abraham et al., 2003b). In the presence of injurious stimulus or cytotoxic drugs, inactive caspases in the cytoplasm of cells are cleaved to become active. Western blot analysis showed that the protein expression of cleaved caspase-3 (15-19 kD) was significantly reduced in HO-1 over-expressing HUVEC treated with 25 and 50 μM cisplatin compared to the Adβ-gal infected cells (Figure 4.2B). The blot also confirmed the successful over-expression of HO-1 (32 kDa) in HUVEC compared to β-gal infected cells (Figure 4.2B) and the uniform expression of β-actin indicated equal loading of total proteins in each well of the gel for the Western blot.

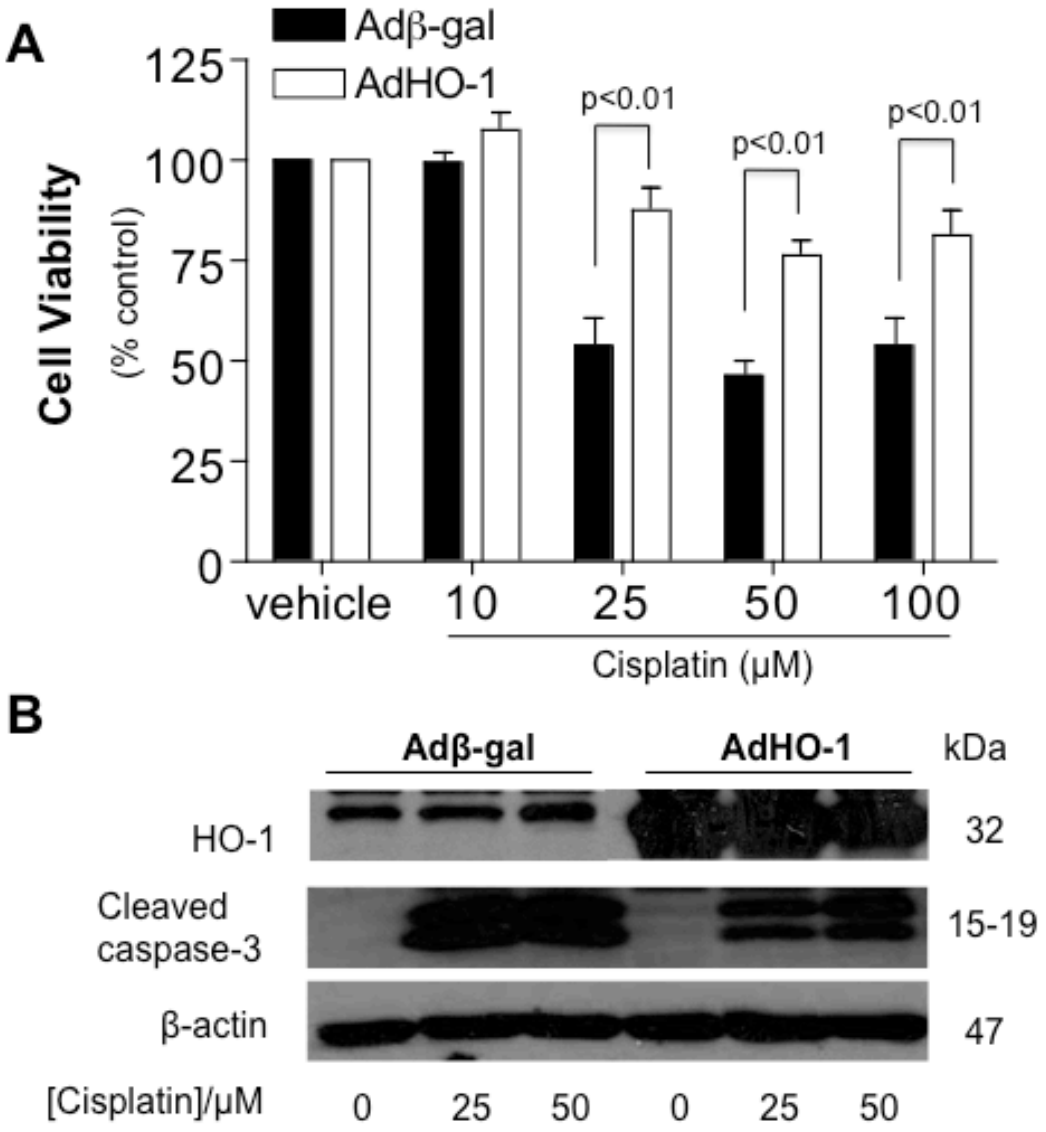

Figure 4.2 **HO-1 protects endothelial cells against cisplatin-induced cytotoxicity.** HUVEC transfected with 50 IFU of AdHO-1 and Adβ-gal were treated with increasing dose of cisplatin for 24 hours. MTT assay was performed to assessed the cell viability and a western blot was done to confirm the expression of HO-1, cleaved caspase-3 and β-actin. (A) HO-1 clearly protects the endothelial against cytotoxic damage by cisplatin because a significant ($P<0.01$) increase in cell viability was obtained at a concentration of 25, 50 and 100 μM of cisplatin compared to β-gal. (B) In the presence of over-expressed HO-1, the level of cleaved caspase-3 was less compared to β-gal. Data are expressed as mean ± SEM of three independent experiments performed in triplicate.

4.2.3 Silencing of HO-1 gene in endothelial cells enhances the cytotoxic effects of cisplatin

To determine whether gene silencing of HO-1 in endothelial cells would exacerbate cisplatin-induced cell death, HUVEC were transfected with HO-1 small interfering RNA (siRNA) and treated with cisplatin for 24 hours. The results showed that knockdown of HO-1 caused a 50% reduction in cell viability after treatment with 25 and 50 μM cisplatin ($p<0.05$) (Figure 4.3A). HO-1 immunoblotting confirmed that the HO-1 siRNA considerably reduced the HO-1 gene expression in HUVEC (Figure 4.3B) and that the reduction in HO-1 increased the level of cleaved caspase-3 compared to the scrambled siRNA (mismatch) control (Figure 4.3B). The equal level of expression of β-actin indicated equal loading of proteins in each well of the gel for the Western blot.

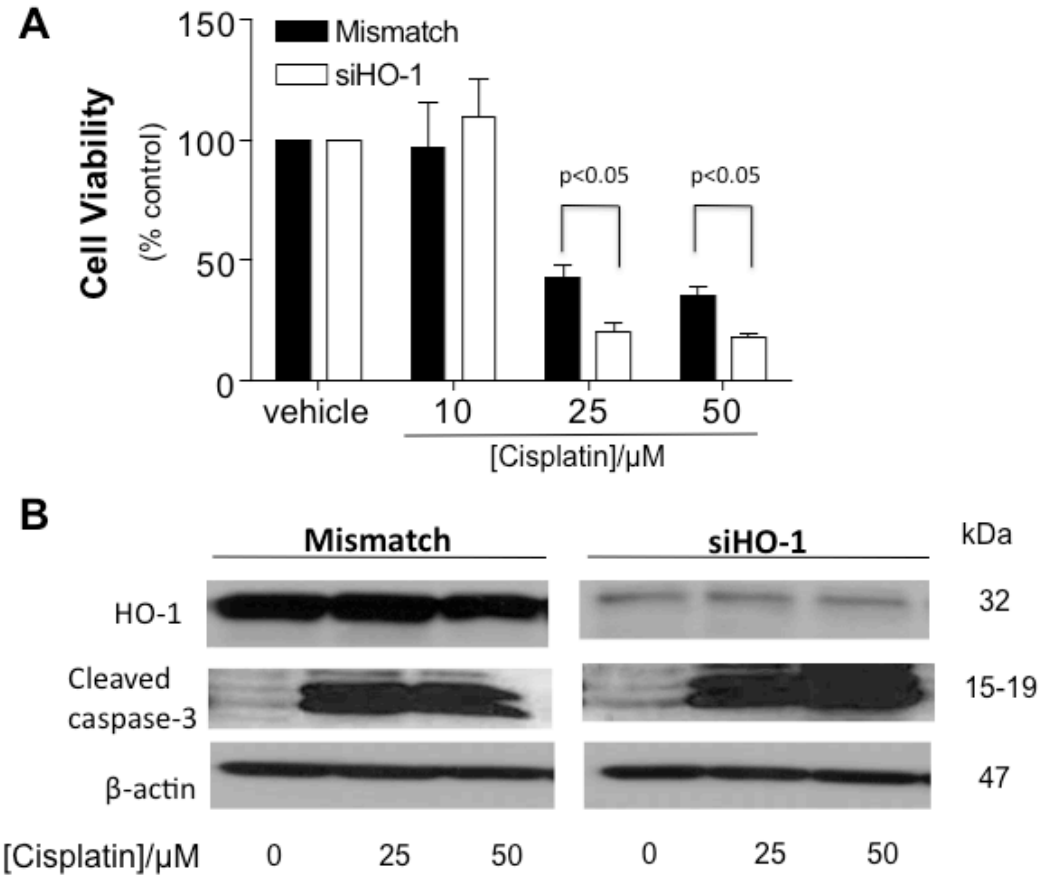

Figure 4.3 **siRNA HO-1 increases the endothelial cells susceptibility to the cytotoxic effect of Cisplatin.** HO-1 gene was knocked-down in HUVECs using siRNA and the cells were incubated with cisplatin for 24 hours. Cell viability was assessed by MTT assay and western blot was done to confirmed knockdown of HO-1 and expression of cleaved caspase-3. (A) The cell viability was significantly lower at 25 and 50 μM of cisplatin in the absence on HO-1 compared to control siRNA (Mismatch) ($p<0.05$). (B) Western blots showed that the expression of HO-1 was considerably silenced with siRNA and that this caused a marked increase in cleaved caspase-3 compared to mismatch with 25 and 50 μM of cisplatin. Data are expressed as mean ± SEM of three independent experiments performed in triplicate.

4.2.4 Overexpression of BVR protects endothelial cells against the toxic effect of cisplatin

Human BVR is present at very low concentration is endothelial cells. Like HO-1, BVR has been reported to confer protection against chemical-induced cell damage (Baranano et al., 2002, Liu et al., 2006). In this study, we tested whether adenoviral over-expression of BVR could rescue endothelial cells from cisplatin-induced damage. The results showed that the over-expression of BVR protects endothelial cells against cisplatin-induced damage by increasing the cell viability at 25 μM and 50 μM cisplatin compared to HUVEC infected with Adβ-gal (Figure 4.4A). Furthermore, anti-cleaved caspase-3 immunoblot demonstrated that the amount of cleaved caspase-3 was significantly reduced in BVR over-expressed HUVEC compared to control cells exposed to 25 and 50 μM cisplatin (Figure 4.4B). Interestingly, the Western blot also showed that the overexpression of BVR upregulated the protein expression of HO-1. Finally, the successful over-expression of BVR (41 kDa) in HUVEC was confirmed by Western blotting, which also showed, through the uniform expression of β-actin that equal amount of proteins were loaded in each well of the gel for the Western blot. (Figure 4.4B).

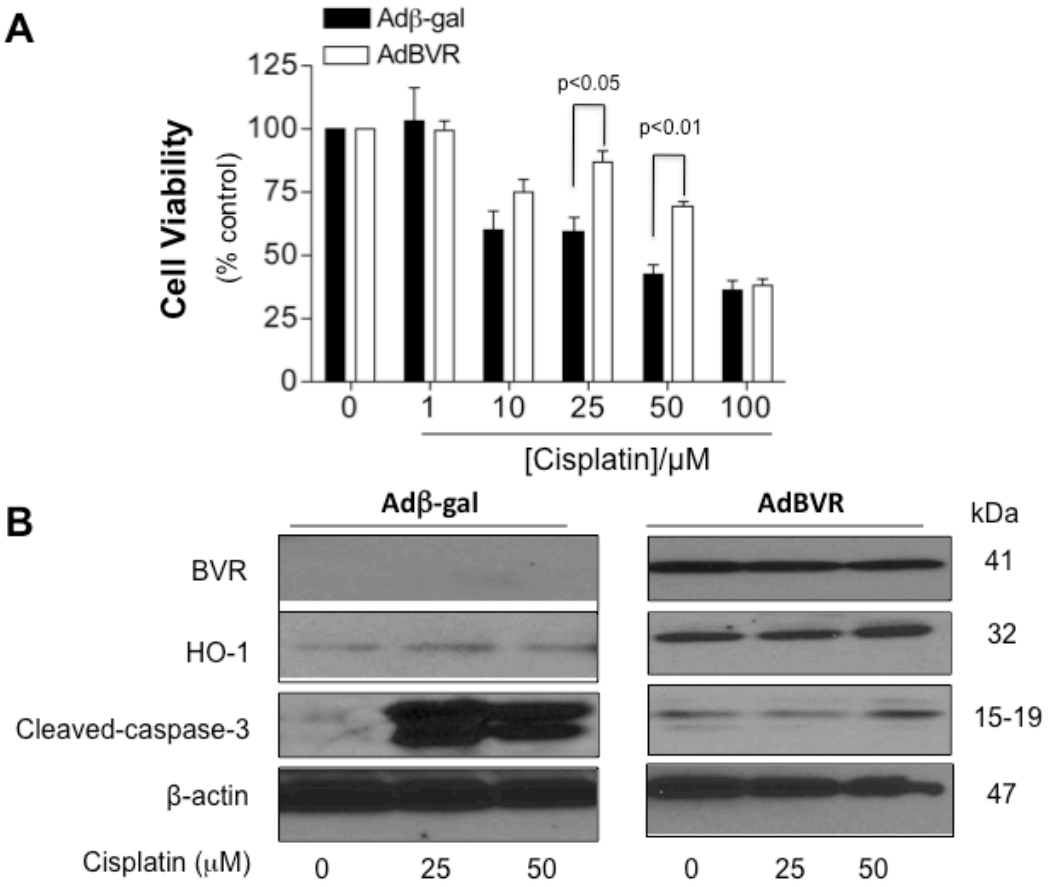

Figure 4.4 **BVR protects endothelial cells against cisplatin-induced cytotoxicity.** HUVECs transfected with 50 IFU of AdBVR and Adβ-gal was treated with increasing dose of Cisplatin for 24 hrs. MTT assay was performed to assess the cell viability and a Western blot confirmed the overexpression of BVR, HO-1, cleaved caspase-3 and β-actin. (A) BVR confers cytoprotection to endothelial cells at a concentration of 25 ($p<0.05$) and 50 μM ($p<0.01$) of cisplatin compared to β-gal. (B) In the presence of overexpressed BVR, the expression of HO-1 is induced and the level of cleaved caspase-3 is reduced with 25 and 50 μM of cisplatin. Data are expressed as mean ± SEM of three independent experiments performed in triplicate.

4.2.5 BVR-mediated cytoprotection is dependent on HO-1

Since we showed above that BVR induces the expression of HO-1 in HUVEC, we further investigated whether the cytoprotective function of BVR against cisplatin-induced endothelial cells damage was dependent on the expression of HO-1. Following adenoviral over-expression of BVR in HUVEC, HO-1 expression was silenced using HO-1 siRNA. Our results showed that the knockdown of HO-1 abrogated the cytoprotective effect of BVR against cisplatin-induced endothelial cell injury by significantly decreasing the cell viability of HUVEC at 25 and 50 μM of cisplatin ($p < 0.05$) (Figure 4.5A). Furthermore, the Western blot analysis demonstrated that the expression of cleaved caspase-3 was increased in the absence of HO-1 and even when BVR was over-expressed in HUVEC. Successful overexpression of BVR and knockdown of HO-1 was also confirmed by the Western blot (Figure 4.5B). These results indicate that the presence of HO-1 is essential for the cytoprotective function of BVR.

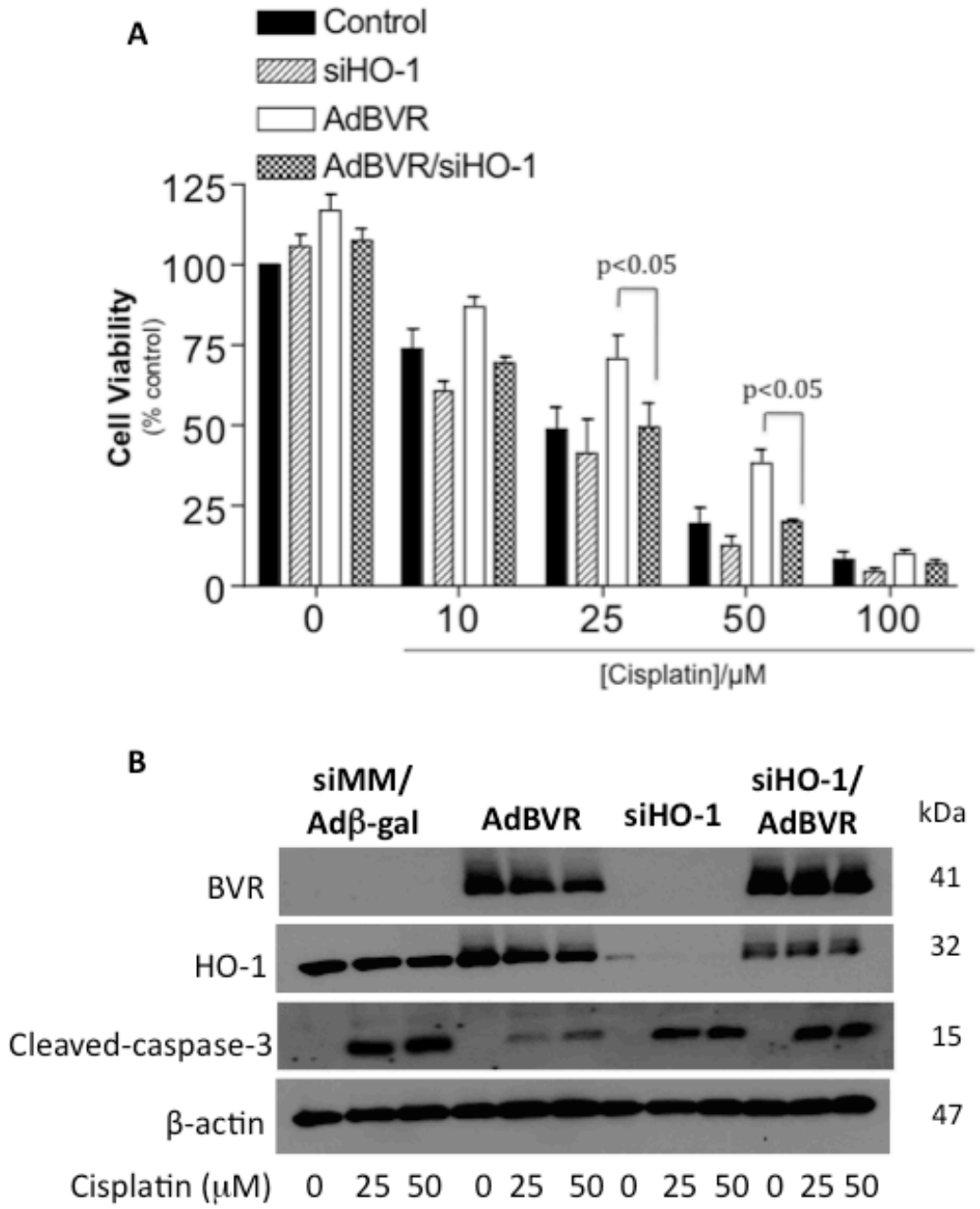

Figure 4.5 **BVR mediated-cytoprotection is dependent on the expression of HO-1.** HUVEC were transfected with 50 IFU of AdBVR or Adβ-gal. Following this, HO-1 gene was silenced in those cells using siRNA and the cells were treated with increasing dose of cisplatin for 24 hrs. MTT assay was performed to assess the cell viability and a Western blot confirmed the expression of BVR, HO-1, cleaved caspase-3 and β-actin. (A) BVR-mediated cytoprotection is significantly reduced at a concentration of 25 ($p<0.05$) and 50 μM ($p<0.01$) of cisplatin when HO-1 gene is silenced compared to AdBVR. (B) Western blot confirms that knockdown of HO-1 increases cleaved caspase-3 at 25 and 50 μM of cisplatin, even in BVR overexpressed cells. Data are expressed as mean ± SEM of three independent experiments performed in triplicate.

4.2.6 Resveratrol rescues endothelial cells from cisplatin-induced damage

Resveratrol is a naturally occurring polyphenol, which has many health benefits, including anti-inflammatory, anti-oxidative and anti-cancer properties (Brisdelli et al., 2009). *In vivo* studies have shown that resveratrol upregulates cellular antioxidant defense mechanisms protecting endothelial cells against oxidative stress in aging, diabetes (Pearson et al., 2008, Espandiari et al., 2010) and cigarette smoking (Kode et al., 2008, Csiszar et al., 2008). In our study, we pre-incubated HUVEC with 50 μM of resveratrol for 8 hrs and subsequently treated them with both resveratrol and cisplatin. Interestingly, we observed that in the presence of resveratrol there was a significant increase in cell viability at 25 and 50 μM of cisplatin compared to controls ($p<0.05$ and $p<0.01$ respectively) (Figure 4.6A). In addition, we showed that the concentration-dependent increase in resveratrol did not affect the viability of HUVEC (Figure 4.6B).

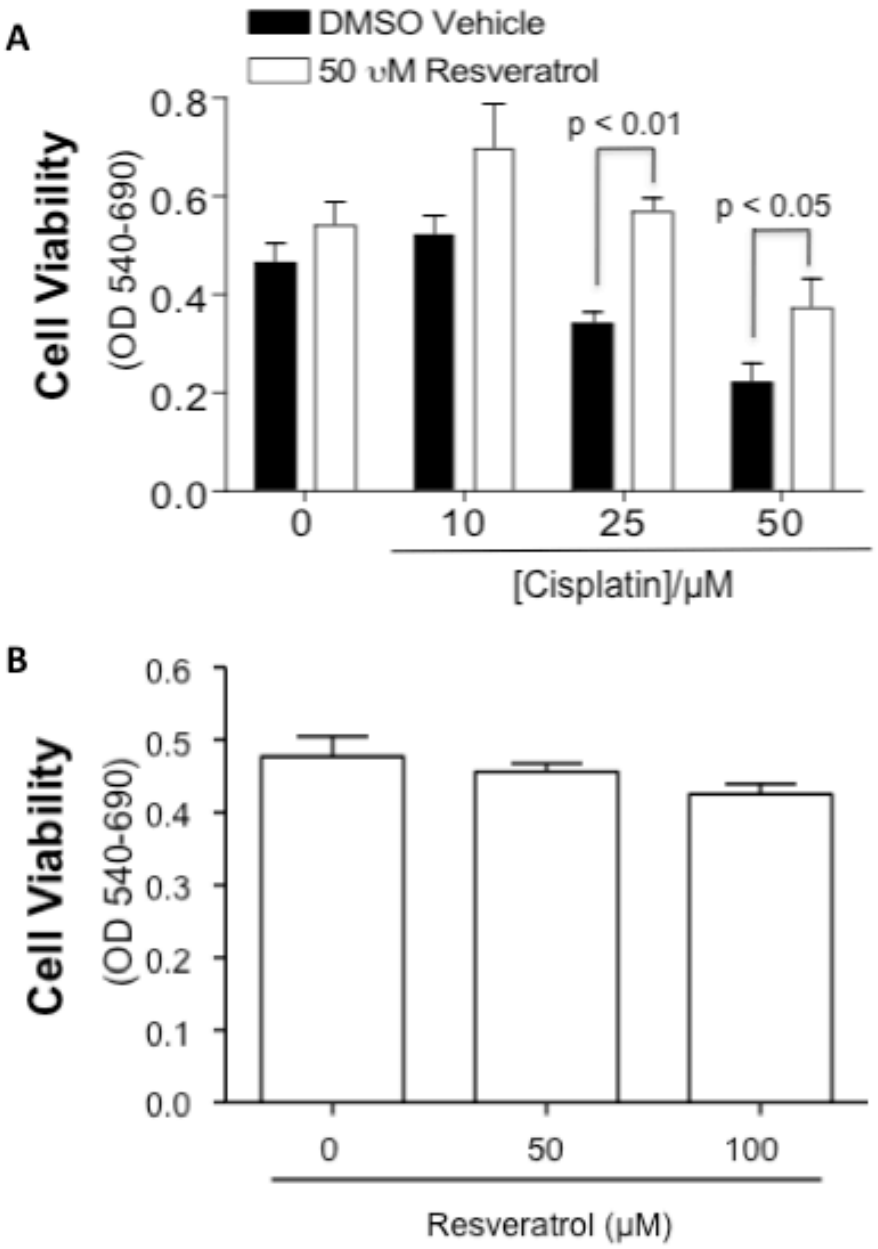

Figure 4.6 **Resveratrol protects endothelial cells against cisplatin-induced cell damage.** MTT assay was performed to determine the cell viability of HUVEC in the presence of cisplatin and Resveratrol (50 μM) together and Resveratrol alone. (A) Resveratrol increased the endothelial cell viability of endothelial cells after treatment with 25 μM ($p<0.01$) and 50 μM ($P<0.05$). (B) The endothelial cells viability was not affected with increasing dose of resveratrol. Data are expressed as mean ± SEM of three independent experiments performed in triplicate.

4.2.7 Resveratrol induces the expression of HO-1 in HUVEC

Resveratrol can protect endothelial cells from damage through the transcription factor Nrf2, which regulates the expression of numerous reactive oxygen species detoxifying genes and antioxidant genes, including HO-1 (Ungvari et al., 2010). Indeed in our study we observed that resveratrol increases both the mRNA level and the protein expression of HO-1 in HUVEC in a concentration-dependent manner in a 24 hour period (Figure 4.7A,B).

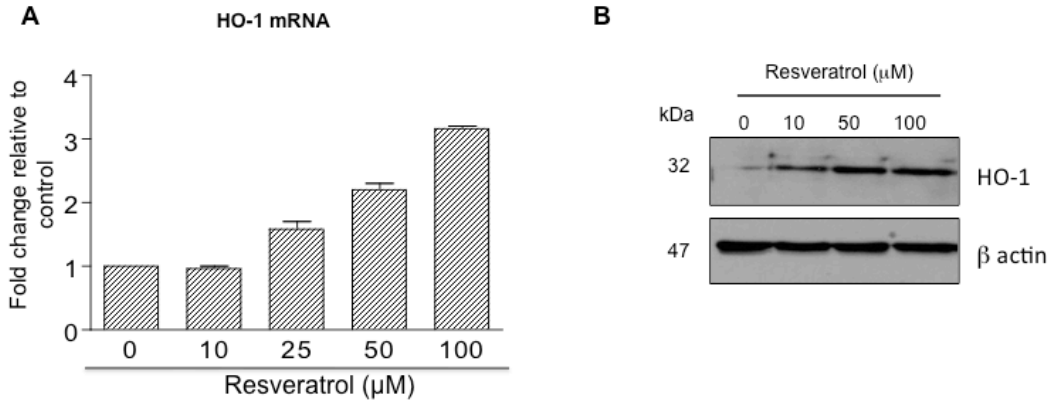

Figure 4.7 **Resveratrol induces the expression of HO-1 in endothelial cells.** (A) Resveratrol induces the expression of HO-1 at the mRNA level in a dose-dependent manner over a 24 hr period. Data are expressed as mean ± SD of three independent experiments. (B) Representative Western blot shows the increase in the protein level of HO-1 in the presence of 10, 50 and 100 μM of resveratrol.

4.2.8 Cisplatin does not induce the release of anti-angiogenic factors

Soluble Fms-like tyrosine receptor-1 (sFlt-1) and soluble endoglin (sEng) are anti-angiogenic factors known to contribute to endothelial dysfunction (Walshe et al., 2009) and to be elevated in hypertensive non-pregnant human (Shah et al., 2010). Since cisplatin induces endothelial cell damage, we determined whether 24-hour exposure to cisplatin induced the release of sFlt-1 and sEng from HUVEC. Our results showed that cisplatin, even at a non-cytotoxic concentration of 1 and 10 μM, did not induced the release of either sFlt-1 or sEng from HUVEC (Figure 4.8). The concentration-dependent decrease in sFlt-1 and sEng observed was due to reduction in cell viability from a concentration of 25 μM onwards of cisplatin as demonstrated previously. Thus it appears that cisplatin does not induce endothelial cell damage through the release of sFlt-1 or sEng release. Furthermore, these findings imply that cisplatin does not possess anti-angiogenic properties.

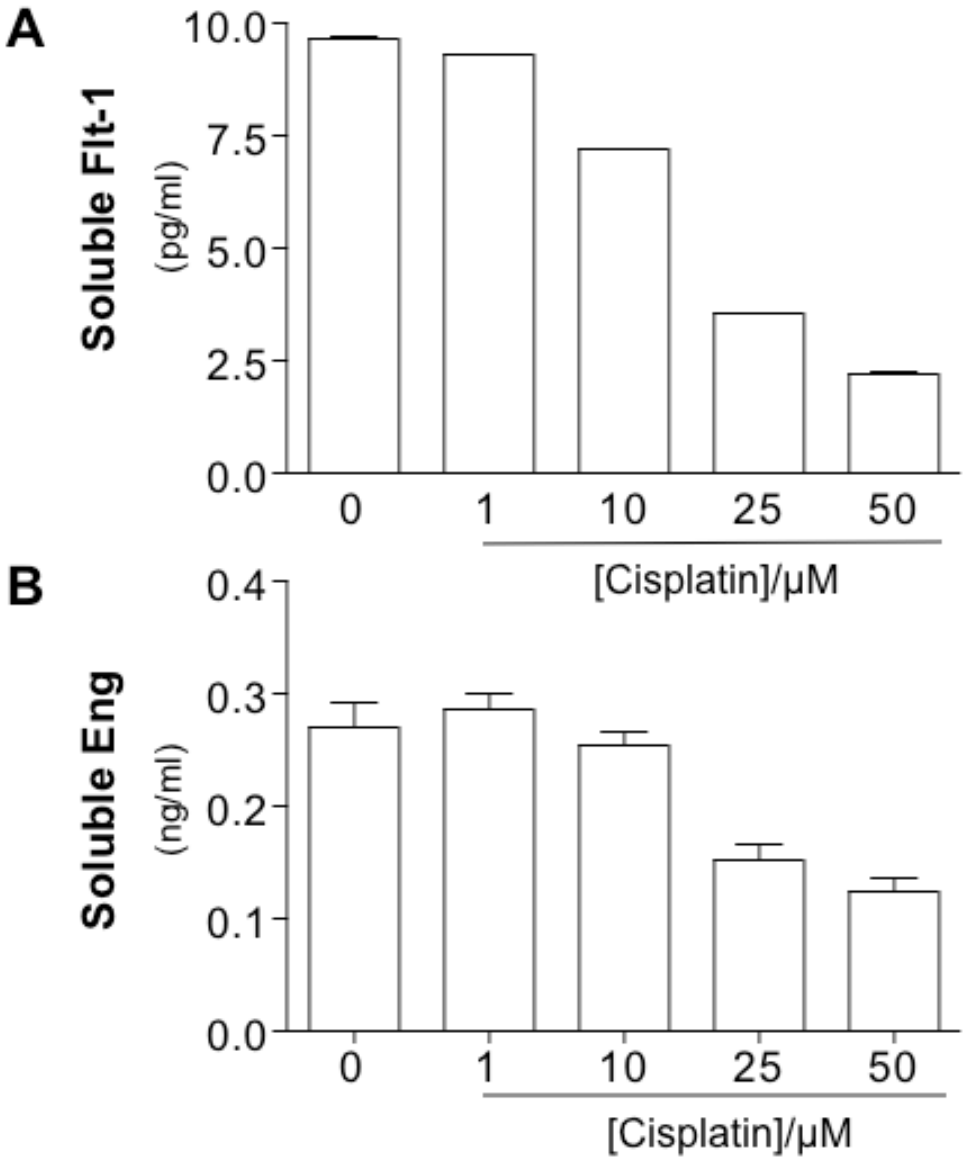

Figure 4.8 **Cisplatin does not stimulate the release of sFlt-1 and sEng from endothelial cells.** The levels of sFlt-1 and sEng were assayed by ELISA after 24 hours incubation with increasing dose of cisplatin in a 24-well plate. (A) and (B) Levels of sFlt-1 and sEng were not affected by cisplatin. Data are expressed as mean ± SEM of three independent experiments performed in duplicate.

4.2.9 Overexpression of HO-1 inhibits cisplatin-induced PlGF release in endothelial cells

PlGF is present at very low concentration in quiescent endothelial cells. However, when the endothelial cells are activated as in pathological conditions, its level is increased (Carmeliet et al., 2001). Since both cisplatin (Nuver et al., 2004) and PlGF (Roncal et al., 2010) are known to play a role in the development of atherosclerosis, we investigated whether cisplatin affects the release of PlGF from endothelial cells. Interestingly, HUVEC, treated with increasing concentration of cisplatin, released significant amount of PlGF as early as 12 hours incubation ($p<0.05$) (Figure 4.9A). The MTT viability assay revealed that at 12 hours, cisplatin was not cytotoxic to HUVEC (Figure 4.9B). However cisplatin-induced PlGF release at both 12 and 24 hours was not dose-dependent, indicating, according to Hill's criteria of causality, that cisplatin was not directly responsible for the release of PlGF from HUVEC. Furthermore, at 24 hrs incubation with cisplatin, we found that the level of PlGF was significantly elevated in Ad-βgal infected cells (Figure 4.10), even at a dose of 25 μM at which cisplatin is known to induce cell death. Interestingly, the over-expression of HO-1 in HUVEC significantly inhibited the release of PlGF in both the untreated cells and the cisplatin-treated cells at 10 and 25 μM of cisplatin ($p<0.05$). These results suggest that cisplatin-induced endothelial PlGF release could promote monocyte infiltration, hence promoting inflammation and increase the risk of atherosclerosis in cisplatin-based chemotherapy.

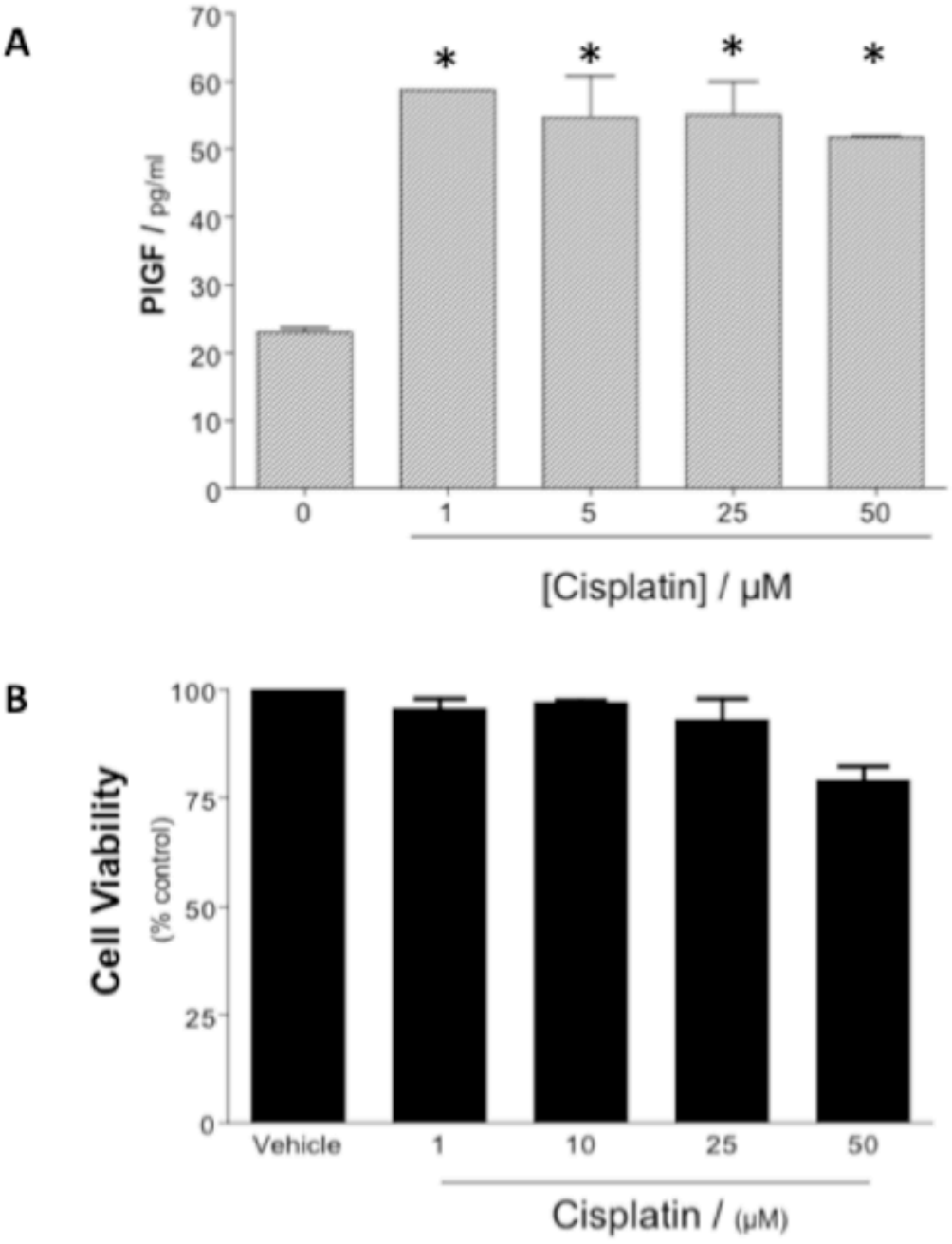

Figure 4.9 **Cisplatin stimulates the release of PlGF from endothelial cells.** (A) PlGF release was measured at 12 hours incubation with increasing dose of cisplatin in a 24-well plate. Cisplatin caused significant increase in PlGF level (*$p<0.05$) compared to control but not in a dose-dependent manner. (B) The viability of the cells was not affected at 12 hours incubation with cisplatin. Data are expressed as mean ± SEM of three independent experiments.

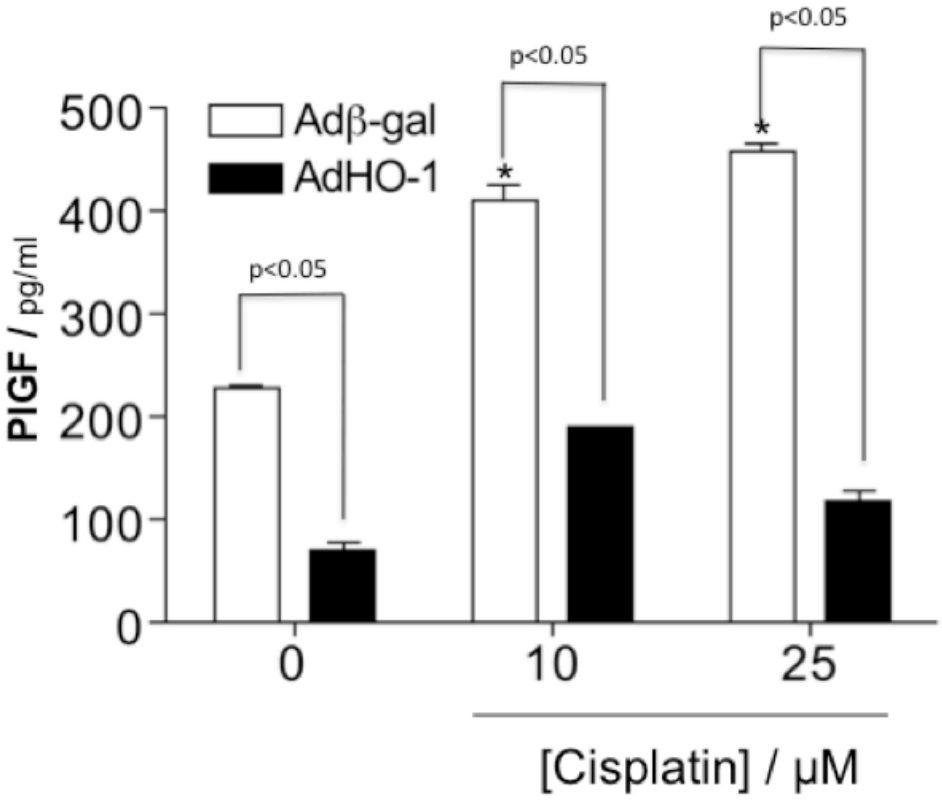

Figure 4.10 **HO-1 prevents cisplatin-induced PlGF release from endothelial cells.** (A) The effect of cisplatin on the release of PlGF was investigated after 24 hours incubation with the drug in a 6-well plate by ELISA. In the control wells (Ad-βgal), cisplatin induced a significant increase in PlGF at 10 and 25 μM compared to untreated cells ($p<0.05$, n=3). Adenoviral overexpression of HO-1 at 50 IFU in endothelial cells reduces the level of PlGF independent of Cisplatin ($p<0.05$). Data are expressed as mean ± SEM of three independent experiments.

4.2.10 HO-1 negatively regulates VEGF-mediated angiogenesis in endothelial cells

Angiogenesis is important for the survival of tumor cells. Since over-expression of HO-1 was shown to protect against cisplatin-induced endothelial damage, it was important to determine whether over-expression of HO-1 could promote or inhibit angiogenesis. We assessed the angiogenic function of HO-1 in HUVEC through capillary tube formation on VEGF-reduced Matrigel. Our results show that siRNA-mediated HO-1 knockdown potentiated the formation of VEGF-induced capillary-like network structure (Figure 4.11). Conversely, adenoviral over-expression of HO-1 in HUVEC significantly inhibited VEGF-induced tube formations compared to β-gal over-expressed cells (Figure 4.12). Collectively, these findings show that HO-1 acts as negative regulators of angiogenesis.

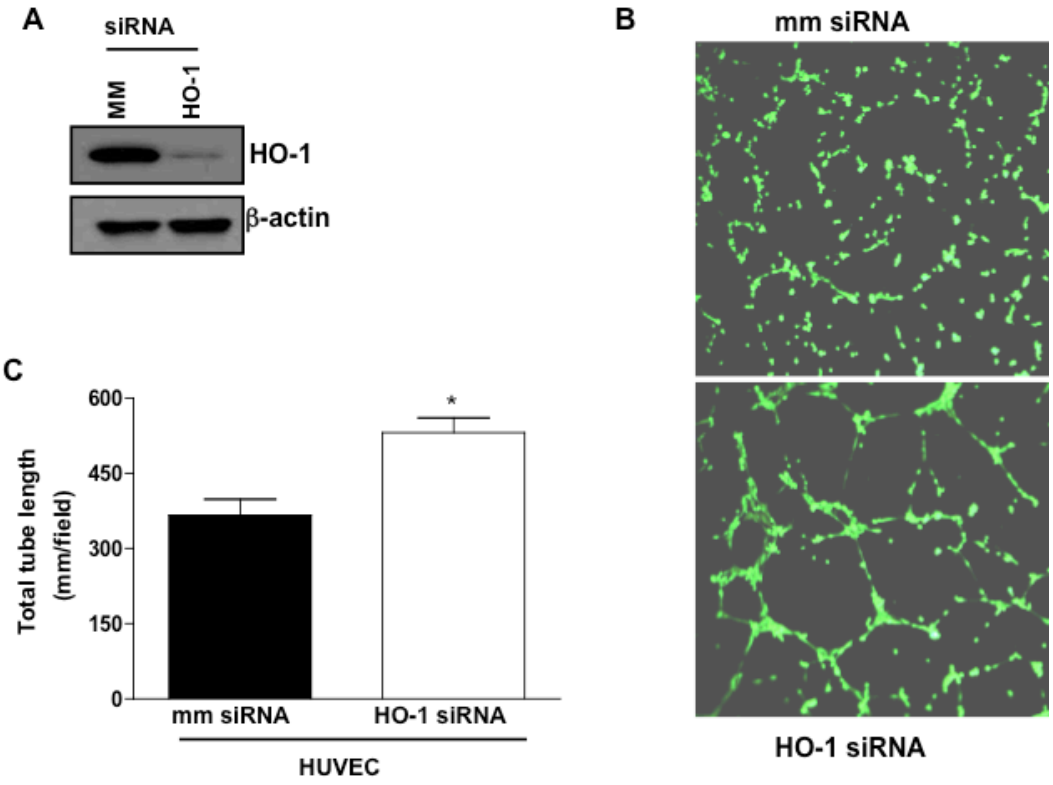

Figure 4.11 **Loss of HO-1 in HUVEC promotes angiogenesis.** (A) Western blot showed that the expression of HO-1 was considerably silenced with siRNA. (B) Representative photomicrographs of capillary networks of HUVEC electroporated with HO-1 siRNA or control siRNA (mm siRNA) after 6 hours on VEGF-reduced Matrigel. (C) Quantification of capillary tubes in the presence of HO-1 siRNA. Data are expressed as mean (±SEM) or representative of five or more separate experiments performed in duplicate. ($p < 0.05$ compared to mm siRNA).

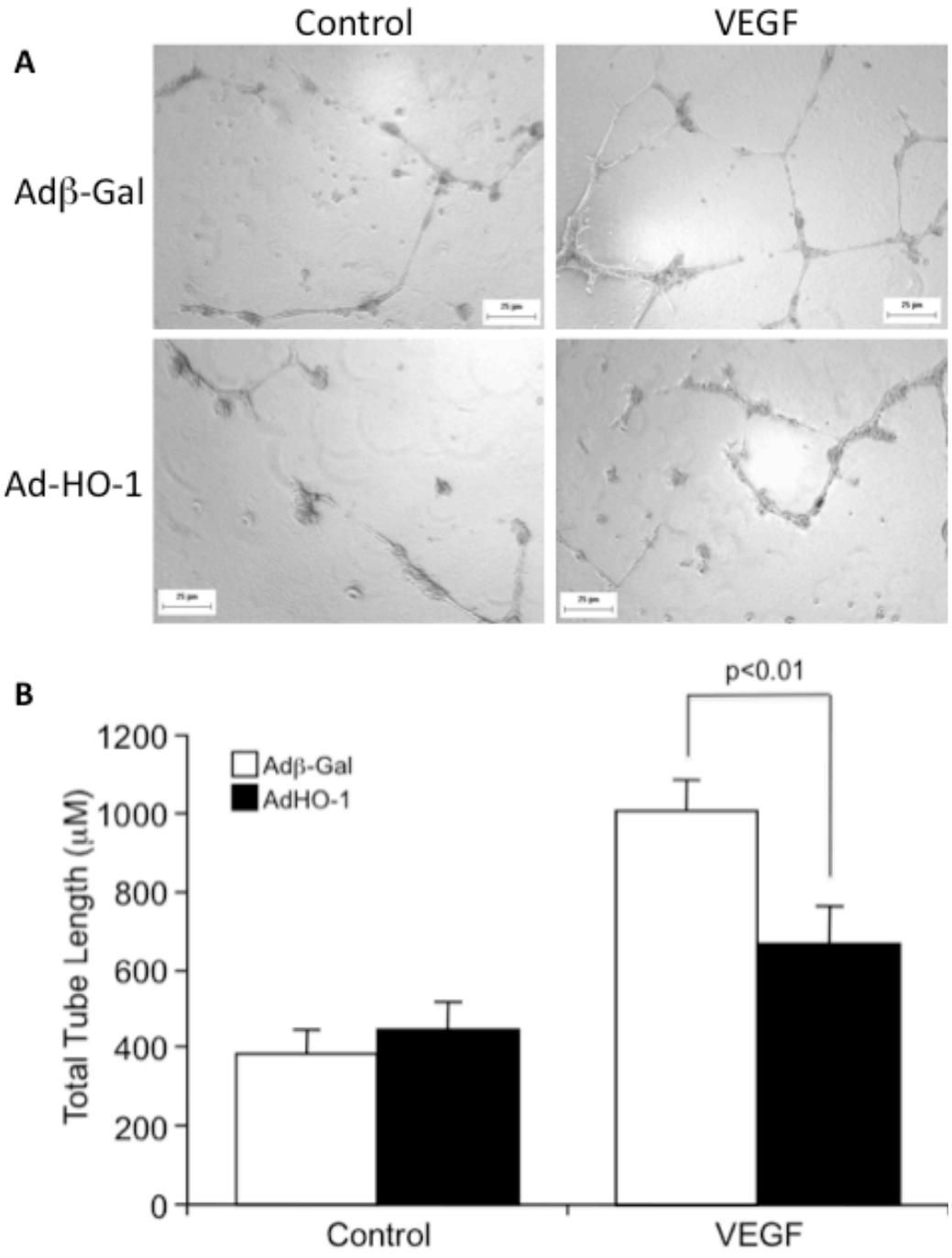

Figure 4.12 **Heme oxygenase-1 negatively regulates growth factor-induced angiogenesis.** (A) Representative photomicrographs of endothelial cell capillary network formation on HUVEC were infected with 50 IFU/cell of adenovirus encoding HO-1 (AdHO-1) or β-gal (Adβ-Gal), plated at a density of $6x10^4$ on growth factor-reduced Matrigel, prior to stimulation with VEGF (20 ng/ml) after 6 hours. (B) Quantification of VEGF induced capillary networks. Data are expressed as mean ± SEM or representative of five or more separate experiments performed in duplicate.

4.2.11 BVR negatively regulates VEGF-mediated angiogenesis in endothelial cells

To date, no studies have investigated the role of BVR in angiogenesis. Hence, similar to HO-1, we assessed the angiogenic behaviour of BVR on VEGF-induced capillary tube formation in HUVEC. Although the protein level of BVR is very low and undetectable in HUVECs, we showed that siRNA knock down of BVR in HUVEC significantly increased the VEGF-mediated capillary tube fomation (Figure 4.12). Conversely, adenoviral overexpression of BVR in HUVEC caused a reduction in VEGF-induced capillary tube formation (Figure 4.13). Taken together, these findings suggest that BVR negatively regulates angiogenesis.

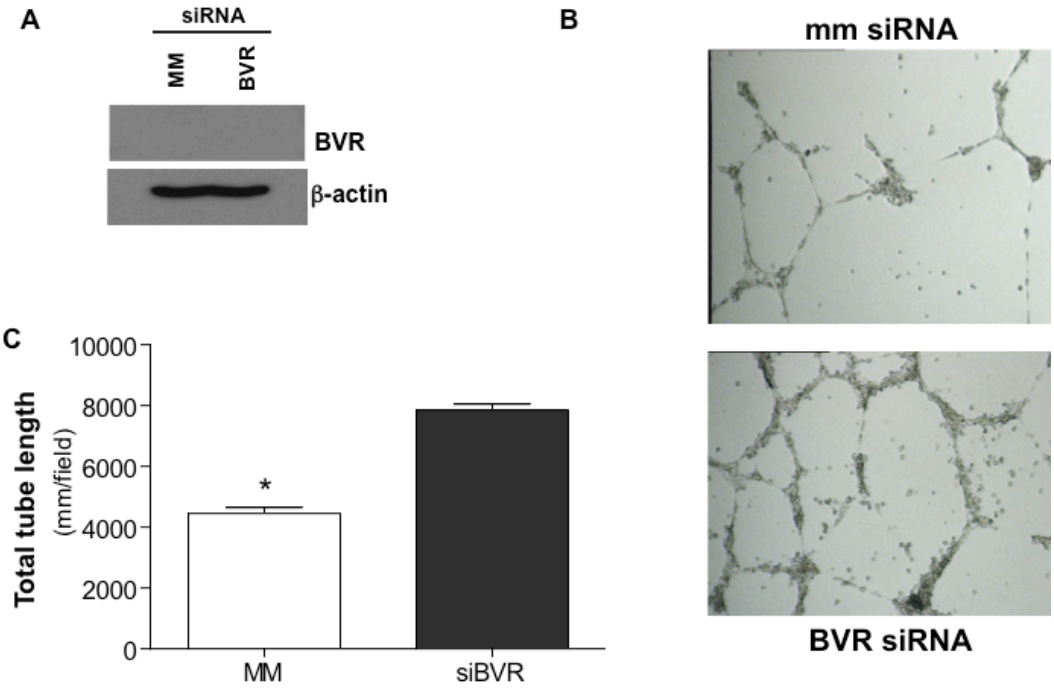

Figure 4.13 **Loss of BVR promotes growth factor-induced angiogenesis.** (A) Western blot showed that the expression of BVR was considerably silenced with siRNA. (B) Representative photomicrographs of capillary networks of HUVEC electroporated with BVR siRNA or control siRNA (mm siRNA) after 4 hours on VEGF-reduced Matrigel. (C) Quantification of capillary tubes in the presence of BVR siRNA. Data are expressed as mean (±SEM) or representative of three or more separate experiments performed in duplicate. ($p < 0.05$ compared to mm siRNA).

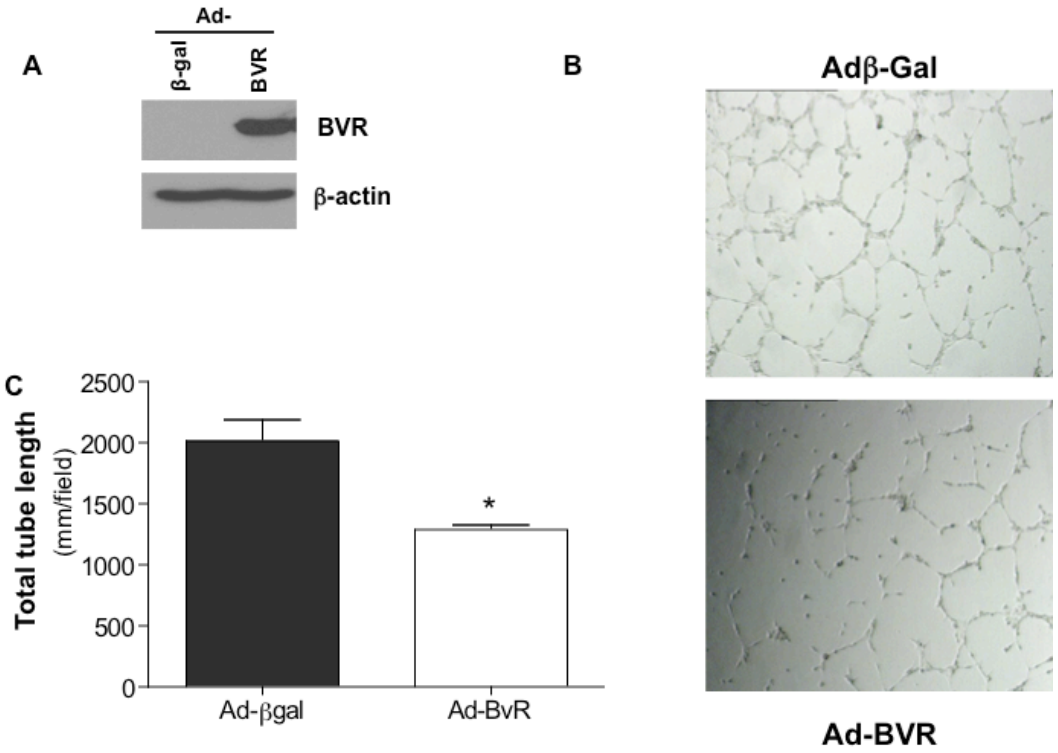

Figure 4.14 **BVR inhibits growth factor-induced angiogenesis.** (A) Western blot to confirm the overexpression of BVR in HUVEC infected with 50 IFU/cell of adenovirus encoding BVR (AdBVR) or β-gal (Adβ-Gal). HUVEC were plated at a density of $6x10^4$ on growth factor-reduced Matrigel. (B) Representative photomicrographs of endothelial cell capillary network formation after 4 hours stimulation of HUVEC with VEGF (20 ng/ml). (C) Quantification of VEGF induced capillary networks. Data are expressed as mean ± SEM or representative of three or more separate experiments performed in duplicate.

4.3 DISCUSSION

This study demonstrates that cisplatin-induced endothelial cell damage, can be attenuated by the endothelial over-expression of HO-1 and BVR. Furthermore, it shows that the ability of BVR to protect against cisplatin-induced cytotoxicity is dependent on the induction of HO-1 by BVR. Cisplatin did not affect the release of anti-angiogenic factors, sFlt-1 and sEng from endothelial cells. However, it induced the release of PlGF, which was inhibited via the over-expression of HO-1 in endothelial cells. Finally, we demonstrated that adenoviral over-expression of HO-1 and BVR are anti-angiogenic as illustrated by the inhibition of capillary-like tube formations in primary human endothelial cells, whereas the knockdown of HO-1 and BVR promotes angiogenesis.

Our *in vitro* findings that cisplatin induces endothelial cell damage is supported by recent studies that demonstrated that cisplatin causes endothelial cell activation by increasing the expression of endothelial intercellular adhesion molecule-1 (ICAM-1) (Yu et al., 2008, Nuver et al., 2010), tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) (Nuver et al., 2010), hence promoting endothelial-leukocyte interactions and inflammation. Furthermore, previous morphological studies of Kohn and colleagues established, through detailed microscopic study of the blood vessels, that cisplatin causes endothelial injury such as mitochondrial inclusions, intra-cytoplasmic vacuole formation,

lipid bodies and constriction of the lumen of the stria vascularis of the guinea pigs (Kohn et al., 1997) as well as damage to the glomerular capillaries of the kidney (Kohn et al., 2002) and the liver (Kohn et al., 2005).

Studies have previously showed that the chemotherapeutic effectiveness of cisplatin on tumours is mediated by apoptosis via the activation of caspase-8, -6 and -3 (Seki et al., 2000). In the present study, Western blot analysis using antibody against cleaved caspase-3 showed that cisplatin indeed caused activation and cleavage of caspase-3 in a dose-dependent manner in cultured endothelial cells with a maximum amount of cleaved caspase-3 generated with 50 μM of cisplatin. Although high doses of cisplatin has been generally associated with necrotic damage, a study by Dursun and colleagues demonstrated that necrotic cell death due to high doses of cisplatin (50 μM) could also cause activation of caspase-3 (Dursun et al., 2006). We further showed that cisplatin does not possess anti-angiogenic properties due to its failure to induce the release of the anti-angiogenic factors, sFlt-1 and sEng, from endothelial cells. Hence our study confirms that the cytotoxic effect of cisplatin on endothelial cells is mediated via apoptosis. Furthermore, together with the previous studies, our study demonstrates that cisplatin causes endothelial injury, which is likely to play a role in the vascular complications associated with cisplatin-based chemotherapy.

Numerous studies have reported that upregulation of HO-1 in the kidney improves pathological conditions such as ischemia-reperfusion injury,

glomerular inflammation, renal failure, angiotensin-mediated hypertension (Agarwal and Nick, 2000) and protects against cisplatin-induced autophagy of kidney cells (Bolisetty et al., 2010). Other studies have shown that agents that upregulates HO-1 can protect against cisplatin-mediated ototoxicity (Gao et al., 2010, Kim et al., 2009). Our study is the first to show that endothelial cells over-expressing HO-1 are protected against the damaging effect of cisplatin. We further report that resveratrol, a polyphenol found in grapes and red wine can upregulate the expression of HO-1 in endothelial cells and that endothelial cells treated with both cisplatin and resveratrol are more resistant to cisplatin-mediated damage. Furthermore, among its many benefits, resveratrol also protects against cardiovascular disease and cancer (Jang et al., 1997, Bhat and Pezzuto, 2002, Pervaiz, 2003). Hence, the inclusion of resveratrol in cisplatin-based chemotherapy may benefit patients undergoing cisplatin-based chemotherapy by protecting them from developing vascular complications.

Similar to HO-1, BVR is known to act as a cytoprotectant (Baranano et al., 2002, Jansen et al., 2010). A recent study where the expression of BVR was induced with doxycycline in NIH 3T3 mouse fibroblastic cell line, demonstrated that over-expression of BVR protects against cisplatin-induced damage by increasing the cell viability. In the present study, we showed for the first time that over-expression of BVR in primary endothelial cells induces the expression of HO-1 and protects against cisplatin-induced cell damage. Furthermore, we demonstrated, through siRNA knockdown of HO-1, that the cytoprotective effect of BVR is dependent on the BVR-mediated

upregulation of HO-1 in HUVEC. In a recent study, Jansen and colleagues demonstrated that the protective effect of pharmacological over-expression of HO-1 was almost completely abrogated in BVR-silenced cells, indicating that BVR is an essential in HO-1-induced cytoprotection (Jansen et al., 2010). Hence, it can be deduced that the presence of both HO-1 and BVR in endothelial cells are essential for cytoprotection. Based on these facts, we can also speculate that the anti-apoptotic and cytoprotective functions of both HO-1 and BVR may be due to bilirubin generated from the reduction of biliverdin by BVR. Indeed, bilirubin has previously been shown to protect against oxidative stress, lipid peroxidation and ROS (Neuzil and Stocker, 1994). We can also speculate that, due to the co-dependency of HO-1 and BVR for cytoprotection, a feedback loop might exist between the two enzymes.

Inflammation plays a major role in the development of atherosclerosis (Manabe, 2011). Nuver and coworkers observed in patients ten years after cisplatin-based chemotherapy that there was an increase in microalbuminuria, a predictive marker of atherosclerosis (Borch-Johnsen et al., 1999) and increased levels of inflammatory marker proteins, which may contribute to the subsequent development of accelerated atherosclerosis and an increased risk for future cardiovascular disease (Nuver et al., 2004). Monocytes and macrophages are keys players in inflammation and in the pathogenesis of atherosclerosis. In atherosclerosis, PlGF can act as a chemoattractant for monocytes and macrophages. It binds to the VEGF receptor-1 (VEGFR-1), which is expressed on monocytes, macrophages as

well as endothelial cells, hence promoting an inflammatory response (Carmeliet et al., 2001). In this context, anti-VEGFR-1 antibody administration has been shown to reduce early lesions and macrophage infiltration in atherosclerosis-prone apolipoprotein-E-deficient ($ApoE^{-/-}$) mice (Luttun et al., 2002). Furthermore, Khurana et al. has shown that PlGF is required for macrophage infiltration in early atherosclerotic lesions in $apoE^{-/-}$ mice (Khurana et al., 2005) and that neutralization of PlGF by a murine anti-PlGF antibody reduced the inflammatory cell infiltration and atherosclerotic lesion size in these mice (Roncal et al., 2010). Our study shows that even low concentration of cisplatin induces a significant release of PlGF from endothelial cells. However, according to the Hill's criteria of causation, the non-dose dependent release of PlGF due to cisplatin treatment suggests that cisplatin is not the direct cause for the increase in PlGF release from endothelial cells. Instead the stress induced by cisplatin may be a trigger for the release of PlGF. Regardless of this lack of causality between cisplatin and PlGF release, we can still speculate that, the increase in PlGF may promote inflammation during cisplatin-based chemotherapy and increase the risk of developing atherosclerosis and cardiovascular complications as has been observed during and post cisplatin chemotherapy.

In this present study, we showed for the first time the negative regulation of HO-1 on cisplatin-induced PlGF release in endothelial cells. Numerous studies using pharmacological or gene induction approach have now demonstrated the cardiovascular protective function of HO-1 (Wu et al., 2010). Indeed, a previous report showed that mice deficient in HO-1 and

apoE developed larger and more advanced lesions than mice deficient in apoE alone fed on a hypercholesterolemic diet (Yet et al., 2003). Thus, agents that up-regulate HO-1 will be effective not only by suppressing cisplatin-induced endothelial cell damage, but also by reducing inflammation caused by the release of PlGF, hence protecting against cardiovascular disease.

Apart from the cytoprotective, anti-apoptotic, anti-inflammatory and cardiovascular protective function of HO-1, we found that the over-expression of HO-1 inhibited VEGF-driven capillary-like tube formation, while HO-1 siRNA gene ablation promoted VEGF-induced capillary-like tube formation in HUVEC, indicating that HO-1 acts as a negative regulator of angiogenesis. Despite being novel, these results are in marker conflict with earlier studies that have attributed a pro-angiogenic function to HO-1 in endothelial cells (Jozkowicz et al., 2003, Malaguarnera et al., 2002, Deramaudt et al., 1998). However, based on the type of cells used in previous studies and the differential mode of induction of HO-1, we feel we can make a cogent argument in support of our data.

Firstly, studies that demonstrated the pro-angiogenic effect of HO-1 primarily used the immortalised cell lines, namely, human dermal microvascular endothelial cell line (HMEC) (Jozkowicz et al., 2003) or coronary endothelial cells that is large vessel endothelium (Deramaudt et al., 1998) or vascular smooth muscle cells (Dulak et al., 2002). As has been reported previously, the behaviour of endothelial cells and their functions

depend on the vascular bed of origin (Page et al., 1992). Indeed, microvascular endothelial cells grown from primary skin (Swerlick et al., 1992) and heart (McDouall et al., 1997) show significant differences compared to HUVEC with respect to their response to inflammatory cytokines. Furthermore, the differences between the angiogenic potential of HUVEC compared to HMEC has been scrutinized and the outcome has revealed that resting or stimulated HUVEC does not produce detectable amount of VEGF, whereas resting HMEC produces about 24.9 ng/ml of VEGF, which can increase in response to specific stimulus (Nanobashvili J, 2003). Indeed, in our study, we were unable to detect any VEGF in HUVEC overexpressed with HO-1. We fully acknowledge, as previously reported, that it is possible that over-expression of HO-1 can induce the release of VEGF in HMEC or in rat lung microvessel endothelial cell line (Jozkowicz et al., 2002, Abdel-Aziz et al., 2003, Abraham et al., 2003b). However, in view of the fact that HUVEC does not produce detectable level of VEGF, the study by Pae et al. (Pae et al., 2005), which showed that the pharmacological induction of HO-1 causes angiogenesis through the increase in VEGF in HUVEC, can be challenged.

Moreover, HMEC and HUVEC differ in their sensitivity to exogenous growth factors. While under basal condition, the proliferation of HUVEC is very low; this can be increased with growth factors such as VEGF or FGF-2. On the contrary, HMEC proliferate spontaneously and their proliferation is *not* enhanced by growth factors. Similarly, the spontaneous outgrowth of capillaries is negligible in unstimulated HUVEC, but well pronounced in

HMEC (Nanobashvili J, 2003). Based on these observations, we feel the studies in HMEC are inappropriate. The study by Jozkowicz et al. attempted to address the pro-angiogenic effect of HO-1 by studying capillary tube formation using HUVEC and outgrowth of capillaries from HUVEC spheroids (Jozkowicz et al., 2003). However, the quality of the representative pictures and quantification was poor, which we could argue renders the findings of the study inconclusive.

Different experimental conditions could also account for the conflict between our study and previous studies. In our study we used adenoviral over-expression of HO-1, while other studies used specific doses of pharmacological inducers of HO-1 (Jozkowicz et al., 2003, Bussolati et al., 2004) or retroviral transduction of HO-1 (Abraham et al., 2003b, Abdel-Aziz et al., 2003, Malaguarnera et al., 2002). In this context, we could argue that the use of pharmacological inducer of HO-1 may not specifically target HO-1 and can also affect other processes unrelated to HO-1. In contrast, the use of adenoviral induction provides is a highly specific method to assess the effect of HO-1 on angiogenesis. Similarly, we cannot discount the fact that adenoviral and retroviral over-expression of HO-1 can affect the function of HO-1 in distinctly different ways.

Using adenoviral over-expression or siRNA knockdown of HO-1 makes it difficult to modulate the expression of HO-1 in a dose-dependent manner. Hence, this limitation in our study raises the question of whether HO-1 has a

dual effect on angiogenesis in endothelial cells depending on its level of expression. Recently a study by Meng et al. showed that the arsenite-induced angiogenesis and capillary tube formation in HMEC is mediated via the dose- and time-dependent upregulation of HO-1 by arsenite. Indeed, the same study revealed that arsenite was only able to induce tube formation at a low concentration ranging from 0.1 to 1 μM, whereas at higher concentration of arsenite, no capillary-tube formation was observed. Hence, these observations raise the question of whether HO-1 promotes angiogenesis at a lower level of induction, whereas it inhibits angiogenesis when substantially over-expressed.

This new concept that HO-1 could inhibit angiogenesis can be of therapeutic relevance in cancer treatments. Tumours depend on a supply of blood for their growth and survival. Since endothelial over-expression of HO-1 inhibits angiogenesis, hence HO-1 may inhibit tumour growth by inhibiting the differentiation of tumour endothelial cells. In contrast, inhibition of angiogenesis will not affect healthy endothelial cells since the rate of angiogenesis in those cells are very low. Further experiments are required to prove our theory that over-expression of HO-1 inhibit angiogenesis. However, if this proves to be accurate, HO-1 will not only have a beneficial role in protecting against cisplatin-induced vascular toxicity, but may also cause tumour regression.

The role of biliverdin and bilirubin in angiogenesis has not been fully determined. In this study, we show for the first time that over-expression of BVR in endothelial cells inhibits capillary-like tube formation, while the gene ablation of BVR induces VEGF-driven angiogenesis. Unlike HO-1, BVR does not induce the expression of VEGF in endothelial cells, whereas it markedly increases VEGF release in keratinocytes (Loboda et al., 2008). To date, the effect of bilirubin on VEGF production or angiogenesis is unknown. Further work is required to determine the mechanism via which BVR exerts its anti-angiogenic effect. However, several mechanisms can be proposed. First, as a transcription factor, BVR could induce the transcription of anti-angiogenic factors. Secondly via its capacity to phosphorylate and autophosphorylate serine and tyrosine kinase residues, it can influence anti-angiogenic signaling pathways.

In summary, this study has demonstrated that HO-1 and BVR protect against cisplatin-induced endothelial cell damage by increasing cell viability. HO-1 also inhibits the endothelial release of the pro-inflammatory chemokine, PlGF. To date, there are no clear and widely accepted recommendations on the prevention of vascular complications during cisplatin therapy, hence we can speculate that agents which upregulate HO-1/BVR should be included in cisplatin-based chemotherapy to improve therapeutic outcome. In addition, the over-expression HO-1 and BVR can potentially help in reducing tumour growth during chemotherapy. Indeed studies in breast cancer cells have shown that HO-1 inhibits breast cancer cell invasion via the suppression of the expression of matrix metalloproteinase-9 (Lin et al.,

2008a). Another study demonstrated that HO-1 and its product, CO, suppress photocarcinogenesis in hairless mouse exposed to UVA radiation (Allanson and Reeve, 2007) and a more recent study illustrated that HO-1 over-expression in prostate cancer cells led to a marked decrease in cell proliferation and migration (Gueron et al., 2009). Collectively, these studies provide support for the beneficial effect of HO-1 in certain type of cancers.

CHAPTER 5

ACTIVATED NEUTROPHILS INDUCE THE RELEASE OF SOLUBLE ENDOGLIN FROM ENDOTHELIAL CELLS

5.1 INTRODUCTION

Endoglin is a 180 kDa integral membrane bound glycoprotein, expressed constitutively on the endothelial cell surface, which served as a co-receptor for transforming growth factor-beta 1 (TGF-β1) and TGF-β3, in the presence of TGF-β type II receptor (Duff et al., 2003, Bernabeu et al., 2009). Its soluble form, soluble endoglin (sEng), which is generated from the proteolytic cleavage of the extracellular domain of endoglin (Gougos and Letarte, 1990), is elevated in various disease conditions, including cancer (Li et al., 2000, Fonsatti et al., 2003), systemic sclerosis (Wipff et al., 2008), malaria (Dietmann et al., 2009), Alzheimer's disease (Juraskova et al., 2010), type II diabetes (Blazquez-Medela et al., 2010), atherosclerosis (Blann et al., 1996) and in obstructive sleep apnea and hypertension (Mohsenin and Urbano, 2011). The role of sEng has been most extensively studied in preeclampsia, a pregnancy and human specific hypertensive disorder. In pregnant women, circulating sEng is elevated many weeks prior to the clinical onset of preeclampsia (Levine et al., 2006, Rana et al., 2007, Romero et al., 2008, Erez et al., 2008, Baumann et al., 2008, Lim et al., 2009, Foidart et al., 2010). Soluble Endoglin functions to limit the activity of TGF-β signalling and endothelial nitric oxide synthase (eNOS) (Jerkic et al., 2004, Toporsian et al., 2005), hence inducing endothelial dysfunction. Together soluble Fms-like tyrosine kinase receptor-1 (sFlt-1), another anti-angiogenic factor, sEng is known to induce preeclampsia-like symptoms in pregnant rats (Venkatesha et al., 2006).

Preeclampsia has been previously described as an excessive maternal inflammatory response to pregnancy (Redman et al., 1999) and studies have shown that neutrophils are activated in preeclamptic patients (Greer et al., 1991, Lee et al., 2003, Sabatier et al., 2000, Tsukimori et al., 2005, Aly et al., 2004). Indeed, neutrophils are the archetypal inflammatory leukocytes and during inflammation, they are the first type of leukocytes to leave the blood and migrate to the injurious site to defend the host in response to chemoattactants, such as IL-8 (Savill, 1993). They induce "endothelial cell activation" by releasing cytokines, such as TNF-α or IFN-ϒ, which in turn upregulate adhesion molecules on endothelial cells, hence amplifying the leukocyte-endothelial cell interaction and the inflammatory process (Lush and Kvietys, 2000, Ley and Reutershan, 2006). Previous studies have shown that pro-inflammatory cytokines such as interferon-γ (IFN-ϒ) or TNF-α (Cudmore et al., 2007) stimulate sEng release while, the stress-responsive enzyme heme oxygenase-1 (HO-1), inhibits its release (Zhou et al., 2010). We hypothesized that, since sEng is formed from the proteolytic cleavage of the extracellular domain of endoglin and activated neutrophils release large quantities of proteinases, including elastase, cathepsin, and metalloproteinases (MMP), which are able to cleave cell surface receptors (Tosi et al., 1990, Champagne et al., 1998), activated neutrophils may contribute to the shedding sEng from endothelial cells. Furthermore, to support the rationale of this study, many studies have proposed that neutrophil activation in preeclampsia may contribute to the pathogenesis of the disorder (Greer et al., 1991, Lee et al., 2003, Sabatier et al., 2000, Tsukimori et al., 2005, Aly et al., 2004) (Belo et al., 2003, Gupta et al., 2007).

However, no study has yet demonstrated the potential link between neutrophil activation and the release of anti-angiogenic factors in preeclampsia. Hence, we tested this hypothesis by co-culturing activated neutrophils with human umbilical vein endothelial cells *in vitro* and assayed for the levels of sEng released in the conditioned media.

Our data demonstrate that the direct contact of LPS-mediated activated neutrophils to the endothelial cells induced the release of sEng from endothelial cells but not sFlt-1. We further demonstrate that the inhibition of MMP using a broad-spectrum non-specific inhibitor reduced both the basal and the activated neutrophil-induced release of sEng from endothelial cells. However, the specific inhibition of MMP-2 and MMP-9 did not affect the release of sEng in co-culture experiments. Hence, it is likely that the membrane bound MMP could mediate the release of sEng from endothelial cells.

5.2 RESULTS

5.2.1 Activated neutrophils increase sEng release from endothelial cells

Neutrophils can be activated by Toll-like receptor (TLR) agonists (Kutsuna et al., 2004, Suzuki et al., 1999, Yuo et al., 1989), which are important mediators of the innate immune system. TLR4 is a receptor for Gram-negative bacteria, lipopolysaccharide (LPS) and some viruses. The concentration of LPS in plasma or blood of patients with sepsis is about 200 ng/ml (Ngo et al., 2009). Previous studies have shown that the level of neutrophil activation in preeclampsia is almost similar to the level of neutrophil activation in sepsis (Sacks et al., 1998). For our in vitro studies, we opted to use a maximum concentration of 100 ng/ml of LPS, which is more appropriate to mimick inflammation in cultured endothelial cells. Hence, to determine whether activated neutrophils induce sEng release from endothelial cells, neutrophils were co-cultured with confluent human umbilical vein endothelial cells (HUVEC) in the presence of the TLR4 ligand, LPS (100 ng/ml). Neutrophils were first stimulated with LPS for 10 minutes before being added to HUVEC for 24 hours. The level of sEng was assayed by enzyme linked immunosorbent assay (ELISA), which showed that sEng release from HUVEC was significantly increased by about 3-fold in the presence of LPS-stimulated neutrophils ($p < 0.05$) compared to LPS-treated HUVEC (Figure 5.1A). Although, previous studies have shown that LPS activates endothelial cells and upregulates adhesion molecules (Zhang et al., 2011), in our study, LPS did not stimulate the release of sEng from HUVEC, indicating that LPS-

induced endothelial cell activation *per se* is not responsible for the cleavage of endoglin from endothelial cells. In contrast, when HUVEC were co-cultured with quiescent neutrophils there was an increase in sEng release, which did not reach statistical significant at 95% confidence interval (Figure 5.1A). A minimum concentration of 0.01 ng/ml of LPS on neutrophils was sufficient to elicit a statistically significant release of sEng from HUVEC and the release of sEng was directly proportional to the increase in concentration of LPS used (Figure 5.1 B). Overall, these results suggest that LPS-mediate neutrophil activation plays a role in the shedding of sEng from endothelial cells.

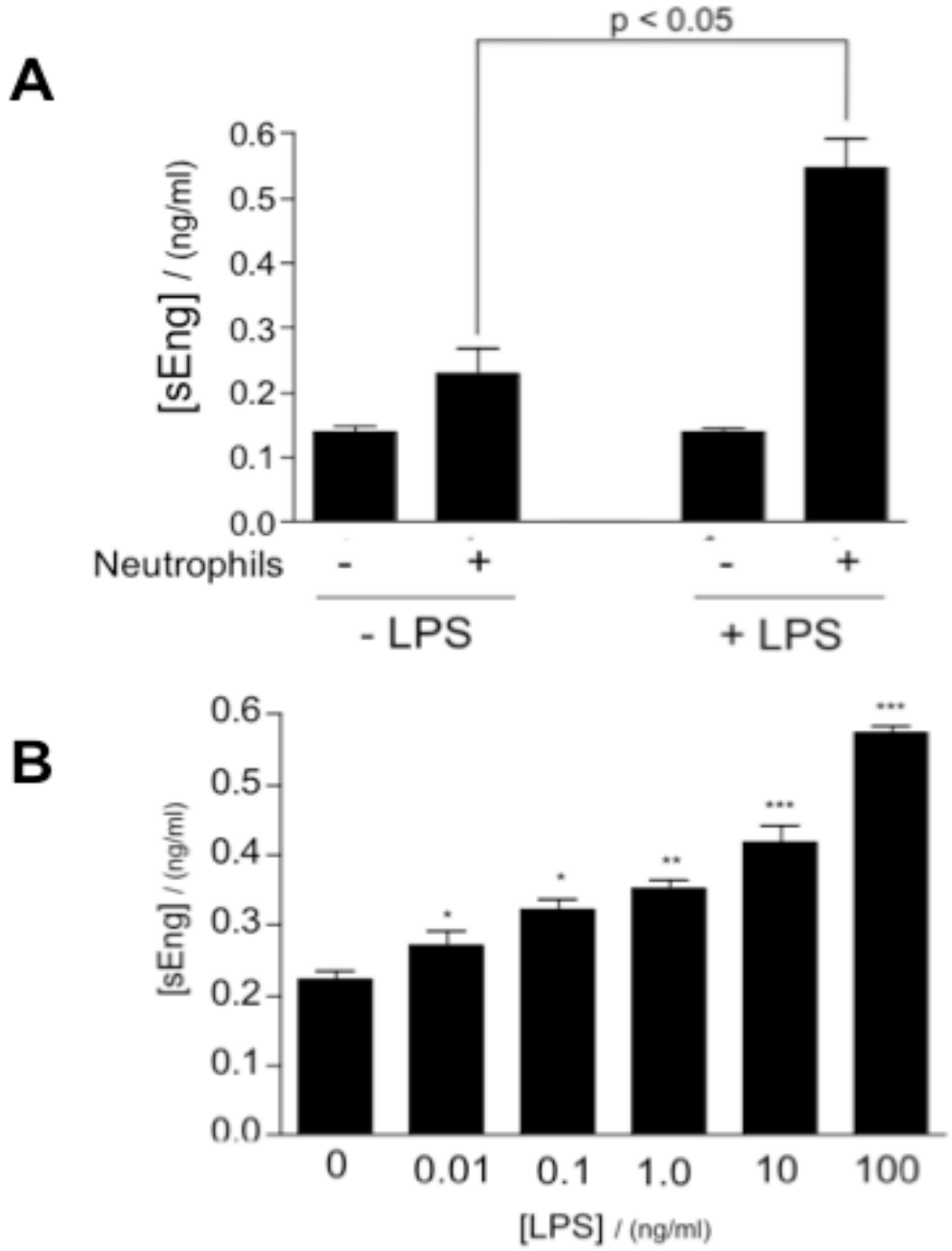

Figure 5.1. **LPS-activated neutrophils increase the release of sEng from endothelial cells.** (A) HUVEC were co-cultured with or without neutrophils for 24 hours in the presence or absence of LPS (100 ng/ml). LPS-stimulated neutrophils co-cultured with HUVECs causes a significant release of sEng of HUVEC compared to quiescent neutrophils ($p<0.05$). (B) Neutrophils were pre-treated with different concentration of LPS for 10 mins and added to HUVEC and co-cultured for 24 hours. Increasing concentration of LPS induces a dose-dependent release of sEng in the neutrophils/HUVECs co-culture. Data are mean ± SEM of 4 independent experiments performed in duplicate.

5.2.2 Activated neutrophils fails to induce sFlt-1 release from HUVEC

Soluble Fms-like tyrosine kinase receptor-1 (sFlt-1) binds to and antagonizes vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), thereby lowering circulating levels of unbound VEGF and PlGF. This altered balance causes generalized endothelial dysfunction resulting in multi-organ disease (Luttun et al., 2004). In pregnant rats, sFlt-1 treatment induces preeclampsia-like symptoms including hypertension, proteinuria, and glomerular endotheliosis (Maynard et al., 2003). Previous studies have reported that complement activation arises in inflammatory conditions and that the products of the complement cascade trigger the release of sFlt-1 from monocytes (Girardi et al., 2006). Since no studies have demonstrated the effect of neutrophil activation on the release of sFlt-1, we investigated whether LPS-induced activation of neutrophils affected the release of sFlt-1 when co-cultured with HUVEC. Our results showed that activated neutrophils do not induce the release of sFlt-1 from endothelial cells or from neutrophils (Figure 5.2).

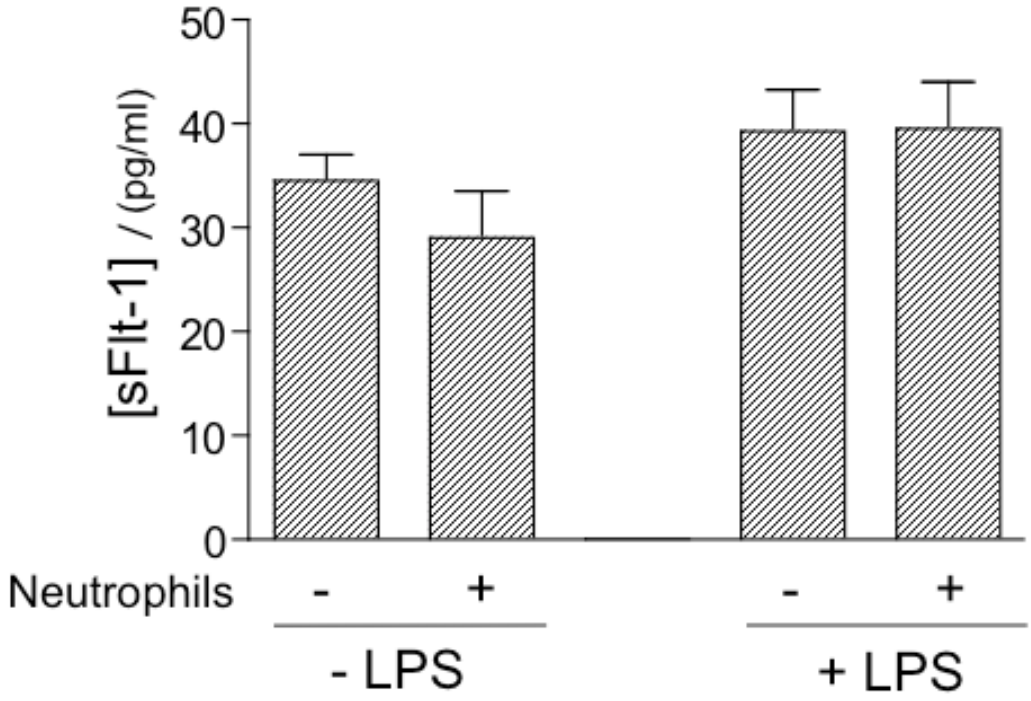

Figure 5.2. **LPS-activated neutrophils have no effect on the release of sFlt-1 from endothelial cells.** HUVECs were co-cultured with or without neutrophils for 24 hours in the presence and absence of LPS (100 ng/ml). The level of sFlt-1 in the supernatant was measured by ELISA. The release of sFLt-1 was not affected by the presence of either quiescent or LPS-activated neutrophils. Data are mean ± SEM of 4 independent experiments performed in duplicate.

5.2.3 Over-expression of HO-1 does not inhibit neutrophil-induced sEng release

Many studies have shown that HO-1 and its products can inhibit neutrophil recruitment and neutrophil-endothelial cell interactions in inflammatory conditions by downregulating selectins and cell adhesion molecules (Freitas et al., 2006, Soares et al., 2004, Otterbein et al., 2003). A recent study demonstrated that the downregulation of ICAM-1 expression by HO-1 and its products could prevent neutrophil recruitment (Dal-Secco et al., 2010). In addition, adenoviral and pharmacological over-expression of HO-1 in HUVEC also inhibits cytokine-induced sEng release from endothelial cells (Cudmore et al., 2007), (Zhou et al., 2010).

To determine whether the inhibitory effect of HO-1 on sEng release was maintained in the presence of activated neutrophils, HO-1 was over-expressed in HUVEC using an adenovirus prior to the addition of activated neutrophils. Our results confirmed that over-expression of HO-1 indeed inhibits the release of sEng from HUVEC in the absence of neutrophils (Figure 5.3A). However, when quiescent or activated neutrophils were co-cultured with HUVEC, HO-1 failed to block the release of sEng from endothelial cells (Figure 5.3B). Neutrophils bind to ICAM-1 via its β_2-integrin receptor (Dustin and Springer, 1988). Hence, this could indicate that the interaction of neutrophils β_2-integrin with ICAM-1 does not participate in the release of sEng from endothelial cells. In addition, since HO-1 is associated

with anti-oxidative and anti-inflammatory properties (Loboda et al., 2008), it is also possible that the production of reactive oxygen from activated neutrophils is not responsible for the release of sEng from HUVEC.

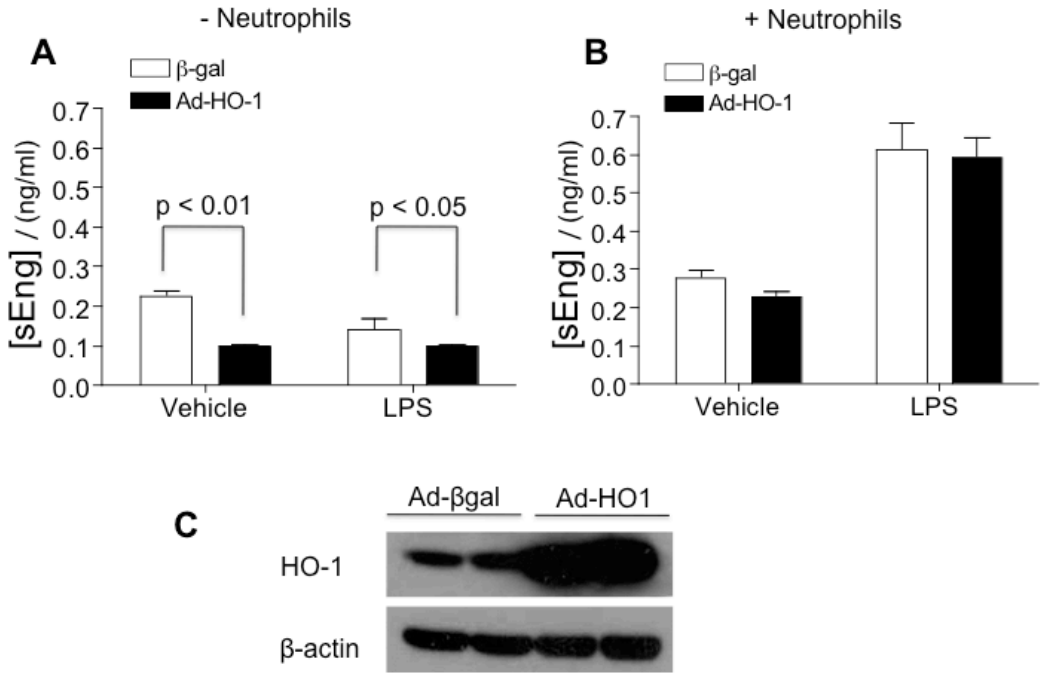

Figure 5.3. **HO-1 overexpression fails to suppress LPS-stimulated neutrophils from inducing endothelial sEng release.** HUVECs were infected with 50 infective units/cell of AdHO-1. (A) HO-1 negatively regulated the level of sEng release in HUVECs ($p< 0.01$) (B) Neutrophils and HUVEC cells coculture were treated with or without TLR-4 ligand (LPS, 100 ng/ml) for 24 hours. In the presence of activated neutrophil, HO-1 did not inhibit the release of sEng from HUVECs. sEng level in culture medium was measured by ELISA. Data are mean ± SEM of 3 experiments performed in duplicate. (C) Representative blots to confirm over-expression of HO-1 in HUVECs.

5.2.4 Neutrophil induced sEng release from endothelial cells is mediated by metalloproteinases

The release of sEng from the endothelial cells is dependent on the direct contact of neutrophils on the endothelial cell surface and not via the release of soluble factors from LPS-activated neutrophils (data not shown). Hence, we postulated that the adherence of neutrophils to the endothelium and the degradation of the basal lamina followed by the subsequent migration of neutrophils across the endothelial layer could participate in the generation of sEng. The degradation of the basal lamina and extracellular matrix of HUVEC require the concerted action of various proteolytic enzymes including serine proteases and MMP (Khandoga et al., 2006). However, pre-incubation with the serine protease inhibitor, phenylmethylsulfonylfluoride (PMSF) did not affect sEng release (data not shown). In contrast, pre-incubation with the broad-spectrum MMP inhibitor GM6001 (also known as Ilomastat or Galardin) reduced the basal release of sEng from HUVEC by 50%. In the presence of activated neutrophils, GM6001 significantly reduces sEng release by 1.5-fold ($p < 0.002$) (Figure 5.4). These results indicate that MMP are likely to be involved in regulating neutrophil-induced sEng cleavage from endothelial cells.

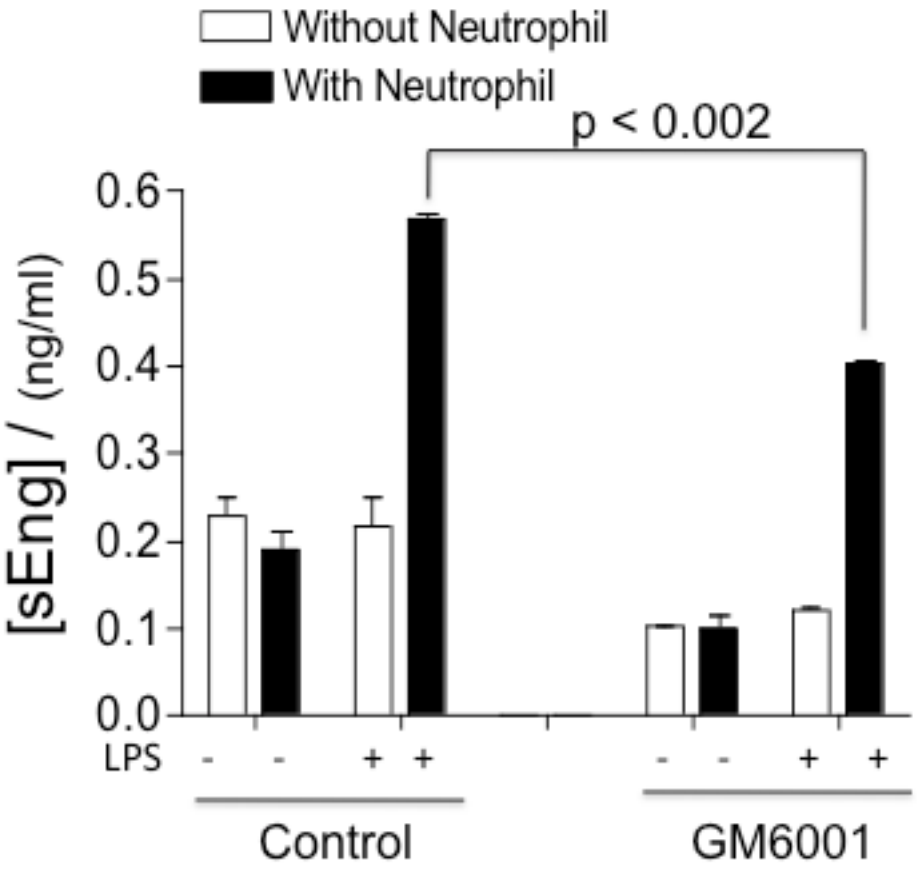

Figure 5.4. **GM6001 partially inhibits activated neutrophil-induced sEng release from HUVEC.** HUVEC co-cultured with LPS-stimulated neutrophils were treated with 25 mM of the MMP inhibitor (GM6001) for 24 hours. Inhibition of MMP significantly reduced the release of sEng from HUVECs ($p < 0.002$). Data are mean ± SEM of 3 experiments performed in duplicate.

5.2.5 MMP-2/9 inhibitor does not affect shedding of sEng

Soluble matrix metalloproteinases-2 and -9 (MMP-2/9) are of the gelatinase class of MMP. Degranulation of activated neutrophils releases large amount of the tertiary granules, MMP-9, which is responsible for the conversion of inactive proMMP-2 produced by HUVEC to the active form MMP-2 (Schwartz et al., 1998). Previous studies have shown that MMP-9 is responsible for the shedding of TNF-α from the membrane (Gearing et al., 1995). Hence, we used a specific MMP-2/9 inhibitor to determine the potential role of these enzymes in regulating the release of sEng from HUVEC/neutrophils co-cultures. Our analysis revealed that the inhibition of MMP-2/9 produced no effect on the release of sEng (Figure 5.5), indicating that gelatinases produced from the degranulation of neutrophils are not involved in the shedding of sEng from HUVEC.

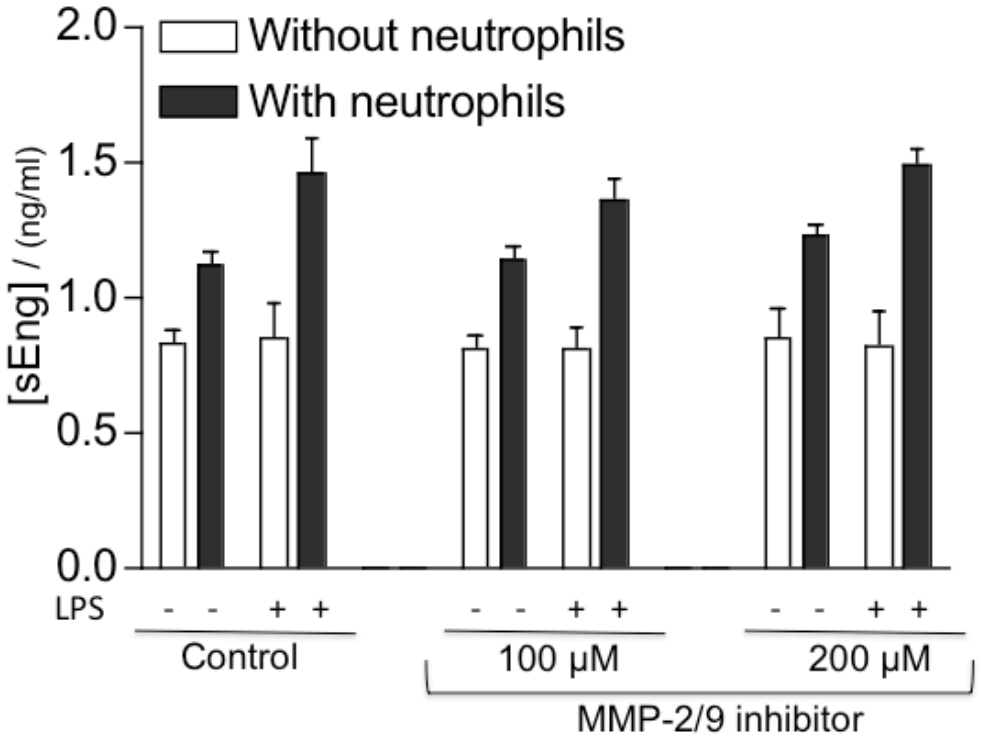

Figure 5.5. **MMP-2/9 does not affect the release of sEng from endothelial cells.** HUVEC co-cultured with LPS-stimulated neutrophils were treated with 100 and 200 μM of the MMP-2/9 inhibitor for 24 hours. Inhibition of MMP-2/9 did not affect the release of sEng from HUVEC. Data are mean ± SEM of 3 experiments performed in duplicate.

5.2.6 Expression of MMP-14 and Eng in the placenta

A recent study identified MMP-14 (or MT1-MMP) as the most abundant membrane bound MMP in HUVEC, which was responsible for the shedding of sEng in endothelial cells and in cancer cells. The same study showed that the co-expression of Endoglin and membrane-bound MMP-14 on the same cell led to a strong increase in soluble Endoglin release from endothelial cells

(Hawinkels et al., 2010). The surface-anchored membrane type 1 matrix metalloproteinase (MT1-MMP or MMP-14) can degrade a wide range of extracellular matrix components that includes collagens, laminins, fibronectin and the structural proteoglycan aggrecan (Polette and Birembaut, 1998). Since the expression of Endoglin has been shown to be elevated in the human placental syncytiotrophoblast and circulating sEng is elevated in preeclampsia (Venkatesha et al., 2006), we investigated the expression of MMP-14 in relation to Endoglin in human placenta tissues. Placental sections were incubated with the catalytic domain of anti-MMP-14 and the extracellular domain of anti-Eng, stained with Vector NovaRed and counterstained with Hematoxylin. Positive immunostaining for MMP-14 and endoglin was detected in the bilayer of the syncytiotrophoblast and cytotrophoblast of normal control samples (Figure 5.6 C, E). Interestingly, preeclamptic placenta section showed strong endoglin staining in the bilayer of syncytiotrophoblast that was accompanied by low MMP-14 expression in the same location. We speculated that the reduction in MMP-14 might be due to active MMP-14 being shed during the process of cleaving endoglin to produce sEng. However, in order to confirm this, the activity of MMP-14 in the circulation and placental tissues needs to be assessed.

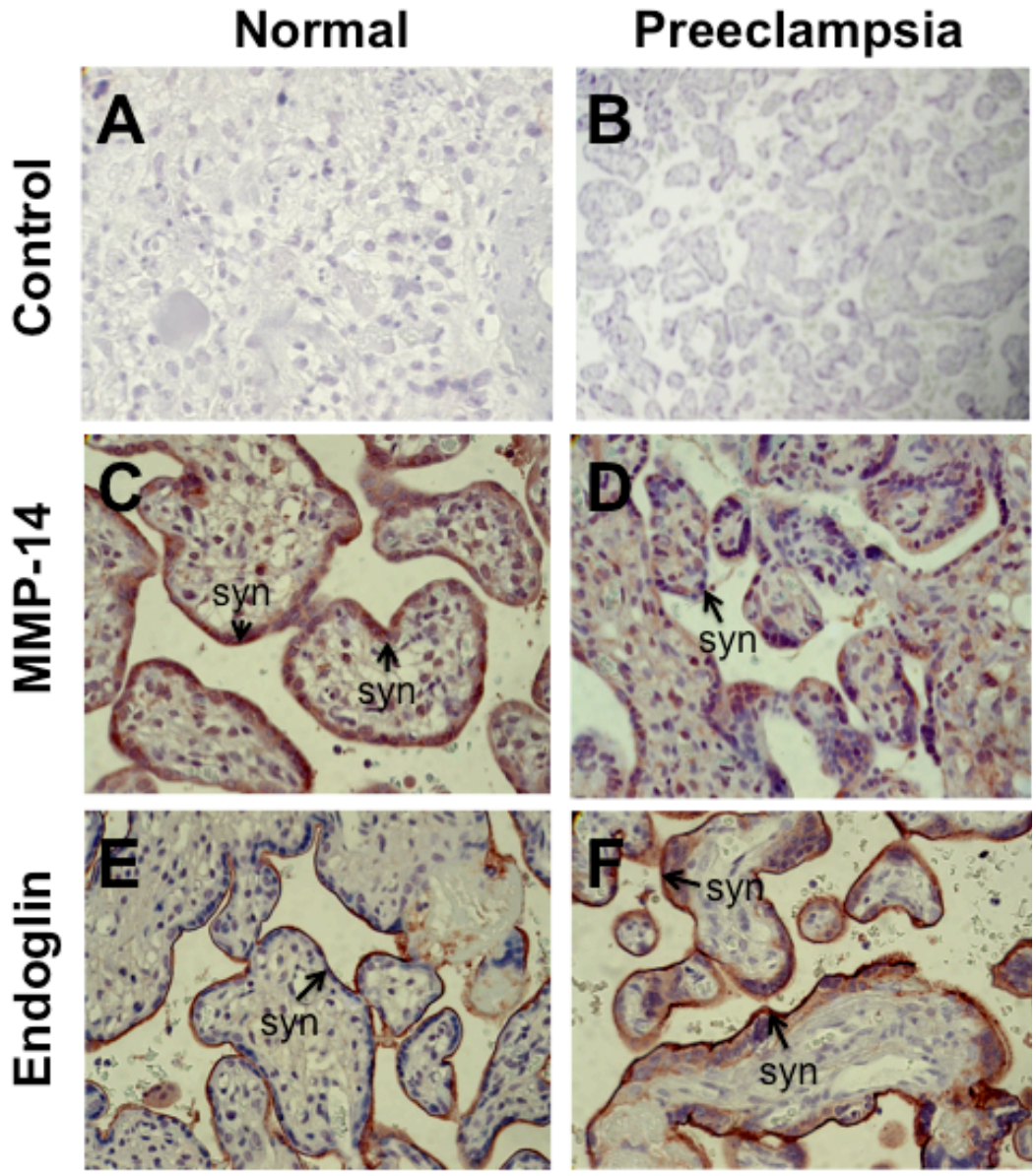

Figure 5.6. **Immunolocalisation of MMP-14 and Eng in the placenta.** (A) and (B) show the negative control from Endoglin and MMP-14 respectively. (C) Intense staining for MMP-14 and (E) moderate staining for endoglin was detected in the bilayer of syncytiotrophoblast (syn) of normal placenta. (D) Weak staining for MMP-14 and (F) intense staining of endoglin in preeclamptic compared to normal were observed in the syncytiotrophoblast. Original magnification: x40.

5.3 DISCUSSION

In the present study, we show that sEng release from endothelial cells was increased as a result of direct contact of LPS-mediated activated neutrophils on endothelial cells, suggesting that activated neutrophils may contribute to systemic endothelial dysfunction by stimulating the release of this soluble anti-angiogenic factor. We further showed that the release of sEng could be partially inhibited with a broad-spectrum inhibitor, but not with a specific inhibitor of MMP-2 and MMP-9. We also show that the over-expression of HO-1 failed to inhibit the release of sEng from endothelial cells in the presence of activated neutrophils.

Our *in vitro* study is the first to demonstrate that LPS-mediated activated neutrophils induce the release of sEng from endothelial cells. LPS activates neutrophils by binding to its receptor TLR-4 on the neutrophils. It regulates important neutrophil functions, including adhesion, generation of reactive oxygen species and activation of the nuclear factor–KB pathway (Hayashi et al., 2003). In atherosclerosis and preeclampsia, where the level of sEng is known to be elevated (Blann et al., 1996, Venkatesha et al., 2006), the expression of TLR-4 on neutrophils is also increased in these two disorders (Satoh et al., 2008, Xie et al., 2010). LPS, produced from gram-negative bacteria, is unlikely to be elevated in non-infectious inflammatory conditions. However, endogenous agonists of TLR-4, such as hyaluronic acid or high mobility group box 1 (HMGB1), may mediate the release of sEng. Hence it would be of interest to determine the levels of these endogenous agonists and their relationship with sEng in the clinical settings in order to validate

our *in vitro* findings.

Although sEng is generated from the cleavage of the extracelluar domain of the membrane bound Endoglin, the potential role of MMP, as a possible mechanism of sEng release has not been extensively studies. MMP are a family of zinc-dependent proteinases that digest specific extracellular matrix components in many physiological and pathological processes, including inflammation, bacterial infection, wound healing, and cancer cell invasiveness (Egeblad and Werb, 2002). They are released from the cells as inactive pro-enzymes (zymogens) and are activated extracellularly by other proteinases (Goetzl et al., 1996, Vaday and Lider, 2000). Activated endothelial cells express a variety of MMP, while activated neutrophils can release large quantities of soluble metalloproteinases upon degranulation (Epstein and Weiss 1989). We showed in the present study that MMP are partially responsible for the release of sEng from endothelial cells mediated by LPS-activated neutrophils. However, the secreted form of MMP, MMP-2 and -9, did not affect the release of sEng from endothelial cells. Hence this led us to think that membrane-bound MMP could instead play a role in the release of sEng. Indeed, a recent study reported that the membrane bound MT1-MMP, most commonly known as the MMP-14, is the most abundant metalloproteinases in endothelial cells (Hawinkels et al., 2010). Hawinkels and colleagues demonstrated that over-expression of MMP-14 caused sEng shedding from the endothelial cell surface and only the membrane bound, but not circulating MMP-14, was able to mediate this process (Hawinkels et al., 2010). Previous studies have reported that the expression of Endoglin is

elevated in the syncytiotrophoblast of the preeclamptic placenta (Venkatesha et al., 2006). In our study, we showed for the first time that in the placenta of preeclamptic patients, a strong expression of Endoglin is accompanied by a low expression of MMP-14 in the syncytiotrophoblast layer. Accordingly, strong MMP-14 expression in the syncytiotrophoblast of control placentas was accompanied with low endoglin levels at the same location. These results are in accordance with the study by Hawinkels and colleagues who observed in cancer cells that strong MMP-14 was accompanied by lower or absence of endoglin staining and vice versa (Hawinkels et al., 2010). Hence the role of MMP-14 in relation to sEng should be further explored in preeclampsia.

It was interesting to see in this study that in the presence of activated neutrophils, the overexpression of HO-1 did not inhibit the release of sEng from endothelial cells. Earlier studies had demonstrated that adenoviral or pharmacological induction of HO-1 inhibits the release of sEng in response to cytokines (Cudmore et al., 2007, Zhou et al., 2010). Our results, however, suggest that the generation of endothelial-derived sEng in response to activated neutrophil is independent of mechanisms, which are responsible for suppressing sEng release by HO-1. Furthermore, the rolling of neutrophils on the endothelium is mediated by mainly E-selectins interacting with their respective carbohydrate ligands on neutrophils. Adhesion and transmigration of neutrophils to the endothelial cells are mediated by β_2-integrins CD11a/CD18, CD11b/CD18, CD11c/CD18 interacting with various ligands, including the endothelial cells intercellular adhesion

molecules (ICAM-1) (Smith, 1993). LPS is a powerful activator of both neutrophils and endothelial cells. It induces the upregulation of cell adhesion molecules, such as ICAM-1 and E-selectins in order to facilitate the binding and transmigration of neutrophils to and across the endothelium (Lorenzon et al., 1998, Bannerman and Goldblum, 1999). Numerous studies have shown that the HO-1 pathway can inhibit leukocyte-endothelial interactions (Freitas et al., 2006, Otterbein et al., 2003, Soares et al., 2004, Vicente et al., 2003). Furthermore a recent study demonstrated that the product of HO-1, CO, regulates neutrophil migration due a reduction in the neutrophil/endothelium interaction by downregulation of LPS-induced ICAM-1/CD54 expression on endothelial cells. The same study showed that HO-1 and its products did not affect the expression of β_2-integrin, the counter-receptor for ICAM-1 (Dal-Secco et al., 2010). Another study showed that biliverdin (another product of HO-1) downregulates the expression of selectins in endothelial cells (Vachharajani et al., 2000). Interestingly, in our study, we showed that over-expression of HO-1 did not inhibit the release of sEng in the presence of activated neutrophils, indicating that sEng release is not mediated via the selectin or β_2-integrin/ICAM-1 signalling pathway.

In summary, these results suggest that in situations where large number of neutrophils are activated by circulating 'danger' signals and subsequently accumulate in the vasculature, sEng may be cleaved from endothelium and released into circulation. Neutralization of TGF-β functions by sEng leads to endothelial dysfunction characterized by impaired endothelium-mediated vasodilatation and elevated expression of surface adhesion molecules, which

results in increased leukocyte adhesion (Walshe et al., 2009), hence promoting inflammatory processes. Although our study shows that LPS-mediated activated neutrophils induce the release of sEng from cultured endothelial cells, these observations need to be validated in the clinical settings of either infectious disorder such as sepsis or in "non-infectious" inflammatory conditions such as atheresclerosis and preeclampsia.

CHAPTER 6

INCREASE IN CIRCULATING ANTI-ANGIOGENIC FACTORS IS INDEPENDENT OF NEUTROPHIL ACTIVATION IN PREECLAMPSIA

6.1 INTRODUCTION

Preeclampsia is a human-specific hypertensive disorder of pregnancy that affects about 5% of all pregnant women (WHO, 2005). It is characterized by the *de novo* development of hypertension (systolic blood pressure ≥ 140 mmHg or diastolic pressure ≥ 90 mmHg) and proteinuria (≥300 mg/24hr) after 20 weeks' of gestation. Extreme variant of preeclampsia may include hemolysis, elevated liver enzymes and low platelets count (HELLP) syndrome. The placenta plays a key role in the genesis of this disorder as its removal at the time of delivery results in rapid resolution of the clinical symptoms (Pijnenborg et al., 1998). Early-onset preeclampsia occurs before 34 weeks of gestation and is associated with placental damage and usually results in premature delivery and fetal growth restricted fetus. In contrast, late-onset preeclampsia happens after 34 weeks of gestation and frequently does not display the classical placental changes normally associated to this disorder (von Dadelszen et al., 2003, Sebire et al., 2005). In the context of this study, all the preeclamptic samples were of early-onset origin.

Although the exact etiology of the disorder is unknown, the loss of vascular endothelial growth factor (VEGF) activity due to increase in soluble Fms-like tyrosine-receptor-1 (sFlt-1) (Maynard et al., 2003, Bergmann et al., 2010, Costantine et al., 2010) and the loss of transforming growth factor-β (TGF-β) activity as a consequence of elevated soluble Endoglin (sEng) (Venkatesha et al., 2006) appear increasingly to be responsible for the clinical signs of early-

onset preeclampsia. A number of studies have also claimed that, as in cardiovascular disease, oxidative stress-induced endothelial activation (Roberts and Redman, 1993, Roberts et al., 1989), endothelial dysfunction due to loss of nitric oxide bioavailability (Chambers et al., 2001, Kinzler et al., 2004) and excessive inflammation (Redman et al., 1999) may cause early-onset preeclampsia.

Indeed, Redman and colleagues showed that the inflammatory changes in peripheral blood leukocytes associated with normal pregnancy and preeclampsia were similar to sepsis (Sacks et al., 1998). They postulated that preeclampsia is the consequence of an excessive inflammatory response to pregnancy and that the intravascular inflammatory response is not an epiphenomenon but is in fact the cause of the clinical syndrome of preeclampsia (Redman et al., 1999). This theory gained support as earlier studies had demonstrated that neutrophil activation is confined to the maternal circulation in pregnancy-induced hypertension, where it may contribute to vascular damage (Greer et al., 1991). Indeed, mild activation of neutrophils was noted during normal pregnancy in response to syncytiotrophoblasts' apoptotic debris that enter the maternal circulation (Sargent et al., 2003) and further evidence of neutrophil activation in preeclampsia included the production of reactive oxygen species (Sacks et al., 1998, Morris et al., 1998, Tsukimori et al., 2008) and the release of neutrophils granules into the circulation (Halim et al., 1996, Rebelo et al., 1996). For example, human α-defensins, a specific marker of neutrophil activation released in the circulation upon the degranulation of neutrophils,

and calprotectin (also called S100A8/S100A9) that is predominantly found in neutrophils (Kostakis et al., 2010), are both increased in preeclampsia (Holthe et al., 2005, Prieto et al., 1997). Likewise, interleukin-6 (IL-6), a pro-inflammatory cytokine secreted by both activated leukocytes and endothelial cells, is elevated in severe preeclampsia (Clark et al., 1998b, Tosun et al., 2010, Szarka et al., 2010). Thus, the idea that inflammation *per se* causes preeclampsia has persisted without solid causational evidence (Hill, 1965).

In contrast, the argument first articulated over a decade ago that preeclampsia may arise due to loss of VEGF activity by the possible elevation of sFlt-1 (Ahmed, 1997) gained momentum when biological evidence showed that adenoviral over-expression of sFlt-1 and sEng to pregnant rats mimicked the full spectrum of the clinical manifestations of preeclampsia (Maynard et al., 2003, Venkatesha et al., 2006). These anti-angiogenic factors compromise endothelial vascular function (Maynard et al., 2003, Venkatesha et al., 2006) and play a pivotal role in the development of the maternal syndrome of preeclampsia (Ahmed, 2011a). Furthermore, the early increase in the maternal circulating levels of sFlt-1 and sEng, which precedes the onset of the disorder, (Levine et al., 2004, Levine et al., 2006) fulfils the Hill's temporal relationship criterion of causation (Hill, 1965).

Numerous studies support the theory that activated neutrophils are likely to contribute to the endothelial damage associated with preeclampsia (Segel et al., 2011), whereas activated endothelium can also release cytokines or

chemokines that can activate neutrophils (Ward and Varani, 1993). Hence, it is unclear whether neutrophil activation is the cause or the consequence of endothelial damage (Clark et al., 1998b) or is associated with a rise in circulating anti-angiogenic factors. No case-control gestational-matched studies have investigated the inter-relationship between neutrophil activation and anti-angiogenic factors in women with severe preeclampsia.

In the previous chapter (Chapter 5), we had shown that activated neutrophils cause the release of sEng from the endothelial cells. Here we investigated whether our in vitro findings would correlate in the clinical setting of preeclampsia. Hence, the aim of this study was to determine whether a direct relationship could be demonstrated between the increase in the anti-angiogenic factors and increases in α-defensins, calprotectin and IL-6 to assess the clinical significance of increased inflammatory status during preeclampsia. In addition, we investigated whether plasma level of fibronectin, a marker of endothelial cell injury, is elevated in preeclampsia as previously demonstrated (Halligan et al., 1994, Sen et al., 1994, Friedman et al., 1995) and whether endothelial cell injury correlated with neutrophils activation/inflammation and release of sEng.

Our findings show a direct correlation between increase in α-defensins and calprotectin, supporting our rational in using these markers as a measure of neutrophil activation. However, although sEng, sFlt-1/PlGF ratio, fibronectin, α-defensins, calprotectin and IL-6 were elevated in preeclampsia,

there was no direct relationship between the increase in these factors and the markers of neutrophil activation. In addition, we found that the increase in sEng and sFlt-1/PlGF ratio bear a direct relationship with diastolic blood pressure in the severe preeclamptics and their matched controls cohort.

6.2 RESULTS

6.2.1 Patient selection

The clinical characteristics of the pregnant women included in our study are shown in Table 6.1. The study population consisted of 27 women with normal pregnancy, 45 women with severe preeclampsia without IUGR (intrauterine growth restriction) and 36 women with chronic hypertension. There was no statistically significant difference in gestational age at blood collection. Women with severe preeclampsia were of lower parity and had significantly higher body weights and blood pressure. All of the control subjects had negative urine dipstick protein reading, whereas the preeclamptic subjects were positive. Patients with chronic hypertension also had positive urine dipstick reading. Five of the patients with severe preeclampsia had evidence of HELLP (hemolysis, elevated liver enzymes and low platelets) syndrome. Control women delivered a healthy baby at term. As expected, severe preeclampsia women delivered at gestational ages significantly lower compared to their controls. The exclusion criteria in the three study groups were the presence of infectious disease or medical complications including autoimmune disorder, diabetes mellitus and inflammatory conditions.

Table 6.1. **Clinical characteristics of patient groups included in the study**

Characteristics	Pregnant control (n=27)	Severe preeclampsia (n=45)	Chronic hypertension (n=13)	P-value
Patient age at delivery (years)	29 (15-40)	27 (14-42)	36 (32-40)	0.0004 *
Weight (kg)	73.90 (62.3-102.7)	87.75 (56.40-149.5)	110 (81.8-147.7	0.0075 *
Parity	1 (0-10)	0 (0-6)	2 (0-5)	0.0042 *
Gestational age at collection (weeks)	29.75 (21.4-33.5)	30.5 (24.1-34.2)	32.3 (23.5-38.6)	ns
Gestational age at delivery (weeks)	39 (37.20-41)	30.5 (24.1-34.2)	38.1 (29.7-40)	<0.0001*
Systolic blood pressure > 20 weeks (mm Hg)	106 (90-140)	168 (130-220)	152 (100-220)	<0.0001*
Diastolic blood pressure > 20 weeks (mm Hg)	63 (50-76)	101 (88-130)	91 (70-120)	<0.0001 *
Dipstick proteinuria	-	3 (0-4)	2.55 (0.15-9.34)	-

Values shown as median and range (minimum and maximum values)
*P <0.05, Mann-Whitney test

6.2.2 Gestational pattern of serum sEng in uncomplicated pregnancy

We first evaluated the gestational patterns associated with the release of sEng in the following groups: non-pregnant, pregnancies in early gestation (10-16 weeks), mid-gestation (21-33 weeks) and at term (37-42 weeks). We confirmed, as previously reported (Levine et al., 2006), that the level of sEng in pregnancy was stable until 33 weeks of gestation and increased significantly to a median concentration of 8.455 ng/ml (95% CI, 4.648-22.65 ng/ml) at term until delivery ($p<0.001$) (Figure 6.1). In the non-pregnant group, the median concentration of serum sEng was 3.722 ng/ml (95% CI, 3.043 – 5.534 ng/ml). During pregnancy at early gestation, the level of sEng appears to increase slightly with a median concentration of 5.496 ng/ml (95% CI, 2.665 – 7.426 ng/ml) and remained approximately stable at mid-gestation (4.271 ng/ml; 95% CI, 2.762 – 12.01 ng/ml). All of these pregnant women were normotensive during pregnancy and delivered appropriately sized infants.

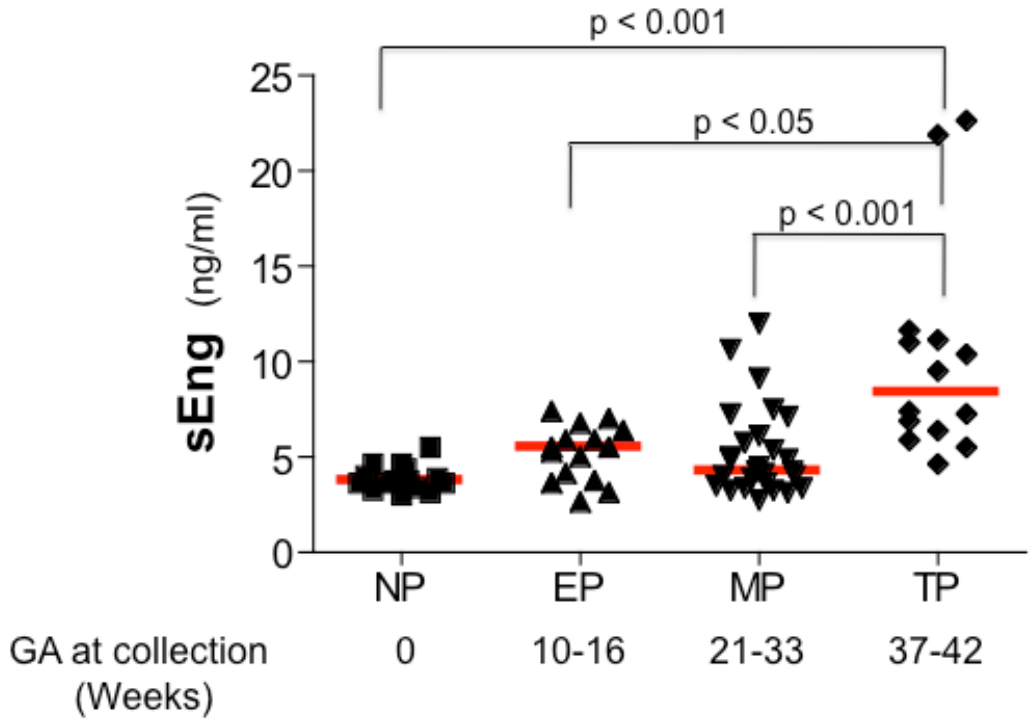

Figure 6.1. **Circulating levels of sEng throughout normal pregnancy.** Serum levels of sEng (nanograms/milliliter) in non-pregnant (NP), early gestation (EP), mid gestation (MP) and at term (TP) pregnancies. Horizontal bars represent median values.

6.2.3 Maternal serum level of sEng in pregnancies complicated by inflammatory conditions

To evaluate the relationship between inflammation and the production of sEng during pregnancy, we compared the level of sEng in pregnancies complicated by disease conditions known to cause inflammation. The median level of sEng in systemic inflammation, subclinical chorioamnionitis and clinical amnionitis were 4.44 ng/ml (95% CI, 0.0837-13.84 ng/ml), 5.274 ng/ml (95% CI, 3.24-26.07) and 4.894 ng/ml (95% CI, 4.108-5.026) respectively. The median level of sEng in each of these inflammatory conditions was low and similar to the level of sEng in the patients at mid-gestation (4.271 ng/ml; 95% CI, 2.762 -12.01 ng/ml) (Figure 6.2). Importantly, we also showed (see Insert in figure 6.2) that there was no significant difference in the median levels of sEng between pregnancy at mid-gestation (21-33 weeks) (4.271 ng/ml; 95% CI, 2.762 -12.01 ng/ml) and the idiopathic pre-term delivery group (7.07 ng/ml; 95% CI, 3.685-61.28 ng/ml). Hence, we decided that it was appropriate to use the non-pathological, the mid-gestation pregnant group, as the gestational age-matched controls for severe preeclampsia rather than the idiopathic pre-term group.

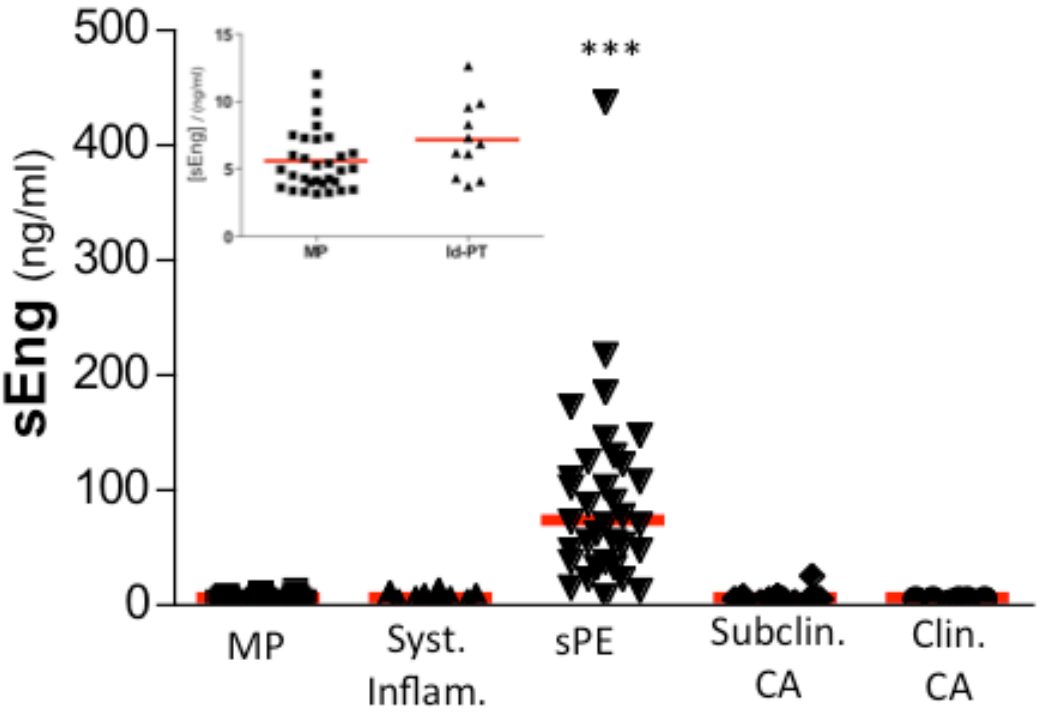

Figure 6.2. **Circulating level of sEng in inflammatory conditions during pregnancy.** Serum levels of sEng (nanograms/milliliter) in pregnancies complicated by systemic inflammation (Syst. Inflam), severe preeclampsia (sPE), subclinical chorioamnionitis (subclinical CA), clinical chorioamnionitis (clinical CA). The “insert” shows that the level of sEng does not vary significantly between mid-pregnancy (MP) and idiopathic preterm (Id-PT) pregnancy. Horizontal bars represent median values.

6.2.4 Maternal serum sEng is elevated in severe preeclampsia

In our case-control study, women, diagnosed with severe preeclampsia, had approximately a 20-fold increase in their median levels of serum sEng (70.10 ng/ml; 95% CI, 6.701-437.9 ng/ml) compared to gestational age-matched controls (4.271 ng/ml; 95% CI, 2.762 -12.01 ng/ml; $p < 0.001$) (Figure 6.3A). In contrast, women with chronic hypertension during pregnancy exhibited only a slight increase in serum sEng (7.413 ng/ml; 95% CI, 3.573-26.98) compared to their gestational age matched controls (4.271 ng/ml; 95% CI, 2.762 -12.01 ng/ml; $p < 0.05$) (Figure 6.3B). These observations suggest that the increase in sEng in our study appears to be more prominent to severe preeclampsia.

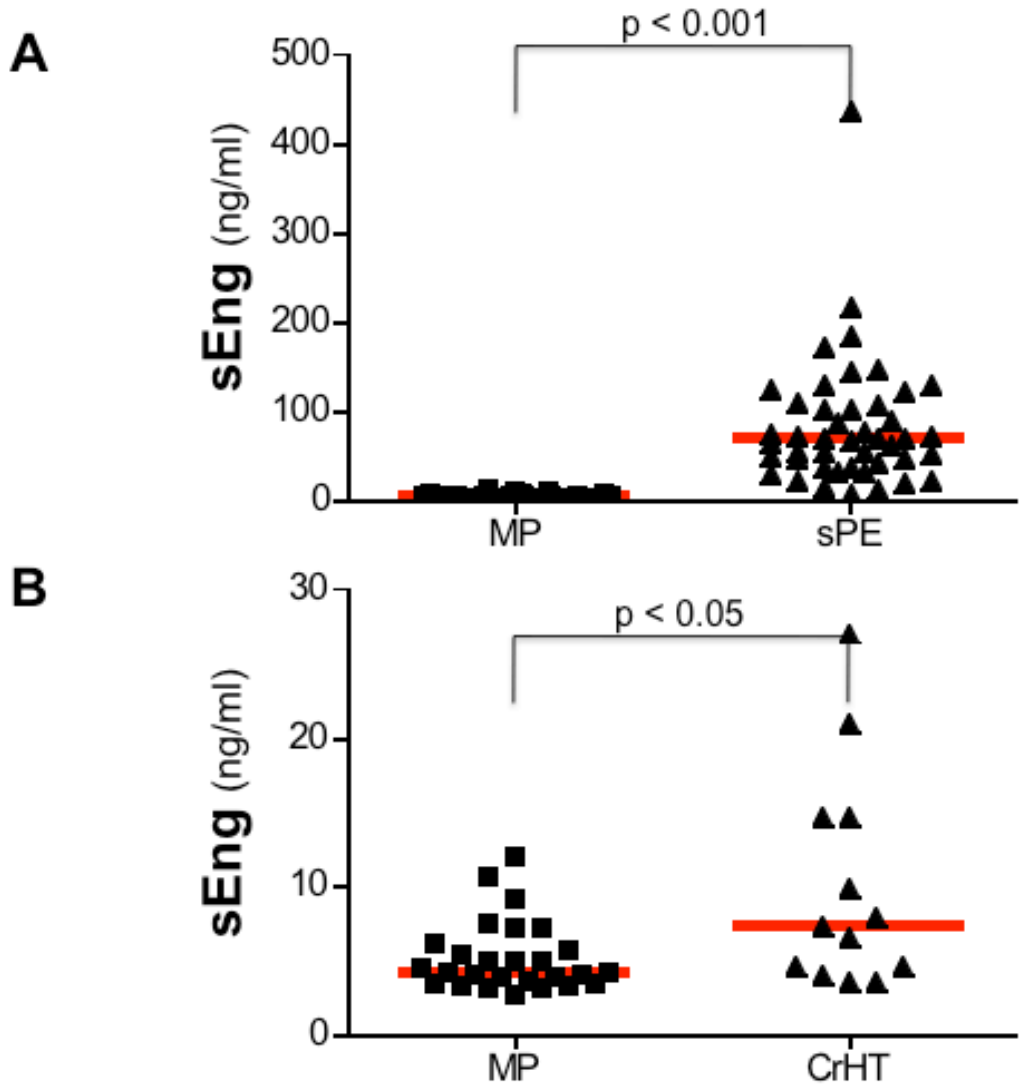

Figure 6.3. **Elevated level of circulating sEng in severe preeclampsia and chronic hypertension.** Serum levels of sEng (nanograms/milliliter) in (A) severe preeclamptics (sPE) compared to gestational age-matched controls (MP) (B) in pregnancies complicated by chronic hypertension (CrHT) compared to gestional age-matched controls (MP). Horizontal bars represent median values.

6.2.5 Plasma levels of markers of neutrophil activation are increased in severe preeclampsia

Human α-defensins, HNP 1-3, are unique to neutrophils and activation of neutrophils leads to their rapid release. Thus, only one cell type, neutrophils, may be the source of HNP 1-3 measured in plasma during infection and inflammation. In normal plasma, low levels of HNP 1-3 are present, ranging from undetectable levels to 50-1000 ng/ml (Zhang et al., 2002a). To determine the extent of neutrophil activation, we assayed the levels of circulating α-defensins in the plasma of pregnant women. In severe preeclampsia, the median plasma level of α-defensins was increased by a modest 1.5-fold (136.1 ng/ml; 95% CI, 118.4-4180 ng/ml) compared to the gestational age-matched normal pregnancy (122.9 ng/nl; 95% CI, 116-507 ng/ml; $p < 0.05$). Interestingly, there was no significant increase in the median level of α-defensins between the non-pregnant (117.7 ng/ml; 95% CI, 116.8-170 ng/ml), the early gestational age (117 ng/ml; 95% CI, 116-221 ng/ml), the mid gestational age (122.9 ng/ml; 95% CI, 116-507 ng/ml) and the gestational age-matched chronic hypertension (118 ng/ml; 95% CI, 115-1120 ng/ml) groups (Figure 6.4 A).

Calprotectin (MRP8/14) is also released from activated neutrophils and is an indication of the severity of inflammation. In normal plasma, the range of calprotectin is 0.5-3.5 μg/ml. In our study, we observed that the median plasma level of calprotectin was increased by approximately 2-fold in severe

preeclampsia (40.74 μg/ml; 95% CI, 3.149 – 503.6 μg/ml) compared to the mid-gestational age control groups (18.84 μg/ml; 95% CI, 4.718 – 108.5 μg/ml; $p < 0.05$) (Figure 6.4B). Together, these results suggest that neutrophil activation is significantly, but only slightly, elevated in severe preeclampsia compared to matched controls.

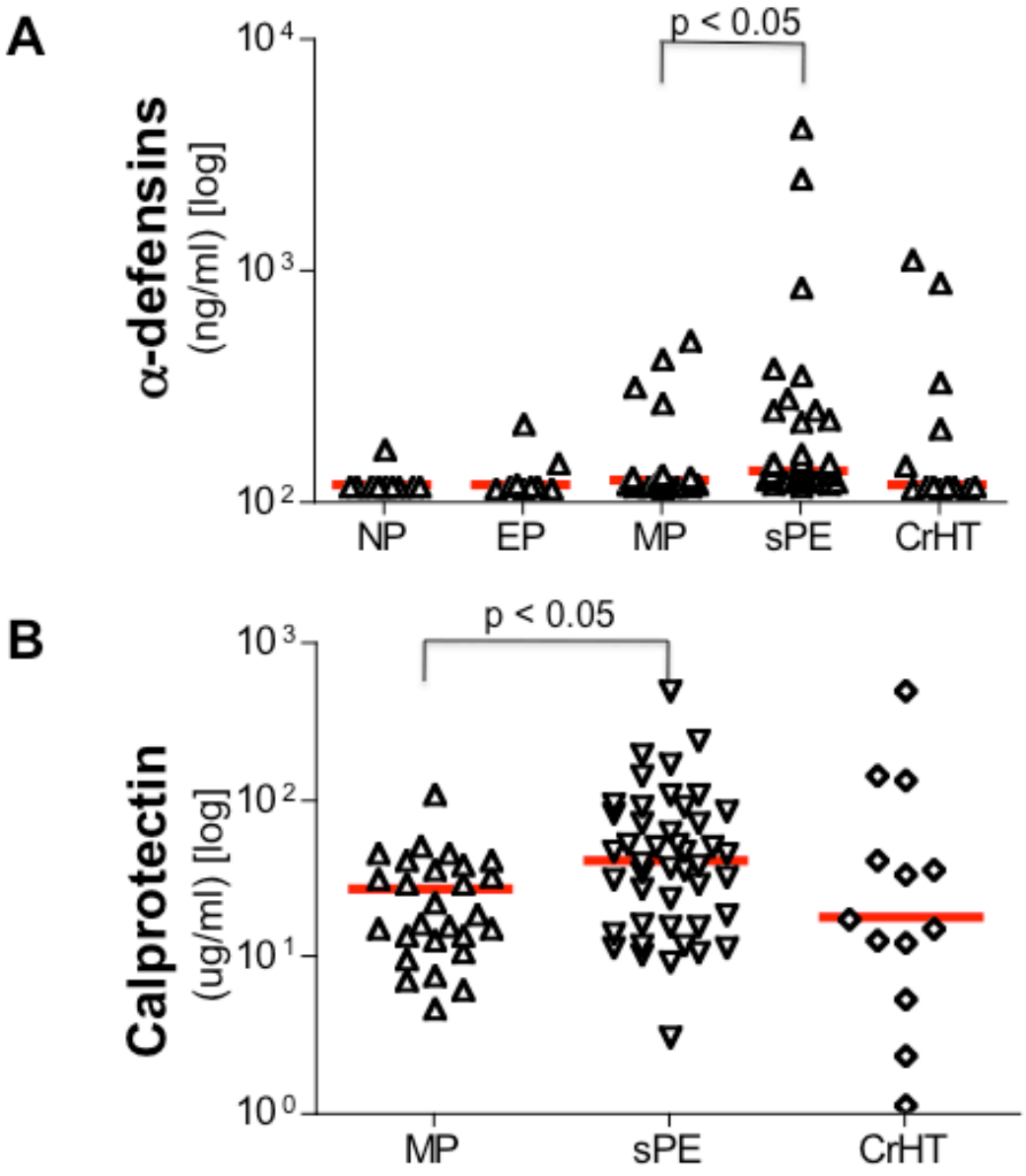

Figure 6.4. **Markers of neutrophil activation are elevated in severe preeclampsia.** (A, B) Plasma levels of α-defensins (nanograms/milliliter) and calprotectin in non-pregnant (NP) and at early (EP), mid (MP) and term (TP) gestation and in pregnancies complicated by severe preeclampsia (sPE) and chronic hypertension (CrHT). Horizontal bars represent median values.

6.2.6 α-defensins is positively correlated with calprotectin in severe preeclampsia

To demonstrate a direct relationship between α-defensins and calprotectin, we used the Spearman rank correlation analysis to determine their degree of association. Our analysis showed a linear relationship (R^2=0.533) and a significant positive correlation between maternal plasma α-defensins and calprotectin (Spearman's correlation, 0.6585; $p < 0.0001$; Figure 6.5). In the mid-gestational age control group, in which both the levels of α-defensins and calprotectin were low, there was a weak positive correlation between α-defensins and calprotectin (Spearman's correlation, 0.4096; p=0.0378) (data not shown). The linear relationship between these two markers of neutrophil activation strongly suggests that degranulation of activated neutrophils leads to the release of both α-defensins and calprotectin.

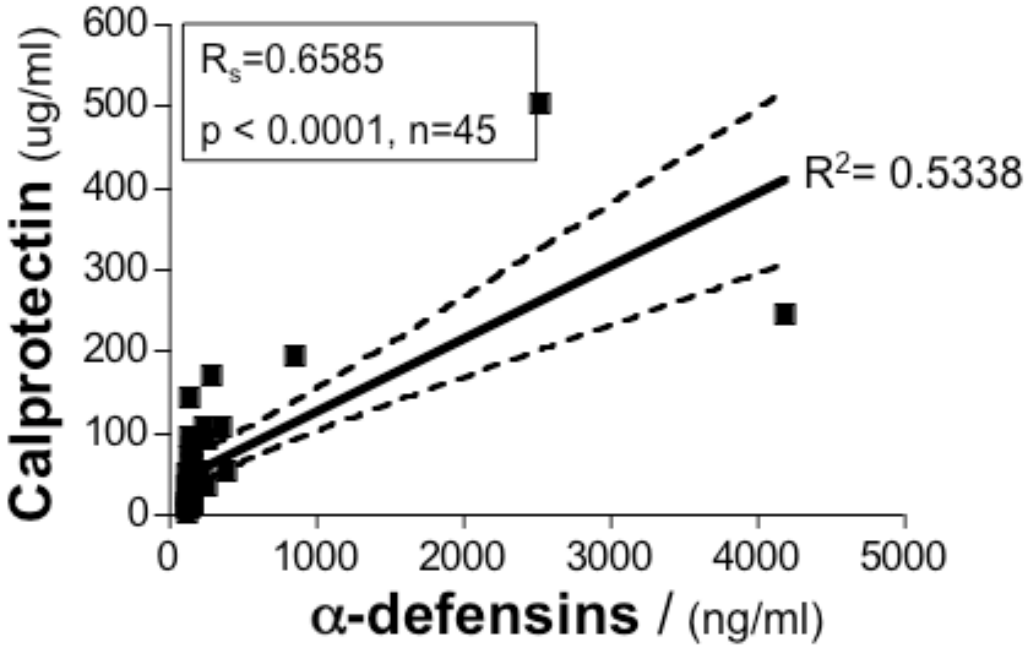

Figure 6.5. **Correlation and linear regression analysis between α-defensins and calprotectin in severe preeclampsia.** Maternal plasma α-defensins concentrations (nanograms/milliliter) correlates positively with maternal plasma calprotectin concentrations (micrograms/milliliter). The *line* represent regression line and the dotted lines represent the 95% CI of the regression line.

6.2.7 Correlation between sEng and neutrophil activation in severe preeclampsia

Since both sEng and the markers of neutrophil activation are elevated in severe preeclampsia, albeit at different magnitudes, we investigated whether neutrophil activation, as demonstrated in our cell culture studies (Chapter 5) plays a significant role in the increase in sEng in severe preeclampsia. No correlation between α-defensins and sEng (Spearman's correlation, 0.1312; p = 0.4136) or calprotectin and sEng (Spearman's correlation, 0.2273; p = 0.1332) was observed in severe preeclampsia (Figure 6.6 A, B), indicating that the increase in and magnitude of neutrophil activation does not play a major role in the dramatic elevation of sEng in severe preeclampsia.

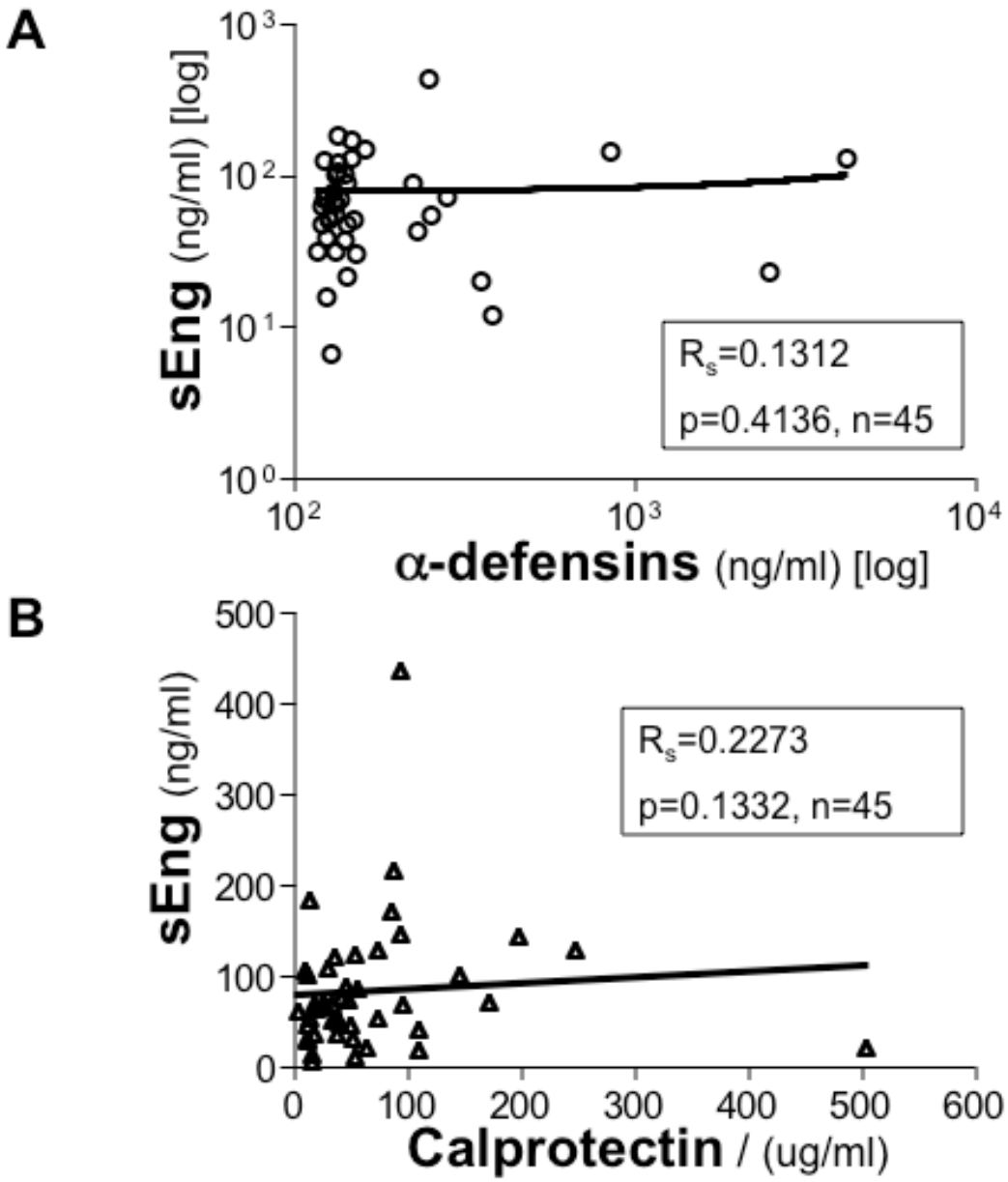

Figure 6.6. **Correlation between the markers of neutrophil activation and sEng in severe preeclampsia.** Plasma α-defensins (nanograms/milliliter) and Plasma calprotectin (micrograms/milliliter) do not correlate with serum sEng concentration (nanograms/milliliter).

6.2.8 IL-6 levels in elevated in preeclampsia and correlates with the markers of neutrophil activation

IL-6 is a pro-inflammatory cytokines secreted by both activated leukocytes and activated endothelial cells. Several studies have previously shown that the pro-inflammatory cytokine IL-6 is elevated in severe preeclampsia and contribute to endothelial cell activation (Clark et al., 1998b, Tosun et al., 2010, Szarka et al., 2010). We confirmed that IL-6 is indeed elevated by 2-fold in severe preeclampsia (1.092 pg/ml; 95% CI, 0.181 – 151.3 pg/ml) compared to gestational age-matched controls (0.6455 pg/ml; 95% CI, 0.141 – 3.874 pg/ml; $p<0.01$). However, we found no significant difference between the non-pregnant group (0.478 pg/ml; 95% CI, 0.058 – 3.392) and the control group for severe preeclampsia (Figure 6.7).

We tested whether the increase in IL-6 in severe preeclampsia correlates with the markers of neutrophil activation, α-defensins and calprotectin. Indeed IL-6 exhibited a positive non-linear relationship with both α-defensins (Spearman's correlation, 0.5672; $p < 0.0001$) and calprotectin (Spearman's correlation, 0.3615; $p = 0.0172$) (Figure 6.7), indicating the neutrophil activation and inflammation are not independent phenomenon in severe preeclampsia.

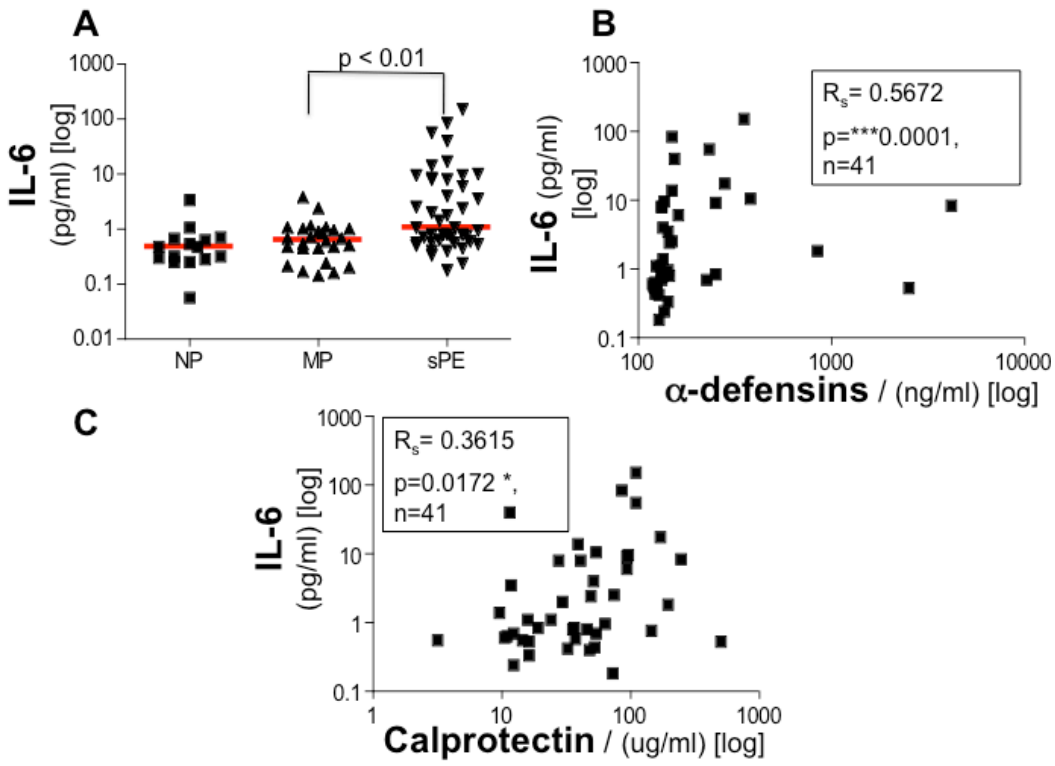

Figure 6.7. **Circulating IL-6 is elevated in severe preeclampsia and correlated with neutrophil activation.** (A) Serum levels of IL-6 (picograms/milliliter) in non-pregnant (NP), severe preeclamptics (sPE) and gestational age-matched control (MP). Serum IL-6 correlates positively with both (B) plasma α-defensins concentrations (nanograms/milliliter) and (C) plasma calprotectin concentrations (micrograms/milliliter).

6.2.9 Correlation between IL-6 and sEng in severe preeclampsia

We investigated whether there is a relationship between IL-6 and sEng. There was no correlation between IL-6 and sEng (Spearman's correlation, 0.05293; p = 0.7360) in severe preeclampsia (Figure 6.8) indicating that inflammation may not directly modulate the increase in sEng in severe preeclampsia.

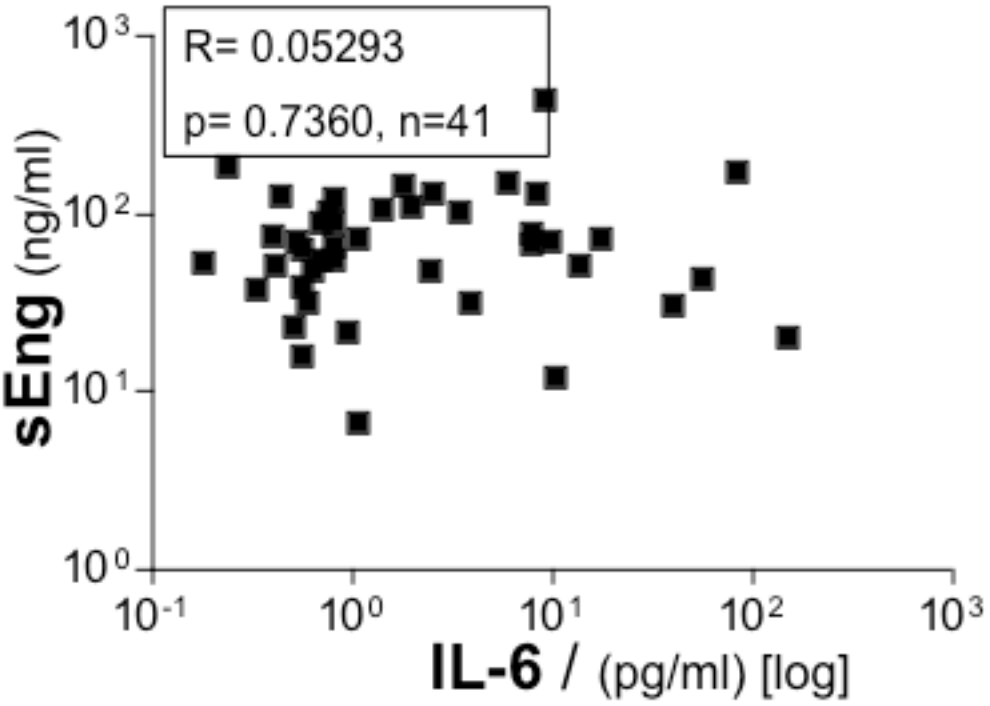

Figure 6.8. **Relationship between IL-6 and sEng.** Serum IL-6 (picograms/millilitre) does not correlate with serum sEng (nanograms/milliliter).

6.2.10 Urine sFlt-1/PlGF ratio in severe preeclampsia

We analysed the urine level of sFlt-1 and PlGF and calculated the ratio of urine sFlt-1/PlGF. As previously demonstrated (Buhimschi et al., 2005), the median urine sFlt-1/PlGF ratio was significantly higher in the severe preeclampsia (5.419; 95% CI, 0.4061-45.42) compared to the control group (0.034; 95% CI, 0-0.26; $p<0.0001$) (Figure 6.9A), and correlated positively with serum sEng (Spearman's correlation, 0.5239; $p = 0.0004$). However, urine sFlt-1/PlGF ratio did not correlate with α-defensins (Spearman's correlation, -0.0626; $p = 0.7009$), calprotectin (Spearman's correlation, -0.1937; $p = 0.2249$) or IL-6 (Spearman's correlation, 0.08624; $p = 0.5919$) (Figure 6.9 B, C, D). Together our data indicate that in severe preeclampsia, neutrophil activation/inflammation is independent of the release of sFlt-1 or the decrease in PlGF.

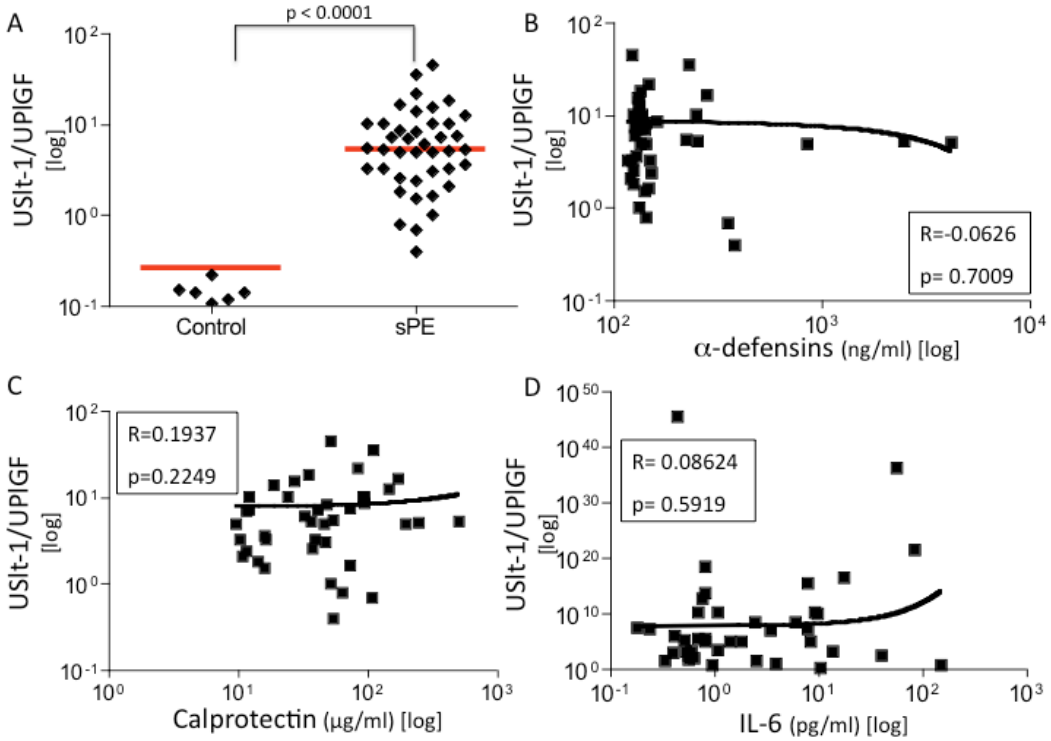

Figure 6.9. **Urine sflt-1/PlGF ratio is elevated in severe preeclampsia.** (A) Urine sflt-1/PlGF ratio is elevated in severe preeclamptics (sPE) compared to gestational age-matched control. (B, C, D) Plasma α-defensins concentrations (nanograms/milliliter), calprotectin concentrations (micrograms/milliliter) and serum IL-6 (picograms/milliliter) do not correlate with urine sFlt-1/PlGF ratio.

6.2.11 Serum sEng and urine sFlt-1/PlGF ratio correlate with diastolic blood pressure

Severe preeclampsia is associated with an increase in blood pressure of >160/90 (systolic/diastolic). We investigated whether the sEng and the markers of neutrophil activation and inflammation correlated with the clinical sign of preeclampsia measured as increase in diastolic blood pressure in the severe preeclamptics and their matched controls cohort. sEng correlated positively with diastolic blood pressure (Spearman's correlation, 0.6017; $p<0.0001$) and sFlt-1/PlGF ratio also correlated positively with diastolic blood pressure (Spearman's correlation, 0.601; $p<0.0001$) (Figure 6.10 A, B). This indicates that sEng and urine sFlt-1/PlGF ratio may play a causal role in the increase of diastolic blood pressure. In contrast, α-defensins (Spearman's correlation, 0.387; $p>0.05$), calprotectin (Spearman's correlation, 0.2526; $p>0.05$) and IL-6 (Spearman's correlation, 0.34; $p>0.05$) did not correlate with the diastolic blood pressure (Figure 6.11 A, B, C), suggesting that the rise in diastolic blood pressure is independent of neutrophil activation/inflammation.

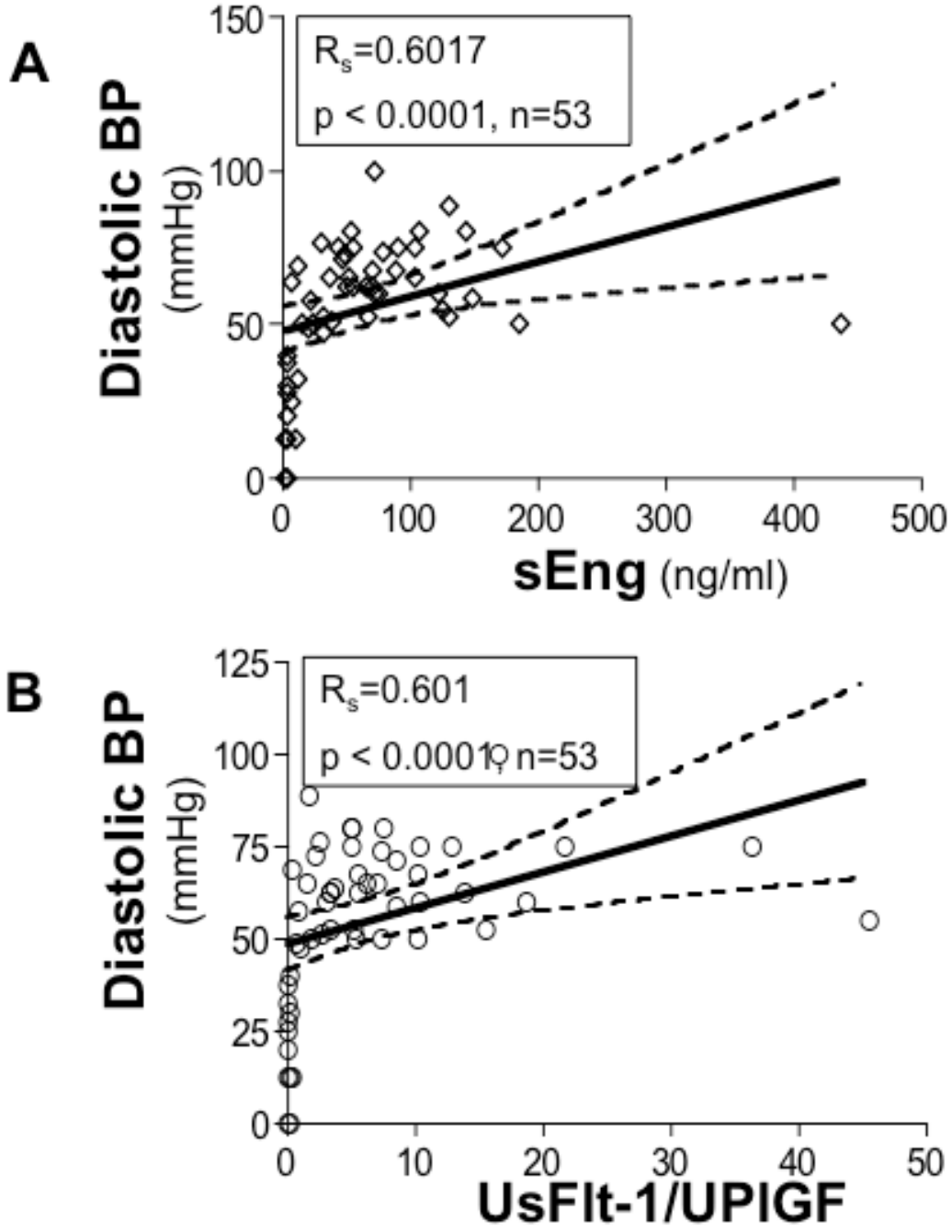

Figure 6.10. **Relationship between sEng, urine sFlt-1/PlGF ratio and diastolic blood pressure in control and severe preeclamptic patients.** Both serum sEng (nanograms/milliliter) and urine sFlt-1/PlGF ratio correlates positively with linear regression with diastolic blood pressure in control and severe preeclamptic patients combined. The *line* represent regression line and the dotted lines represent the 95% CI of the regression line.

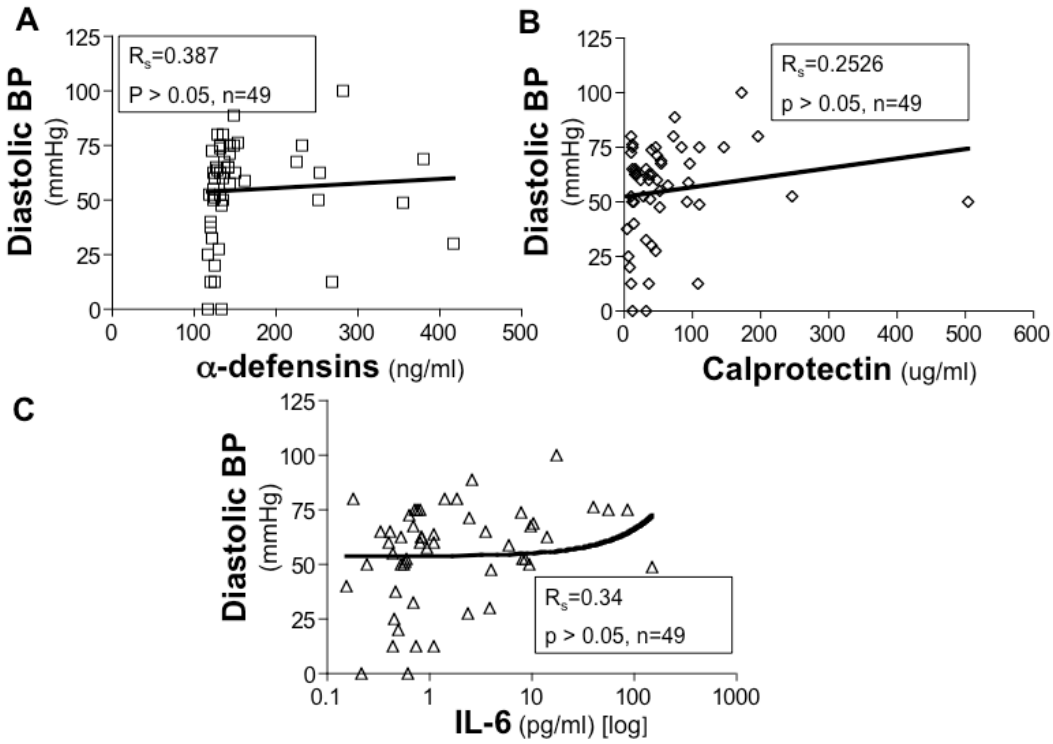

Figure 6.11. **Relationship between α-defensins, calprotectin, IL-6 and diastolic blood pressure in control and severe preeclamptic patients.** α-defensins (nanograms/milliliter), calprotectin (micrograms/milliliter) or IL-6 (picograms/milliliter) does not correlate with diastolic blood pressure in control and severe preeclamptic patients combined.

6.2.12 Fibronectin levels in elevated in preeclampsia but does not correlate with neutrophil activation

Cellular fibronectin is an important extracellular matrix glycoprotein of the endothelium that mediates important cell-to-cell interactions (Ruoslahti, 1988). Activated neutrophils and their products can cause degradation of fibronectin on the endothelial surface (Forsyth and Levinsky, 1990). Hence, fibronectin has been used as a marker of endothelial injury (Stubbs et al., 1984, Halligan et al., 1994, Sen et al., 1994, Friedman et al., 1995). Previous studies have shown that the total cellular fibronectin levels are elevated in preeclamptic patients (Uzun et al., 2010). Since endothelial dysfunction is associated with preeclampsia, we chose to examine whether neutrophil activation in severe preeclampsia plays a significant role in endothelial cell injury via the generation of cellular fibronectin. Our data confirmed that a plasma level of cellular fibronectin is indeed increased by approximately 2-fold in severe preeclampsia (175.5 μg/ml; 95% CI, 35.14 – 1099 μg/ml) compared to mid-gestation controls (93.17 μg/ml; 95% CI, 53.73 – 193.3 μg/ml; $p < 0.001$). There was no increase in cellular fibronectin between the non-pregnant group (88.79 μg/ml; 95% CI, 48.31- 406.4 μg/ml) and the pregnancies at mid gestation (Figure 6.12A).

The increase in circulating cellular fibronectin indicates that endothelial cell injury is a specific feature of severe preeclampsia. However the Spearman rank correlation analysis showed that there was no correlation between α-defensins and fibronectin in severe preeclampsia (Spearman's correlation, 0.003833; $p = 0.98$) (Figure 6.12 B) or between calprotectin and fibronectin

(Spearman's correlation, 0.06159; p = 0.6912) (Figure 6.12 C), probably indicating that the magnitude of neutrophil activation in severe preeclampsia is not sufficient to directly contribute to endothelial cell injury observed in preeclampsia.

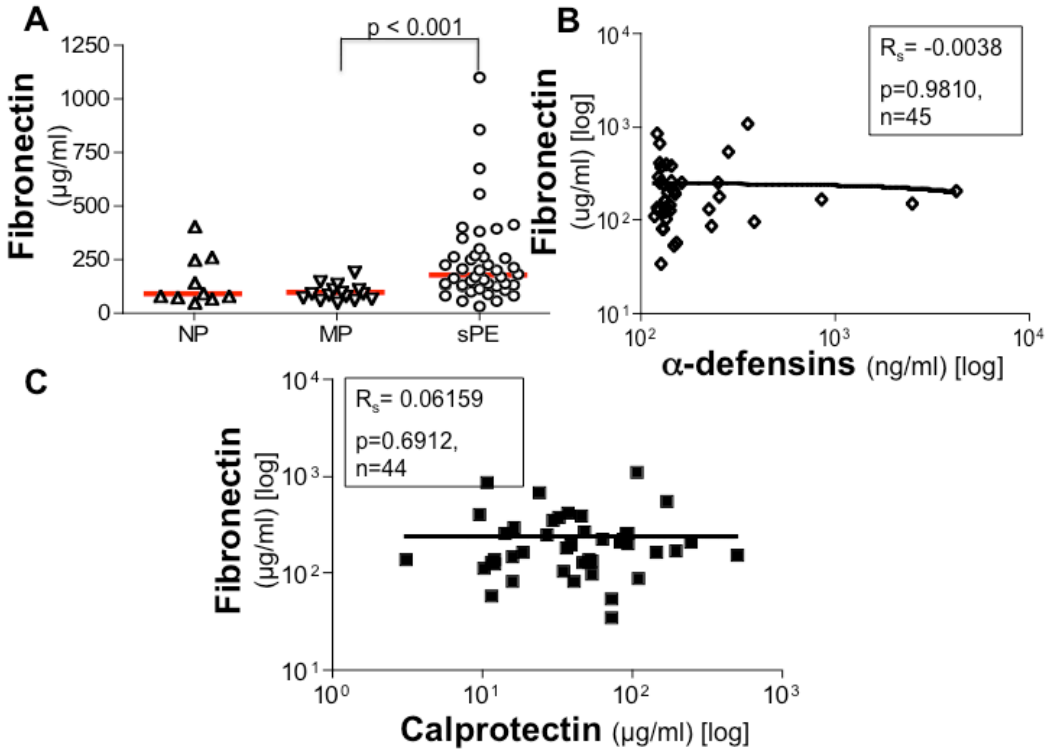

Figure 6.12. **Circulating level of fibronectin is elevated in severe preeclampsia.** (A) Serum levels of fibronectin (micrograms/milliliter) in severe preeclamptics (sPE) compared to gestational age-matched controls (MP) and non-pregnant (NP). Horizontal bars represent median values. (B) Plasma α-defensins concentrations (nanograms/milliliter) and plasma calprotectin concentrations (micrograms/milliliter) do not correlate with serum fibronectin concentration (micrograms/milliliter).

6.2.13 Correlation between fibronectin and soluble endoglin in severe preeclampsia

Since both cellular fibronectin (Halligan et al., 1994, Sen et al., 1994, Friedman et al., 1995) and sEng (Walshe et al., 2009) are involved in endothelial cell injury and dysfunction. We investigated the relationship between fibronectin and sEng. sEng and fibronectin did not correlate with each other in severe preeclampsia (Spearman's correlation, 0.07639; p = 0.6221) (Figure 6.13), indicating that the increase in sEng in severe preeclampsia is not caused by endothelial injury or that sEng does not directly cause endothelial injury.

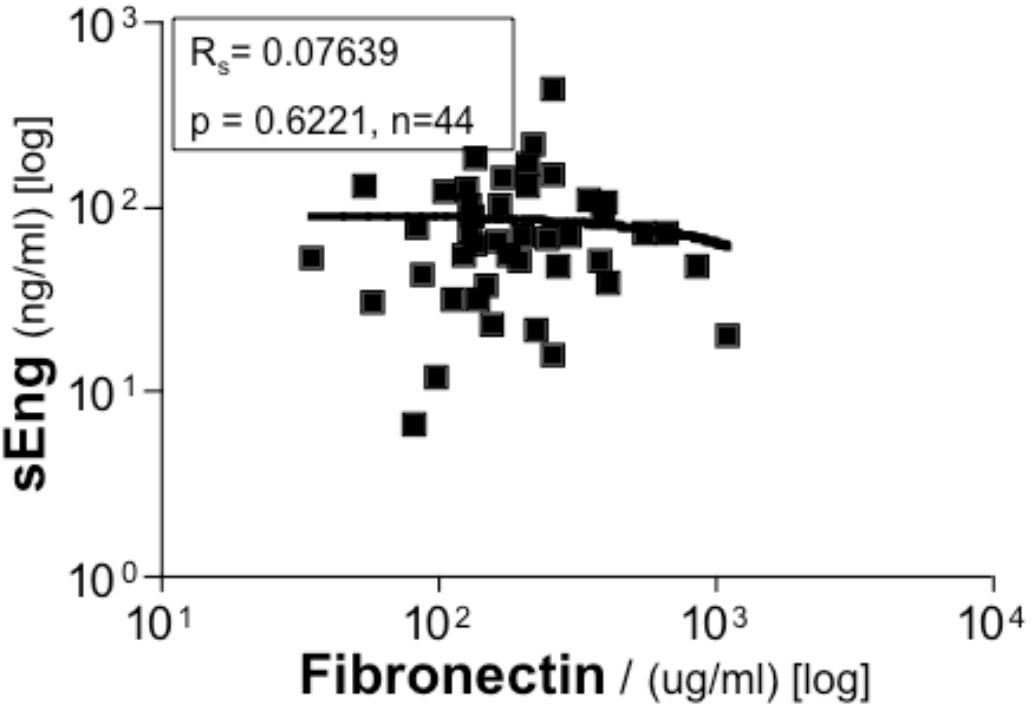

Figure 6.13. **Relationship between fibronectin and α-defensins and sEng in the maternal circulation.** Plasma α-defensins concentrations (nanograms/milliliter) do not correlate with serum fibronectin concentration (micrograms/milliliter).

6.2.14 sEng is negatively correlated with PlGF in severe preeclampsia

To confirm that the groups of patients showed the similar characteristics of preeclampsia as previously reported (Staff et al., 2007), we measured the serum level of PlGF. In the non-pregnant group, the level of PlGF was negligible (0 pg/ml; 95% CI, 0 – 15.66 pg/ml). As expected, there was a 4-fold reduction in PlGF in severe preeclampsia (85.15 pg/ml; 95% CI, 7.87 – 294.8 pg/ml) compared to the mid-gestation control group (401.2 pg/ml, 95% CI, 126.3 -1528 pg/ml; $p < 0.0001$) (Figure 6.14 A). We also confirmed that there was a negative correlation between PlGF and sEng (Spearman's correlation, 0.4666; p=0.0021). The linear regression analysis demonstrates this correlation (Figure 6.14 B). This confirmed that the population of patients used for this study was appropriate.

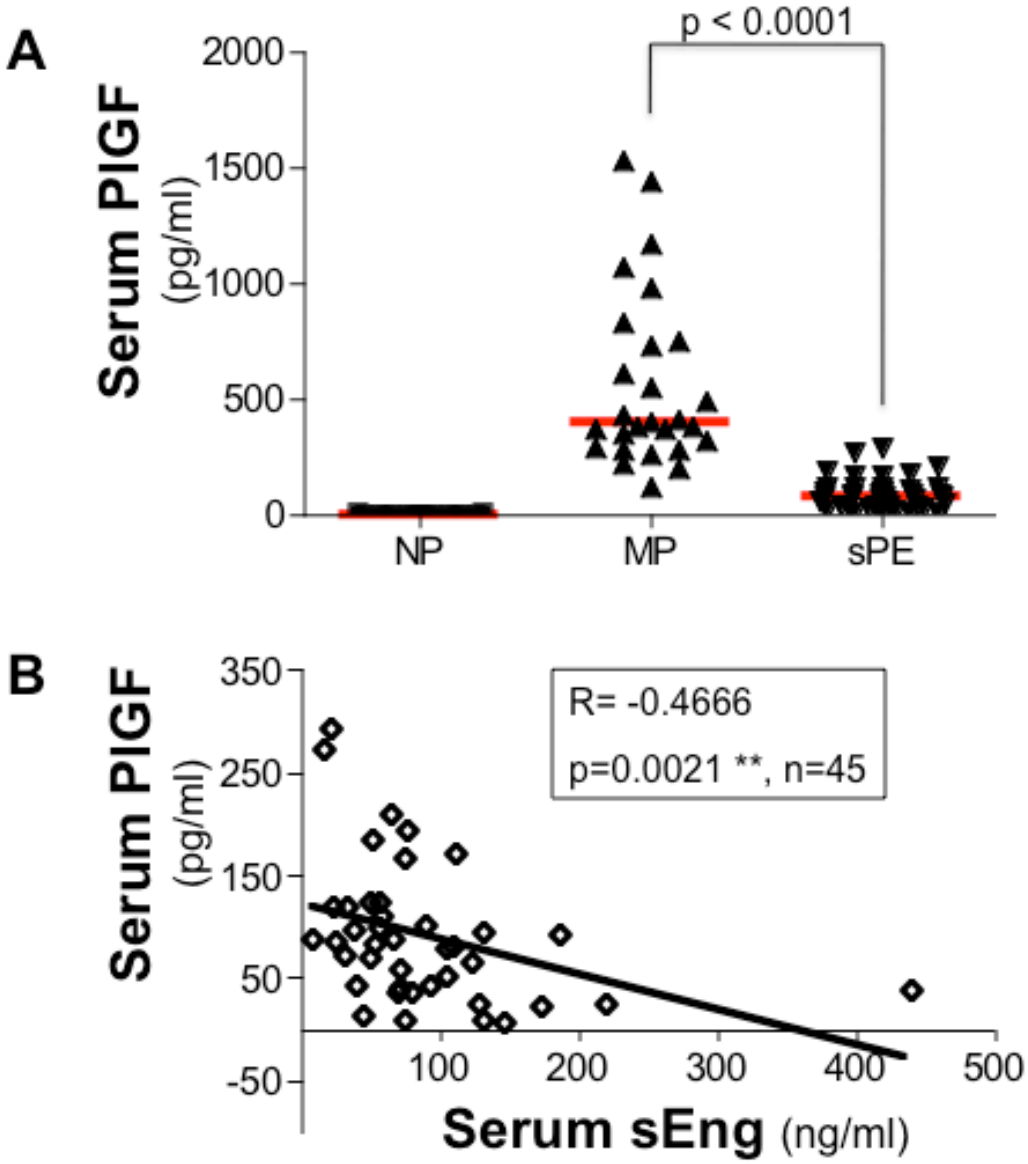

Figure 6.14. **Circulating levels of serum PlGF is reduced in severe preeclampsia and negatively correlates with serum sEng.** (A) Serum levels of PlGF (picograms/milliliter) in non-pregnant (NP), mid-gestation pregnancy (MP), severe preeclamptics (sPE) and chronic hypertension (CrHT). Horizontal bars represent median values. (B) Negative correlation between circulating levels of sEng (nanograms/milliliter) and PlGF (picograms/milliliter).

6.3 DISCUSSION

This study shows a direct correlation between increase in α-defensins and calprotectin demonstrating neutrophil degranulation during preeclampsia and a significant correlation between these markers of neutrophil activation and IL-6 indicating increased inflammatory status at the time of the clinical manifestation of the disease. Although sFlt-1/PlGF ratio and sEng, IL-6 and α-defensins and calprotectin were all elevated in preeclampsia, the present study found that there was no relationship between the increase in the anti-angiogenic factors and neutrophil activation/inflammation demonstrating that it is highly unlikely that inflammation is the cause of the maternal signs of preeclampsia. Furthermore, while sEng and sFlt-1/PlGF ratio both correlated positively with diastolic blood pressure, none of the markers of neutrophil activation/inflammation correlated with blood pressure. This further strengthens the argument that neutrophil activation/inflammation does not play a major role in preeclampsia.

Oxidative stress is an inflammatory stimulus mediated by several factors including those associated with the activation and degranulation of neutrophils resulting in the generation of reactive oxygen species. Recently, Redman and Sargent hypothesized that the main placental problem that leads to the onset of preeclampsia is oxidative stress and the authors proposed that oxidative stress induces the release of sFlt-1 and sEng via nuclear factor kappa-B (NFκB) to a similar or greater extent than hypoxia

(Redman and Sargent, 2009). Univariate analysis of our data showed that women with preeclampsia had a 20-fold increase in serum sEng and approximately a 2-fold increase in the markers of neutrophil activation, α-defensins and calprotectin, as well as in the circulating levels of pro-inflammatory IL-6. This is consistent with the results of similar earlier studies on sEng (Venkatesha et al., 2006), α-defensins (Prieto et al., 1997), calprotectin (Holthe et al., 2005) and IL-6 (Luppi et al., 2006). However, increase in maternal circulatory levels of the aforementioned factors does not demonstrate direct causation of clinical symptoms of preeclampsia. Correlation analysis showed that there was no meaningful relationship between neutrophil activation and the increase in sEng observed in preeclampsia. This is in contrast to a previous report that claimed a positive correlation between calprotectin and sEng in preeclampsia (Staff et al., 2007). The observed differences are likely to be the consequence of gestational age differences among groups as acknowledged by the authors (Staff et al., 2007). Indeed, consistent with the study by Levine and colleagues (Levine et al., 2006), we showed that circulating maternal sEng concentration increases with gestational age, likely reflecting growth in placental volume and production of sEng from the placenta. Hence, to account for this possible confounder, the patient groups were matched for gestational age in our study.

Clinical manifestation of severe preeclampsia is also associated with increased urinary output of sFlt-1 and a decreased output of PlGF (Buhimschi et al., 2005) and sEng is elevated in the urine of women who

develop early-onset preeclampsia (Buhimschi et al., 2010). The present study found that elevated sEng in the maternal serum was associated with elevated urine sFlt-1/PlGF ratios in severe early-onset preeclampsia**.** Furthermore, these results are supported by a strong negative correlation between elevated circulating levels of sEng and PlGF in the maternal circulation. Nevertheless, the urinary sFlt-1/PlGF ratio did not correlate with circulating levels of α-defensins, calprotectin or IL-6, which suggests that the degree of neutrophil activation and inflammation that occurs in severe preeclampsia plays an insignificant role in the increase in anti-angiogenic factors. Hence, these results refute the proposition that the release of anti-angiogenic factors and the clinical signs of preeclampsia are due to excessive neutrophil activation/inflammation during preeclampsia.

In addition, our results shed important clues on the role of inflammation in the pathogenesis of preeclampsia. The pro-inflammatory cytokine IL-6 is secreted by activated leukocytes and is classically known to activate acute phase response genes such as C-reactive protein and adhesion molecules (Kvale et al., 1992), indicating its potential role in the initiation and propagation of inflammation in preeclampsia. Indeed, many studies including ours (Vince et al., 1995, Conrad et al., 1998, Greer et al., 1994) have shown that IL-6 is elevated in preeclampsia, however a few studies have reported no change in plasma IL-6 in preeclampsia compared to uncomplicated pregnancy (Al-Othman et al., 2001, Ellis et al., 2001). Orshal et al. reported that IL-6 has the ability to directly modulate the mechanisms of contraction and relaxation in systemic vessels of pregnant rats by impairing

the endothelium-dependent nitric oxide-mediated relaxation (Orshal and Khalil, 2004) indicating a potential association between IL-6, endothelial dysfunction and hypertension during preeclampsia. We observed no correlation between the increase in blood pressure and the increase in IL-6 in the cohort of severe preeclamptic patients and their matched controls indicating that the magnitude of inflammation in severe preeclampsia is not sufficient to cause increase in blood pressure. To further support the present findings that neutrophil activation/inflammation is unlikely to be directly involved in the release of maternal sEng levels in preeclampsia, a recent study showed that despite the increase in complement activation in preeclampsia (Derzsy et al., 2010), there was no relationship between complement activation and the release of angiogenesis related factors in preeclamptic women (Lynch et al., 2010). In support of that, we showed that although the level of α-defensins is elevated in systemic inflammation, sEng level remained unchanged compared to controls.

We agree that a correlation cannot simply imply causation. However, if there is no relationship between two variables, then there is no causal connection. Hence, in view of our correlation analysis and supported by the findings of other groups (Lynch et al., 2010), we can conclude that inflammation does not play a causative role in the release of anti-angiogenic factors in preeclampsia. Furthermore, by considering the Hill's criteria of causation, we can further support this conclusion. In prognostic research, factors associated with the outcome, whether they are causal or not, are of interest. In contrast, causality is of significant importance in aetiological research (Sheehan et al., 2008). The

determination of causality by observational data is tricky as it is not always clear which of the two associated variables is the cause and which is the effect, or whether both are a common effect of a third unobserved variable or confounder. However, according to Hill, causation can be determined using the temporal relationship, which implies that the cause of a disorder must precede the clinical signs of that disorder (Hill, 1965, Sheehan et al., 2008). Indeed previous studies, including ours has demonstrated that neutrophil activation (Greer et al., 1991, Barden et al., 1997) and the humoral mediators of inflammation (Peracoli et al., 2007, Szarka et al., 2010, Greer et al., 1994) are elevated at the time of diagnosis of preeclampsia. However, a previous prospective nested case control study revealed that at 18 weeks of gestation, the levels of inflammatory parameters including IL-6 were not elevated in women who later developed preeclampsia compared to matched healthy controls (Djurovic et al., 2002). Hence, also according to the temporal criteria of causality, we can confute the long held concept that preeclampsia is caused by excessive inflammation (Redman et al., 1999). In contrast, numerous studies have shown that maternal circulatory sFlt-1 is elevated as 5-10 weeks before the onset of preeclampsia (Levine et al., 2004, Levine et al., 2006, Chaiworapongsa et al., 2005) and sEng is elevated as early as 11-13 weeks of gestation prior to the development of preeclampsia (Rana et al., 2007, Romero et al., 2008, Erez et al., 2008, Baumann et al., 2008, Lim et al., 2009, Foidart et al., 2010). Decrease in urinary PlGF also precedes the onset of preeclampsia (Levine et al., 2005). Collectively, these observations provide evidence of the cause and effect relationship between sFlt-1, sEng and PlGF and preeclampsia. Furthermore, increasing experimental data also shows that the

clinical signs of preeclampsia can be largely attributed to high levels of sFlt-1 and sEng (Levine et al., 2004, Levine et al., 2006, Maynard et al., 2003, Venkatesha et al., 2006, Makris et al., 2007, Roberts et al., 2006, Agunanne et al., 2010) and that neutralization of sFlt-1 below a critical threshold eliminates the signs of preeclampsia in mice (Li et al., 2007, Bergmann et al., 2010).

Endothelial dysfunction and injury has been reported to be a key component of the pathophysiology of preeclampsia (Wallenburg and Visser, 1994). Activated neutrophils and their degranulation products can degrade the endothelial extracellular matrix, cleaving collagen and cellular fibronectin (Hynes, 1986, O'Reilly et al., 2008). Under normal circumstances, the level of cellular fibronectin is low in the plasma (<2%), but rises in conditions characterized by endothelial injury. Several studies (Halligan et al., 1994, Sen et al., 1994, Friedman et al., 1995), including ours has shown that the plasma levels cellular fibronectin is elevated in preeclampsia. Previously, Madazli et al. reported that the levels of fibronectin and VCAM-1 levels positively correlated with each other in preeclampsia and that the elevation in fibronectin also positively correlated with diastolic blood pressure (Madazli et al., 2000). In contrast, another study showed that there was no correlation between plasma levels of cellular fibronectin and diastolic blood pressure in preeclamptic patients and their controls (Aydin et al., 2006). We showed that there was no correlation between the levels of neutrophil activation and the increase in plasma levels of cellular fibronectin in severe preeclampsia, indicating that the magnitude of neutrophil activation plays a minor role in endothelial cell injury in preeclampsia. Although, some studies have

attributed the elevation in circulating VCAM-1 as a measure of leukocyte activation (Lyall et al., 1994), this is unlikely to reflect neutrophil activation since neutrophils lack the counterreceptor for VCAM-1 and cannot bind to VCAM-1. Hence, increased expression of VCAM-1 reflects endothelial cell activation, but not neutrophil activation. Furthermore, in our study, the increase in fibronectin did not correlate with diastolic blood pressure in the preeclamptic and matched control groups combined (data not shown).

The conflicting results between different groups could be explained by the fact that the reported correlations or absence of correlations may be confounded by several variables, including the type of test used to evaluate the levels of fibronectin, the study population, and the type of fibronectin measured. Indeed, it has been previously reported that the plasma level of *intact* cellular fibronectin, as measured in our study, could be fragmented in severe preeclampsia, hence leading to an underestimation of the plasma levels of *intact* cellular fibronectin detected by ELISA (De Jager et al., 1996). Furthermore, many studies, which have reported that women with preeclampsia have higher levels of plasma fibronectin, have failed to provide details about the type of fibronectin or the total fibronectin measured. In our study, although we measured the plasma level of total cellular fibronectin, we acknowledge that further measurements and analysis of the different fragments of fibronectin in the plasma should be done before any conclusion of the role of fibronectin in preeclampsia can be drawn.

We also reported that the increase in sEng and fibronectin in severe preeclampsia did not correlate with each other. This is not surprising given the evidence from the rodent study by Venkatesha and colleagues who reported that sEng by itself induced only mild endothelial dysfunction assessed by the increase in blood pressure and proteinuria in pregnant mice, in comparison to the concerted action of sEng and sFlt-1 which cause severe kidney damage, hypertension and proteinuria as well as elevated liver enzymes in pregnant mice. They also showed that the combination of sEng and sFlt-1 caused an increase in capillary permeability in the lungs and liver of the same mice, indicating disruption in endothelial integrity and considerable vascular damage and leakage (Venkatesha et al., 2006). Furthermore, extensive vascular damage of the placenta was only observed in sFlt-1 and sEng treated mice and not in mice treated with either agent alone (Venkatesha et al., 2006). Therefore in theory, we would expect the combined effect of circulating sFlt-1 and sEng to correlate with the levels of fibronectin. However, considering the magnitude of elevation of fibronectin in preeclampsia compared to the substantial elevation of both sEng and sFlt-1, it is highly unlikely that a positive correlation between sEng/sFlt-1 and fibronectin will be observed.

In conclusion, our study shows that inflammation does not play a central role in the release of anti-angiogenic factor or is responsible for preeclampsia. However, we do acknowledge that genetic, environmental or other external conditions can affect an individual's susceptibility to inflammation. Hence in order to confirm this new hypothesis, similar studies in various centres need

to be conducted. Nevertheless, these results question the long held view that inflammation is the cause of preeclampsia and warrant new thinking in regards to the pathogenesis of this hypertensive disorder specific to human.

CHAPTER 7

GENERAL DISCUSSION AND FUTURE WORK

7.1 General discussion and future work

Cytoprotective function of HO-1 and BVR

The results presented in the first part of this thesis demonstrate the multifunctional role of HO-1 in preventing endothelial injury, which is commonly associated with cisplatin chemotherapy and preeclampsia. We showed that the over-expression of HO-1 as well as substances known to upregulate HO-1 expression could prevent drug-induced endothelial damage. Specifically, we showed that resveratrol, a dietary antioxidant polyphenol, found in grapes, red wine and peanuts, upregulates HO-1 in endothelial cells and protects against cisplatin-induced injury. Since, resveratrol have protective effects against cardiovascular disease and cancer, including all stages of carcinogenesis (Jang et al., 1997, Bhat and Pezzuto, 2002, Pervaiz, 2003), the use of resveratrol in cisplatin-based chemotherapy merits further investigation.

An essential feature of this study is that we provide the first evidence that BVR induces the expression of HO-1 in endothelial cells. Furthermore, we show that the cytoprotective function of BVR against cisplatin-induced damage is dependent on the upregulation of HO-1 by BVR. Previous studies had demonstrated that BVR induces the expression of HO-1 in HEK293A cells (Kravets et al., 2004) and that silencing of BVR gene dramatically reduces the cytoprotective effect of HO-1 against superoxide anion and arsenite, indicating the essential role of BVR in the HO-1-mediated

cytoprotection. Furthermore, it has been reported that the expression of BVR in primary endothelial cells, even at very low level, is important for the protective function of HO-1 (Jansen et al., 2010). We show that the ability of BVR to protect against cisplatin-induced endothelial cell damage is dependent on its upregulation of HO-1 in endothelial cells. Accordingly, we can speculate that there may exist in endothelial cells a feedback loop between HO-1 and BVR (Figure 7.1). Further work is required to determine the mechanism through which BVR induces HO-1 gene expression in endothelial cells, whether via direct DNA binding, in its capacity as a transcription factor, or via its ability to phosphorylate serine and threonine residues. Furthermore, since the cytoprotective effect of BVR depends on the upregulation of HO-1, it is likely that bilirubin which also possesses antioxidant and cytoprotective properties (Stocker et al., 1987) would also prevent cisplatin-induced endothelial cell damage. The determination of bilirubin level is required to determine the mechanism through which BVR over-expression confers protection to endothelial cells.

Ideally, it would be important to show in an *in vivo* setting the importance of HO-1 and BVR in endothelial cells against cytototoxic damage using the cre-Lox system, which will allow in vivo endothelial-specific over-expression of HO-1 or BVR. In this way, it could be confirmed that endothelial specific over-expression of HO-1 or BVR protects against cisplatin induced injury in vivo and that the loss of endothelial HO-1 or BVR exacerbates the damage. Furthermore, there is a need to identify drugs that can induce BVR. This approach will allow the translation of our findings into the clinical setting.

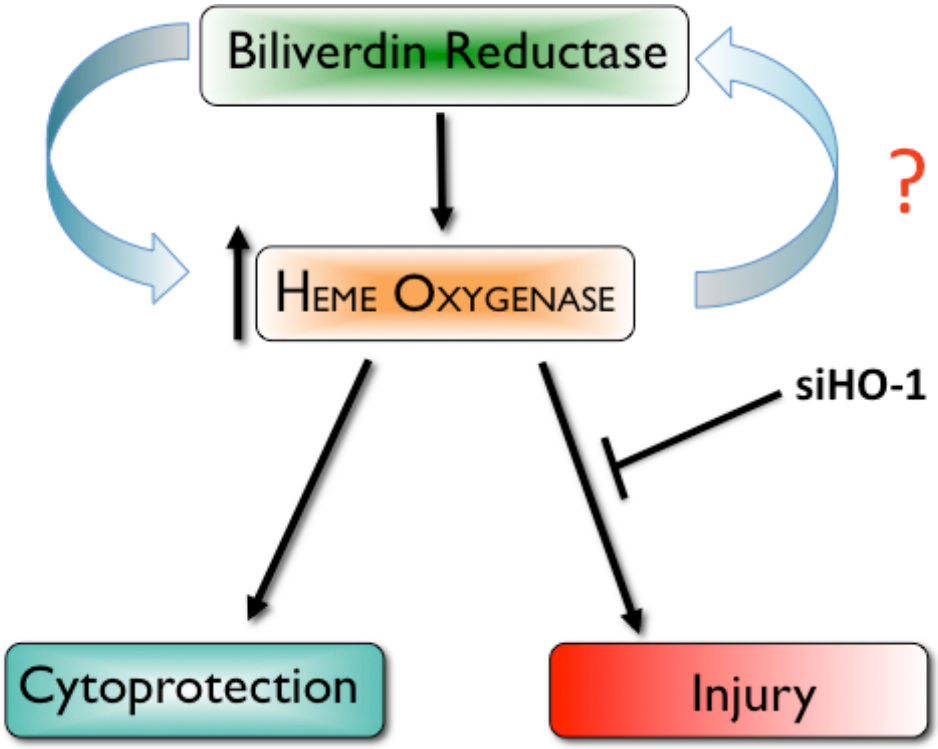

Figure 7.1. **Schematic diagram illustration the possible feedback loop between HO-1 and BVR**.

Cancer patients treated with cisplatin have an increased risk of developing coronary arterial disease and atherosclerosis (Nuver et al., 2004). Cisplatin also upregulates the expression of ICAM-1 in endothelial cells, hence promoting the interaction between leukocytes and the endothelium in a time- and dose-dependent manner (Yu et al., 2008), thereby potentiating inflammatory responses. HO-1 has been shown to downregulate the expression of ICAM-1 in endothelial cells of the microcirculation (Dal-Secco et al., 2010). Hence over-expression of HO-1 would also protect against cisplatin-mediated inflammatory processes. PlGF has been implicated in vascular remodelling, atherosclerosis, and adverse ischemic events in animal

models and in humans. As discussed before, neutralization of PlGF by an anti-PlGF antibody reduced the inflammatory cell infiltration and atherosclerotic lesion size in ApoE$^{-/-}$ mice (Roncal et al., 2010). Hence, the cardiovascular complications observed during and post cisplatin chemotherapy may be aggravated by increase in PlGF levels. Hence it might be important to evaluate the effect of cisplatin in PlGF $^{-/-}$ mice fed on a high cholesterol diet. We would predict that the incidence of atherosclerosis and damage would be less in the PlGF $^{-/-}$ mice treated with cisplatin compared to the wild type animals.

In addition to its cytoprotective, anti-apoptotic, anti-oxidative and anti-inflammatory properties, HO-1 have recently been shown to have a role to play in angiogenesis (Dulak et al., 2004). In this study, we showed that adenoviral over-expression of HO-1 and BVR in endothelial cells inhibits VEGF-induced capillary-tube formation, while the siRNA knockdown of HO-1 and BVR promotes capillary-tube formation. Despite being novel, these novel results are in marked conflict with earlier studies that have attributed a pro-angiogenic function to HO-1 in endothelial cells (Jozkowicz et al., 2003, Deramaudt et al., 1998). Studies have shown that HO-1 and CO, but not biliverdin or bilirubin, induces VEGF in vascular smooth muscle cells and HMEC (Dulak et al., 2002). Furthermore, adenoviral over-expression of HO-1 in the mice myocardium has been associated with an increase in VEGF levels as well as increased in vascularization as demonstrated by the detection of higher capillary and arteriole densities in the peri-infarct region (Lin et al., 2008b). These evidences prompted researchers to attribute the pro-

angiogenic properties of HO-1 to the increase in VEGF. However, while a few studies have demonstrated the pro-angiogenic effect of HO-1 and CO in endothelial cells (Deramaudt et al., 1998, Jozkowicz et al., 2003), the outcome of these reports warrants further investigations based on a number of discrepancies in relation to the origin of endothelial cell types, the method of induction of HO-1 or the concentration of HO-1/CO used. Since none of the previous studies have provided quantitative measurement of the expression or activity of HO-1 or BVR and that adenoviral over-expression or siRNA knockdown of HO-1 makes it difficult to modulate the expression of HO-1 in a dose-dependent manner, the limitation of previous studies including ours raises the question of whether HO-1 has a dual effect on angiogenesis in endothelial cells depending on its level of expression. Hence we propose that future work on this should focus on manipulating the expression of HO-1 in a dose-dependent way using specific dose of pharmacological inducers of HO-1 (such as hemin) in HO-1 deficient cells and evaluating HO-1 activity through the measurement of CO release and determining at which concentration of hemin used does HO-1 inhibits tube formation. A cell viability assay to determine the maximum dose of hemin, which is not cytotoxic to cells, should be performed beforehand. Alternatively, our group has shown has also shown that CO, at a concentration of 250 ppm, inhibits angiogenesis as well as inhibits VEGFR-2 phosphorylation. VEGFR-2 is a tyrosine kinase receptor, which upon phosphorylation as a result of binding of its ligand VEGF-A promotes angiogenesis. CO inhibits VEGFR-2 phosphorylation. Hence we could also determine the minimum dose at which inhibition of phosphorylation of VEGFR-2 occurs, i.e. the dose at

which CO will inhibit angiogenesis, and use that specific dose to determine VEGF-induce capillary tube formation, endothelial cell migration or proliferation.

Inflammation and preeclampsia

Although our *in vitro* studies by themselves would have led us to postulate that neutrophil activation in preeclampsia could be directly responsible for the release of sEng, as shown in chapter 5, the same observations were not reflected in our clinical data indicating *in vitro* studies can give rise to false positive concepts.

The clinical findings shed important clues on the role of inflammation and anti-angiogenic factors in the pathogenesis of preeclampsia. We conclusively demonstrated that neutrophil activation and inflammation does not play a causal role in the development of preeclampsia. While sEng and urine sFlt-1/PlGF ratio bear a positive linear relationship with diastolic blood pressure, no such relationship was observed with the markers of neutrophil activation or inflammation. A previous prospective nested case control study revealed that at 18 weeks of gestation, the levels of inflammatory parameters including IL-6 were not elevated in women who later developed preeclampsia compared to healthy controls (Djurovic et al., 2002). Hence, also according to the temporal criteria of causality, we can confute the long held concept that preeclampsia is caused by excessive inflammation (Redman et al., 1999). In contrast, numerous studies have shown that maternal circulatory sFlt-1 is elevated as 5-10 weeks before the onset of preeclampsia (Levine et al., 2004,

Levine et al., 2006, Chaiworapongsa et al., 2005) and sEng is elevated as early as 11-13 weeks of gestation prior to the development of preeclampsia (Rana et al., 2007, Romero et al., 2008, Erez et al., 2008, Baumann et al., 2008, Lim et al., 2009, Foidart et al., 2010). Decrease in urinary PlGF also precedes the onset of preeclampsia (Levine et al., 2005). Collectively, these observations provide evidence of the cause and effect relationship between sFlt-1, sEng and PlGF and preeclampsia. Furthermore, increasing experimental data also shows that the clinical signs of preeclampsia can be largely attributed to high levels of sFlt-1 and sEng (Levine et al., 2004, Levine et al., 2006, Maynard et al., 2003, Venkatesha et al., 2006, Makris et al., 2007, Roberts et al., 2006, Agunanne et al., 2010) and that neutralization of sFlt-1 below a critical threshold eliminates the signs of preeclampsia in mice (Li et al., 2007, Bergmann et al., 2010).

In conclusion, our study shows that inflammation does not play a central role in the release of anti-angiogenic factor or is responsible for preeclampsia. Since various factors including genetic, environmental or other external conditions can affect an individual's susceptibility to inflammation, we recognise that in order to confirm this new hypothesis, similar studies in various centres need to be conducted. Nevertheless, these results question the long held view that inflammation is the cause of preeclampsia and warrant new thinking in regards to the pathogenesis of this hypertensive disorder specific to human.

Role of HO-1 in preeclampsia

All diseases have an inflammatory component and preeclampsia is no exception. However, as we demonstrated, it is not the increased in inflammation *per se* that causes preeclampsia. In 2000, a new concept was proposed as to the cause of preeclampsia. Ahmed and colleagues proposed that it is the loss of endogenous protective factors that may predispose women during pregnancy to preeclampsia (Ahmed et al., 2000). They demonstrated that the loss of HO activity promoted placental damage induced by TNF-α, which could be prevented by increasing HO activity by the pharmacological inducer, hemin (Ahmed et al., 2000). This led them to propose that a lack of HO/CO activity could be the predisposing factor during pregnancy leading to preeclampsia. They subsequently showed that HO-1 inhibits the release of sFlt-1 and sEng and that HO-1 deficient mouse with systemic endothelial damage, has significant elevation in circulating sEng (Cudmore et al., 2007). Furthermore, a recent publication showed that the angiotensin receptor agonistic auto-antibody stimulate sEng *in vivo* by the upregulation of TNF-α and this upregulation can be prevented by induction of HO-1 using hemin (Zhou et al., 2010). Further experimental studies have confirmed that cigarette smoke extract induces HO-1 expression in trophoblasts (Ahmed et al., 2000) and decreases sFlt-1 release from placental villous explants without altering placental apoptotic status (Mehendale et al., 2007). In addition, CO treatment has been shown to enhance HO-1 expression in endothelial cells (Thom et al., 2000).

To confirm the *in vitro* data, clinical studies have shown that preeclamptic

women have significant decreased CO concentrations in their exhaled breath compared to those with healthy pregnancies indicating a decreased HO activity (Baum et al., 2000, Kreiser et al., 2004). Paradoxically, although, smoking during pregnancy is associated with spontaneous abortion; stillbirth, preterm labor, fetal growth restriction and placental abruption, the incidence of preeclampsia is reduced by a third in smokers (Conde-Agudelo et al., 2008), compared to snuff (smokeless tobacco) users (England et al., 2002). This indicates that it is the combustible product of tobacco, carbon monoxide that confers the protection. Furthermore, women who smoke have reduced circulating sFlt-1 and increased PlGF (Levine et al., 2006).

The hypothesis that HO-1 mediated CO release protects against preeclampsia is strengthen by mounting evidence that this stress response gene and its gaseous product confer protection during pregnancy in both in vitro and animal studies (Cudmore et al., 2007, Zhou et al., 2010, Ahmed et al., 2000, Acevedo and Ahmed, 1998). A study has shown that the HO-1 mRNA is decreased in the blood of preeclamptic women at term and that this reduction was inversely correlated with disease severity (Nakamura et al., 2009). However, the most compelling evidence, which can lead us to think of HO-1 as a cause for preeclampsia, comes from a recent study using fetal placental cells (chorionic villous sampling, CVS) from women at 11 weeks gestation. Farina and colleagues showed that the expression of HO -1 mRNA was decreased in CVS from women who went on to develop preeclampsia compared to controls (Farina et al., 2008). This data opens up the possibility that this very early decrease in HO-1 could lead, at least in

part, to the elevated anti-angiogenic factors seen in preeclamptic women later in pregnancy (Figure 7.2). The HO enzyme system generates three molecules (Biliverdin, Fe^{2+} and CO), which are unique in that they all have biological activity. Biliverdin is an antioxidant, which is rapidly reduced by biliverdin reductase to bilirubin, another potent antioxidant. The interactive role of these in preeclampsia still needs to be evaluated.

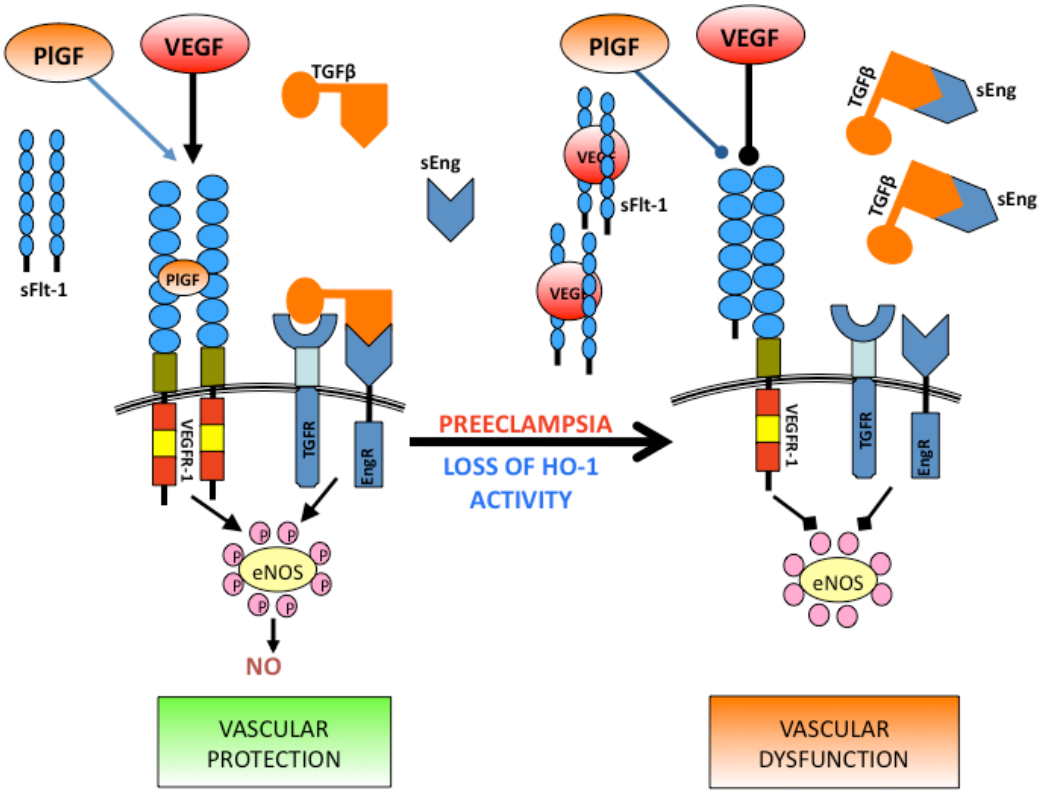

Figure 7.2 **Schematic diagram illustrating that loss of HO-1 may trigger preeclampsia.** This diagram shows that loss of HO results in the rise of the anti-angiogenic factors, soluble Flt-1 (sFlt-1) and soluble Endoglin (sEng), which then mops up the angiogenic growth factors (VEGF and TGF-β). As a consequence of this, enzymes called endothelial nitric oxide synthase (eNOS) that produce nitric oxide (NO) are not fully activated. This results in the generation of superoxide and free radicals, hence promoting vascular dysfunction.

APPENDIX I

Chemical Reagents and Suppliers

Acetic acid (glacial):	Sigma, Poole, U.K.
Acrylamide solution (40%):	Bio-Rad, Hemel Hempstead, U.K.
Adenovirus:	
Adβ-gal:	**Gift**: C.Kontos, Duke University, U.S.A.
AdHO-1:	**Gift**: J.Alam, Louisiana State University, U.S.A.
AdBVR:	Vector Biolabs, Philadelphia, U.S.A.
α-defensins ELISA	Hycult Biotech, Uden, Netherlands
β-mercaptoethanol:	Sigma, Poole, U.K.
Bio-Rad protein assay:	Bio-Rad, Hemel Hempstead, U.K.
Bovine serum albumin:	Sigma, Poole, U.K.
Bromophenol blue:	Sigma, Poole, U.K.
Calcein AM Fluorescent dye	BD Biosciences, Oxford , U.K.
Calprotectin (MRP8/14) ELISA	BMA Biomedicals, Switzerland
Cell scrapers:	Sarstedt, Leics, U.K.

Cisplatin	Sigma, Poole, U.K.
Collagenase A:	Boehringer Mannheim, Sussex, U.K.
DABCO:	Sigma, Poole, U.K.
DAKO StreptABC Complex/HRP Duet kit:	Dako, Denmark.
Decon-90:	Phillip Harris, Staffs, U.K.
Dextran	Sigma, Poole, UK.
Diaminobenzidine:	Sigma. Poole, U.K.
DMEM:	ICN, Basingstoke, U.K.
DMEM (phenol red free):	GibcoBRL, Paisley, UK.
DMSO:	Sigma, Poole, U.K.
DPEX mounting medium:	BDH, Poole, U.K.
DTT:	Pharmacia, Herts., U.K.
ECL detection kit:	Amersham, Buckinghamshire, U.K.
EDTA:	Sigma, Poole, U.K.
Endothelial cell growth supplement	Sigma, Poole, U.K.
EGF:	Peprotech, London, U.K.
Ethanol (99.7-100%):	BDH, Poole, U.K.
Ethyl acetate:	BDH, Poole, U.K.
Fibronectin ELISA	American Diagnostica, CT, U.S.A.

Foetal calf serum:	GibcoBRL Life Technologies, Scotland.
Formaldehyde:	Sigma, Poole, U.K.
Gelatin:	Sigma, Poole, U.K.
Glycerol:	Sigma, Poole, U.K.
Glycine:	CN Biosciences, Nottingham, U.K.
Goat serum:	Sigma, Poole, U.K.
HAMS-F12:	ICN, Basingstoke, U.K.
Hanks buffered saline solution HBSS:	Sigma, Poole, U.K.
Hematoxylin	Vector, Burlingame, CA, U.S.A.
Hybond ECL nitrocellulose membrane:	Amersham Int., Buckinghamshire, U.K.
Hydrogen chloride:	Sigma, Poole, U.K.
Hydrogen peroxide	JT Baker Inc., CA, U.S.A.
Human IL-6 ELISA	Pierce-Endogen, IL, U.S.A
Isopropanol:	Sigma, Poole, U.K.
Kaleidoscope pre-stained standards:	Bio-Rad, Hemel Hempstead, U.K.
Kodak, Biomax MR film:	Anachem, Luton, U.K.
L-Glutamine:	Sigma, Poole, U.K.

L-NNA:	CN Biosciences, Nottingham, U.K.
Lipopolysaccharide	Sigma, Poole, U.K.
Leupeptin:	Sigma, Poole, U.K.
M199 (Earles buffer):	Sigma, Poole, U.K.
Marvel dried milk:	Sainsbury's, U.K.
Mayer's Haematoxylin:	Sigma, Poole, U.K.
Matrigel	BD Biosciences, Oxford, UK
Methanol:	BDH, Poole, U.K.
MOPS:	Sigma, Poole, U.K.
MMP 2/9 inhibitor	Calbiochem, UK.
MTT	Sigma, Poole, U.K.
Nitrogen (industrial):	BOC, Surrey, U.K.
NP-40:	Sigma, Poole, U.K.
OCT embedding medium:	Agar Scientific, Essex, U.K.
Oxygen (industrial):	BOC, Surrey, U.K.
Penicillin:	GibcoBRL Life Technologies, Scotland.
Percoll	Sigma, Poole, U.K.
Phenylmethylsulfonyl fluoride (PMSF):	Sigma, Poole, U.K.

Phosphatase Inhibitor Cocktail I:	Sigma, Poole, U.K.
Phosphatase Inhibitor Cocktail II:	Sigma, Poole, U.K.
Phosphate buffered saline tablets (PBS):	Sigma, Poole, U.K.
PlGF Duoset ELISA:	R&D systems, Abingdon, U.K.
PlGF Quantikine ELISA	R&D systems, Minneapolis, U.S.A.
Potassium chloride:	Sigma, Poole, U.K.
Protease Inhibitor Cocktail:	Sigma, Poole, U.K.
Resveratrol	Sigma, Poole, U.K.
RNA easy columns	Qiagen, West Sussex, UK
Scott's solution:	Sigma, Poole, U.K
sEng Duoset ELISA:	R&D systems, Abingdon, U.K.
sEng Quantikine ELISA	R&D systems, Minneapolis, U.S.A.
sFlt-1 Duoset ELISA	R&D systems, Abingdon, U.K.
sFlt-1 Quantikine ELISA	R&D systems, Minneapolis, U.S.A.
Sodium acetate:	Sigma, Poole, U.K.
Sodium chloride:	Sigma, Poole, U.K.
Sodium citrate:	Sigma, Poole, U.K.
SDS:	Sigma, Poole, U.K.
Sodium hydroxide:	Sigma, Poole, U.K.

Sodium iodide:	Sigma, Poole, U.K.
Sodium nitrite:	Sigma, Poole, U.K.
Special Gases:	
0% O_2, 5% CO_2, bal N_2:	Air products, U.K.
1% O_2, 5% CO_2, bal N_2:	Air products, U.K.
5% O_2, 5% CO_2, bal N_2:	Air products, U.K.
20% O_2, 5% CO_2, bal N_2:	Air products, U.K.
40% O_2, 5% CO_2, bal N_2:	Air products, U.K.
Streptomycin:	GibcoBRL Life Technologies, Scotland.
sVEGFR-1 Duoset ELISA:	R&D systems, Abingdon, U.K
SYBR green	Quantace, London, UK
TCA:	Sigma, Poole, U.K.
TEMED:	Bio-Rad, Hemel Hempstead, U.K.
Triethanolamine:	Sigma, Poole, U.K.
Tris:	CN Biosciences, Nottingham, U.K.
TRITC-phalloidin:	Sigma, Poole, U.K.
Triton X-100:	Sigma, Poole, U.K.
Trypsin/EDTA:	Sigma, Poole, U.K.

Tween-20:	Sigma, Poole, U.K.
$VEGF_{165}$:	RELIATech, Brauschweig, Germany.
Whatman 3 MM paper:	Whatman, Kent, U.K.
Wortmannin:	Calbiochem, Nottingham, U.K.
Xylene:	JT Baker Inc., CA, U.S.A.

APPENDIX II

Equipment and Suppliers

Cell culture Pipettes (5ml and 10 ml):	Fahrenheit Lab Supplies, U.K.
Centrifuge (Sigma 2K 15):	Sigma, Poole, U.K
Class II cell culture cabinets:	Triple Red, Oxfordshire, U.K.
Conical tubes (15 ml):	GibcoBRL, Paisley, U.K
Coverglass:	Surgipath, St Neots, U.K
Cryovials:	GibcoBRL, Paisley, U.K
Developing Cassettes:	Amersham Int., Buckinghamshire, U.K
Disposable Scalpels:	Appleton Woods, Birmingham, U.K
Eppendorfs:	Sarstedt, Leicester, U.K
Falcon tubes (14 and 50 ml):	Falcon/BDH, Poole, U.K
Filter units (swinnex 47 and 22):	Millipore, Hertfordshire, U.K
Filters (0.22 mm):	Millipore, Hertfordshire, U.K
Flasks (25 and 80 cm^2):	GibcoNUNC, Paisley, Scotland
Gilson pipettes:	Anachem, Luton, U.K
Gilson tips (blue):	Appleton Woods, Birmingham, U.K

Gilson tips (yellow):	Sarstedt, Leicester, U.K
Glass Pasteur pipettes 9":	Fisher Scientific, Loughborough, U.K.
Glassware:	Phillip Harris Scientific, U.K.
Horizontal gel electrophoresis system:	GibcoBRL Life Technologies, Scotland
Intensifying screens:	Amersham, Buckinghamshire, U.K
Micro-centrifuge:	Phillip Harris Scientific, U.K.
Microscope slides (Superfrost):	Surgipath, St Neots, U.K
Mini sub DNA gel:	Bio-Rad, Hemel Hempstead, U.K.
Mini-monitor (900):	Mini-instruments, Essex, U.K
Modular Incubators:	ICN, Basingstoke, U.K.
Multiwell Plates (6, 12 and 24-wells):	Fahrenheit Lab Supplies, U.K.
NOA 270/280B Analyser:	Analytix, Durham, U.K.
Orbital shaker:	Phillip Harris Scientific, U.K.
Petri dishes (30 and 90 mm^2):	GibcoBRL Life Technologies, Scotland.
pH meter:	Corning costar, High Wycomb, U.K.
Polytron Homogeniser PT1200:	Phillip Harris Scientific, U.K.

Rotary shaker (R100):	Luckham, Basingstoke, U.K
Round petri dishes:	Fahrenheit Lab Supplies, U.K.
Scintillation counter:	Canberra Packard, Pangbourne, U.K.
Shaking water bath:	Grant Instruments, Cambridge, U.K
Sonicator (T460):	Camlab, Cambridge, U.K.
Spectrophotometer 8452A:	Hewlett Packard, Bracknell, U.K.
Spinmix:	Sanyo-Gallenkamp, Leicester, U.K.
Square petri dishes:	Fahrenheit Lab Supplies, U.K.
Syringes (1ml - 50 ml):	Appleton Woods, Birmingham, U.K

Transfer-blot electrophoresis transfer cell: Bio-Rad, Hemel Hempstead, U.K.

Universals (30 ml):	Phillip Harris Scientific, U.K.
Vertical gel electrophoresis units:	Bio-Rad, Hemel Hempstead, U.K.
Water-Jacketed Incubator:	Sanyo-Gallenkamp, Leicester U.K.
Weight Balance:	Sartonius Limited, Surrey, U.K

APPENDIX III

Solutions and Buffers

Antibiotics:	100 μg/ml Streptomycin, 100 μg/ml Penicillin.
4% Dextran solution	4 g of Dextran in 96 ml of 0.9% NaCl
Formaldehyde gel-loading buffer:	50% Glycerol, 1 mM EDTA, 0.25% Bromophenol blue, 0.25% Xylene Cyanol FF
Mops buffer 10x:	200 mM MOPS, 50 mM Sodium acetate, 10 mM EDTA pH 7.0
90% Percoll solution	9 ml Percoll, 1 ml 9% NaCl
75% Percoll solution	4 ml 90% Percoll, 1 ml 0.9% NaCl
56% Percoll solution	14 ml 90% Percoll, 11 ml 0.9% NaCl
4% Polyacrylamide gels:	2.5 ml of a 38% acrylamide, 2% bis-acrylamide solution, 12 g urea, 2.5 ml 10 x TBE in a final volume of 25 ml in ultrapure water, 150 μl 10% APS and 25 μl temed
Phosphate buffered saline:	5 Phosphate buffered saline tablets into 1000 ml *d*.H_2O results in pH 7.4, 10mM $NaPO_4$, 2.7mM KCl, 127mM NaCl

Resolving gel 7.5%:	3.64 ml of 40% Acrylamide, 2 ml of 2% Bis/Acryl, 5 ml of 1 M Tris, 4.06 ml *d*.H_2O, 5 ml of 0.4% Gelatin solution, 10 µl TEMED, 100 µl 10% Ammonium persulphate
RIPA buffer	50mM Tris Hcl pH7.4, 1% IGEPAL (NP-40), 0.25% Na deoxycholate, 150nM NaCl, 1mM EGTA, 1mM PMSF 1µg/ml aprotinin, pepstatin, leupeptin, 1mM Na_3VO_4, 1mMNaF
Running buffer:	0.05% Tris, 0.384 M Glycine, 0.1% SDS
Sample buffer 2X:	0.02 M tris-HCL pH 8, 2mM EDTA, 2% SDS, 10% Mercaptoethanol 20% Glycerol Bromophenol Blue to colour
Sample buffer 4x:	0.04 M Tris, 4 mM EDTA, 4% SDS, 40% Glycerol, 0.02% Bromophenol blue
Separating buffer:	1.875 M Tris, 0.5% SDS, pH 8.8
Separating gel:	4.55 ml 40% Acrylamide, 2.5 ml Bis-Acryl, 3.75 ml Separating buffer, 7.6 ml *d*.H_2O, 19 µl TEMED, 188 µl 10% Ammonium persulphate
Solubilisation solution:	1 mM bicarbonate buffer pH 7.6, 1 mM EDTA, 0.01% Triton X-100

Stacking gel buffer:	0.625M Tris, 0.5% SDS, pH 6.8
Stacking gel:	1.5 ml of 40% Acrylamide, 0.8 ml Bis-Acryl, 3 ml Stacking buffer, 9.6 ml *d*.H_2O, 15 ml TEMED, 150 ml Ammonium persulphate.
Transfer buffer:	190mM Glycine, 25mM Tris, 40% Methanol.
TTBS:	0.1M Tris, 0.3M NaCl, 0.1% Tween-20 pH *7.5*.

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Hepatitis C Virus Infection Reduces Hepatocellular Polarity in a Vascular Endothelial Growth Factor–Dependent Manner

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BACKGROUND & AIMS: Hepatitis C virus (HCV) infection leads to progressive liver disease, frequently culminating in fibrosis and hepatocellular carcinoma. The mechanisms underlying liver injury in chronic hepatitis C are poorly understood. This study evaluated the role of vascular endothelial growth factor (VEGF) in hepatocyte polarity and HCV infection. **METHODS:** We used polarized hepatoma cell lines and the recently described infectious HCV Japanese fulminant hepatitis (JFH)-1 cell culture system to study the role of VEGF in regulating hepatoma permeability and HCV infection. **RESULTS:** VEGF negatively regulates hepatocellular tight junction integrity and cell polarity by a novel VEGF receptor 2–dependent pathway. VEGF reduced hepatoma tight junction integrity, induced a re-organization of occludin, and promoted HCV entry. Conversely, inhibition of hepatoma expressed VEGF with the receptor kinase inhibitor sorafenib or with neutralizing anti-VEGF antibodies promoted polarization and inhibited HCV entry, showing an autocrine pathway. HCV infection of primary hepatocytes or hepatoma cell lines promoted VEGF expression and reduced their polarity. Importantly, treatment of HCV-infected cells with VEGF inhibitors restored their ability to polarize, showing a VEGF-dependent pathway. **CONCLUSIONS: Hepatic polarity is critical to normal liver physiology. HCV infection promotes VEGF expression that depolarizes hepatoma cells, promoting viral transmission and lymphocyte migration into the parenchyma that may promote hepatocyte injury.**

Keywords: VEGF; Tropism; HCC; Angiogenesis.

Hepatitis C virus (HCV), the sole member of the Hepacivirus genus in the Flaviviridae, poses a global health burden with an estimated 170 million infected individuals. The acute phase of infection is often subclinical and a majority of individuals develop persistent infection with progressive liver pathology, frequently culminating in fibrosis and hepatocellular carcinoma (HCC). HCV infection is the leading indication for liver transplantation in many parts of the world. The mechanisms underlying liver injury in HCV infection are poorly understood with 2 nonexclusive models being proposed. The "immunopathogenic" model argues that disease is largely mediated by the host immune response, whereas the "cytopathic" model suggests that HCV replication and protein expression may induce cell injury.[1] The recent discovery that the Japanese fulminant hepatitis (JFH)-1 strain of HCV can replicate and release infectious particles in cultured cells (HCVcc)[2–4] allows studies to assess the effect(s) of virus replication on hepatocellular properties.

HCV has a short positive sense RNA genome encoding 3 structural, Core, E1, and E2 glycoproteins, and 7 nonstructural proteins (p7, NS2–NS5).[5] The E1E2 glycoproteins interact with cell surface receptors to facilitate particle entry via low pH and clathrin-dependent endocytosis.[6] Recent evidence suggests that a number of host cell molecules are important for HCV entry: tetraspanin CD81, scavenger receptor class B member I, and several members of the tight junction (TJ) protein family including Claudin-1, -6, and -9,[7] and occludin.[8] Recent data from our laboratory show that hepatoma polarity limits HCV entry, suggesting that agents that disrupt hepatocyte permeability may promote HCV infection.[9]

Vascular endothelial growth factor (VEGF) originally was discovered for its effect(s) on endothelial cell permeability.[10] The critical role of VEGF in pathologic angiogenesis has lead to the development and clinical testing of VEGF inhibitors to limit tumor growth.[11] However, recent research suggests a diversity of roles for VEGF in maintaining normal adult tissue.[12,13] We show a role for VEGF in regulating hepatocyte TJ integrity, polarity, and permissivity to HCV infection. Neutralization of endogenous HepG2-expressed VEGF promotes polarization and significantly inhibits HCV entry, confirming that an autocrine pathway is in operation. HCV infection increases primary hepatocyte and hepatoma VEGF expres-

Abbreviations used in this paper: BC, bile canaliculi; CLDN1, claudin-1; CMFDA, 5-chloromethylfluorescein diacetate; HCVcc, cell culture-derived HCV; HCVpp, HCV pseudotype particles; IFN, interferon; JFH, Japanese fulminant hepatitis; MRP2, multidrug-resistant protein 2; PHH, primary human hepatocytes; TJ, tight junction; VEGF-A, vascular endothelial growth factor A; VEGFR, vascular endothelial growth factor receptor; ZO-1, zona occludens-1.

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sion, which reduces their polarity. Importantly, VEGF antagonists restore the ability of infected hepatoma cells to polarize. In summary, our data support a model in which HCV up-regulation of VEGF expression induces a localized disruption of hepatocellular TJs that promotes viral transmission in the liver, providing a potential therapeutic opportunity for the use of VEGF antagonists to treat chronic hepatitis C infection.

Materials and Methods

Cell Lines and Antibodies

HepG2 and Huh-7.5 cells (C. Rice, Rockefeller University, NY) were propagated in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1% nonessential amino acids. WIF-B9 cells (D. Cassio, Centre National de la Recherche Scientifique, Paris, France) were maintained in Coon's F12 media supplemented with 5% fetal bovine serum, 1% nonessential amino acids and hypoxanthine, aminopterin, and thymidine. Primary human hepatocytes (PHH) were isolated and cultured as previously reported.[14] Liver sinusoidal endothelial cells were cultured in endothelial basal media supplemented with 10% human serum and human growth factor (Peprotech, London, UK). All cells were maintained at 37°C, 5% CO_2, with the exception of WIF-B9 cells that require 7% CO_2. HepG2 cells expressing CD81, AcGFP.CD81, and AcGFP.claudin-1 (CLDN1) were generated by lentiviral transduction as previously described.[15]

The following primary antibodies were used: anti–multidrug-resistant protein 2 (MRP2) (M2 III-6; Abcam, Cambridge, UK); anti-NS5A 9E10 (C. Rice, Rockefeller University); anti-CD81 (2s131); anti-occludin (Zymed, San Francisco, CA); anti-CLDN1 JAY.8 (Invitrogen, Carlsbad, CA); anti-CLDN1 1C5-D9 (Novus, Littleton, CO), and anti–zona occludens-1 (ZO-1) (Zymed). Secondary labeled antibodies: Alexa 488 goat anti-mouse immunoglobulin (Ig)G; Alexa 488 goat anti-rabbit IgG, Alexa 633 goat anti-mouse IgG, and Alexa 633 goat anti-rabbit IgG were purchased from Invitrogen.

Pharmacologic Treatments and Compounds

HepG2 cells were seeded at $4–6 \times 10^4/cm^2$ on tissue culture plastic or glass coverslips and allowed to polarize for 3 days before incubating with the following treatments at nontoxic concentrations: recombinant human VEGF-A, placental-induced growth factor, interferon-γ (IFNγ) (Peprotech) and VEGF-E (RELIATech, Wolfenbüttel, Germany); neutralizing anti-VEGF monoclonal antibody VG76e;[16] Sorafenib, a small molecular inhibitor of tyrosine protein kinases (D. Palmer, University of Birmingham, UK); VEGFR-2 kinase inhibitor Ki8751 (Calbiochem, Nottingham, UK); and anti-VEGF monoclonal antibody 2c3 (R. Brekken, UT Southwestern Medical Center) that inhibits VEGFR-2 activation.[17] Human VEGF was measured by enzyme-linked immunosorbent assay following the suppliers recommended instructions (Peprotech).

Cell Polarity Determination

HepG2 and WIF-B9 cells were allowed to grow for 3 and 11 days, respectively, to polarize before fixing with 3% paraformaldehyde for 30 minutes at room temperature. Cells were permeabilized with 0.1% Triton/0.5% bovine serum albumin in phosphate-buffered saline (PBS) and stained with anti-MRP2 and Alexa-Fluor 488 goat anti-mouse. Nuclei were visualized using 4′, 6′-diamidino-2-phenylindole (Invitrogen) and their polarity index was determined by counting the number of MRP2-positive apical structures per 100 nuclei using a Nikon Eclipse TE2000-S fluorescence microscope (Nikon, Tokyo, Japan).

Determination of TJ Barrier Function

To determine the functionality of TJs and whether they restrict paracellular diffusion of solutes from the bile-canalicular (BC) lumen to the basolateral medium (barrier function), HepG2 cells were incubated with 5 mmol/L 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen) at 37°C for 10 minutes to allow translocation to the BC lumen. After washing extensively with PBS, the capacity of BC to retain CMFDA was enumerated using a fluorescence microscope. WIF-B9 cells were treated with 5 mmol/L fluorescein diacetate (Sigma, Poole, UK) at 37°C for 10 minutes before washing and quantifying the frequency of BC retaining fluorescein diacetate.

HCV Pseudotype Particles Generation and Infection

Pseudoviruses were generated by transfecting 293T cells with plasmids encoding a human immunodeficiency virus provirus expressing luciferase and HCV strain H77 E1E2 region, the murine leukemia virus envelope, or a no-envelope control, as previously described.[18] Virus-containing media were added to target cells for 4 hours, unbound virus was removed, and the media was replaced with Dulbecco's modified Eagle medium/3% fetal bovine serum. At 72 hours postinfection the cells were lysed, luciferase substrate was added, and luciferase activity was measured for 10 seconds in a luminometer (Lumat LB 9507; Berthold, Bad Wildbad, Germany). Specific infectivity was calculated by subtracting the mean no-envelope control pseudotype particle relative light unit signal from the HCV pseudotype particle (HCVpp) or murine leukemia virus pseudotype particle signals. Infectivity was presented relative to untreated control cells (ie, the mean luciferase value of the replicate untreated cells was defined as 100%).

HCVcc Generation and Infection

J6/JFH virus was generated as previously described.[2] Briefly, RNA was transcribed in vitro from full-

length genomes using the Megascript T7 kit (Ambion, Austin, TX) and electroporated into Huh-7.5 cells. At 72 and 96 hours after electroporation supernatants were collected and stored immediately at −80°C. Virus-containing media were added to target cells plated as described earlier and infected cells were detected by methanol-fixation and staining for NS5A with monoclonal antibody 9E10 and Alexa-488 anti-mouse IgG. Infection was quantified by enumerating $NS5^+$ foci and infectivity was defined as the number of focus forming units/mL.

Immunoprecipitation and Western Blotting

Polarized HepG2 cells were harvested in lysis buffer (PBS, 1% Brij97, 20 mmol/L Tris [pH 7.5], 300 mmol/L NaCl, 2 mmol/L $CaCl_2$, and 2 mmol/L $MgCl_2$) containing protease and phosphatase inhibitors (Roche, Burgess Hill, UK). Lysates were clarified by centrifugation (20,000*g*, 10 min), precleared with Protein G–Sepharose (GE Healthcare, Little Chalfont, UK), and 100 μg was incubated with Protein G–Sepharose beads precoated with anti-occludin or irrelevant isotype-matched control antibody at 4°C for 90 minutes. The beads were collected by centrifugation, washed thoroughly in lysis buffer, and the precipitated proteins eluted with Laemmli buffer. Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with anti-occludin, antiphosphoserine, antiphosphothreonine, or antiphosphotyrosine (Millipore, Watford, UK) (1 μg/mL) and horseradish-peroxidase–conjugated anti-rabbit or anti-mouse IgG antibodies. Proteins were detected by enhanced chemiluminescence (Geneflow, Fradley, UK).

Quantification of CD81, Claudin-1, and Occludin Localization

Parental HepG2 cells and those transduced to express AcGFP tagged CD81 and CLDN1 were allowed to polarize on glass coverslips, fixed in ice-cold methanol, and TJs were localized by staining for occludin and ZO-1 as previously reported.[9] Coverslips were mounted on glass slides and viewed on a Zeiss Meta Head Confocal Microscope with a 63× water immersion objective (Zeiss, Jena, Germany). Cells were imaged with the microscope settings optimized for each fluorescent protein to obtain the highest signal-to-noise ratio. Protein expression at the basolateral, cytoplasmic, and TJ locations (arbitrary fluorescence units/pixel) provides 500–1000 measurements per cell. The data from 10 cells were normalized, and the localized expression was calculated.

Statistical Analysis

Results are expressed as the mean ± 1 standard deviation of the mean, except where stated to the contrary. Statistical analyses were performed using the Student *t* test in Prism 4.0 (GraphPad, San Diego, CA) with a *P* value of less than .05 considered statistically significant.

Results

VEGF Regulates Hepatocellular TJ Integrity and Polarity

Hepatocytes in the liver are polygonal and multipolar, with at least 2 basal surfaces facing the circulation and a branched network of grooves between adjacent cells constituting the apical or BC surface. A majority of hepatocyte-derived cell lines and primary hepatocytes dedifferentiate in culture and fail to develop a polarized phenotype.[19] Human HepG2 and human–rat hybrid WIF-B9 hepatoblastoma cell lines polarize and develop apical cysts that are equivalent to BC in the liver. Polarization can be quantified by enumerating MRP2-positive BC per 100 nuclei (polarization index) and TJ integrity can be assessed by determining the frequency of BC retaining CMFDA or fluorescein diacetate (Figure 1*A*). VEGF-A induced a dose-dependent decrease in HepG2 and WIF-B9 polarity and TJ integrity (Figure 1*B* and *C*). Because VEGF is expressed in many tumor-derived cell lines, we were interested to investigate whether HepG2 or WIF-B9 cells express VEGF and whether endogenous protein can act in an autocrine manner to regulate cell polarity. Both cell lines express VEGF (Figure 1*D*) and neutralization of endogenous VEGF with anti-VEGF VG76e or receptor kinase antagonist Sorafenib for 24 hours promoted HepG2 polarization (Figure 1*E*). Similar data were observed after inhibitor treatment of WIF-B9 cells (data not shown). Neutralization of endogenous HepG2-expressed VEGF had a more significant effect on polarity than expected from the recombinant VEGF-A dose-response titrations (Figure 1*B*), which may represent differences between extracellular and cell-bound forms of VEGF that will not be measured accurately by enzyme-linked immunosorbent assay or altered expression of VEGF splice variants in hepatoma cells. To ascertain whether PHHs express VEGF we isolated cells from 2 independent donors and quantified soluble VEGF levels. PHHs expressed VEGF at comparable levels with WIF-B9, whereas liver sinusoidal endothelial cells failed to secrete detectable levels of VEGF (Figure 1*D*). PHHs failed to polarize sufficiently (<3% polarization index) to analyze the effect(s) of VEGF inhibitors on TJ integrity.[9]

VEGF acts through binding 2 high-affinity tyrosine kinase receptors, VEGF receptor (VEGFR)-1 (Fms-like tyrosine kinase 1) and VEGFR-2 (fetal liver kinase-1/kinase insert domain receptor). To investigate the receptor dependency of VEGF-mediated effects on hepatocellular TJs, HepG2 cells were pretreated with the broad-spectrum inhibitor Sorafenib or VEGFR-2 antagonist Ki8751[20] for 24 hours followed by VEGF-A or IFNγ for 1 hour. Treating HepG2 cells with VEGF for a short time period of 1 hour reduced TJ integrity by 25%–40%. Pretreatment with both inhibitors ablated the effects of VEGF-A on TJ integrity, while having no detectable ef-

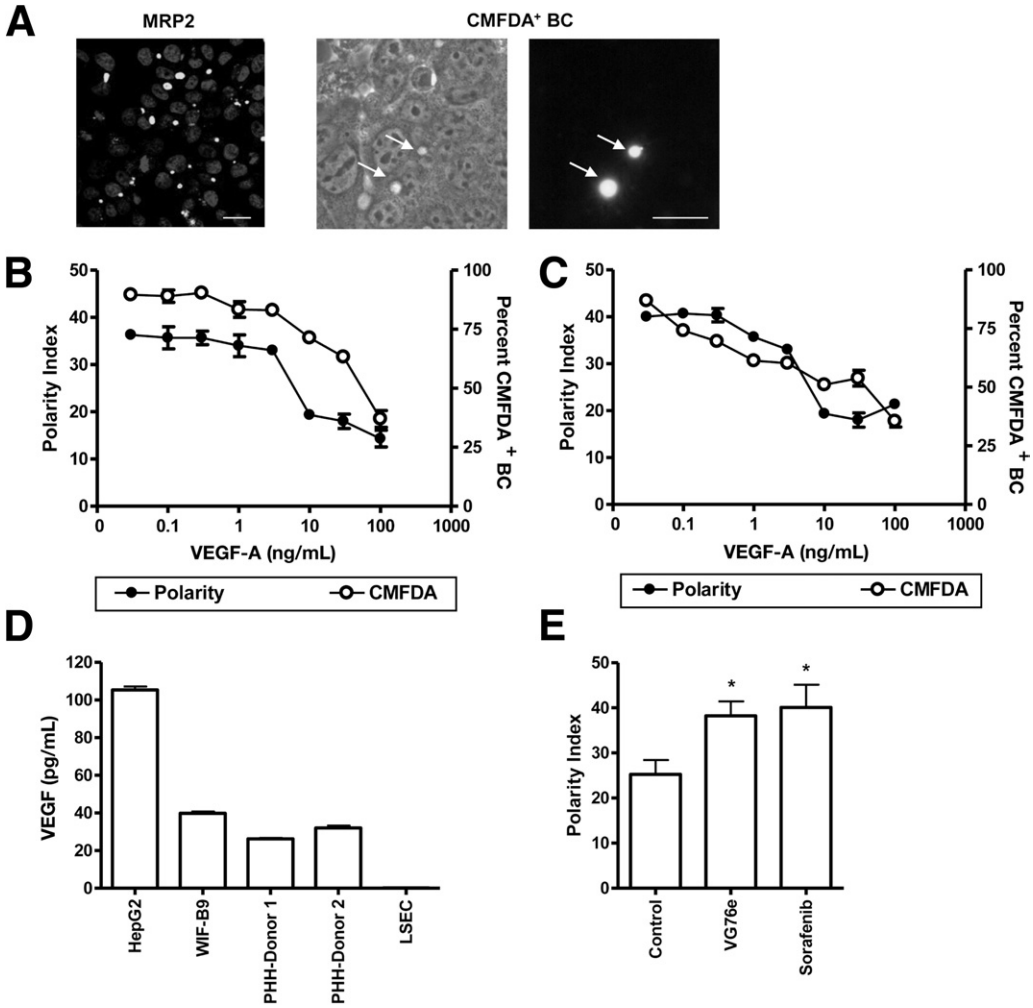

Figure 1. VEGF regulates hepatocellular TJ integrity and polarity. (*A*) HepG2 polarity was quantified by fixing the cells in 3% paraformaldehyde and staining for the BC marker MRP2. Enumerating the frequency of MRP2$^+$ BC per 100 stained nuclei using CMFDA (4′, 6′-diamidino-2-phenylindole) enabled us to determine a polarity index. TJ barrier function was measured by quantifying the frequency of BC retaining CMFDA. Representative images depict apical expressed MRP2 and BC annotated with *arrows* and their retention of fluorescent CMFDA. Scale bar, 10 μmol/L. (*B*) HepG2 cells were allowed to polarize for 3 days and treated with VEGF-A for 24 hours. Treated and untreated cells were stained for MRP2 to quantify their polarity or incubated with CMFDA to measure TJ barrier function. Polarity index and TJ integrity were determined by quantifying the number of MRP2$^+$ BC per 100 nuclei that retained CMFDA in 5 fields of view on 3 replicate coverslips. (*C*) WIF-B9 cells were grown for a minimum of 11 days to develop hepatic polarity and treated with VEGF-A for 24 hours; polarity and TJ integrity were assessed as described earlier. (*D*) HepG2, WIF-B9, and PHHs from 2 independent donors, and liver sinusoidal endothelial cells (LSEC) were plated and the extracellular media was collected from 100,000 cells over a 24-hour time period and assessed for VEGF using a human VEGF enzyme-linked immunosorbent assay. (*E*) HepG2 cells were allowed to polarize for 3 days and were pre-incubated in serum-free Dulbecco's modified Eagle medium for 4 hours before treating with control dimethyl sulfoxide, neutralizing anti–VEGF-A antibody VG76e (1.3 mg/mL), or receptor kinase antagonist Sorafenib (10 nmol/L) for 24 hours. Cells were fixed and their polarity index was measured. *$P < .01$ (*t* test).

fect(s) on IFNγ-induced depolarization, suggesting a VEGFR-2–dependent pathway (Figure 2*A*). Furthermore, incubation of VEGF-A with a 5-fold molar excess of anti-VEGF monoclonal antibody 2c3, which blocks VEGF association with VEGFR-2,[17] abrogated the effect of VEGF-A but not IFNγ on HepG2 TJ integrity (Figure 2*B*). Finally, when HepG2 cells were treated with VEGF-E, a specific VEGFR-2 ligand, we noted a reduction in TJ integrity whereas the VEGFR-1 selective ligand placental-induced growth factor had no effect, confirming that VEGF regulates TJ integrity via a VEGFR-2–dependent pathway (Figure 2*D*).

VEGF Regulates Hepatocellular Permissivity for HCV Entry

We previously reported that polarization of HepG2-CD81 limits HCV entry.[9] To study the effects of VEGF on HCV infectivity, HepG2 cells were allowed to polarize over 3 days and were treated with VEGF-A or VEGF-E and challenged with HCV J6/JFH. Both treatments led to a significant increase in HCV infectivity (Figure 3*A*) and concomitant decrease in TJ integrity. VEGF-A had no effect on HCV-RNA replication in HepG2 or Huh-7.5 cells (data not shown). To ascertain

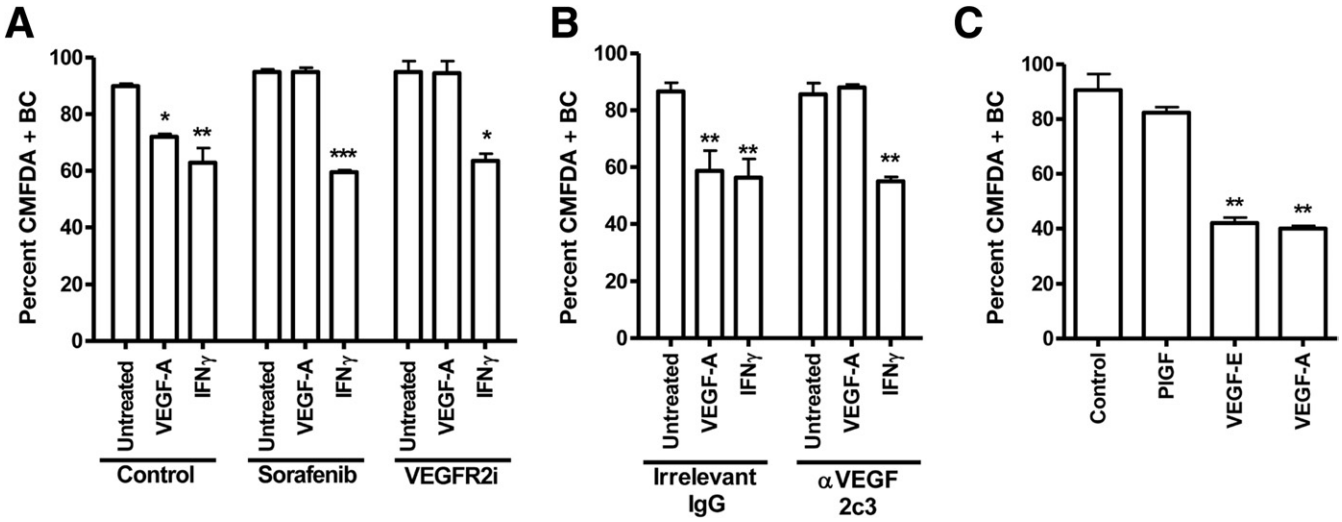

Figure 2. VEGF regulation of hepatoma TJ integrity and polarity is VEGFR-2–dependent. HepG2 cells were allowed to polarize for 3 days, incubated in serum-free Dulbecco's modified Eagle medium for 4 hours followed by: (*A*) control dimethyl sulfoxide, Sorafenib (10 nmol/L), or VEGFR-2 antagonist Ki8571 (10 nmol/L) for 24 hours, followed by treatment with VEGF-A (10 ng/mL) or IFNγ (10 ng/mL) for 1 hour; (*B*) VEGF-A (10 ng/mL) or IFNγ (10 ng/mL) pretreated with irrelevant IgG (5 mg/mL) or anti–VEGF 2c3 (5 mg/mL) for 1 hour, or (*C*) treated with mock (control), placental-induced growth factor (PlGF) (50 ng/mL), VEGF-E (50 ng/mL), or VEGF-A (50 ng/mL) for 1 hour. TJ barrier function was assessed by quantifying the number of $CMFDA^+$ BC in a minimum of 5 fields of view on 3 replicate coverslips, where $^*P < .01$, $^{**}P < .001$, $^{***}P < .0001$ (*t* test).

whether VEGF modulates HCV entry, we studied HCVpp infection, which measures HCV glycoprotein specific–receptor dependent internalization. VEGF-A and VEGF-E promoted a significant increase in HCVpp entry but had no detectable effect on murine leukemia virus pseudotype particle infection, confirming VEGF modulation of an HCV glycoprotein-specific pathway (Figure 3*A*). To investigate whether endogenous VEGF affects HepG2 permissivity to support HCV replication, polarized HepG2-CD81 cells were treated with VG76e or Sorafenib for 16 hours and challenged with HCVcc J6/JFH and HCVpp. Both inhibitors significantly reduced HCVcc and HCVpp infection, with no detectable effect on murine leukemia virus pseudotype particle entry (Figure 3*B*).

We recently reported that HCV can transmit by cell-free particle infection of naive target cells and by cell-to-cell transfer.[21] To assess the role of cell polarity and VEGF in HCV transmission, we monitored the frequency of HCV-infected HepG2-CD81 over time in comparison with the permissive Huh-7.5 cell line. We previously reported that HepG2-CD81 cells are approximately 700-fold less permissive at supporting HCV replication than

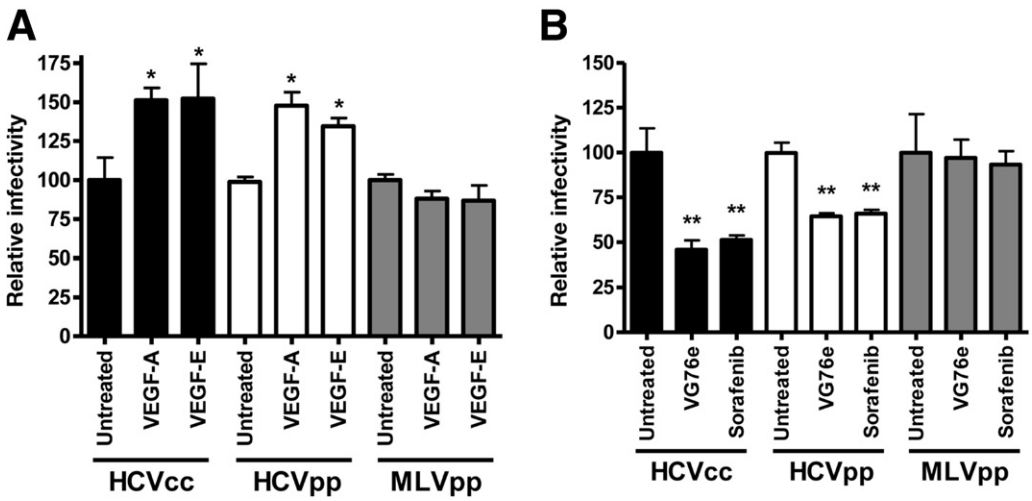

Figure 3. VEGF regulates HCV entry into polarized HepG2 cells. (*A*) HepG2-CD81 cells at 3 days postplating were untreated or treated with VEGF-A (10 ng/mL) or VEGF-E (10 ng/mL) for 1 hour and challenged with HCVcc J6/JFH (*black bars*), HCVpp (*white bars*), or murine leukemia virus pseudotype particles (MLVpp) (*gray bars*). $^*P < .01$ (*t* test). (*B*) HepG2-CD81 cells at 3 days postplating were untreated or treated with neutralizing anti–VEGF-A antibody VG76e (1.3 mg/mL) or receptor kinase antagonist Sorafenib (10 nmol/L) for 16 hours and challenged with HCVcc J6/JFH (*black bars*), HCVpp (*white bars*), or MLVpp (*gray bars*). Infectivity is expressed relative to control. $^{**}P < .001$ (*t* test).

Huh-7.5 cells.[9] Thus, reduced levels of virus inocula were used to infect Huh-7.5 cells in this comparative transmission experiment. The number of NS5A-expressing HepG2-CD81 cells in the culture remained constant after 48 and 96 hours postinfection (167 ± 7.0 at 48 hours to 186 ± 7.4 at 96 hours). In contrast, the number of NS5A-expressing Huh-7.5 cells increased from 579.5 ± 19.5 to 875 ± 27 between 48 and 96 hours postinfection. Similarly, the levels of HCV RNA remained constant in HepG2-CD81 cells. In summary, these data show that HCV can initiate a primary infection of HepG2-CD81 cells, however, there are no second-round infection events, which most likely is explained by the low permissivity of HepG2 cells to support HCV-RNA replication and protein expression levels that may be required for particle assembly. Thus, we were unable to study the effect of VEGF antagonists on HCV transmission in polarized HepG2 cells.

HCV Infection Perturbs Hepatocellular Polarity in a VEGF-Dependent Manner

Several recent reports have suggested that HCV infection induces VEGF expression in Huh-7 cells;[22,23] we confirm and extend these observations, showing that J6/JFH infection of HepG2, Huh-7.5, and PHH induces VEGF expression (Figure 4*A*). Similar increases in VEGF expression were observed in JFH-1- and H77/JFH-infected Huh-7.5 cells (data not shown). To ascertain whether infection perturbs hepatocellular polarity, HepG2-CD81 cells were infected with J6/JFH and monitored for their ability to polarize over a 48-hour time period. Naive HepG2-CD81 cells showed a 91.4% increase in polarization over 48 hours (Figure 4*B*). HCV J6/JFH-infected cells showed a significantly reduced frequency of polarized cells after 48 hours (50.5% increase) (Figure 4*B*). This is particularly noteworthy if one considers that the frequency of NS5A-expressing HepG2-CD81 cells was less than 2% of the population, suggesting a significant bystander effect. Treatment of naive and infected HepG2-CD81 cells with VG76e or Sorafenib promoted their polarization and abrogated the depolarizing effects of HCV, confirming a VEGF-dependent pathway (Figure 4*B*).

Effect of VEGF on TJ Protein Localization

To define the mechanism(s) of VEGF-induced changes in HepG2 TJ integrity and polarity that promote HCV entry, we studied the effect(s) of VEGF-A on occludin, CLDN1, CD81, and ZO-1 localization. We previously reported that occludin and ZO-1 localize as a discrete band surrounding the BC in polarized HepG2 cells. In contrast, CLDN1 and CD81 were detected at the basolateral and apical surfaces.[9] VEGF-A treatment significantly reduced occludin expression at TJs, promoting basolateral and intracellular pools (Figure 5*A*). In contrast, there was no significant change in CLDN1, CD81, or ZO-1 localization (Figure 5*B*). Occludin, similar to many TJ proteins, is a phosphoprotein and phosphorylation/dephosphorylation of both tyrosine but predomi-

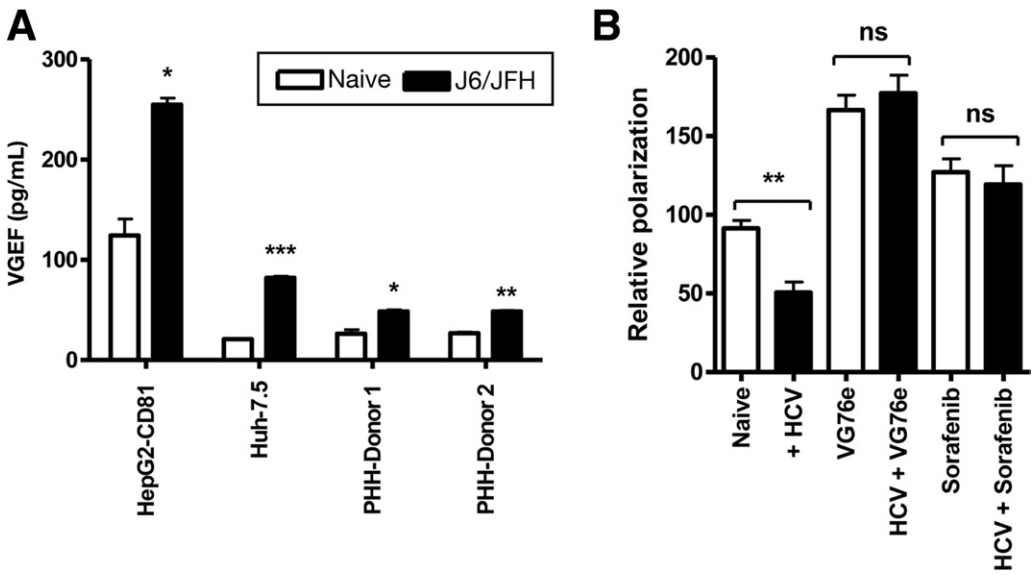

Figure 4. HCV infection increases VEGF expression, which reduces hepatoma polarity. (*A*) HepG2-CD81, Huh-7.5, and PHHs from 2 donors were inoculated with mock or HCVcc J6/JFH, pretitrated to achieve comparable levels of infection in the different target cells. Infected HepG2-CD81, Huh-7.5, and PHHs contained 2.6×10^7, 2.9×10^7, and 1.9×10^7 HCV RNA copies/10^6 cells, respectively. Extracellular media was collected from 100,000 uninfected (*white bars*) or HCV-infected cells (*black bars*) over a period of 24 hours and assessed for VEGF expression using a human VEGF-A enzyme-linked immunosorbent assay. $^*P < .01$, $^{**}P < .001$, $^{***}P < .0001$ (*t* test). (*B*) HepG2-CD81 cells were infected with mock or HCV J6/JFH for 4 hours, unbound virus was removed by extensive washing, and the cells were untreated or treated with anti–VEGF-A antibody VG76e (1.3 mg/mL) or Sorafenib (10 nmol/L) for 48 hours. Cells were fixed and the frequency of NS5A+ and polarized cells in duplicate coverslips was assessed. Less than 2% of infected HepG2-CD81 cells expressed NS5A antigen at 48 hours postinfection. Relative polarity is presented as the increase in polarized cells over 48 hours for uninfected (*white bars*) and J6/JFH-infected (*black bars*) cells. $^{**}P < .001$ (*t* test).

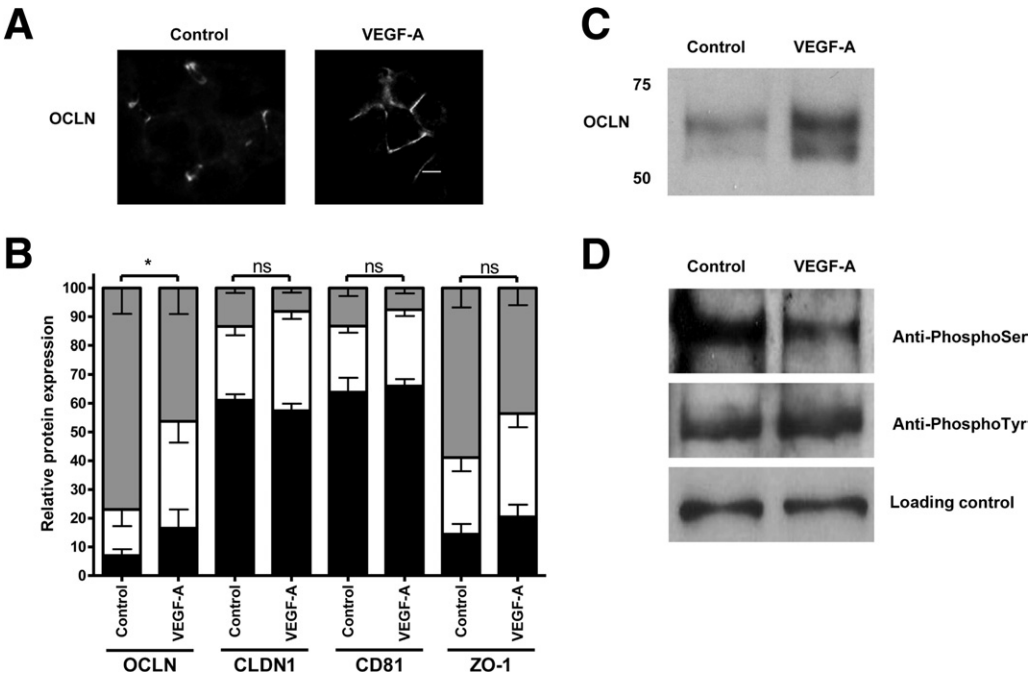

Figure 5. VEGF modulates occludin localization and phosphorylation. (*A*) HepG2 cells were allowed to polarize for 3 days and were untreated (control) or treated with VEGF-A (10 ng/mL) for 1 hour, TJ integrity was assessed, and occludin (OCLN) localization was ascertained by staining with an OCLN-specific antibody. VEGF-A reduced the frequency of $CMFDA^+$ BC by 48% and representative images show a re-organization of OCLN to basolateral membranes. (*B*) To quantify the effect of VEGF on OCLN localization and to ascertain the effect on other TJ-associated proteins and viral co-receptor CD81, polarized HepG2 cells expressing AcGFP.CLDN1 and AcGFP.CD81 were untreated (control) or treated with VEGF-A (10 ng/mL) for 1 hour and stained with anti-OCLN or anti–ZO-1. OCLN, CLDN1, CD81, and ZO-1 localization at basolateral (*black*), intracellular (*white*), and TJ (*grey*) locations was quantified. (*C*) Polarized HepG2 cells were untreated or treated with VEGF-A (10 ng/mL) for 1 hour and OCLN immunoprecipitates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting with anti-OCLN. (*D*) To determine the phosphorylation status of precipitated OCLN after VEGF-A treatment, sodium dodecyl sulfate–polyacrylamide gel electrophoresis separated proteins were probed with antiphosphoserine or antiphosphotyrosine and protein loading confirmed using anti–β-actin.

nantly serine/threonine residues, play an important role in regulating occludin localization and its subsequent role in TJ assembly.[24–26] Western blotting shows that occludin runs predominantly as a single band in lysates prepared from polarized HepG2 cells, whereas VEGF-A treatment results in a second lower molecular weight band that may represent less phosphorylated occludin (Figure 5*C*). To investigate the effects of VEGF on occludin phosphorylation, we immunoprecipitated occludin from untreated and VEGF-A–treated HepG2 cells and probed the precipitates with antiphosphoserine or antiphosphotyrosine by Western blotting. VEGF-A treatment reduced the levels of serine-phosphorylated occludin and had no effect on tyrosine-phosphorylated occludin levels (Figure 5*D*).

Discussion

We show that VEGF regulates TJ integrity and hepatocellular polarity through a VEGFR-2–dependent pathway (Figures 1 and 2). We previously reported that hepatoma polarization limits the availability of basolateral-expressed TJ protein viral receptors and reduces HCV entry,[9] hypothesizing that cytokine or growth factor–induced changes in hepatocyte permeability will promote HCV entry. Indeed, VEGF treatment of polarized HepG2 led to a significant increase in HCV entry (Figure 3*A*). Furthermore, neutralization of hepatoma-expressed VEGF with inhibitors targeting extracellular VEGF or intracellular VEGF-R kinases significantly inhibited HCV entry (Figure 3*B*). HCV infection of primary hepatocytes or hepatoma cells increased VEGF expression and reduced cell polarization. Importantly, VEGF inhibitors restored the ability of infected hepatoma cells to polarize, confirming a VEGF-dependent pathway. Hepatic polarity is critical to the function of the liver and cholestatic disease occurs when TJs, which seal the bile canaliculi, lose function, permitting leakage of proteins and bile acids between apical and basal compartments.[27–29] Cholestatic HCV disease occurs in the early posttransplant period and generally is associated with high levels of intrahepatic HCV RNA and may reflect viral-induced cytopathic injury.[30–32] The data presented in this article support a role for viral encoded proteins promoting VEGF-dependent alterations in TJ integrity and hepatocyte polarity.

VEGF initially was recognized as an endothelial-specific growth factor that regulated vascular permeability and angiogenesis. A majority of investigations studying

the role of VEGF in HCC have characterized the effect of VEGF antagonists on tumor development and metastasis. Indeed, several recent reports have shown that VEGF inhibitors significantly can increase the life expectancy of subjects with HCC.[33–35] A systematic study of VEGF expression in adult VEGF–β-galactosidase mice showed a defined subset of cells expressing VEGF, including hepatocytes in the liver.[36] Our data support a role for VEGF in regulating hepatocyte polarity, consistent with an earlier report from Schmitt et al[37] who showed a protein kinase C-α–dependent pathway underlying VEGF perturbation of HepG2 TJs. The protein kinase C dependency of this pathway is consistent with our earlier report that phorbol ester activation of protein kinase C depolarized HepG2 and increased HCV entry.[9] The observation that PHHs secrete VEGF (Figure 1) suggests that this pathway is in operation in nontumor cells; however, given the difficulties in studying primary hepatocyte polarization[19] we were unable to assess the effects of VEGF on hepatocyte polarity.

HCV infection of hepatoma cells has been reported to induce oxidative stress and calcium signaling that stabilizes hypoxia inducible growth factor 1a, a transcription factor that regulates angiogenic cytokines, including VEGF,[22,23,38,39] suggesting a role for HCV in HCC development and metastasis. Hassan et al[23] recently reported increased VEGF immunostaining of liver biopsy specimens from HCV-infected subjects, suggesting a role for HCV core protein in hepatic angiogenesis. Several oncogenic viruses, hepatitis B virus, human papillomavirus-16, and Kaposi's sarcoma herpes virus have been reported to activate hypoxia inducible growth factor 1a and promote VEGF expression, suggesting an important pathway in tumor development.[40] In addition to the angiogenic properties of increased VEGF expression, our data support a model in which HCV-infected hepatocytes secrete VEGF, which induces a localized depolarization of hepatocytes that promotes viral transmission between adjacent hepatocytes, consistent with the recent report by Liang et al[41] showing infected foci in the liver of HCV chronically infected subjects.

Proinflammatory cytokines modulate epithelia and endothelia permeability by a variety of mechanisms.[42] We previously reported that tumor necrosis factor-α and IFNγ reduced HepG2 TJ integrity and yet had no demonstrable effect on HCV entry,[9] suggesting independent pathway(s) to perturb TJ protein localization, some of which modulate HCV entry. Our recent data suggest that VEGF inhibitors will promote hepatocellular TJ integrity and reduce HCV infection of naive cells within the context of an inflamed liver and may have minimal effect(s) on cytokine-mediated changes to epithelial permeability.

VEGF has been reported to stimulate the phosphorylation/dephosphorylation of occludin via protein kinase C and mitogen-activated protein kinase pathways in a cell-type–dependent manner.[37,43,44] VEGF stimulation of brain microvessel endothelial cells leads to a dephosphorylation of occludin, disruption of TJ protein localization, and increased permeability.[45] In contrast, VEGF stimulates occludin phosphorylation at serine 490 and increases permeability of bovine retinal endothelial cells.[44,46,47] Our data show that VEGF reduces occludin localization at TJs in HepG2 cells, while having no significant effect(s) on CLDN1, CD81, or ZO-1 localization (Figure 5*A* and *B*). The VEGF-A–dependent reorganization of occludin is accompanied by an altered serine phosphorylation pattern. In summary, our data support a model in which HCV up-regulation of VEGF expression disrupts occludin localization, reduces TJ integrity, and promotes viral transmission. These data highlight a potential role for VEGF antagonists to help control HCV infection in addition to their known properties of regulating HCC growth and development.

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Conflicts of interest

The authors disclose no conflicts.

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Activation of Proteinase-Activated Receptor 2 Stimulates Soluble Vascular Endothelial Growth Factor Receptor 1 Release via Epidermal Growth Factor Receptor Transactivation in Endothelial Cells

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Abstract—The proteinase-activated receptor 2 (PAR-2) expression is increased in endothelial cells derived from women with preeclampsia, characterized by widespread maternal endothelial damage, which occurs as a consequence of elevated soluble vascular endothelial growth factor receptor-1 (sVEGFR-1; commonly known as sFlt-1) in the maternal circulation. Because PAR-2 is upregulated by proinflammatory cytokines and activated by blood coagulation serine proteinases, we investigated whether activation of PAR-2 contributed to sVEGFR-1 release. PAR-2–activating peptides (SLIGRL-NH_2 and 2-furoyl-LIGRLO-NH_2) and factor Xa increased the expression and release of sVEGFR-1 from human umbilical vein endothelial cells. Enzyme-specific, dominant-negative mutants and small interfering RNA were used to demonstrate that PAR-2–mediated sVEGFR-1 release depended on protein kinase C-β_1 and protein kinase C-ε, which required intracellular transactivation of epidermal growth factor receptor 1, leading to mitogen-activated protein kinase activation. Overexpression of heme oxygenase 1 and its gaseous product, carbon monoxide, decreased PAR-2–stimulated sVEGFR-1 release from human umbilical vein endothelial cells. Simvastatin, which upregulates heme oxygenase 1, also suppressed PAR-2–mediated sVEGFR-1 release. These results show that endothelial PAR-2 activation leading to increased sVEGFR-1 release may contribute to the maternal vascular dysfunction observed in preeclampsia and highlights the PAR-2 pathway as a potential therapeutic target for the treatment of preeclampsia. **(*Hypertension*. 2010;55:689-697.)**

Key Words: PAR-2 ■ sVEGFR-1/sFlt-1 ■ endothelium ■ factor Xa ■ HO-1 ■ preeclampsia

Preeclampsia is a pregnancy specific multiorgan syndrome characterized by widespread maternal endothelial damage with a clinical presentation of hypertension and proteinuria after 20 weeks' gestation.[1] Women with preeclampsia are at an increased risk of developing cardiovascular disease.[2] The antiangiogenic factors, soluble vascular endothelial growth factor receptor 1 (VEGFR; sVEGFR-1, also known as sFlt-1) and soluble endoglin, are increased dramatically before the clinical onset of preeclampsia.[3] Elevated sVEGFR-1 antagonizes the action of vascular endothelial growth factor and placenta growth factor resulting in impaired human placental angiogenesis[4] and glomerular endothelial cell damage, proteinuria, and hypertension in rodent models,[5] indicating that it is a major contributory factor to the development of preeclampsia. Furthermore, the anti-inflammatory enzyme heme oxygenase 1 (HO-1), which is decreased in preeclamptic placentas[6] and regulates inflammatory angiogenesis,[7] suppresses sVEGFR-1 release from endothelial cells.[8]

During placentation, the trophoblasts invade the maternal tissues but avoid immune rejection.[9] Preeclampsia is associated with a failure to switch from the T helper 1 cytokine profile (eg, interferon-γ, tumor necrosis factor [TNF]-α, interleukin [IL]-8, and IL-18) to T helper 2 cytokine profile (eg, IL-4 and IL-10), indicating a lack of immune tolerance.[10] A rise in circulating levels of proinflammatory cytokines (eg, TNF-α and IL-1β) upregulates tissue factor expression leading to activation of the coagulation system, which can result in disseminated intravascular coagulation, particularly in early onset severe preeclampsia[11] and eclampsia.[12]

The main physiological activators of the proteinase-activated receptors (PAR-1 and PAR-2) are serine proteinases, such as thrombin and factors VIIa and Xa (FXa).[13] PAR-2 plays an

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important role in inflammation and regulates vascular function.[14,15] Proinflammatory cytokines, including interferon-γ and TNF-α, induce PAR-2 expression and, in turn, PAR-2 activation promotes the production of interferon-γ, TNF-α, IL-8, and IL-18 in various cell types, including the endothelium.[16] Indeed, T-cell proliferation, interferon-γ, and IL-18 levels are significantly reduced in PAR-2 knockout mice,[16,17] whereas endotoxin-stimulated macrophages show significantly greater IL-10 expression[18] and enhanced IL-4 secretion[19] in PAR-2 null mice. PAR-2 expression is reported to be increased in human umbilical vein endothelial cells (HUVECs) derived from preeclamptic pregnancies, and the conditioned medium from preeclamptic placental villous tissue explants upregulates PAR-2 in cultured endothelial cells.[20] Although PAR-2 activity is known to be upregulated in the vasculature in inflammatory conditions,[21] the potential relationship between PAR-2 activation and sVEGFR-1 release is unknown. Therefore, we speculated that the activation of PAR-2 could increase endothelial sVEGFR-1 release. In this study, we report the ability of PAR-2 agonists to increase sVEGFR-1 release from endothelial cells via protein kinase C (PKC)–mediated intracellular transactivation of epidermal growth factor (EGF) receptor (EGFR) 1 and subsequent downstream mitogen-activated protein (MAP) kinase signaling. Furthermore, we show that PAR-2–stimulated sVEGFR-1 release was suppressed by HO-1 overexpression and enhanced by HO-1 knockdown, indicating that HO-1 is a central regulator of sVEGFR-1 expression.

Materials and Methods

A full description of materials and methods used can be found in the online Data Supplement (please see http://hyper.ahajournals.org).

Cell Culture

HUVECs were isolated and cultured as described.[8] Human embryonic kidney cells (HEK-293) were maintained in DMEM containing 10% FCS, whereas porcine aortic endothelial cells (PAECs) expressing PAR-2 (PAEC-PAR-2) and cells containing the vector alone (PAEC-pCDNA3.1B) were propagated in G418-containing F12-HAM nutrient mix supplemented with 10% FCS.

Adenoviruses

The recombinant, replication-deficient adenoviruses encoding rat HO-1[8] and dominant-negative PKC (dnPKC) isozymes[22] were amplified and titered and the optimal multiplicity of infection determined by Western blotting as 50 infectious units (ifu) per cell for HO-1 and 100 ifu per cell for the dnPKC isozyme adenoviruses. HUVECs were infected overnight with adenoviruses and then incubated for 24 hours in basal medium containing 5% FCS.

Small Interfering RNA-Mediated Gene Knockdown

The small interfering RNAs (siRNAs) targeted against c-Src,[23] HO-1,[8] and $PKC_{\beta I}$ (sense: 5′-GGGAGAAACUUGAACGCAAtt-3′; antisense: 5′-UUGCGUUCAAGUUUCUCCCtt-3′) and a universal control siRNA (Dharmacon) were introduced into HUVECs using the Amaxa Nucleofector HUVEC II kit (Amaxa) and incubated overnight before treatment.

ELISAs

The sVEGFR-1 concentration in cell supernatants was determined as described.[4] EGFR was measured using the EGFR DuoSet IC ELISA (R&D Systems) and phosphorylated EGFR by a sandwich ELISA using an anti-EGFR capture antibody and phosphotyrosine detection antibody.

Western Blotting

After stimulation, cells were lysed in radioimmunoprecipitation assay buffer and 30 μg of protein were Western blotted using rabbit antiphospho-extracellular signal–regulated kinase (ERK)1/2, anti–Src phospho-Y416, or anti–Raf-1-phospho-S338 (Cell Signaling) antiactivated EGFR (BD Biosciences) antibodies.[4]

VEGFR-1 Promoter Reporter Assays

A 1.3-Kb fragment of the human VEGFR-1 promoter-luciferase construct was used to determine the ability of PAR-2 to activate the VEGFR-1 gene. The reporter plasmid was constructed by cloning a PCR fragment corresponding with sequences from −1214 to +155 relative to the first exon in the VEGFR-1 gene into the *Bgl*II and *Hind*III sites of pGL2 (Promega). HEK293 cells, which express functional PAR-2,[24] and porcine aortic endothelial cells were transfected with the VEGFR-1 promoter construct using Exgen 500 (Fermentas). For details see the online Supplemental Methods.

Statistical Analysis

All of the data are expressed as the mean±SEM. Statistical analysis was performed using the 2-tailed Student *t* test. $P<0.05$ was considered statistically significant.

Results

PAR-2 Activation Stimulates sVEGFR-1 Release

Endothelial cells derived from patients with preeclampsia exhibit increased PAR-2 expression.[20] To determine the effect of PAR-2 activation on sVEGFR-1 production, HUVECs were stimulated with the PAR-2 selective activating peptides (SLIGRL-NH_2 and 2f-LIGRLO-NH_2) or FXa for 24 hours and sVEGFR-1 quantified in the culture medium by ELISA. PAR-2 activating peptides induced sVEGFR-1 release, whereas the corresponding reverse-control peptides (LRGILS-NH_2 and 2f-OLRGIL-NH_2) failed to induce sVEGFR-1 release (Figure 1A). Similarly, FXa (100 nmol/L) induced sVEGFR-1 release. A 1.3-Kb human VEGFR-1 promoter luciferase reporter was used to assess the ability of PAR-2 to activate the VEGFR-1 gene in PAECs and HEK293 cells. Activation of PAR-2 significantly increased VEGFR-1 promoter activity, indicating that PAR-2 regulates the production of sVEGFR-1 transcription (Figure 1B and Figure S2A, available in the online Data Supplement). To confirm that the sVEGFR-1 release was generated through PAR-2 activation, HUVECs were coincubated with 2f-LIGRLO-NH_2 and the PAR-2 antagonist, FSLLRY-NH_2,[25] which abolished both the sVEGFR-1 release and VEGFR-1 promoter activity (Figure 1C and 1D). Moreover, specificity of PAR-2-stimulated VEGFR-1 promoter activity was demonstrated in PAECs engineered to express PAR-2 (Figure S1) but not in PAECs transfected with empty vector (Figure 1E and 1F). Furthermore, PAR-2 activation induced robust activation of VEGFR-1 promoter and also increased sVEGFR-1 release from trophoblasts (Figure S2C), and PAR-2 activators did not significantly alter cellular activity, confirming that the effect on sVEGFR-1 expression was not because of an increase in endothelial cell proliferation or survival (Figure S2B).

PAR-2–Induced sVEGFR-1 Release Depends on PKC and Src Activity

Both PKC[26] and Src[27] are involved in PAR-2 signaling. Soluble VEGFR-1 release and VEGFR-1 promoter activity

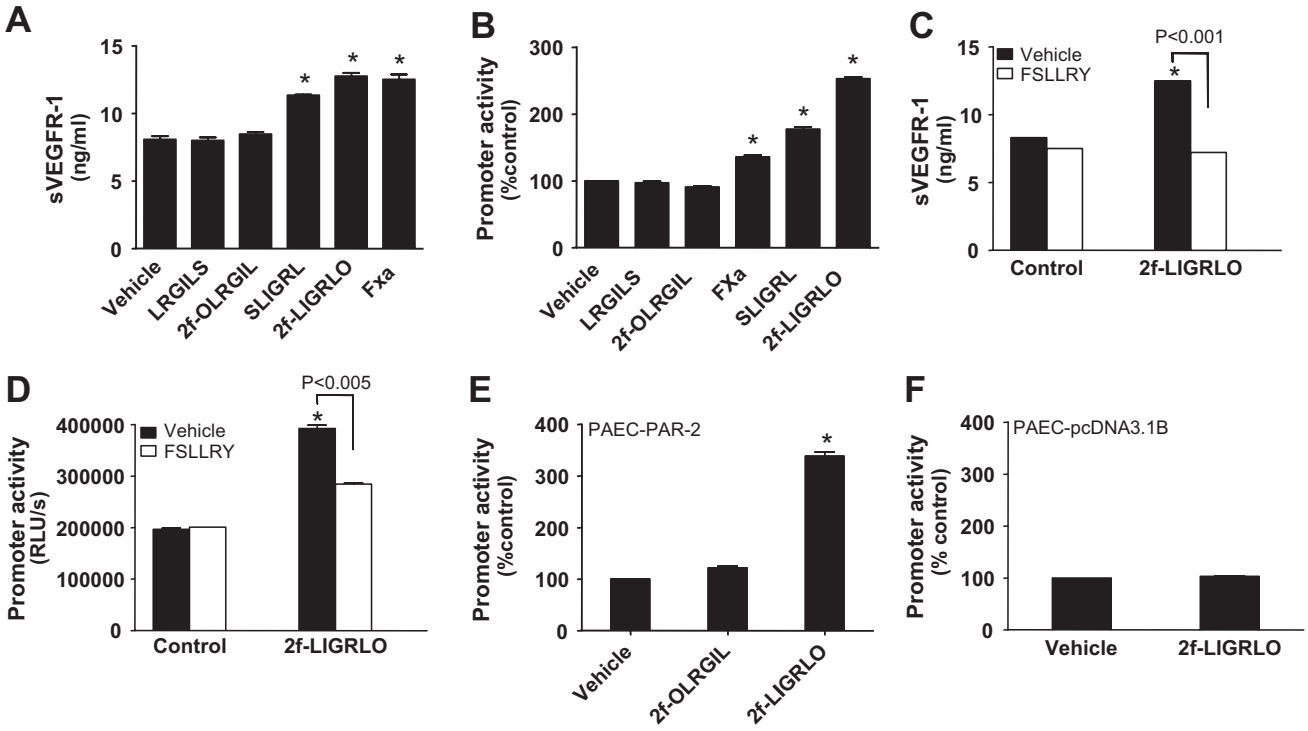

Figure 1. Selective PAR-2 activation stimulates sVEGFR-1 release in endothelial cells. A, Confluent HUVECs and (B) HEK-293 cells transfected with a ≈1.3-kb fragment of the VEGFR-1 promoter with a luciferase reporter were incubated with PAR-2 activating peptide (100 μmol/L of SLIGRL-NH_2 or 50 μmol/L of 2f-LIGRLO-NH_2) or FXa (100 nmol/L) for 24 hours, and the cell supernatants assayed for sVEGFR-1 by ELISA and VEGFR-1 promoter activity in cell lysates were determined by luciferase assay. The corresponding reverse peptides (LRGILS-NH_2 or 2f-OLRGIL-NH_2) were used as negative controls. C, HUVECs and (D) HEK-293 cells transfected with VEGFR-1 promoter were incubated for 24 hours with 2f-LIGRLO-NH_2 (10 μmol/L) in the presence or absence of a PAR-2 antagonist (400 μmol/L of FSLLRY-NH_2), and sVEGFR-1 levels in cell supernatants (C) or promoter activity (D) were determined. E, Porcine aortic endothelial cells expressing PAR-2 (PAEC-PAR-2) or (F) control cells (PAEC-pcDNA3.1B) were transfected with the VEGFR-1 promoter and stimulated for 24 hours with PAR-2 activating peptide (10 μmol/L of 2f-LIGRLO-NH_2) or control peptide (10 μmol/L of 2f-OLRGIL-NH_2), and luciferase activity in the cell lysates was determined. Results are the mean (±SEM) of 3 experiments. *$P<0.01$ (A and C), $P<0.05$ (B), and *$P<0.001$ (D and E) vs control.

were blocked by a PKC inhibitor (Ro-32-0432), indicating its involvement in PAR-2–mediated sVEGFR-1 production (Figure 2A and 2B). HUVECs express PKCα, PKCβ_1, PKCδ, and PKCε isozymes.[28] To evaluate the PKC subtype involved in PAR-2–mediated sVEGFR-1 release, HUVECs were infected with adenoviruses encoding dominant-negative isozymes of PKC. PAR-2–induced release of sVEGFR-1 was inhibited by both PKCα and PKCε, and the basal level of sVEGFR-1 was suppressed by the overexpression of PKCβ in endothelial cells (Figure 2C). PKCβ1 knockdown in HUVECs abrogated PAR-2–mediated sVEGFR-1 release (Figure 2D). Western blot analysis confirmed endogenous expression of PKC isozymes and the modulation by adenovirus overexpression or knockdown (Figure 2C). Similarly, Src family kinase inhibitor PP2 inhibited sVEGFR-1 release and VEGFR-1 promoter activity, implicating its involvement in PAR-2–stimulated sVEGFR-1 expression (Figure 3A and 3B). These results were confirmed using siRNA-mediated knockdown of Src (Figure 3C and 3D).

MAP Kinase Activation and EGFR Transactivation Are Required for PAR-2–Induced sVEGFR-1 Expression

The activation of G protein–coupled receptors including PAR-1, PAR-2, and angiotensin II receptors, is widely reported to phosphorylate MAP kinase via PKC-mediated transactivation of EGFR.[29–31] To investigate whether MAP kinase activation is required for PAR-2–induced sVEGFR-1 release, HUVECs were preincubated with MAP kinase kinase (MEK)1/2 inhibitor (U0126) and stimulated with PAR-2 ligand. Inhibition of MEK-1/2, which is immediately upstream of ERK-1/2 in the MAP kinase pathway, resulted in a complete loss of PAR-2–mediated sVEGFR-1 release (Figure 4A) and VEGFR-1 promoter activity in HEK-293 (Figure 4B) and caused a loss of PAR-2–mediated ERK-1/2 phosphorylation (Figure 4C). Furthermore, the overexpression of dominant-negative PKCβ and PKCε or Src-kinase inhibition suppressed PAR-2–mediated ERK-1/2 phosphorylation (Figure 4D and 4E).

To determine the sequence of events leading to PAR-2–mediated sVEGFR-1 release, we examined the phosphorylation of Src at Y416 and Raf-1 at S338 in relation to downstream activation of ERK-1/2 and whether PKC activation is upstream of Src and Raf-1 in PAR-2–stimulated VEGFR-1 release. Overexpression of dominant-negative PKCβ or PKCε attenuated PAR-2–stimulated Src phosphorylation and completely inhibited the phosphorylation of Raf-1 (Figure 4F). In addition, the PKC (GF109203X) and Src (PP2) inhibitors completely abrogated 2f-LIGRLO-NH_2–stimulated phosphorylation of Raf-1 (Figure 4G). Collectively, these data demonstrate that PAR-2 stimulates sVEGFR-1 expression and release by activat-

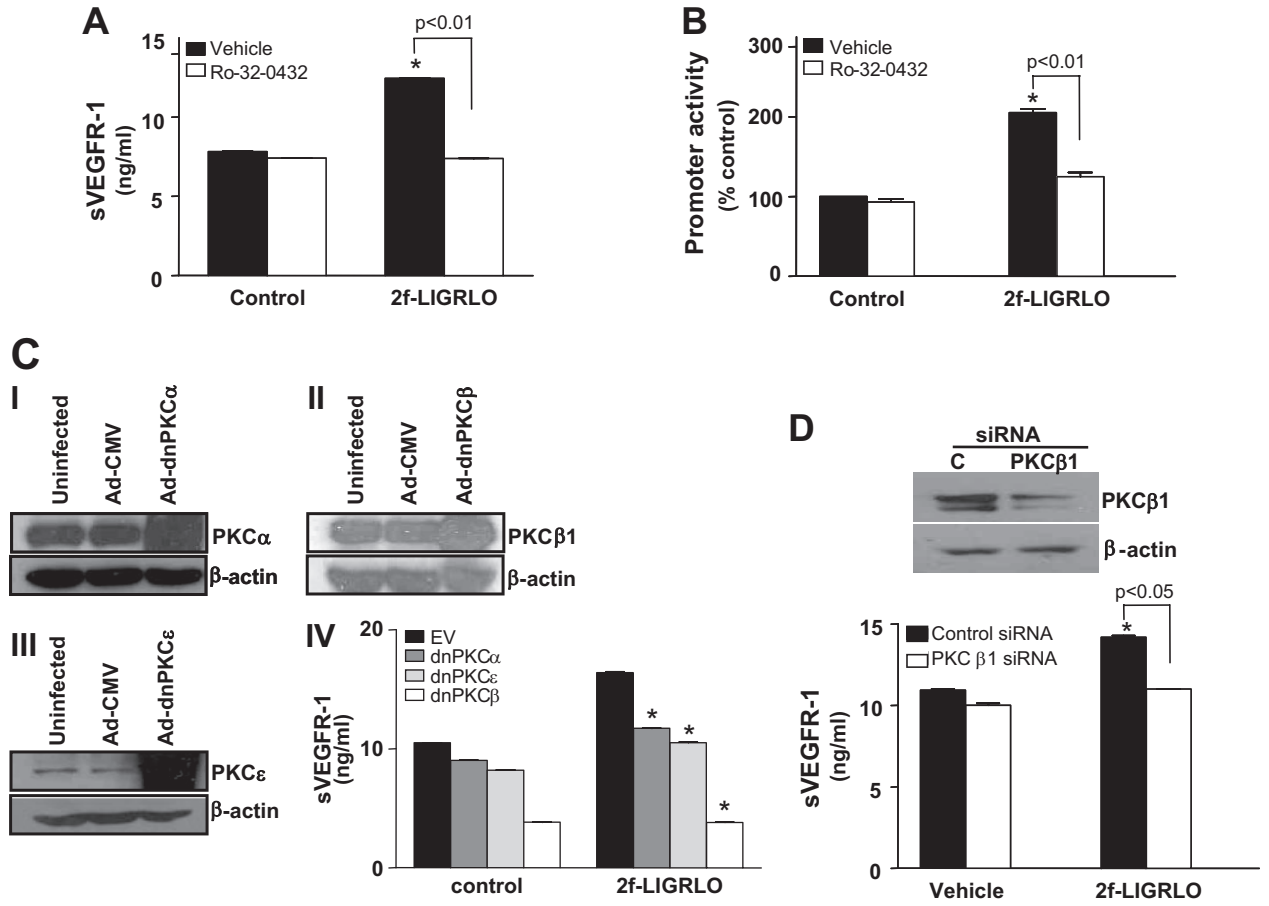

Figure 2. PAR-2–induced sVEGFR-1 release from endothelial cells depends on PKC activity. A, HUVECs and (B) HEK293 cells transfected with a VEGFR-1 promoter luciferase reporter construct were pretreated with the PKC inhibitor (Ro-32-0432; 1 μmol/L) for 45 minutes and then stimulated with PAR-2 activating peptide (10 μmol/L of 2f-LIGRLO-NH_2) for 24 hours, and the cell culture supernatants were assayed for sVEGFR-1 by ELISA (A) and cell lysates assayed for luciferase activity (B), respectively. C, HUVECs were infected overnight with 100 ifu per cell of adenoviruses expressing dominant-negative PKCα (dnPKCα), PKCβ (dnPKCβ), PKCε (dnPKCε), or empty vector (EV), incubated for 24 hours, and the expression of PKC isoforms were examined in cell lysates by Western blotting. These cells were stimulated with PAR-2–activating peptide (10 μmol/L of 2f-LIGRLO-NH_2) for 24 hours, and the conditioned medium was assayed for sVEGFR-1 by ELISA. D, PKC-β1 siRNA was introduced into HUVECs using an Amaxa nucleofector and the knockdown of PKCβ1 confirmed by Western blotting. PAR-2–mediated sVEGFR-1 release was inhibited in cells treated with PKC-β1 siRNA. Results represent the mean (±SEM). *$P<0.01$ (A and B vs control and C vs EV+2f-LIGRLO); *$P<0.05$ (D vs control).

ing PKC, leading to sequential Src, Ras, Raf-1, and ERK-1/2 activation.

Subsequently, we investigated whether EGFR transactivation is required for PAR-2–induced ERK1/2 activation and sVEGFR-1 expression. However, early studies reported the absence of EGFR in endothelial cells,[32] and EGFR-1 has only recently been detected in HUVECs.[33] ELISA and Western blot analysis confirmed the presence of functional EGFR in HUVEC lysates (Figure S3). To investigate whether PAR-2 activation can lead to EGFR transactivation, HUVECs were stimulated with PAR-2 peptide or EGF, and EGFR phosphorylation was determined by ELISA. PAR-2 activation increased EGFR phosphorylation in HUVECs, which was inhibited by the EGFR kinase inhibitor AG1478 (3 μmol/L; Figure 5A).[34] In addition, 2f-LIGRLO-NH_2, FXa, and EGF induced similar levels of Raf-1^{S338} phosphorylation in endothelial cells (Figure S3C). EGFR inhibition abrogated sVEGFR-1 release and downstream ERK-1/2 phosphorylation in response to the PAR-2 agonists demonstrating the requirement of EGF transactivation for PAR-2–mediated sVEGFR-1 release (Figure 5B). Furthermore, the inhibition EGFR resulted in the loss of ERK-1/2 activity after the acute stimulation of HUVECs with either 2f-LIGRLO or FXa (Figure 5C). As anticipated, the inhibition of ERK-1/2 prevented sVEGFR-1 production in response to FXa and EGF (Figure S3D).

Src activity has been reported to act both upstream and downstream of EGFR transactivation after PAR-2 stimulation in different cell types.[27,35] PAR-2–induced EGFR phosphorylation was inhibited by the Src inhibitor PP2, indicating that Src activity is required for EGFR transactivation (Figure 5D). In addition, the PAR-2–mediated activation of Src was not inhibited by AG1478, supporting these findings (Figure 5E). Transactivation of the EGFR by PAR-2 can occur through the release of EGFR agonists, such as transforming growth factor-α or heparin-binding EGF from the cell surface through the activation of matrix metalloproteinases (MMPs).[30] To determine whether the transactivation of the EGFR by PAR-2 observed in our studies occurred via a similar extracellular route, HUVECs were preincubated with the MMP inhibitor, GM6001, or the reverse-control peptide (rGM6001; 10 μmol/L), before stimulation with

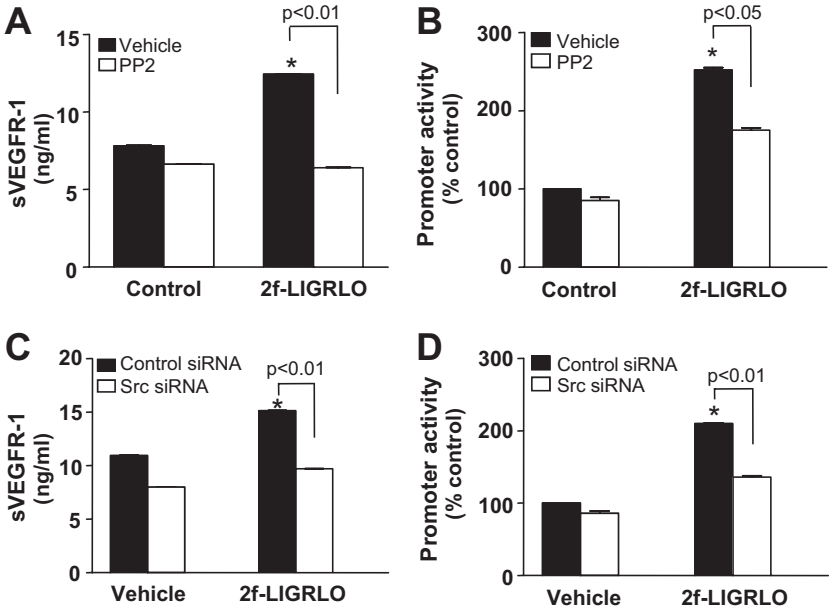

Figure 3. PAR-2–induced sVEGFR-1 production requires Src activation. A, HUVECs and (B) HEK-293 cells transfected with a VEGFR-1 promoter luciferase reporter construct were pretreated with an Src inhibitor (PP2; 10 μmol/L) for 45 minutes and then stimulated with PAR-2–activating peptide (10 μmol/L of 2f-LIGRLO-NH_2) for 24 hours. The cell culture supernatants were assayed for sVEGFR-1 by ELISA (A) and cell lysates were assayed for luciferase activity (B), respectively. C, HUVECs and (D) HEK293 cells containing the VEGFR-1 luciferase reporter were electroporated with Src siRNAs using an Amaxa Nucleofector and incubated overnight. After stimulation with PAR-2–activating peptide (10 μmol/L of 2f-LIGRLO-NH_2) for 24 hours, sVEGFR-1 was measured in the cell culture supernatants by ELISA (C) and cell lysates were assayed for luciferase activity (D), respectively. Results represent the mean (±SEM). *$P<0.01$ (A and D) and *$P<0.05$ (D and C) vs control.

2f-LIGRLO-NH_2 (10 μmol/L) or FXa (200 nmol/L; Figure 5F). The MMP inhibitor did not significantly suppress sVEGFR-1 release, indicating that MMPs are not involved in PAR-2–stimulated sVEGFR-1 release. On the basis of these results, we conclude that the PAR-2–mediated transactivation of EGFR occurs through an intracellular route via a PKC- and Src-dependent pathway.

Statins and HO-1 Activity Downregulate PAR-2s-Induced sVEGFR-1 Release

Statins (which upregulate HO-1), HO-1, and its gaseous product CO act as negative regulators of sVEGFR-1 release in endothelial cells.[8] Consistent with this concept, simvastatin inhibited PAR-2–mediated sVEGFR-1 release (Figure 6A) and VEGFR-1 promoter activity (Figure 6B and 6C). The overexpression of HO-1 also significantly inhibited the release of sVEGFR-1 (Figure 6D), whereas loss of HO-1 enhanced VEGFR-1 promoter activity (Figure 6E). The lipid soluble CO-releasing molecule (CORM-2) reduced PAR-2–induced sVEGFR-1 release, whereas the inactive CORM-2 had no significant effect (Figure 6F). These results further support a potentially beneficial role for HO-1 and its product CO in preeclampsia, as reported previously.[8]

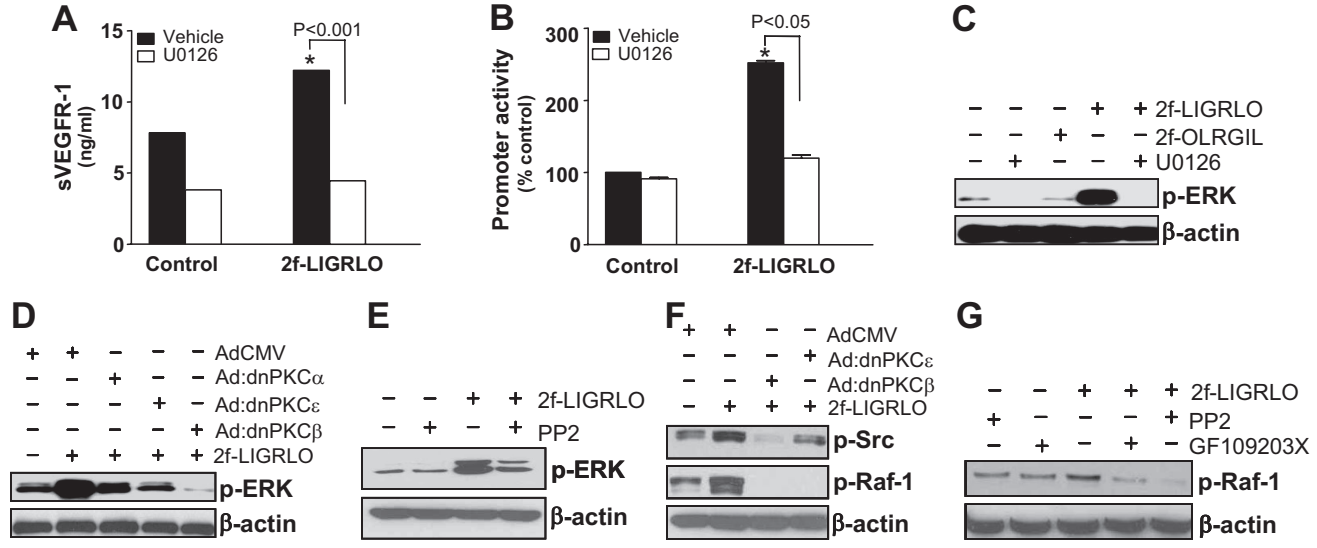

Figure 4. ERK-1/2 activation is required for PAR-2–induced sVEGFR-1 release. A, HUVECs and (B) HEK293 cells transfected with a VEGFR-1 promoter luciferase reporter construct were pretreated with an MEK-1/2 inhibitor (U0126; 10 μmol/L) for 45 minutes, stimulated with PAR-2–activating peptide (2f-LIGRLO-NH_2; 10 μmol/L) for 24 hours, and the conditioned medium was assayed for sVEGFR-1 by ELISA (A) and luciferase activity determined in cell lysates (B), respectively. C through G, Western blot analysis: cell lysates were immunoblotted for phosphor–ERK-1/2 (p-ERK), phosphor-Src (pY416Src), and phospho–Raf-1 (pS338Raf-1); HUVECs were either preincubated for 45 minutes with (C) MEK-1/2 (U0126; 10 μmol/L), (E) Src (PP2; 10 μmol/L), and (G) PKC (GF109203X; 5 μmol/L) inhibitors or (D and F) infected overnight with adenoviruses expressing dominant-negative PKCα (dnPKCα), PKCβ (dnPKCβ), or PKCε (dnPKCε) and stimulated with PAR-2–activating peptide (2f-LIGRLO-NH_2; 10 μmol/L) for 10 minutes. *$P<0.01$ (A) and *$P<0.05$ (B) vs control.

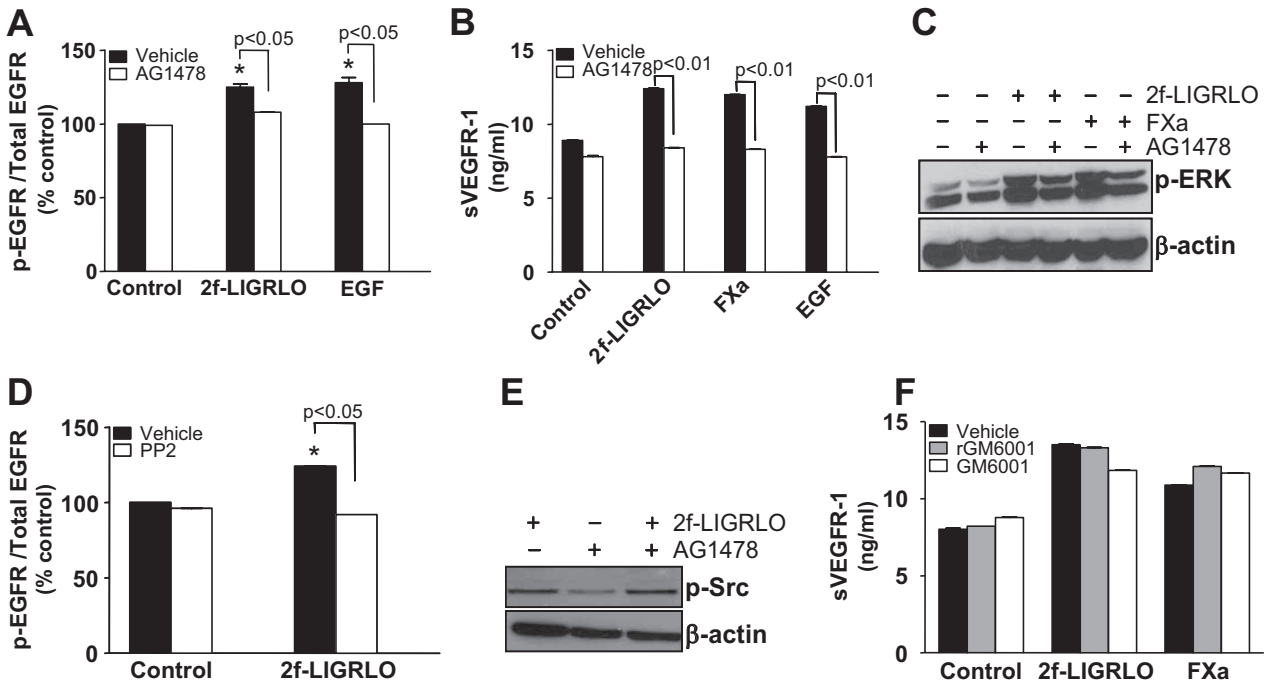

Figure 5. EGFR transactivation is required for PAR-2–induced sVEGFR-1 release. A, HUVECs were pretreated for 45 minutes with AG1478 (3 μmol/L), stimulated for 10 minutes with PAR-2–activating peptides (2f-LIGRLO-NH_2; 10 μmol/L) or EGF (50 ng/mL), and the level of phosphorylated and total EGFRs were determined in cell lysates (100 μg per well) by ELISA (R&D Systems). B, HUVECs were pretreated with AG1478 and stimulated for 24 hours with PAR-2–activating peptide (2f-LIGRLO-NH_2), FXa (200 nmol/L), or EGF (50 ng/mL), and sVEGFR-1 was assayed in the cell culture supernatants by ELISA. C, After pretreatment with the EGFR inhibitor (AG1478), HUVECs were incubated with 2f-LIGRLO-NH_2 and FXa (200 nmol/L) for 10 minutes, and cell lysates (30 μg per lane) were subjected to Western blotting for phosphor-ERK1/2 (p-ERK) and β-actin as a loading control. D, HUVECs were incubated with the Src inhibitor PP2 for 45 minutes and then stimulated for 10 minutes with 2f-LIGRLO-NH_2, and the levels of EGFR and phosphorylated EGFR were determined in cell lysates (100 μg per well) by ELISA. E, HUVECs were treated with AG1478 before incubation with 2f-LIGRLO-NH_2 for 10 minutes, and cell lysates were Western blotted for phosphorylated-Src (p-Src). F, HUVECs were preincubated with the metalloproteinase inhibitor (GM6001; 10 μmol/L) or control (rGM6001; 10 μmol/L) for 45 minutes before stimulation with 2f-LIGRLO-NH_2 or FXa (200 nmol/L), and sVEGFR-1 was assayed in cell supernatants by ELISA. Results represent the mean (±SEM); n=3. *$P<0.05$ (A and D) vs control.

Discussion

This study shows that receptor-selective PAR-2 activation induces VEGFR-1 promoter activity and sVEGFR-1 release from endothelial cells through the sequential activation of PKC, Src, Raf-1, and ERK-1/2 and depends on EGFR transactivation (Figure 7). Furthermore, it demonstrates that upregulation of HO-1 with Simvastatin or overexpression of HO-1 or CO suppresses PAR-2–mediated sVEGFR-1 release and supports our earlier study showing that the HO-1/CO pathway inhibits cytokine-induced sVEGFR-1 release.[8]

The transactivation of the EGFR by G-coupled protein receptors, including PAR-1, PAR-2, and PAR-4, is well established.[29,30,36] Inhibition of Src or EGFR completely abrogated PAR-2–mediated sVEGFR-1 expression, indicating that both Src and EGFR activation is required in concert with PKC for the efficient release of sVEGFR-1 in response to PAR-2. In this study, Src activity was required for PAR-2–mediated EGFR transactivation. This is consistent with a recent report showing that EGFR transactivation and MAP kinase activity in PAR-2–induced chloride secretion in intestinal epithelial cells depended on Src activation[35] and a similar mechanism in cardiomyocytes after PAR-4 stimulation.[36] The inhibition of either the Src or EGFR did not completely block PAR-2–stimulated ERK-1/2 phosphorylation, suggesting that ERK-1/2 may also be activated directly by PKC via Raf-1. However, activation of ERK-1/2 appears to be the final pathway for PAR-2–mediated release of sVEGFR-1. In many cell systems, EGFR transactivation is mediated by the proteolytic cleavage of cell membrane–bound EGFR ligands, including transforming growth factor-α and heparin-binding EGF by MMP such as the TNF-α–converting enzyme.[29,30] PAR transactivation of EGFR was reported to occur in an MMP-independent manner, requiring Src activation in cardiac fibroblasts[36] and intestinal epithelial cells.[35] In this study, MMP inhibition did not prevent PAR-2–mediated sVEGFR-1 release supporting EGFR transactivation occurring via an intracellular route. The observation that EGFR transactivation leads to increased sVEGFR-1 release may have broader significance in preeclampsia. The infusion of angiotensin II selectively upregulates the production of sVEGFR-1 in pregnant mice.[37] Angiotensin II type 1 receptor density increases in preeclamptic placentas,[38] and angiotensin II type 1 activating autoantibodies induce a preeclampsia-like condition in mice.[39] Given the ability of angiotensin II to transactivate the EGFR[31] and the signal transduction pathway identified for PAR-2 in this study, we suggest that this represents a common mechanism by which G protein–coupled receptors could induce sVEGFR-1 production.

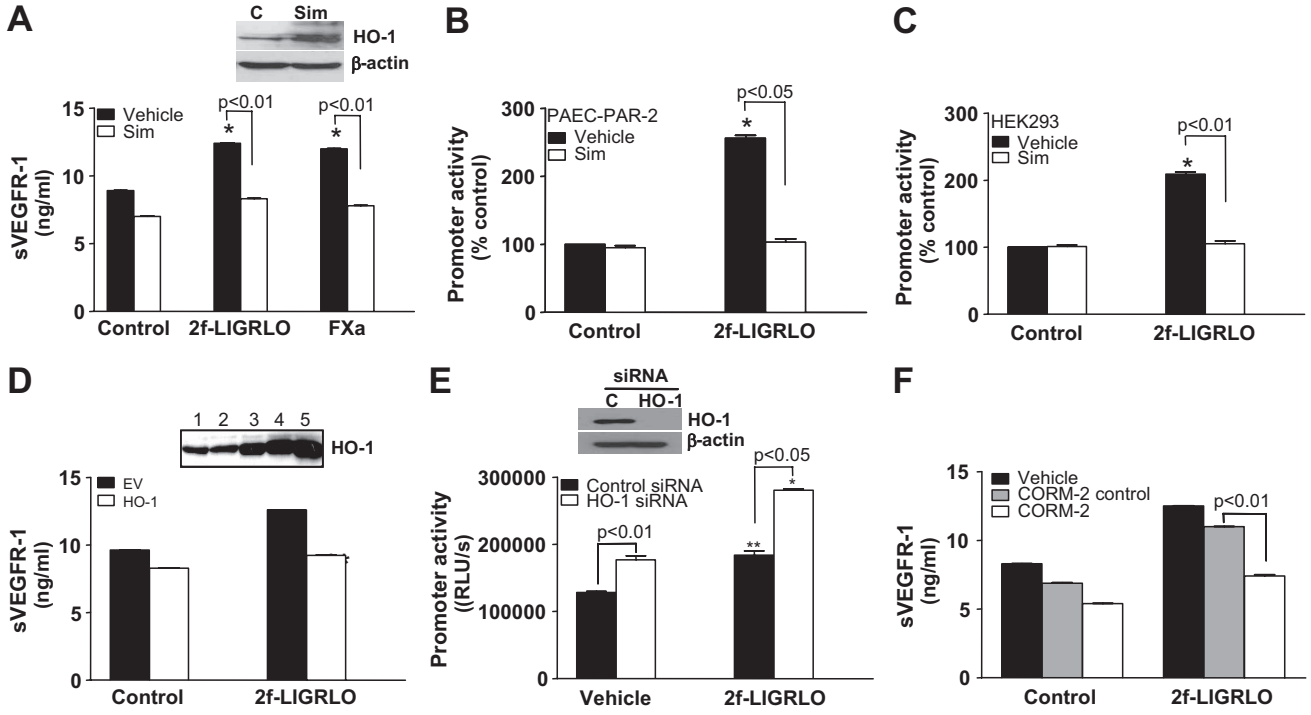

Figure 6. PAR-2-induced sVEGFR-1 release is inhibited by statins, HO-1, and CO. A, HUVECs were stimulated with PAR-2–activating peptide (2f-LIGRLO-NH_2; 10 μmol/L) or FXa (200 nmol/L) in the presence or absence of Simvastatin (10 μmol/L) for 24 hours, and sVEGFR-1 was quantified in cell supernatants by ELISA. HO-1 upregulation in HUVECs incubated with Simvastatin (10 μmol/L) for 24 hours was confirmed by Western blotting (inset). B, PAEC-PAR-2 and (C) HEK-293 cells were transfected with the VEGFR-1 promoter-reporter construct, stimulated with PAR-2–activating peptide (2f-LIGRLO-NH_2) for 24 hours in the presence or absence of Simvastatin (10 μmol/L), and luciferase activity was determined in cell lysates. D, HUVECs were then infected with 50 ifu per cell of HO-1 or control (EV) adenovirus for 48 hours before stimulation with 2f-LIGRLO-NH_2 for 24 hours (*$P<0.001$ vs control). HUVECs infected with low (5 ifu per cell; lanes 2 and 4) or high (50 ifu per cell; lanes 3 and 5) concentrations of HO-1 adenovirus for 24 (lanes 2 and 3) or 48 (lanes 4 and 5) hours or uninfected control cells (lane 1) were immunoblotted for HO-1 (inset). E, HEK293 cells containing the VEGFR-1 promoter-reporter construct were electroporated with HO-1 siRNA and incubated overnight. After stimulation with 2f-LIGRLO-NH_2 (10 μmol/L) for 24 hours, luciferase activity was determined in cell lysates. The medium was then collected and assayed for sVEGFR-1 by ELISA. F, HUVECs were treated with 50 μmol/L of CORM-2 or CORM-2 control for 45 minutes and then stimulated for 24 hours with 2f-LIGRLO-NH_2. Results represents mean (±SEM); n=3. A through C, *$P<0.01$ vs control; D, *$P<0.001$ vs control; **$P<0.001$ vs EV+2f-LIGRLO; E, *$P<0.05$, *$P<0.001$, and **$P<0.05$ vs control.

PAR-2 inhibition can suppress TNF-α expression in inflammatory settings[40] and improve wound healing in mice by reducing inflammation.[41] Given the increased procoagulant activity observed in preeclampsia and the ability of the coagulation proteases factors VIIa and FXa to activate PAR-2, coupled with the reported increased PAR-2 expression on endothelium derived from preeclamptic women,[20] we suggest that PAR-2 activation may be a contributing factor to the increases in circulating sVEGFR-1 in this syndrome. Furthermore, the reported increased expression of PAR-1 in the endothelium[20] and placenta[42] of preeclamptic women, combined with the observed excessive generation thrombin, the ligand for PAR-1, in preeclampsia, indicates that other PAR receptors may also be involved in sVEGFR-1 production in this setting. Although the trophoblast is the main source of sVEGFR-1, and PAR-2 activation increases sVEGFR-1 release from trophoblasts, this study confirms that the endothelium may be a significant source of sVEGFR-1. PAR-2 activation leading to sVEGFR-1 release from the endothelium is relevant not only in the placental-based perturbation in preeclampsia, but inflammatory conditions, such as cardiovascular diseases and sepsis, may contribute directly to the endothelial dysfunction.

A recent report showed that, in mice lacking PAR-2, exposure to antiphospholipid antibodies did not induce fetal injury or miscarriage. Furthermore, statin treatment reduced the adverse effects of PAR-2 activation induced by antiphospholipid antibodies and prevented pregnancy loss.[43] HO-1 activity is essential for the successful outcome of pregnancy,[44] and HO protein expression is reduced in preeclamptic placentas.[6] The concentration of CO in the exhaled breath of women with preeclampsia is significantly less than in normal pregnancy,[45] indicating lower HO activity in these patients. HO-1 and CO inhibit VEGF-stimulated sVEGFR-1.[8] In this study, we demonstrate that upregulation of HO-1 with Simvastatin or activation of the HO-1 pathway suppresses PAR-2–mediated sVEGFR-1 release. More importantly, these studies highlight the potential efficacy of statins in controlling complications of pregnancy, which are being investigated in a randomized, placebo-controlled trial (Statins to Ameliorate early onset Pre-eclampsia [StAmP]) for use of statins to ameliorate early onset preeclampsia.

Perspectives

The antiangiogenic soluble factor sVEGFR-1 (commonly know as sFlt-1) appears to be “the final common pathway”

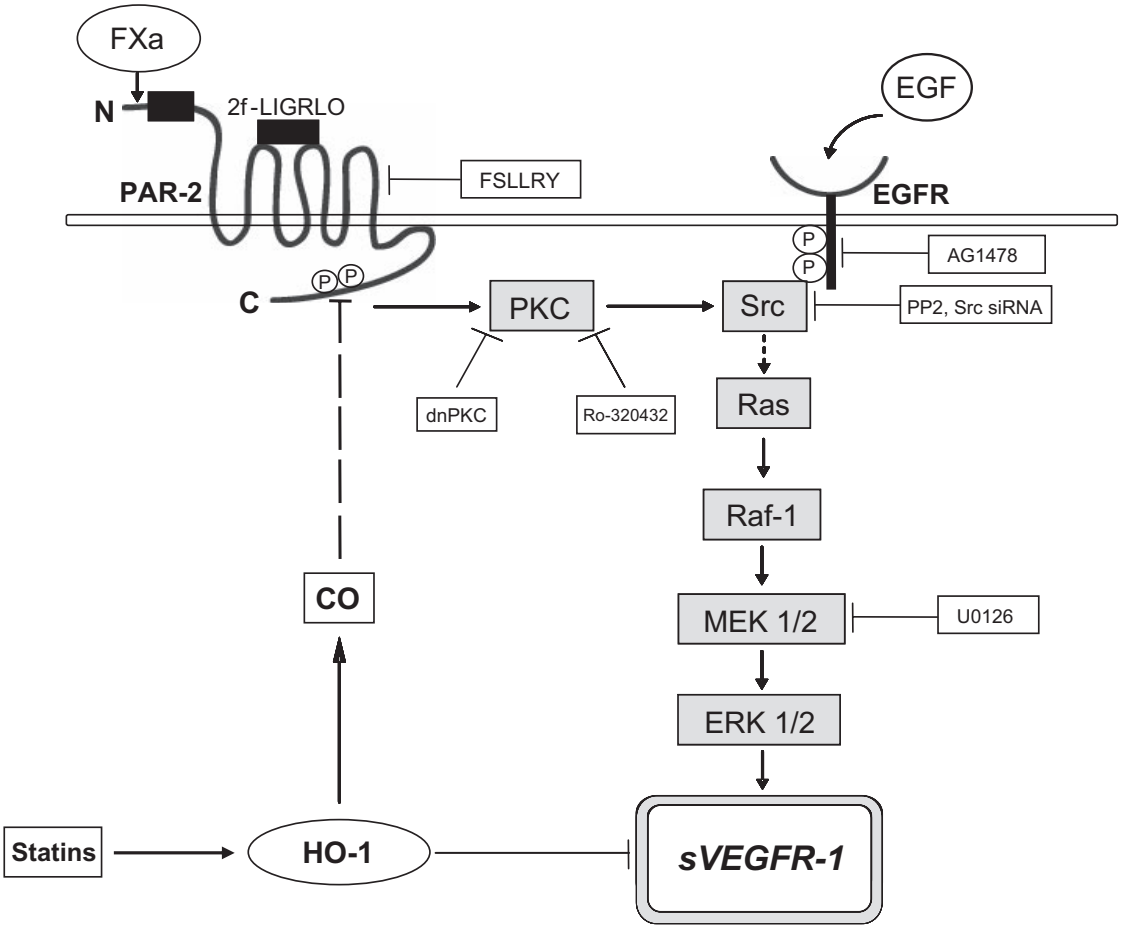

Figure 7. Proposed model for PAR-2–induced sVEGFR-1 release via transactivation of the EGFR. The proposed PAR-2 signaling pathway places the activation of PKC upstream of Src-family enzymes and the EGFR, which leads to the activation of the MAP kinase pathway, including Ras, Raf-1, and ERK-1/2 expression and release of sVEGFR-1. The PAR-2 activator FXa and synthetic PAR-2–activating peptide (2f-LIGRLO), along with the specific PAR-2 inhibitor (FSLLRY) and the various points of inhibition, are indicated. HO-1 and CO negatively regulate sVEGFR-1 production in response to PAR-2 activation, and statins upregulate HO-1.

inducing the maternal clinical signs of preeclampsia. This study demonstrates that activation of the proinflammatory receptor PAR-2 caused the endothelium to release sVEGFR-1, and the lipid-lowering statin, simvastatin was found to completely block sVEGFR-1 expression. Recently, Redecha et al[43] showed that PAR-2 activation caused trophoblast injury and fetal death, which was also blocked by simvastatin. Collectively, the findings indicate that PAR-2 activation leading to increased sVEGFR-1 release may contribute to vascular dysfunction in pregnancy and identifies the PAR-2 pathway as a potential therapeutic target.

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Disclosures

None.

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CLINICAL RESEARCH

Loss of Akt activity increases circulating soluble endoglin release in preeclampsia: identification of inter-dependency between Akt-1 and heme oxygenase-1

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Aims Endothelial dysfunction is a hallmark of preeclampsia. Desensitization of the phosphoinositide 3-kinase (PI3K)/Akt pathway underlies endothelial dysfunction and haeme oxygenase-1 (HO-1) is decreased in preeclampsia. To identify therapeutic targets, we sought to assess whether these two regulators act to suppress soluble endoglin (sEng), an antagonist of transforming growth factor-β (TGF-β) signalling, which is known to be elevated in preeclampsia.

Methods and results Vascular endothelial growth factor-A (VEGF-A), fibroblast growth factor (FGF-2), angiopoietin-1 (Ang-1), and insulin, which all activate the PI3K/Akt pathway, inhibited the release of sEng from endothelial cells. Inhibition of the PI3K/Akt pathway, by overexpression of phosphatase and tensin homolog (PTEN) or a dominant-negative isoform of Akt (Akt_{dn}) induced sEng release from endothelial cells and prevented the inhibitory effect of VEGF-A. Conversely, overexpression of a constitutively active Akt (Akt^{myr}) inhibited PTEN and cytokine-induced sEng release. Systemic delivery of Akt^{myr} to mice significantly reduced circulating sEng, whereas Akt_{dn} promoted sEng release. Phosphorylation of Akt was reduced in preeclamptic placenta and this correlated with the elevated level of circulating sEng. Knock-down of Akt using siRNA prevented HO-1-mediated inhibition of sEng release and reduced HO-1 expression. Furthermore, HO-1 null mice have reduced phosphorylated Akt in their organs and overexpression of Akt^{myr} failed to suppress the elevated levels of sEng detected in HO-1 null mice, indicating that HO-1 is required for the Akt-mediated inhibition of sEng.

Conclusion The loss of PI3K/Akt and/or HO-1 activity promotes sEng release and positive manipulation of these pathways offers a strategy to circumvent endothelial dysfunction.

Keywords Endothelium • Soluble endoglin • HO-1 • PI3K/Akt • HO-1 • Preeclampsia

Introduction

Neutralization of transforming growth factor (TGF)-β leads to endothelial dysfunction characterized by impaired endothelium-mediated vasodilatation and elevated expression of surface adhesion molecules, resulting in increased leucocyte adhesion.[1] Endoglin (CD105), a transmembrane co-receptor for TGF-β1 and TGF-β3, is predominantly expressed by activated, proliferating

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endothelium during angiogenesis[2,3] and regulates the activity of endothelial nitric oxide synthase (eNOS).[4,5] Proteolytic cleavage of the extracellular domain of endoglin gives rise to soluble endoglin (sEng), which functions to neutralize TGF-β signalling.[6]

It has been shown that an increase in circulating sEng has direct, significant, negative effects on endothelial health *in vivo*.[1] Soluble endoglin was also shown to abrogate *in vitro* tube formation, prevent TGF-β1 induction of eNOS phosphorylation, and abolish activation of TGF-β1-mediated Smad 2/3-dependent luciferase reporter activity.[6] In addition, sEng was shown to enhance lung and liver microvascular permeability, cause focal endotheliosis in kidney glomeruli, and block TGF-β-induced rat arterial vasodilation.[6] High levels of plasma sEng have been associated with vascular disorders, such as systemic sclerosis,[7] atherosclerosis,[8] familial hypertension,[7] malaria,[9] and most notably preeclampsia;[10] a novel risk factor for cardiovascular disease in women.[11] Recently, sEng was implicated as a likely cause of the reduced number of regulatory T cells observed in the systemic circulation of preeclamptic women.[12] In addition, sEng was shown to act synergistically with soluble Flt-1 (sFlt-1), the natural antagonist of vascular endothelial growth factor (VEGF), to induce maternal endothelial dysfunction and severe preeclampsia in animal studies.[6]

Haeme oxygenase-1 (HO-1) is an inducible, cytoprotective, and anti-inflammatory enzyme. It is widely acknowledged to provide a defence against oxidant damage[13,14] and to be protective against ischaemia-reperfusion injury.[15–18] Haeme oxygenase-1 null mice have systemic endothelial damage and have greatly elevated circulating sEng.[19] Haeme oxygenase-1 inhibits sEng release, from the placenta and the endothelium, mediated by proinflammatory cytokines, such as, tumour necrosis factor (TNF-α) and interferon-γ (IFN-γ).[19] Significantly, a recent publication showed that the angiotensin receptor agonistic auto-antibody stimulates sEng, *in vivo*, by upregulation of TNF-α and this upregulation can be prevented by induction of HO-1 using haemin[20] confirming our earlier study.

Cellular systems employ a number of endogenous protective mechanisms to defend against cell damage and death. The phosphoinositide 3-kinase (PI3K)/Akt and HO pathways are two important examples. Activation of the PI3K/Akt pathway is crucial for endothelial cell homeostasis and survival after vascular injury.[21] Numerous growth factors, including VEGF-A,[22] basic fibroblast growth factor (FGF-2),[23] angiopoietin-1 (Ang-1),[24] and insulin,[25] exert their protective effect via activation of the PI3K/Akt pathway.

To date, the mechanism responsible for sEng release has not been addressed. In this study, we sought to understand the mechanistic regulation of sEng release and investigated the involvement of two central regulators of vascular homeostasis; the PI3K/Akt and inducible HO-1 pathways.

Methods

Reagents and antibodies

Recombinant VEGF and FGF-2 were purchased from RELIA*Tech* (Brauschweig, Germany). Angiopoietin-1 was purchased from R&D Systems (Abingdon, UK). Monoclonal antibody, anti-PTEN (A2B1) and polyclonal antibodies, anti-Endoglin (C-term), anti-Endoglin (N-term) were from Autogen Bioclear Ltd (Wiltshire, UK). Monoclonal antibody, anti-HO-1 was purchased from Abcam (UK). Intracellular signalling protein antibodies anti-Akt and anti-phospho-Akt (ser 473) antibodies were purchased from New England Biolabs Ltd (Hertfordshire, UK). Polyclonal rabbit anti-HO-1 antibody was purchased from StressGen Biotechnologies Corporation (Canada). Human TNF-α and IFN-γ, monoclonal anti-β-actin, insulin, and all other cell culture reagents and chemicals purchased from Sigma-Aldrich Company Ltd (Dorset, UK).

Soluble endoglin ELISA

Soluble endoglin was measured in culture supernatants using the commercial ELISA kits according to manufacturer's instructions (R&D Systems, UK).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated, characterized, and cultured as previously described.[26] Experiments were performed on third or fourth passage cells. Human umbilical vein endothelial cells were stimulated with VEGF (20 ng/mL), TNF-α (10 ng/mL), or IFN-γ (10 ng/mL) and media collected and assayed for sEng by ELISA.

Adenoviral gene transfer

Recombinant, replication-deficient adenoviruses directing the expression of wild-type (WT) human PTEN (AdPTEN), catalytically inactive human PTEN ($AdPTEN_{dn}$), dominant-negative Akt (Thr308 to Ala and Ser473 to Ala, $AdAkt_{dn}$), and constitutively active, myristoylated Akt ($AdAkt^{myr}$) were generously provided by Dr Christopher Kontos (Duke University, USA) and AdCMV (empty vector used for control infections) adenoviruses were amplified in HEK-293A cells and purified using the BD Adeno-X^{TM} purification kit (BD Biosciences). Viral titres were estimated by using the BD Adeno-X^{TM} rapid titer kit. Human umbilical vein endothelial cells were infected by incubation with adenovirus in M199 containing 5% fetal calf serum (FCS) overnight at 37°C prior to addition of stimulants or vehicle control for up to 24 h. Optimal multiplicity of infection for the adenoviruses was determined by western blotting. The recombinant, replication-deficient adenovirus encoding rat HO-1 (AdHO-1) was used as described previously.[27]

siRNA transfection

Human umbilical vein endothelial cells were trypsinized and $\sim 1 \times 10^6$ cells electroporated with ~3 μg of HO-1,[28] Akt-1, or control siRNA using the HUVEC kit II and Nucleofector (Amaxa GmbH, Cologne, Germany) as described previously.[29]

Quantitative real-time PCR

Sample preparation and real-time PCR was performed as described previously.[29] Briefly, mRNA was prepared using TRIzol and DNase-1 digestion/purification on RNAeasy columns (Qiagen), and reverse transcribed with the cDNA Synthesis Kit (Promega). Triplicate cDNA samples and standards were amplified in SensiMix containing SYBR green (Quantace) with primers specific for endoglin (Forward: GTC-TCA-CTT-CAT-GCC-TCC-AGC-T; Reverse: GG-CTG-TCC-ATG-TTG-AGG-CAG-T) or β-actin. The mean threshold cycle (C_T) for HO-1 was normalized to β-actin and expressed relative to control.

Western blotting

Proteins were extracted from HUVEC with RIPA buffer and subjected to SDS–PAGE on 10% gels, transferred to nitrocellulose membranes (Amersham-Pharmacia, UK). Membranes were incubated with

appropriate antibodies at 4°C overnight. Antibody reactions were detected using the ECL detection kit (Amersham-Pharmacia, UK). Ratios of protein expression to loading control were determined by densitometry using ImageJ software.

Placental tissue and serum collection and preparation

Institutional Ethics Committee approved the tissue and serum collection and written informed consent was obtained. Eligible cases were singleton pregnancies with a diagnosis of preeclampsia. Preeclampsia as diagnosed if a previously normotensive woman had two repeat (4 h apart) diastolic blood pressure measurements of $\geq$90 mmHg after week 20 of gestation, together with proteinuria of $>$300 mg in a 24-h urine specimen or 2+ protein dipsticks in two repeat measurements (4 h apart). Human placental tissue and serum were obtained from normal pregnancies and gestationally matched pregnancies complicated by preeclampsia.

Animals

All procedures and animal care were approved by Institutional Ethics Committees and were in accordance with UK Home Office licensing regulations. C57/Bl6J animals with targeted deletion of the HO-1 gene by neomycin resistance gene insertion[30,31] were supplied by Prof Anupam Agarwal (University of Alabama, Birmingham, USA) and rederived in accordance with local regulations. Mice were injected in the tail vein with AdAkt^{myr} (5×10^9 pfu), AdAkt$_{dn}$ or control Adβgal. Five days post-injection blood was harvested by cardiac puncture and organs collected for histology, western blotting, and liver explant culture.

Ex vivo liver explant culture

Mice were sacrificed and their livers excised and cut into 1 mm^2 pieces. Six to ten pieces of liver were equilibrated for 4 h in phenol red-free DMEM containing 5% FCS in 24-well plate. Medium was changed to fresh phenol red-free DMEM containing 5% FCS and after 24 h conditioned medium was collected and stored at and liver explants were collected and stored at −80°C prior to assay for sEng by ELISA. The explant protein was also harvested and protein content assayed.

Statistical analysis

All data are expressed as the mean (±SEM). Statistical comparisons were performed using one-way ANOVA followed by the Student–

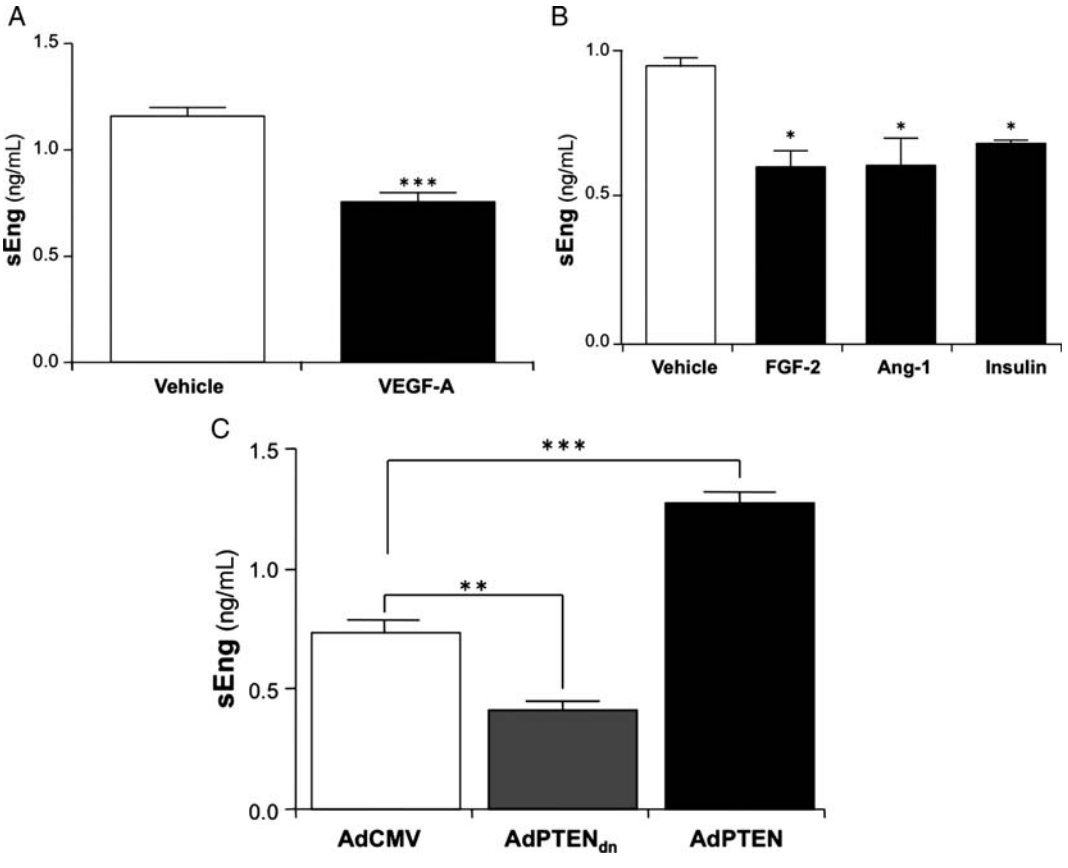

Figure 1 Survival factors and Akt activation repress soluble endoglin release from endothelial cells. All experiments were conducted using confluent human umbilical vein endothelial cells. Cells were incubated with (*A*) VEGF-A (20 ng/mL), (*B*) FGF-2 (20 ng/mL); angiopoieitn-1 (Ang-1;400 ng/mL), or insulin (200 nmol/L). (*C*) Human umbilical vein endothelial cells infected with adenoviruses encoding PTEN (AdPTEN), a dominant-negative PTEN mutant (AdPTEN$_{dn}$) or empty vector (AdCMV). All experiments were conducted in M199/5%FCS for 24 h and cell supernatants collected for soluble endoglin (sEng) quantification by ELISA. All results are the mean (±SEM) of three experiments performed in triplicate ($n = 9$). ***$P < 0.001$, *$P < 0.05$ vs. vehicle. **$P < 0.01$ and ***$P < 0.001$ vs. AdCMV.

Newman–Keuls test as appropriate. Statistical significance was set at a value of $P < 0.05$.

Results

Soluble endoglin release is suppressed by survival factors via activation of the phosphoinositide 3-kinase/Akt pathway in endothelial cells

To assess the impact of pro-survival factors on sEng release in a model system, isolated endothelial cells were incubated with VEGF-A, FGF-2, Ang-1, and insulin, which all activate the PI3K/Akt signalling.[22–25] These factors reduced the release of sEng from endothelial cells (*Figure 1A* and *B*) suggesting that vascular protection reduces shedding of endothelial membrane-bound endoglin. To examine whether the PI3K/Akt pathway regulates endoglin shedding, HUVEC were infected with adenoviruses encoding phosphatase and tensin homolog (PTEN), the phosphatase that inhibits PI3K signalling (AdPTEN) or inactive PTEN ($AdPTEN_{dn}$)[32] (see *Figures 3C* and *5A* for overexpression). $AdPTEN_{dn}$, which potentiates the PI3K pathway, thus activating Akt, significantly decreased the release of sEng (*Figure 1C*), whereas overexpression of PTEN, which depletes the cell of phosphatidylinositol 3,4,5-trisphosphate, the substrate required for Akt activation, induced a two-fold increase in sEng release (*Figure 1C*).

Inhibition of Akt activity using an adenovirus encoding a dominant-negative Akt construct, ($Ad\text{-}Akt_{dn}$)[33] increased endoglin mRNA expression in HUVEC (*Figure 2A*) and prevented VEGF-A-mediated repression of sEng release (*Figure 2B*). However, when $AdAkt_{dn}$ was co-infected with $AdPTEN_{dn}$, the ability of $AdPTEN_{dn}$ to inhibit sEng release was lost (*Figure 2C*) suggesting that PI3K is acting via Akt to modulate sEng release. Tail vein injection of $AdAkt_{dn}$ into mice resulted in increased Akt expression in liver tissue after six days (*Figure 2D* inset). Liver explants established from $AdAkt_{dn}$ infected mice showed increased secretion of sEng (*Figure 2D*). These data demonstrate that inhibition of the survival protein, Akt, *in vitro* and *in vivo*, augments the levels of cleaved endoglin released from cells.

To further investigate this phenomenon, we went on to examine whether positive modulation of Akt, could suppress sEng release. Interestingly, overexpression of constitutively active myristilated Akt (Akt^{myr}) did not inhibit endoglin mRNA expression, nor did it inhibit basal sEng release from HUVEC (*Figure 3A* and *B*). However, when co-expressed with PTEN, Akt^{myr} completely abrogated PTEN-mediated upregulation of endoglin mRNA

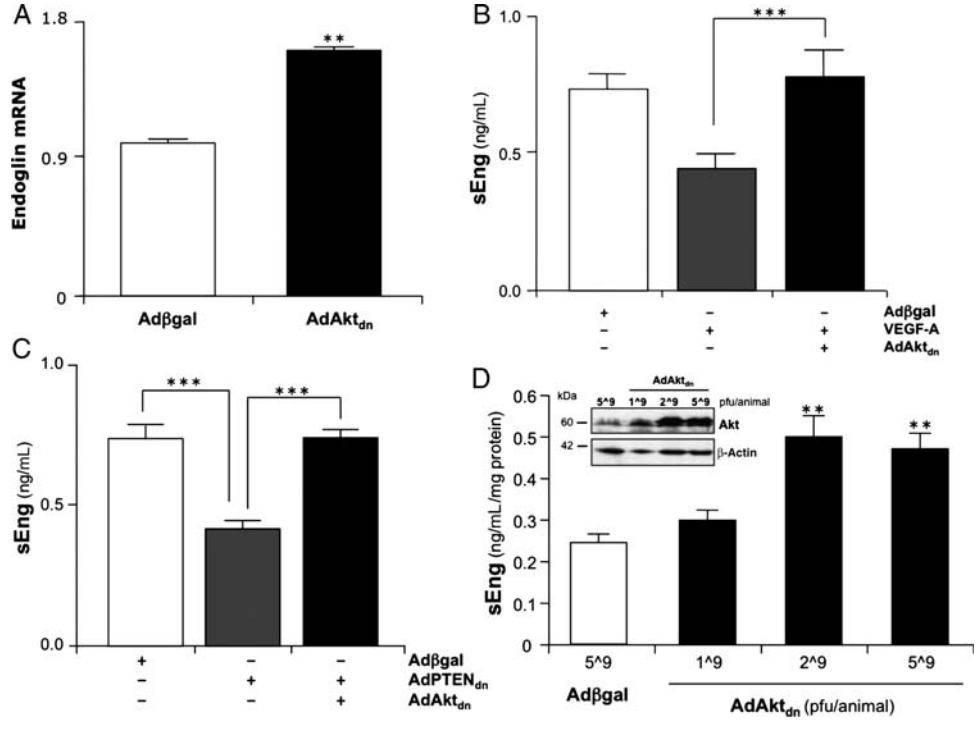

Figure 2 Akt inhibition induces the release of soluble endoglin. (*A*) Relative endoglin mRNA levels in human umbilical vein endothelial cells after infection with an adenovirus encoding dominant-negative Akt ($AdAkt_{dn}$) or β-galactoside control adenovirus (Adβgal). $^{**}P < 0.01$ vs. Adβgal. Soluble endoglin (sEng) level from Akt_{dn} overexpressing human umbilical vein endothelial cells (*B*) stimulated with VEGF-A (20 ng/mL) or (*C*) co-infected with $AdPTEN_{dn}$.(*D*) C57/B6J mice were injected i.v. with $AdAkt_{dn}$ or Adβgal and 5 days later blood and organs analysed. Soluble endoglin levels in conditioned medium of liver explants from mice at 24 h. Soluble endoglin release was normalized to total protein content of the explants and soluble endoglin quantified by ELISA. All results are the mean (± SEM) of three experiments performed in triplicate ($n = 9$). $^{**}P < 0.01$ vs. Adβgal. Inset—immunoblot of mouse liver lysates for Akt.

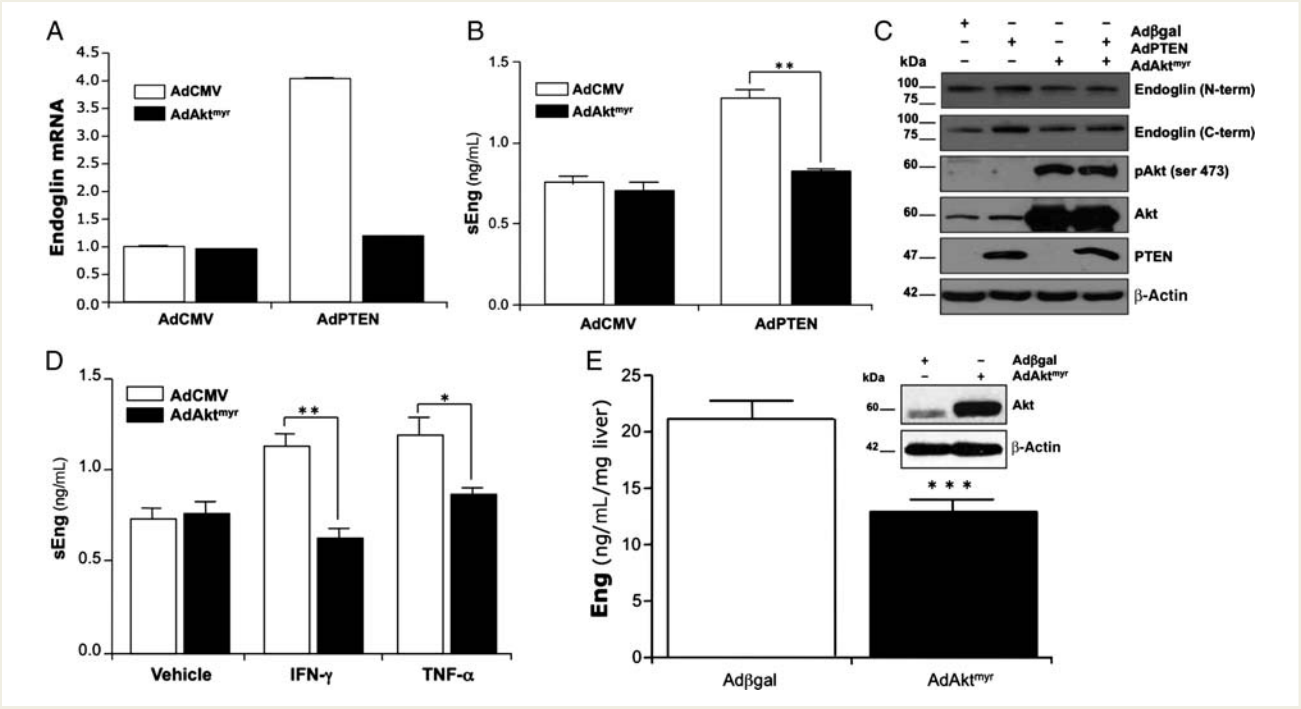

Figure 3 Akt activation inhibits the release of soluble endoglin. (*A*) Relative endoglin mRNA and (*B*) soluble endoglin protein levels after human umbilical vein endothelial cells were infected with AdCMV, AdPTEN, and/or myristylated Akt (AdAkt^{myr}). (*C*) Immunoblot with antibodies against; endoglin (N-terminus), endoglin (C-terminus), phosphorylated Akt [pAkt (ser 473)], Akt, PTEN, and β-actin. (*D*) Akt^{myr} overexpressing cells incubated with interferon-γ (IFN-γ;10 ng/mL) or tumour necrosis factor-α (TNF-α; 50 ng/mL). All experiments (unless stated otherwise) were conducted in M199/5%FCS for 24 h and cell supernatants collected for soluble endoglin quantification by ELISA. All results are the mean (±SEM) of three experiments performed in triplicate ($n = 9$). *$P < 0.05$, **$P < 0.01$. Mice were injected i.v. with AdAkt^{myr} or Adβgal and 5 days later blood and organs analysed. (*E*) Endoglin levels in liver from mice at 24 h. Endoglin was normalized to total protein and quantified by ELISA. All results are the mean (±SEM) of three experiments performed in triplicate ($n = 9$). ***$P < 0.001$ vs. Adβgal.

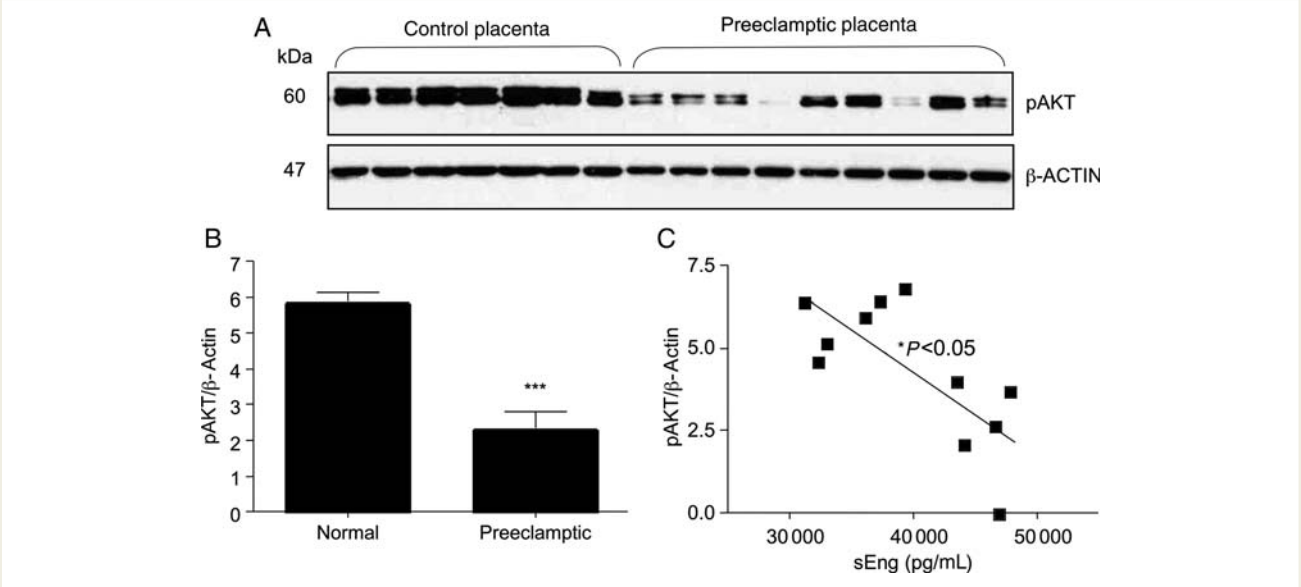

Figure 4 Phosphorylation of Akt is decreased in preeclamptic placenta and correlates inversely with soluble endoglin. (*A*) Lysates of placenta from normal or preeclamptic pregnancies were immunoblotted with antibodies against phosphorylated Akt (pAkt-ser 473) and β-actin. (*B*) Densitometric analysis showing ration of pAkt:β-actin in A. (*C*) Correlation between plasma soluble endoglin and pAkt:β-actin ratio of placenta. *$P < 0.05$.

(*Figure 3A*) and release of the soluble protein (*Figure 3B*). Human umbilical vein endothelial cell lysates immunoblotted for the N and C termini of endoglin confirmed the upregulation of endoglin following PTEN overexpression and Akt^{myr}, which increased the level of phosphorylated Akt, inhibited this upregulation (*Figure 3C*). Consistent with these findings, the pro-inflammatory cytokine-mediated release of sEng was inhibited by Akt^{myr} (*Figure 3D*) and systemic administration of AdAkt^{myr} to mice resulted in decreased endoglin protein in liver tissues compared with control animals (*Figure 3E*).

Akt is reduced in the preeclamptic placenta and inversely correlates with maternal soluble endoglin

Preeclampsia is characterized by widespread endothelial cell dysfunction and the progressive elevation of circulating sEng. Western blot analyses of placental lysates demonstrated that phosphorylation of Akt was significantly reduced in preeclamptic placenta compared with placenta from normal pregnancies (*Figure 4A* and *B*). Furthermore, the level of placental Akt phosphorylation inversely correlated with maternal plasma sEng levels (*Figure 4C*). Thus, the rise in circulating sEng paralleled the fall in Akt activity.

Haeme oxygenase-1 suppresses soluble endoglin release via Akt

We previously demonstrated that HO reduces the release of sEng under basal and cytokine-stimulated conditions.[19] This has been recently confirmed in a study showing that TNF-α-induced sEng release from endothelial cells and placental explants could be abrogated by upregulation of HO activity by haemin.[20] Interestingly, activation of Akt by overexpression of AdPTEN$_{dn}$, which induced Akt phosphorylation, also upregulated HO-1 protein in endothelial cells (*Figure 5A*). Knockdown of Akt1, using siRNA, significantly induced sEng release (*Figure 5B*) and more importantly, prevented the inhibition of sEng caused by overexpression of HO-1 (*Figure 5B*). In addition, HO-1 expression in HUVEC was also prevented by siRNA-mediated knockdown of Akt1 (*Figure 5C*). We previously demonstrated that knockdown of HO-1, using siRNA, induced sEng release,[19] here we show that knockdown of HO-1 potentiates the IFN-γ and TNF-α-induced sEng release (*Figure 5D*). In addition, overexpression of Akt^{myr} could not prevent the upregulation of sEng after loss of HO-1 (*Figure 6A*), suggesting that HO-1 and Akt regulate sEng release interdependently.

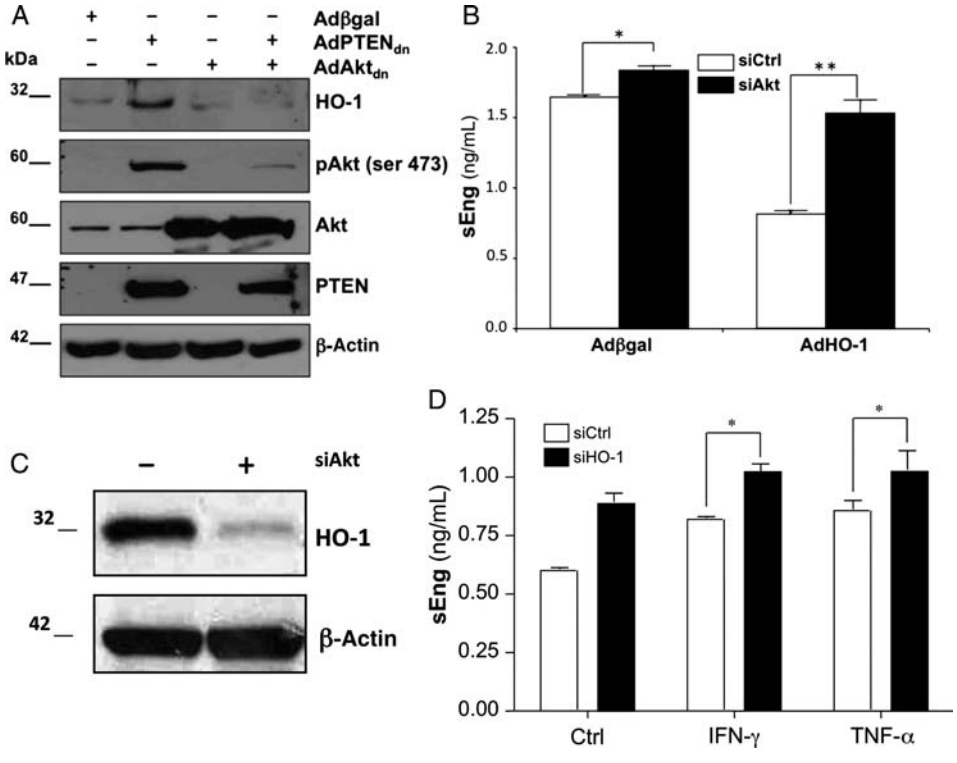

Figure 5 Haeme oxygenase-1 requires Akt to inhibit soluble endoglin release. (*A*) Lysates from human umbilical vein endothelial cells infected with AdCMV, AdPTEN$_{dn}$, and/AdAkt$_{dn}$ were immunoblotted with antibodies against phosphorylated Akt [pAkt (ser 473)], Akt, PTEN, HO-1, and β-actin. (*B*) Knockdown of Akt1 (siAkt) in human umbilical vein endothelial cells overexpressing HO-1 or βgal. (*C*) Immunoblot of Akt siRNA treated human umbilical vein endothelial cells for HO-1 and β-actin. (*D*) Knockdown of HO-1 (siHO-1) in human umbilical vein endothelial cells and stimulation with interferon-γ (10 ng/mL) or tumour necrosis factor-α (TNF-α; 50 ng/mL). All experiments were conducted in M199/5%FCS for 24 h and cell supernatants collected for soluble endoglin (sEng) quantification by ELISA. All results are the mean (±SEM) of three experiments performed in triplicate ($n = 9$). *$P < 0.05$, **$P < 0.01$.

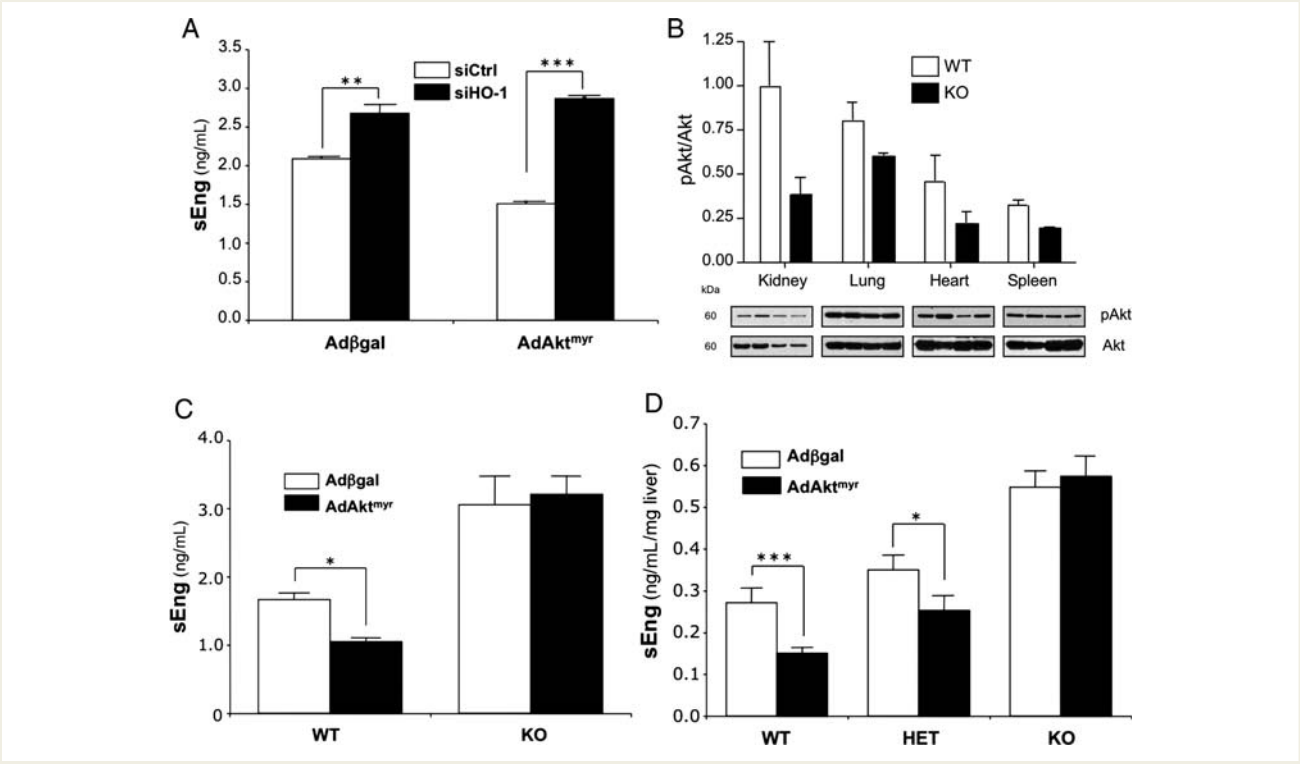

Figure 6 Akt requires haeme oxygenase-1 to inhibit soluble endoglin release. (*A*) Soluble endoglin (sEng) release from human umbilical vein endothelial cells after knockdown of haeme oxygenase-1 (siHO-1), and control (siCtrl) in human umbilical vein endothelial cells overexpressing Akt^{myr} or βgal. (*B*) pAkt levels in organs of haeme oxygenase-1 wild-type and haeme oxygenase-1-deficient mice. Mice were injected i.v. with AdAkt^{myr} or Adβgal and 5 days later blood and organs analysed. (*B*) Plasma soluble endoglin levels in wild-type and haeme oxygenase-1-deficient mice. (*C*) Soluble endoglin levels in conditioned medium of liver explants from haeme oxygenase-1 wild-type, heterozygous, and haeme oxygenase-1-deficient mice at 24 h. In explant studies, soluble endoglin release was normalized to total protein content of the explants and soluble endoglin quantified by ELISA. All results are the mean (±SEM) of three experiments performed in triplicate ($n = 9$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

HO-1-null mice exhibit elevated circulating sEng[19] and western blotting of organs from HO-1 null mice showed a decrease in phosphorylation of Akt (*Figure 6B*). Furthermore, Akt^{myr} overexpression in WT animals resulted in reduced circulating sEng (*Figure 6C*). Moreover, Akt^{myr} overexpression failed to suppress the circulating levels of sEng in HO-1 null mice (*Figure 6C*). To investigate this further, liver explants were established from adenoviral-infected mice, cultured for 24 h and supernatants assayed for sEng. Explants from AdAkt^{myr} infected WT and HO-1 heterozygous mice, produced significantly less sEng compared with Adβgal-infected controls (*Figure 6D*). Liver explants from HO-1 null mice released significantly more sEng than WT and heterozygous mice and consistent with our *in vitro* studies, overexpression of AdAkt^{myr} had no effect on sEng release from liver explants in HO-1 null mice (*Figure 6D*) demonstrating that HO-1 and Akt play pivotal, interdependent roles in suppressing the release of sEng *in vivo*.

Discussion

Serum sFlt-1 and sEng are increased in pregnant women prior to the clinical symptoms of preeclampsia.[10] Inhibition of VEGF or TGF-β signalling by high circulating sEng activates the endothelium to promote vascular dysfunction.[1,6] The salient finding highlighted by this study is the identification of PI3K/Akt signalling, in concert with HO-1, as a central negative regulator of endoglin shedding *in vivo*. The significance of this finding is reinforced by the observation that Akt phophorylation is decreased in the preeclamptic placenta and inversely correlates with the maternal circulating levels of sEng. In addition, survival factors that exert their protective effects via Akt, such as VEGF-A, FGF-2, Ang-1, and insulin, all negatively regulate sEng release from endothelial cells. Our finding that VEGF suppresses sEng release from endothelial cells suggests that in preeclampsia, the increase in placental sEng[6,19] is likely to be further compounded by the loss of VEGF-A activity due to the concomitant rise in its antagonist, sFlt-1, in the maternal circulation.

Knockdown of HO-2 reduces Akt phosphorylation *in vivo*[34] and carbon monoxide, the gaseous product of HO, stimulates Akt phosphorylation in hepatocytes[35] and endothelial cells[36] supporting a positive feedback loop between the HO and PI3K/Akt pathways. In this regard, it is important to remember that the loss of HO activity may be a causative factor in preeclampsia, as HO-1 protects against TNF-α-induced placental damage[37] and

suppresses cytokine-mediated sEng and sFlt-1 release.[19] The most compelling evidence for this comes from a recent study using faetal placental cells from women at 11 weeks gestation. Farina *et al.*[38] showed that the expression of HO-1 mRNA decreased in chorionic villous samples (faetal cells) from women who went on to develop preeclampsia. This very early decrease in HO-1 could explain, at least in part, the elevated levels of anti-angiogenic factors seen later in pregnancy in preeclamptic women. Transforming growth factor-β1 stimulates HO-1 expression via the PI3K/Akt pathway in human lung epithelial cells.[39] Thus, loss of TGF-β1 signalling, due to the rise in sEng in preeclampsia, may further compromise maternal endothelial HO activity. Our data show that PI3K/Akt activation is decreased in the organs of HO-1 null mice and that increased PI3K/Akt activation induces HO-1 expression in endothelial cells and loss of such a positive feedback system may lead to greater loss of endothelial integrity under conditions of high circulating sEng, observed in a number of vascular disorders. The predominant upstream regulator of HO-1 expression is Nuclear factor-like 2 (Nrf-2). Nuclear accumulation of Nrf-2 and HO-1 expression was shown to be PI3K-dependent and MEK-MAPK independent in the endothelium.[40] The interdependency between PI3K/Akt and HO-1 identified in this study needs further investigation to determine whether they are regulated at the level of Nrf-2.

It has been shown that an increase in circulating sEng has direct, significant, negative effects on endothelial health *in vivo*.[1] It abrogates TGF-β-mediated signalling, enhances lung and liver microvascular permeability, causes focal endotheliosis in kidney glomeruli and blocks TGF-β1-induced vasodilation.[6] The involvement of sEng in a number of wide-ranging pathologies demonstrates that sEng is not only marker of endothelial integrity but also a contributing factor of endothelial dysfunction. Our discovery of a co-dependency between HO-1 and Akt in relation to sEng release implies that dysfunction of only one of these factors in the endothelium may explain the resultant increase in sEng in these disorders. Interestingly, both TNFα and sEng are elevated in malaria[41] and the level correlates with disease severity,[9] whereas HO-1 and CO protect against malaria progression.[42]

In conclusion, the discovery that PI3K/Akt and HO-1 provide key co-dependent and inhibitory signals required to suppress sEng release strongly suggests that the positive manipulation of PI3K/Akt and/or HO pathways would provide potential therapeutic targets in preventing excessive sEng release in vascular disorders including preeclampsia.

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Conflict of interest: none declared.

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