MONASH University

Novel Therapeutic Strategies for Preeclampsia

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Abstract

Preeclampsia remains a leading cause of maternal and perinatal mortality and morbidity worldwide. Whilst the use of anti-hypertensives to control maternal blood pressure and magnesium sulfate for seizure prevention has significantly improved maternal and perinatal outcomes by prolonging pregnancy, delivery of the placenta remains the only definitive treatment for preeclampsia. A therapy that targets the underlying disease itself is very much needed, particularly for women who develop preeclampsia early in gestation. The overall aim of this project was to identify potential therapies or therapeutic strategies that target the underlying disease development process in preeclampsia.

The clinical features of preeclampsia arise from widespread maternal endothelial dysfunction, which in turn is triggered by several vasoactive factors produced by a placenta undergoing ischemia-reperfusion injury. Blocking one or more of the vasoactive factors could significantly ameliorate maternal endothelial dysfunction and thus the clinical features of preeclampsia. Studies have shown that some of the key factors, the agonistic autoantibodies for the angiotensin II type-1 receptor, pro-inflammatory cytokines, and the anti-angiogenic factors, closely interact with each other during disease development. Activin A is another placental-derived factor that contributes to the development of endothelial dysfunction in preeclampsia. While it is well known that pro-inflammatory cytokines can trigger activin A production, the relationship between activin A and the anti-angiogenic factors, soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng), is unknown. Therefore, the first project examined the interactions between activin A and the anti-angiogenic factors, during placental production and the development of endothelial dysfunction, in order to determine whether blocking activin A could disrupt the vasoactive factors and be an effective treatment strategy for preeclampsia. The *in vitro* studies demonstrated that activin A does not stimulate placental production of the anti-angiogenic factors or vice versa. Furthermore, activin A triggered endothelial dysfunction by increasing endothelial NADPH Oxidase 2 expression and consequently, endothelial oxidative stress, whilst the anti-angiogenic factors did not. Therefore, activin A and the anti-angiogenic factors, sFlt1 and sEng, do not appear to interact with each other either during placental production or the development of endothelial

dysfunction. Hence, blocking activin A alone may not rescue maternal endothelial dysfunction sufficiently to completely alleviate the clinical features of preeclampsia.

An alternative treatment strategy would be to alleviate the damage at the two sites of injury in preeclampsia, the maternal endothelium and the placenta. Activation of the Nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor and its downstream anti-oxidant enzymes, such as heme oxygenase-1 was investigated to determine its efficacy in mitigating placental and endothelial dysfunction. Melatonin, an Nrf2 activator, reduced certain hallmarks of endothelial dysfunction *in vitro*, such as endothelial activation marker expression and endothelial monolayer permeability, but in the placenta, melatonin only reduced oxidative stress and not the production of vasoactive factors. Another Nrf2 activator, resveratrol, reduced all *in vitro* hallmarks of endothelial function examined, that is endothelial activation marker expression, vasoconstrictor expression and endothelial monolayer permeability. Additionally, resveratrol reduced oxidative stress and production of sFlt1 and activin A by term placental explants. Sulforaphane, one of the most potent Nrf2 activators, also significantly mitigated all the above-mentioned *in vitro* hallmarks of endothelial function. These experiments also worked towards establishing the sFlt1 and sEng animal model of preeclampsia in our laboratory. Preliminary data from this study suggest that melatonin could potentially mitigate the features of preeclampsia *in vivo*.

In conclusion, findings from this project demonstrate that inhibition of one vasoactive factor may not be a sufficient treatment strategy for preeclampsia, as some placental-derived vasoactive factors act independently of others during disease development. An alternative treatment strategy is to activate Nrf2 and its downstream anti-oxidant enzymes, which has the potential to improve endothelial function, whilst also reducing placental oxidative stress and production of vasoactive factors. Therefore, Nrf2 activation warrants further investigation as a potential therapeutic strategy for preeclampsia.

Publications during enrolment

Published journal articles:

Gurusinghe S, Wallace EM, Lim R. The relationship between Activin A and anti-angiogenic factors in the development of pre-eclampsia. *Pregnancy Hypertension.* 2014;4(1):3-6.

Lim R, Muljadi R, Koulaeva E, Vodoganes P, Chan ST, Acharya R, Gurusinghe S, Ritvos O, Pasternack A, Wallace EM. Activin A contributes to the development of hyperoxia-induced lung injury in neonatal mice. *Pediatric Research*. 2015;77(6):749-56.

Lim R, Adhikari S, Gurusinghe S, Leaw B, Acharya R, Rahman R, Ciayadi R, Potdar M, Kelso GF, Hearn MT, Wallace EM. Inhibition of activin A signaling in a mouse model of pre-eclampsia. *Placenta.* 2015;36(8):926-31.

Abstracts:

Gurusinghe S, Wallace EM, Lim R. The relationship between Activin A, anti-angiogenic factors and inflammatory cytokines in the pathogenesis of preeclampsia. Australian Health and Medical Research Congress. 2012

Gurusinghe S, Chan ST, Leaw B, Wallace EM. Lim R. Melatonin and Resveratrol as potential treatment options for preeclampsia. Perinatal Society of Australia and New Zealand (PSANZ). 2015

Gurusinghe S, Chan ST, Leaw B, Rahman R, Wallace EM, Lim R. Melatonin and Resveratrol as potential treatment options for preeclampsia. Society of Reproductive Investigation. 2015

Gurusinghe S, Chan ST, Leaw B, Rahman R, Singh, H, Wallace EM, Lim R. Melatonin and Resveratrol as potential treatment options for preeclampsia. International Federation of Placenta Associations. 2015

Thesis including published works: General Declaration

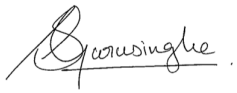
I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in a peer-reviewed journal and one unpublished publication. The core theme of the thesis is preeclampsia. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Ritchie Centre under the supervision of Dr Rebecca Lim.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.
In the case of chapters 2 and 4 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent (%) of students contribution
2	The relationship between Activin A and anti-angiogenic factors in the development of pre-eclampsia	Published	95%
4	Resveratrol activation of nuclear factor erythroid 2-related factor-2 (nrf2): potential therapy for preeclampsia	Submitted	90%

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature:  **Date: 31/03/2016**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributions to this work.

Main Supervisor signature:  **Date: 31/03/2016**

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List of Abbreviations

APR-1	ambient pressure reference monitor
ARE	antioxidant response element
AT_1 receptor	angiotensin type-1 receptor
AT1-AA	agonistic autoantibodies for the angiotensin II type-1 receptor
COMT	catechol-O-methyltransferase deficient
ELISA	Enzyme linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
ET_A receptor	endothelin type A receptor
EVT	Extravillous cytotrophoblast
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GST	glutathione S-transferases
HBSS	Hank's Balanced Salt Solution
HIF-1	hypoxia-inducible factor-1
HO-1	heme oxygenase-1
HUVECs	Human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule 1
IL	interleukin
IFNγ	interferon-γ
Keap1	Kelch-like ECH-associating protein 1
M199	Medium 199
MAP	Mean arterial pressure
MLT	melatonin
MRI	magnetic resonance imaging
NADPH oxidase	nicotinamide adenine dinucleotide phosphate-oxidase enzymes
NF-κB	Nuclear factor- κB
NO	nitric oxide
NOX	nicotinamide adenine dinucleotide phosphate-oxidase enzymes
NQO1	NADPH quinone oxidoreductase

Nrf2	Nuclear factor erythroid 2-related factor 2
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate-buffered saline
PlGF	placental growth factor
RES	resveratrol
ROS	reactive oxygen species
RUPP	reduced uterine perfusion pressure
sEng	soluble endoglin
sFlt1	soluble fms-like tyrosine kinase 1
SFN	sulforaphane
siRNA	small interfering RNA
SIRT1	sirtuin 1
TGF	transforming growth factor
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor-2
X	xanthine
XO	xanthine oxidase
ZO-1	Zonula occludens-1

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Chapter 1

Literature Review

1.1 Preeclampsia

Preeclampsia is a pregnancy-specific disorder that affects over 4 million women globally, every year [1]. It is the third leading cause of maternal mortality in the world, resulting in over 63,000 maternal deaths annually, with developing countries being the most affected [1]. Preeclampsia poses a significant risk to the fetus as well, as it considerably increases the risk of neonatal morbidity and mortality. It is currently the leading cause of iatrogenic preterm birth and is responsible for nearly 15% of all preterm births annually [2].

Preeclampsia is associated with a wide spectrum of clinical features, however the critical clinical feature of the disease is new-onset hypertension that arises after 20 weeks of gestation [3, 4]. Diagnosis of preeclampsia requires this cardinal feature to be present along with one or more of the following signs of organ dysfunction: pulmonary oedema, renal insufficiency as indicated by proteinuria or elevated serum creatinine levels, liver impairment as indicated by raised serum levels of liver transaminases or severe epigastric pain, haematological dysfunction as suggested by thrombocytopenia or haemolysis and neurological impairment as indicated by visual disturbances or headaches [3, 4]. The Australian and New Zealand guidelines for the diagnosis of preeclampsia also includes fetal growth restriction as a clinical feature indicative of preeclampsia, however in the US it was removed as an indicator in 2013 [3, 4]. The presence of haemolysis, elevated liver enzyme levels and low platelet count, which is collectively known as HELLP syndrome, is a severe form of preeclampsia that requires immediate treatment. Approximately 2% of women with preeclampsia can also develop generalized seizures and this form of the disease is widely known as eclampsia [5]. Headaches and visual blurring, the signs of neurological impairment mentioned above, can

precede eclampsia, but not always. The symptoms in patients diagnosed with preeclampsia vary greatly from one another with regards to clinical presentation, time of onset and severity.

Thus far, the only definitive treatment for preeclampsia that can alleviate all of the above-mentioned clinical features and prevent the maternal progression to multiple organ failure is delivery of the placenta. However, this is a treatment option that can often lead to fetal mortality or complications associated with preterm birth for the fetus, especially when the disease develops very early in gestation. Hence, current management of preeclampsia requires the severity of the maternal clinical features to be weighed against the maturity of the fetus.

Once preeclampsia is diagnosed, disease management is based on the severity of the maternal disease and gestation at diagnosis [3]. Patients with mild to moderate clinical features are closely monitored to ensure that the disease has not progressed to the severe form [4]. Maternal blood pressure is controlled via anti-hypertensive medication if necessary, and the baby is delivered as soon as feasible [6]. In general, preeclampsia is considered to be severe when there is difficulty controlling blood pressure and there is a deterioration of the clinical condition; involving the development of HELLP syndrome, pulmonary edema, worsening kidney and liver function or persistent neurological symptoms suggesting impending eclampsia [3, 7]. In such cases of severe preeclampsia, delivery is organized as soon as possible in order to prevent significant maternal morbidity and mortality [4]. However, depending on the gestational age of the fetus, this can lead to significant neonatal morbidity and mortality. The onset of severe preeclampsia at $\leq$ 24 weeks gestation results in 80-100% perinatal mortality and considerable maternal morbidity, with rates over 65% [8, 9]. Perinatal survival rates significantly improve if preeclampsia develops at or after 24 weeks gestation, and almost all neonates have been shown to survive if the onset of severe preeclampsia occurs after 27 weeks gestation, provided there are appropriate neonatal intensive care facilities [8, 10]. However, these neonates are at significant risk of developing preterm birth related complications, including neurological impairments and respiratory problems [11, 12]. In developing countries, the lack of appropriate neonatal intensive care facilities further increases the rate of perinatal mortality and morbidity. In addition, patients in many developing countries lack regular antenatal care and have limited access to hospitals. This results in late detection of the disease when severe preeclampsia has already set in, thus significantly increasing the incidence of both maternal and neonatal morbidity, as well as mortality in these countries.

Due to the significant maternal and neonatal consequences that result from preeclampsia, a treatment that can alleviate the clinical features for the mother, whilst allowing the pregnancy to continue till term is very much needed. However, the biggest obstacle to finding therapies for preeclampsia is our limited understanding of the mechanisms that contribute to disease development.

1.2 Pathophysiology of preeclampsia

In preeclampsia, it is evident that the placenta is the underlying source of the disease, where delivery remains the only effective treatment to-date. Since preeclampsia develops in molar pregnancies as well, it is clear that it is the placenta rather than the fetus that is necessary for disease progression [13]. However, the clinical features of preeclampsia are known to arise from widespread maternal endothelial dysfunction [14]. Thus, disease progression has been suggested to take place in two stages [15]. The initial asymptomatic phase involving the placenta occurs in the first trimester of pregnancy, when the maternal uterine spiral arteries that supply blood to the placenta fail to undergo adequate remodeling, a process required for sufficient placental perfusion. The resulting alteration in blood flow to the placenta is hypothesized to lead to placental ischemia-reperfusion injury. The second phase is the development of maternal endothelial dysfunction, and the consequent manifestation of the clinical features of preeclampsia. These two phases are linked together by a wide variety of factors that are released by the stressed placenta into the maternal circulation, and which in turn induce maternal endothelial dysfunction. The exact nature and mechanisms of action of these vasoactive factors are currently an area of intense investigation.

1.2.1 Uterine spiral artery remodeling and preeclampsia.

During pregnancy, the placenta receives its blood supply via the uterine spiral arteries. In a non-pregnant uterus, these spiral arteries are narrow, high-resistance vessels with a low blood flow, in order to prevent significant blood loss during menstruation. Thus, in early pregnancy these arteries need to be remodeled into large flaccid high-flow vessels, to ensure a continuous and adequate blood supply reaches the placenta and the developing fetus.

Remodeling is done by the extravillous cytotrophoblast (EVT) during placental development. They invade the uterine spiral arteries and induce the loss of the endothelial and smooth muscle cell lining and also significantly widen the vessels until they reach the inner third of the myometrium [16-18]. These changes increase the size of each spiral arterial lumen up to 10-fold, allowing 3-4 times more blood to be delivered to the intervillous space of the placenta [19]. Due to the loss of the endothelial and smooth muscle lining, the vessels are also non-responsive to maternal endocrine stimuli, which ensures that this large blood flow to the placenta is uninterrupted and at a low pressure [19-21].

In preeclampsia, the degree of spiral artery remodeling is significantly less compared to healthy pregnancies (Figure 1.1). Histological studies of spiral arteries from preeclamptic pregnancies revealed remodeling to be limited only to the decidual segments with no remodeling in the myometrial segments, whilst about one third of the vessels showed no signs of remodeling at all [22-27]. In fact, in severe preeclampsia only 10% of the spiral arteries were found to be fully converted as opposed to 96% in healthy pregnancies [28, 29]. Hence, in preeclampsia, most spiral arteries are extremely narrow, whilst also retaining the smooth muscle lining that enables regular vasoconstriction in response to maternal endocrine stimuli [25]. This results in a significantly reduced, as well as intermittent, blood flow to the placenta, which ultimately causes placental ischemia-reperfusion injury [30]. The resulting molecular changes in the placenta lead to the excessive production of the vasoactive molecules that trigger systemic maternal endothelial dysfunction in preeclampsia.

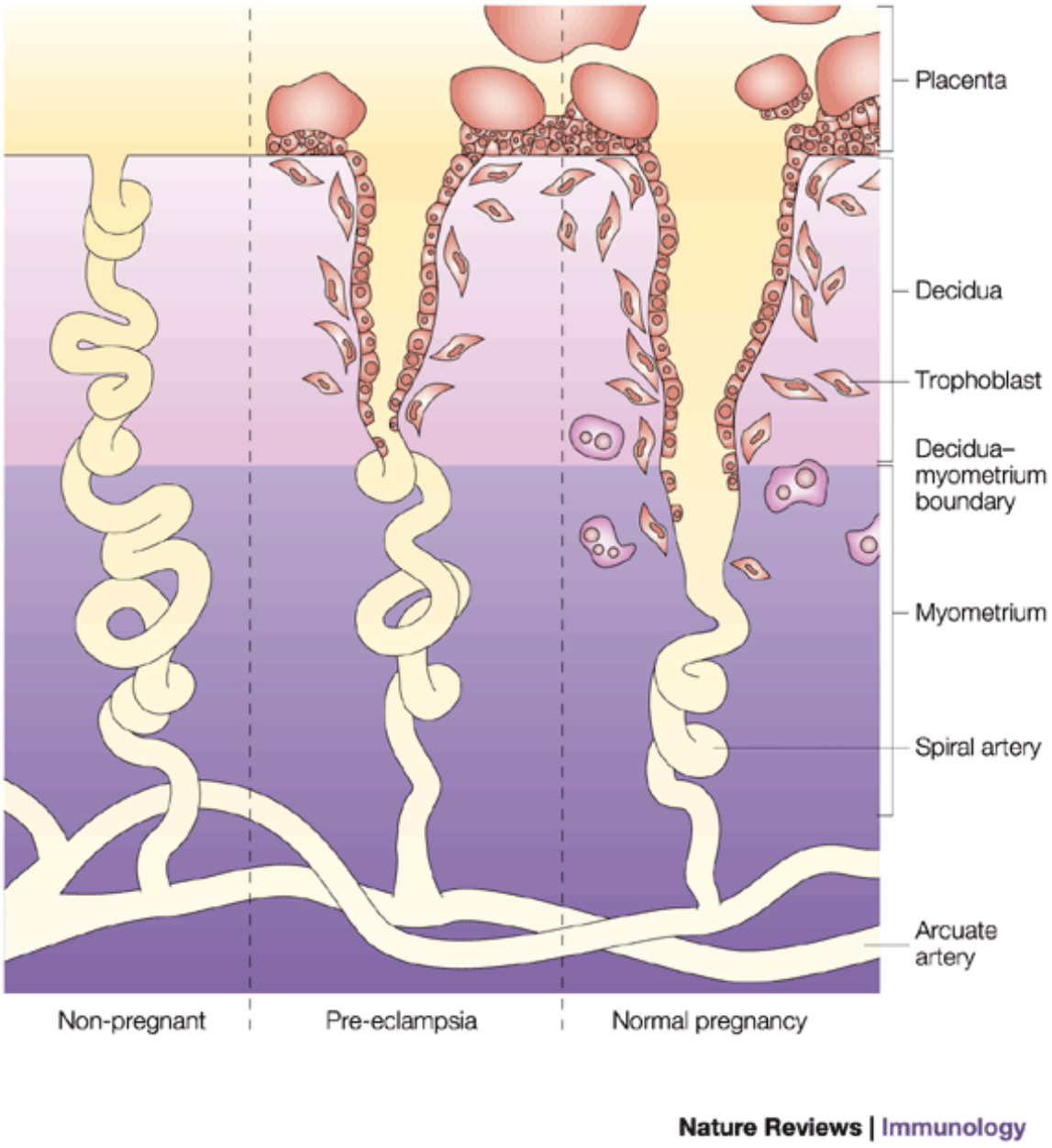

Figure 1.1: Uterine spiral arteries in non-pregnant women, in women with preterm preeclampsia and in normal pregnancies. In the non-pregnant state the uterine spiral arteries are narrow and tightly coiled. In healthy pregnancies, invading trophoblast cells remodel the spiral arteries by uncoiling and widening them, whilst also removing their endothelial and smooth muscle lining to make the vessels unresponsive to maternal stimuli. These changes continue into the myometrial segment of the spiral arteries and they ensure a large and continuous blood supply to the placenta and the growing fetus. In pregnancies affected by preterm preeclampsia, remodeling of the spiral arteries is incomplete and therefore the vessels are narrower than those of a healthy pregnancy with most remodeling being limited only to the decidual segment of the spiral arteries. Image adapted from Moffett-King, A. Natural killer cells and pregnancy. Nature Reviews Immunology. 2002; 2(9): 656-663.

The exact causes underlying impaired spiral artery remodeling in preeclampsia are still under investigation. The key factor appears to be a failure of adequate trophoblast invasion [22, 24,

31]. In fact, Meekins *et al.* observed complete trophoblast invasion in only 46% of decidual spiral arteries and 18% of myometrial spiral arteries from preeclamptic pregnancies, as opposed to 100% and 68% respectively in healthy pregnancies [24]. This deficient trophoblast invasion is mainly thought to arise from either EVT defects that affect their ability to invade the decidua, or from failed cross talk between the EVT and the maternal immune cells (decidual natural killer cells and macrophages). Current research suggests that there are multiple different causes underlying defective spiral artery remodeling in preeclampsia, and it is very likely that the exact cause would vary from one woman to another, with the end result being impaired placental perfusion.

1.2.2 Placental ischemia-reperfusion injury

The significantly reduced and intermittent placental perfusion ultimately leads to placental ischemia-reperfusion injury. The resulting impact on the placenta arises via two key pathways, that is, hypoxia and oxidative stress (Figure 1.2). During periods of ischemia, the placental tissue endures tissue hypoxia and responds by activating mechanisms that allow the tissue to adapt to the low oxygen levels (Figure 1.2). Conversely, during reperfusion, the sudden increase in oxygen levels leads to the production of high concentrations of free radicals in the placental tissue and subsequently to the development of placental oxidative stress (Figure 1.2).

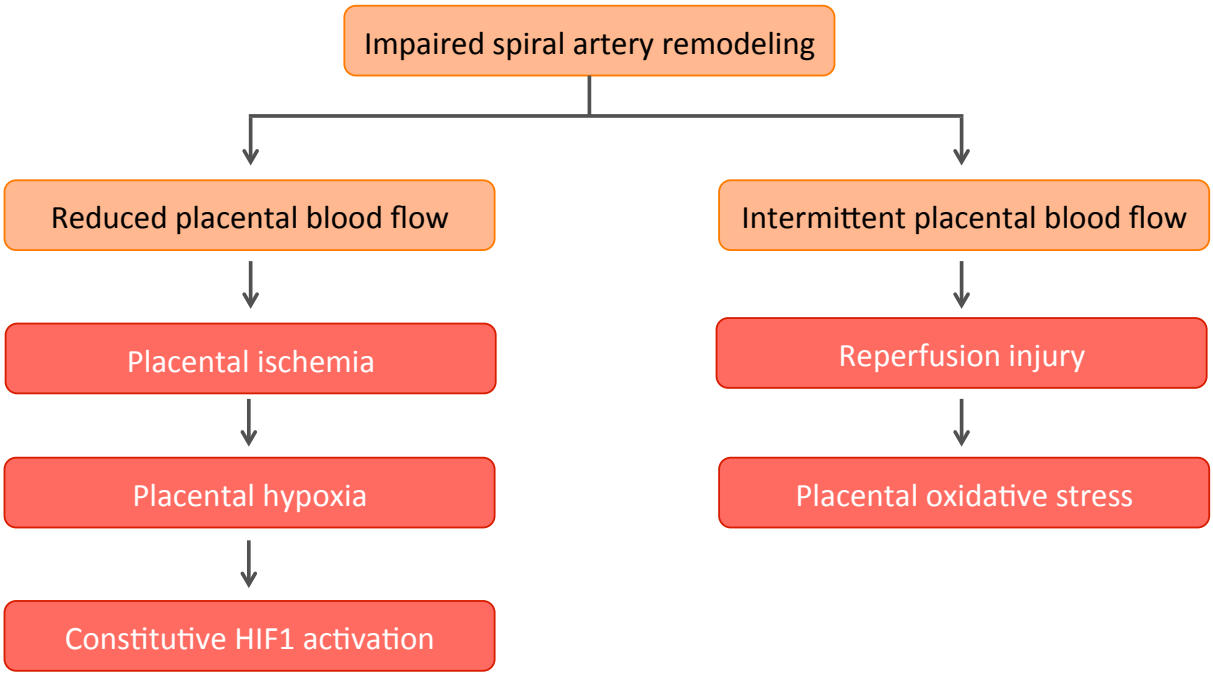

Figure 1.2: A schematic diagram of the process by which impaired spiral artery remodeling give rise to placental hypoxia and oxidative stress in pregnancies affected by preterm preeclampsia. Orange represents changes taking place in uterine arteries, whilst red represents changes taking place within placental tissue.

There is considerable evidence supporting the presence of placental hypoxia in preeclampsia. Placental perfusion have been shown to be significantly reduced in severe preeclampsia, sometimes even up to 50% lower than healthy pregnancies [32, 33]. Correspondingly, placentas from preeclamptic women have an increased incidence of infarcts compared with placentas from normal pregnancies [34]. Furthermore, the incidence of preeclampsia is significantly higher in populations living in high altitude areas [35]. Finally, animal models of reduced uterine placental perfusion have been shown to develop several clinical features of preeclampsia, such as hypertension and proteinuria [36-38].

Tissue hypoxia leads to constitutive activation of hypoxia-inducible factor-1 (HIF-1), a transcription factor that regulates the cellular response to low oxygen levels (Figure 1.2). It is a heterodimeric protein consisting of a constitutively expressed β subunit and an oxygen-sensitive α subunit, which is rapidly degraded during normoxia [39]. In normal pregnancies, HIF-1α is highly expressed in the low-oxygen environment of the placenta in early gestation [39]. However, as spiral artery remodeling is completed and placental oxygen levels rise, HIF-1α levels fall, starting at around week 9 of gestation [39]. In contrast, in placentas from

preeclamptic women, HIF-1α remains elevated throughout gestation, due to the periods of placental ischemia [40, 41]. In addition, preeclamptic placentas fail to sufficiently down regulate HIF expression once re-oxygenation begins [40]. This prolonged activation of HIF-1α in the placenta plays a significant role in the development of preeclampsia, as it results in the overexpression of several molecules that ultimately contribute to the development of systemic endothelial dysfunction [42, 43] (more details in section 1.2.3). This has been confirmed by a study, which showed that HIF-1α overexpression in pregnant mice increases the circulatory levels of certain vasoactive factors, and the development of many of the characteristic features of preeclampsia, such as hypertension, proteinuria, fetal growth restriction and HELLP syndrome [44].

Several studies have also provided evidence in support of increased placental oxidative stress in preeclampsia, which arises due to the changes in oxygen levels that occur during ischemia-reperfusion [30]. Preeclamptic placentas have been shown to have higher levels of free radicals, such as peroxynitrite and superoxide, along with decreased expression and activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase [45-49]. These data were complemented by studies that demonstrated high concentrations of lipid peroxides and protein carbonyls in preeclamptic placentas indicating oxidative damage to lipids and proteins respectively [46, 47, 50-53]. The damage induced by oxidative stress can eventually lead to cell death, and indeed increased placental apoptosis and necrosis is observed in preeclampsia [54, 55]. The fact that the placental oxidative stress is induced by ischemia-reperfusion is supported by *in vitro* studies, that have shown normal placental tissue exposed to ischemia-reperfusion to develop oxidative damage as well [56, 57].

The degree of placental hypoxia and oxidative stress greatly depends on the extent of spiral artery remodeling which varies significantly between patients, which in turn contributes to the variations in disease severity, time of onset and clinical presentation observed among patients with preeclampsia. The molecular cascades triggered in the placenta in response to the hypoxia and oxidative stress lead to the production of many factors that enter the maternal circulation and trigger systemic endothelial dysfunction.

1.2.3 Vasoactive factors released by preeclamptic placentae

Identification of the placental vasoactive factors that trigger maternal endothelial dysfunction in preeclampsia has been an area of intense investigation. Evidence to support the presence of such factors in preeclampsia has come from studies that have shown serum from women with preeclampsia to induce endothelial damage and dysfunction *in vitro* [58, 59]. Whilst previous research was focused on identifying a single predominant circulating factor, to-date several molecules have been identified that are present in high concentrations in the circulation of women with preeclampsia that can also cause significant endothelial damage. Considering the heterogeneity of the disease in terms of severity, time of onset and clinical presentation, it is not surprising that a number of different factors would be involved. It is likely that the extent of placental ischemia-reperfusion injury determined by the degree of spiral artery remodeling, would establish the nature and the volume of the factors released into the maternal circulation which in turn would influence the severity, time of onset and clinical presentation of the disease. Whilst many factors have been suggested to play a role in the development of preeclampsia, this review will focus on a few of the major vasoactive factors, such as the inflammatory cytokines, agonistic autoantibodies for the angiotensin II type-1 receptor (AT1-AA), anti-angiogenic factors and Activin A.

1.2.3.1 Inflammatory mediators

In preeclampsia, circulatory levels of inflammatory cytokines are significantly altered compared with healthy pregnancies. Serum levels of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-18 (IL-18) and interleukin-8 (IL-8) are significantly elevated in women with preeclampsia, whilst serum concentrations of the anti-inflammatory cytokine interleukin-10 (IL-10) is significantly reduced when compared with healthy pregnant women [60-65]. Correspondingly, placental production of these same pro-inflammatory cytokines has been shown to be significantly higher, while that of the anti-inflammatory cytokines is significantly lower in preeclampsia compared with healthy pregnancies [64, 66-70]. These changes to placental cytokine production could be mediated by prolonged HIF-1α activation due to ischemia, as well as oxidative stress generated due to reperfusion in the preeclamptic placenta, as both HIF-1 and reactive oxygen species (ROS) have been shown to increase

production of pro-inflammatory cytokines [56, 71-73]. Furthermore, AT1-AAs from preeclamptic patients have also been shown to stimulate placental production of TNF-α and increase circulatory TNF-α levels in pregnant mice injected with these autoantibodies, when compared with control mice [74]. The role of AT1-AAs in the pathophysiology of preeclampsia is discussed in more detail in section 1.2.3.2. In addition to the above, microparticles released from the syncytiotrophoblast contribute to the inflammatory response in preeclampsia. These microparticles are usually produced throughout pregnancy. The outer syncytiotrophoblast layer of the placental villi undergoes constant turnover, and the apoptotic cell fragments from the outer layers are released into the circulation in the form of microparticles in a process known as trophoblast deportation. Significantly higher levels of these microparticles are found in the plasma of women with preeclampsia compared with normal pregnant women [75]. This is due to the fact that in preeclampsia, placental oxidative stress along with the strong blood flow from the still muscular spiral arteries contributes to syncytiotrophoblast cell death, in addition to the normal process of trophoblast deportation [76]. Furthermore, syncytiotrophoblast microparticles from preeclamptic patients differ in their properties to those from normal pregnancies [17, 77]. Whilst microparticles from normal pregnancies stimulate the secretion of proinflammatory cytokines such as TNF-α, IL-12 and IL-18, they also have immunosuppressive properties as evidenced by their ability to inhibit interferon-γ (IFNγ) production by monocytes [77]. In contrast, microparticles from preeclamptic pregnancies stimulate the production of pro-inflammatory cytokines (TNF-α, IL-12, IL-18) to a greater extent than those from normal pregnancies and do not suppress IFNγ production by monocytes [77]. Therefore, syncytiotrophoblast microparticles contribute to the systemic inflammatory response in preeclampsia.

Infusion of TNF-α into pregnant rats increases mean arterial pressure and alters renal function, suggesting that pro-inflammatory cytokines play a critical role in giving rise to endothelial dysfunction and the subsequent clinical features of preeclampsia [78, 79]. Inflammatory cytokines, such as TNF-α and IL-6 can cause endothelial dysfunction via several pathways. One such pathway is by stimulating the maternal endothelial cells to produce the potent vasoconstrictor, endothelin-1 (ET-1) [78, 79]. Pregnant rats treated with TNF-α have elevated expression of ET-1 in the kidneys, placentas and the vascular cells [79]. Furthermore, TNF-α-induced hypertension and renal impairment in these rats was abolished by treatment with an antagonist to the ET-1 receptor, endothelin type A receptor (ET_A receptor) [79]. Another mechanism by which pro-inflammatory cytokines contribute to the

development of endothelial dysfunction is by inducing ROS production in vascular endothelial cells via the activation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase/NOX) enzymes [80, 81]. This leads to endothelial activation with the expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), as well as impaired endothelium-dependent vasodilation due to the depletion of nitric oxide (NO) [82, 83]. The reduction in NO levels results from the uncoupling of endothelial nitric oxide synthase (eNOS) to generate superoxide rather than NO, and also due to the reaction of NO with superoxide to form peroxynitrite [83]. In addition, intracellular ROS can increase endothelial cell permeability by inducing tyrosine phosphorylation of VE-cadherin and thereby reducing the integrity of the endothelial cell junctions [84]. Therefore, pro-inflammatory cytokines, especially TNF-α, directly cause endothelial dysfunction by increasing vasoconstrictors, impairing endothelium-dependent vasodilation, activating the endothelium and increasing endothelial permeability.

Apart from these direct mechanisms, inflammatory cytokines contribute to the development of endothelial dysfunction by stimulating placental production of other vasoactive factors, such as the anti-angiogenic factors, Activin A and possibly the AT1-AAs as well [85-88].

1.2.3.2 Angiotensin II type-1 receptor agonistic autoantibodies (AT1-AA)

Wallukat *et al.* (1999) were the first to identify the presence of autoantibodies capable of activating the angiotensin type-1 receptor (AT1-AA) in the serum of preeclamptic women [89]. These AT1-AAs can be detected as early as 20 weeks gestation in the majority of women that ultimately develop preeclampsia [90]. Interestingly, the autoantibodies are present only in pregnancies with an abnormal uterine perfusion pattern [90]. Correspondingly, pregnant rats subjected to a reduced uterine perfusion pressure have significantly higher levels of AT1-AAs in circulation compared with normal pregnant rats [88]. These data suggest that placental hypoxia/ischemia is important in the production of AT1-AAs. Furthermore, TNF-α treatment also significantly increases the level of circulatory AT1-AAs in pregnant rats, suggesting that inflammatory cytokines are also involved in stimulating placental production of AT1-AAs [88]. Hence, inflammatory cytokines resulting from placental hypoxia could be a trigger for the placental production of AT1-AAs in preeclampsia, however the exact molecular mechanisms are not known.

It is well accepted that autoantibodies play a key role in the development of systemic endothelial dysfunction in preeclampsia. When affinity-purified AT1-AAs from preeclamptic women are injected into pregnant mice, they develop some of the key features of preeclampsia, such as hypertension, proteinuria, the classical renal lesion of preeclampsia known as glomerular endotheliosis and fetal growth restriction [91]. Hypertension is induced by the autoantibodies via several distinct pathways. The primary pathway is via the activation of the angiotensin type-1 receptor (AT_1 receptor), which triggers a variety of mechanisms in the vasculature and kidneys that result in an increase in blood pressure [91]. AT_1 receptor activation by AT1-AAs also stimulates endothelial cell production of ET-1, which contributes to the hypertension in women with preeclampsia [92]. The final pathway by which AT1-AAs induce hypertension in preeclampsia is by stimulating an increase in circulatory levels of the anti-angiogenic factor, soluble fms-like tyrosine kinase 1 (sFlt1) [85, 91, 93]. Zhou *et al.* (2008) found that pregnant mice injected with AT1-AAs from women with preeclampsia had nearly 40-fold higher levels of circulatory sFlt1 than non-pregnant mice injected with the same autoantibodies, indicating that AT1-AA-stimulated sFlt1 is produced by the placenta [91]. Interestingly, whilst both these groups of mice develop hypertension, only the pregnant mice that also have high levels of sFlt1 developed proteinuria and glomerular endotheliosis [91]. This suggests that whilst AT1-AAs can induce hypertension by itself, AT1-AA-stimulated placental production of sFlt1 is necessary for the development of the renal pathologies associated with preeclampsia. The mechanisms by which sFlt1 contributes to the development of these clinical features will be described in section 1.2.3.3.

AT1-AAs further contribute to the pathophysiology of preeclampsia by stimulating placental production and increasing circulatory levels of two other vasoactive factors, that is, TNF-α (as previously mentioned in section 1.2.3.1), and the anti-angiogenic factor, soluble endoglin (sEng) [74, 85]. The role of sEng in the pathophysiology of preeclampsia will be discussed in more detail in section 1.2.3.3.

1.2.3.3 Anti-angiogenic factors

The anti-angiogenic factors, sFlt1 and sEng, are the most well-known and extensively studied vasoactive factors in the field of preeclampsia. Signaling from pro-angiogenic factors is

essential for the survival and growth of endothelial cells, and thus the presence of these circulating anti-angiogenic factors that inhibit pro-angiogenic factors contributes to the systemic endothelial dysfunction in preeclampsia.

sFlt1 is the alternatively spliced soluble form of the membrane-bound receptor, fms-like tyrosine kinase-1 (Flt-1), which is a receptor for the pro-angiogenic molecules vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). VEGF is a well-known promoter of endothelial cell survival, proliferation, migration, as well as endothelial NO and prostacyclin production, whilst PlGF augments the effects of VEGF during angiogenesis [94, 95]. Flt1 is thought to act as a negative regulator of VEGF, by sequestering it from vascular endothelial growth factor receptor-2 (VEGFR-2), the receptor that mediates most of the cellular responses to VEGF. sFlt1 consists of only the ligand-binding extracellular domain and thus binds to circulatory VEGF, reducing its interaction with endothelial VEGFR-2. This effectively diminishes the beneficial pro-angiogenic effects of VEGF on the vasculature. Circulatory sFlt1 levels are significantly higher in women with preeclampsia than in healthy pregnant women [96-98]. This rise in serum sFlt1 levels is evident from 17-20 weeks of gestation in women that develop early-onset preeclampsia [99].

Several studies have shown sFlt1 mRNA expression to be upregulated in preeclamptic placentas, indicating that the excess sFlt1 is derived from the placenta [100, 101]. Placental ischemia appears to be the main trigger for placental production of sFlt1 in preeclampsia, since pregnant rats subjected to reduced uterine perfusion pressure have elevated placental and plasma sFlt1 levels [102]. There is evidence to suggest that this increase in sFlt1, in response to placental hypoxia is mediated by HIF-1. Nevo *et al.* (2006) showed that first-trimester villous explants exposed to low oxygen conditions produce significantly more sFlt1, whilst HIF-1α knockdown in these explants mitigated the increase in sFlt1 [42]. Furthermore, HIF-1α overexpression in pregnant mice led to a significant increase in circulatory sFlt1 levels in these mice, compared to controls [44]. The elevated levels of pro-inflammatory cytokines present in preeclampsia could also trigger placental sFlt1 production, as TNF-α was shown to stimulate the release of sFlt1 from placental explants in a dose-dependent manner [103]. Another known mechanism of sFlt1 release is by the AT1-AAs present in women with preeclampsia, as mentioned in section 1.2.3.2.

A seminal study by Maynard *et al.* (2003) showed that adenoviral administration of sFlt1 to pregnant rats led to the development of several clinical features of preeclampsia, such as elevated blood pressure, proteinuria and glomerular endotheliosis [100]. These data clearly demonstrate that sFlt1 plays a key role in giving rise to systemic endothelial dysfunction and subsequently, the clinical features of preeclampsia. sFlt1 causes endothelial dysfunction mainly via sequestration of circulatory VEGF and PlGF, thus reducing their beneficial effects on the endothelium. As previously mentioned, VEGF promotes vasodilation by stimulating endothelial production of NO and prostacyclin [94]. Therefore, inhibition of these vasodilator effects of VEGF by sFlt1 contributes to the systemic vasoconstriction observed in preeclampsia [104-106].

The renal pathologies associated with excess circulatory sFlt1, proteinuria and glomerular endotheliosis, can also be attributed to inhibition of VEGF signaling, as VEGF knockdown in mouse kidneys results in proteinuria and glomerular endotheliosis [107]. Glomerular endotheliosis is a distinct renal lesion, in which the endothelial cells of the glomerular capillaries swell causing both occlusion of the capillary lumen and loss of endothelial fenestrations [108-110]. VEGF signaling is necessary for maintenance of endothelial fenestrations, and thus its inhibition leads to a reduction in glomerular endothelial fenestrations and the development of endotheliosis [111]. VEGF is also a major inducer and regulator of nephrin expression [112]. Nephrin is a component of the podocyte slit diaphragm and is the charge and size selective barrier of the glomerulus. Reduced expression of nephrin greatly disrupts normal glomerular permeability resulting in proteinuria [112]. Correspondingly, women with preeclampsia have significantly reduced expression of nephrin in their glomeruli and this is considered to be a contributory factor for the development of proteinuria in preeclampsia [113]. This decrease in nephrin expression in preeclampsia is most likely mediated by diminished VEGF signaling due to excess circulatory sFlt1.

Apart from sequestering VEGF and diminishing its actions, sFlt1 also contributes to systemic vasoconstriction in preeclampsia by increasing circulatory endothelin-1 levels. The expression of preproendothelin, the precursor protein for endothelin-1, was 3-fold higher in the kidneys of sFlt1-treated pregnant rats compared with normal pregnant rats [114]. Furthermore, endothelin type A (ET_A) receptor inhibition completely abolished the sFlt1-induced increase in arterial pressure in the pregnant rats, suggesting that endothelin-1 plays an important role in the development of hypertension due to excess sFlt1 [114]. Some studies also suggest that

sFlt1 contributes to the development of endothelial dysfunction by inducing ROS production, as sFlt1-treated pregnant rats were found to have elevated ROS levels in blood vessels, placentas and the kidneys [115, 116]. Correspondingly, impaired endothelium-dependent vasodilatation in sFlt1-treated rats was rescued by a superoxide scavenger, Tiron [116]. sFlt1-induced hypertension in pregnant rats was also significantly attenuated by another superoxide scavenger, Tempol [115].

sEng is another anti-angiogenic factor involved in the pathophysiology of preeclampsia. Endoglin is a membrane-bound co-receptor for transforming growth factor (TGF) β1 and β3, which is highly expressed in endothelial cells, as well as in syncytiotrophoblast cells [117, 118]. sEng consists only of the extracellular domain of membrane-bound endoglin, and is thus capable of binding to circulatory TGF-β1 and TGF-β3 and sequestering them from the membrane-bound receptors and subsequent cell signaling [119]. TGF-β is involved in the regulation of many complex and diverse pathways. It is important for vascular development during embryogenesis, as it can regulate endothelial cell proliferation and migration [120]. TGF-β1 and TGF-β3 can also stimulate vasodilation [121].

Circulatory levels of sEng in women that developed preeclampsia before term were found to be more than 4-fold higher than matched controls [122]. This increase begins at 17-20 weeks of gestation, with serum sEng levels nearly double at this stage in women who later developed preeclampsia than in matched controls [122]. Interestingly, the increase in serum sEng levels in preeclampsia has been shown to correlate with disease severity. Venkatesha *et al*. (2006), found that serum sEng levels were 3-fold higher in patients with mild preeclampsia, 5-fold higher in those with severe preeclampsia and 10-fold higher in those with HELLP syndrome, compared with gestation-matched controls [121]. This is corroborated by other studies that have also shown serum sEng levels to be higher in patients that develop severe and early-onset (<32 weeks) preeclampsia rather than mild and late-onset preeclampsia [122, 123]. These clinical data indicate that sEng is most likely associated with the more severe forms of the disease.

The placenta is considered to be a major source of excess sEng in preeclampsia. Placentas from preeclamptic patients have significantly higher levels of sEng protein than gestation-matched control placentas, particularly in the syncytiotrophoblast [121]. Furthermore, after placental delivery, serum sEng levels decline by 55-70% in preeclamptic patients [121, 124].

However, since the circulatory levels of sEng do not completely normalize after delivery in preeclamptic women and remain significantly higher than in healthy women, extra placental sources, such as the maternal endothelium cannot be ruled out [121, 124]. Similar to sFlt1, circulatory sEng release is also triggered by placental ischemia [125]. Pregnant rats subjected to reduced uterine perfusion pressure (RUPP) have significantly higher levels of circulatory sEng levels compared with normal pregnant rats [125]. This ischemia-induced increase in sEng is most likely mediated by HIF-1, since HIF-1α overexpression in pregnant mice leads to a significant increase in circulatory sEng levels compared with control rats [44]. AT1-AAs in preeclamptic patients may also contribute to placental sEng production, as they have been shown to elevate circulatory sEng levels in pregnant rodents [74, 85]. This is most likely mediated by TNF-α, as the increase in sEng expression in human placental explants treated with AT1-AAs from preeclamptic patients was significantly diminished when also treated with a TNF-α inhibitor [74]. Furthermore, TNF-α has also been shown to significantly increase sEng production by endothelial cells as well, and this could the mechanism underlying extra placental production of sEng [74].

Venkatesha *et al.* (2006) were the first to discover the contribution of sEng in the development of preeclampsia [121]. They found that adenoviral overexpression of sEng in pregnant rats induced a significant increase in blood pressure, which was confirmed by another study that found sEng overexpression in transgenic mice to lead to increased arterial pressure [126]. The mechanism by which sEng disrupts endothelial dysfunction is by binding to circulatory TGF-β1 and TGF-β3, thus sequestering them from exerting their beneficial effects on the endothelial cells [44]. Both TGF-β1 and TGF-β3 have been shown to induce vasodilation in a dose-dependent manner [121]. TGF-β1 appears to mediate this effect via the activation of eNOS and stimulation of prostacyclin production [121, 127, 128]. Thus, the inhibition of TGF-β signaling by sEng would eliminate its vasodilator effects, leading to vasoconstriction and consequently to an increase in blood pressure, as observed in sEng-treated pregnant rats.

Interestingly, a systemic increase in both sFlt1 and sEng resulted in the pregnant rats developing features of severe preeclampsia, including severe hypertension, nephrotic-range proteinuria and evidence of HELLP syndrome [121]. This data indicate that the effects of sFlt1 and sEng on endothelial dysfunction in preeclampsia are additive. Correspondingly, other studies have also shown that the combination of sFlt1 and sEng, but neither alone, can

induce loss of endothelial fenestrae, swelling of endothelial cells and cerebral edema, further confirming that together, sFlt1 and sEng can cause severe endothelial damage [129].

Since VEGF and TGF-β are critical for placental vasculogenesis and angiogenesis, it is often considered that the increase in sFlt1 and sEng could contribute to the impaired spiral artery remodeling during placentation in preeclampsia. However, this is not the case, as first trimester sFlt1 and sEng levels are similar between women who later on develop preeclampsia and women who do not develop the disease [99]. As previously mentioned, differences in the levels of these anti-angiogenic factors between the two groups of women can only be seen from 17-20 weeks [99]. Therefore, it appears that the placental abnormalities associated with preeclampsia precede the increase in anti-angiogenic levels, and as such altered anti-angiogenic levels could not be a cause of the placental defects.

1.2.3.4 Activin A

Activin A is a member of the TGF-β superfamily and is involved in cell proliferation, differentiation and apoptosis in a variety of organ systems, while also being a key regulator of inflammation [130]. Activin A is a homodimer consisting of two Inhibin βA subunits. It plays a critical role in the establishment of pregnancy by promoting pre-implantation embryo development, EVT invasion, syncytial fusion and production of placental hormones [131-134]. In preeclampsia, maternal circulatory Activin A levels are significantly higher than in healthy pregnancies, and in cases of severe early-onset preeclampsia this increase is evident as early as 11-13 weeks of gestation [135-138]. The placenta is the main source of Activin A in healthy pregnancies and this appears to be the case in preeclampsia as well [139]. Both Inhibin βA subunit mRNA expression and Activin A protein levels have been found to be significantly elevated in the placentas of women with preeclampsia compared with matched controls [140, 141]. Studies have shown this increase to be stimulated by placental oxidative stress rather than by placental exposure to hypoxia [142-144]. Placental explants cultured with free radical stimulators produce nearly 2-fold more Activin A than the untreated placental explants [143], whilst exposure to low oxygen conditions was actually found to reduce Activin A production by placental explants when compared with those exposed to 20% oxygen [142, 144]. In addition, the pro-inflammatory cytokines, TNF-α and IL-1β, also stimulate Activin A production from trophoblast cells [86, 145]. Since placental oxidative

stress also triggers placental production of pro-inflammatory cytokines, it is possible that the placental Activin A production in response to oxidative stress is mediated by the pro-inflammatory cytokines.

It is evident that the increased maternal serum Activin A levels significantly contribute to the development of preeclampsia, as pregnant mice treated with Activin A have been shown to develop hypertension, proteinuria and reduced fetal growth [146]. Activin A appears to disrupt endothelial function and give rise to these features of preeclampsia mainly by inducing endothelial oxidative stress via the up-regulation of endothelial NOX2 [146]. NOX2 is highly expressed in vascular endothelial cells and has the capacity to generate very high levels of ROS [147]. Consistently, endothelial NOX2 expression was found to be nearly 4-fold higher in women with preeclampsia compared with healthy controls [146]. Significantly higher levels of NOX2 in the vasculature can generate almost toxic levels of ROS. As previously described in section 1.2.3.1, elevated levels of ROS in endothelial cells impair endothelial-dependent vasodilatation by reducing NO bioavailability, and can also increase endothelial cell permeability by inducing tyrosine phosphorylation of VE-cadherin and reducing the integrity of the endothelial cell junctions. Furthermore, Activin A treatment was shown to decrease endothelial expression of eNOS, which could also decrease NO bioavailability [146]. Thus, it appears that Activin A contributes to the systemic endothelial dysfunction in preeclampsia by decreasing eNOS expression and by stimulating vascular oxidative stress via the up-regulation of endothelial NOX2.

1.2.3.5 The relationship between the vasoactive factors involved in preeclampsia

It is evident that there is a certain degree of cross talk between all of these vasoactive factors (represented in figure 1.3). The placental production of pro-inflammatory cytokines, such as TNF-α, is triggered by both placental hypoxia and oxidative stress. TNF-α in turn, has been shown to stimulate placental production of all of the other vasoactive factors. Hence, it is possible that the placenta produces AT1-AAs, anti-angiogenic factors and Activin A in response to TNF-α, following placental ischemia-reperfusion injury. AT1-AAs have then been shown to trigger placental production of both sFlt1 and sEng in preeclampsia, via the further activation of TNF-α [74, 92, 148]. Thus, there is strong evidence to suggest that AT1-AAs, anti-angiogenic factors and pro-inflammatory cytokines interact with each other along

one pathway in the development of preeclampsia (Figure 1.3). An understanding of these interactions between the vasoactive factors is important, as it enables us to determine which vasoactive factors, if any, should be inhibited, in order to significantly improve endothelial function and attenuate the clinical features of preeclampsia.

As previously mentioned, placental production of Activin A is triggered by oxidative stress, as well as by pro-inflammatory cytokines, such as TNF-α and IL-1β. Thus, the oxidative stress-induced increase in placental Activin A production is most likely mediated by the pro-inflammatory cytokines. However, it is not yet known whether Activin A interacts with AT1-AAs or the anti-angiogenic factors in the development of preeclampsia. Hence, the relationship between Activin A and the anti-angiogenic factors, sFlt1 and sEng, will be explored in detail in chapter 2.

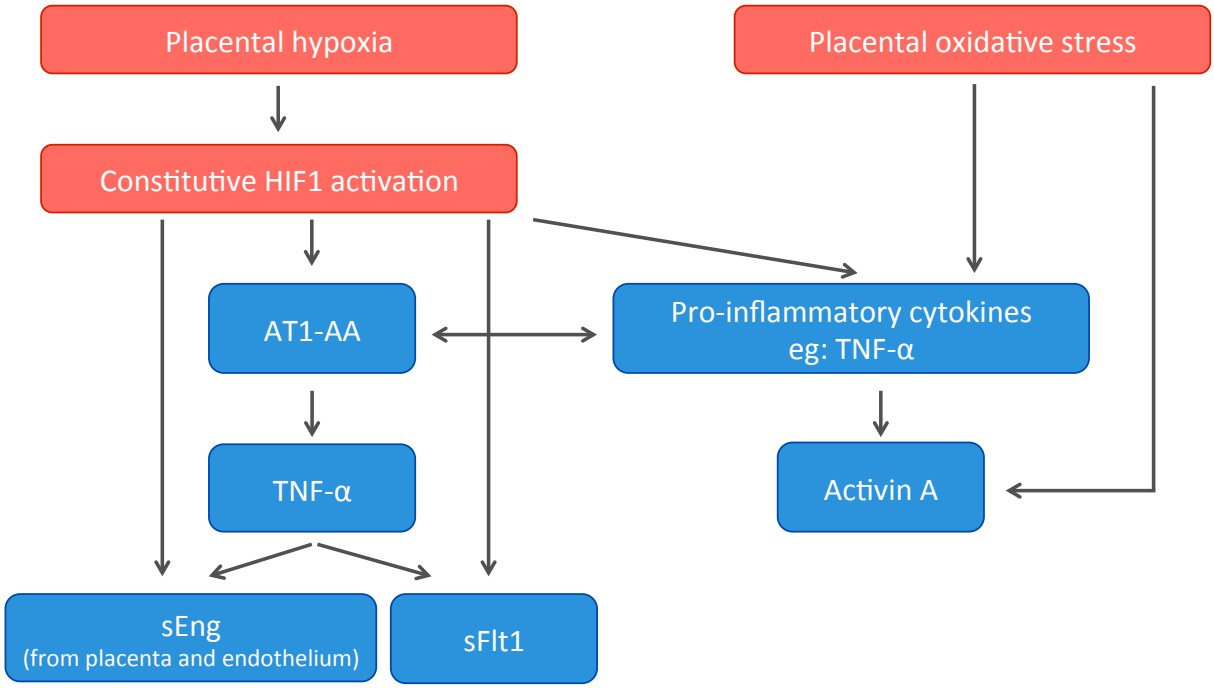

Figure 1.3: A schematic representation of the pathways that trigger the placental production of the key vasoactive factors. The red boxes represent the placental changes that result in the production of the vasoactive factors shown in blue. The diagram demonstrates the pathways by which the vasoactive factors are produced by the placenta, except for sEng, which is also produced by the endothelium in response to TNF-α.

1.2.4 Endothelial dysfunction in preeclampsia

Despite the significance of the placenta in disease development, the clinical features of preeclampsia are attributed to maternal endothelial dysfunction and activation. This is strengthened by the fact that maternal risk factors for preeclampsia, such as diabetes, obesity, increased insulin resistance, kidney disease, rheumatoid arthritis and lupus all involve altered endothelial function and are also risk factors for other endothelial diseases, specifically arthrosclerosis [149]. Furthermore, endothelial dysfunction is a known deleterious result of ischemia-reperfusion [150].

The endothelium plays a critical role in regulating vascular tone and vascular permeability, as well as in mediating inflammatory responses [151]. Endothelial dysfunction is characterized by a shift in the properties of the endothelium towards reduced vasodilatation and a proinflammatory, prothrombotic state [151]. There is ample evidence for the presence of endothelial dysfunction in women with preeclampsia. Plasma concentrations of biomarkers of endothelial activation and injury, such as VCAM-1, ICAM-1, E-selectin, von Willebrand factor, fibronectin, thrombomodulin, soluble tissue factor, plasminogen activator inhibitor-1 (PAI-1) and platelet-derived growth factor are all elevated in preeclamptic women [152-161]. Furthermore, endothelium-dependent vasodilatation is significantly impaired in preeclamptic women compared with healthy pregnant women [162-169].

1.2.4.1 Development of hypertension in preeclampsia

During normal pregnancy there is an increase in plasma volume and cardiac output to accommodate the significant increase in blood flow to the placenta and the fetus [170]. To prevent a consequent increase in mean arterial pressure, there is a reduction in total vascular resistance, ultimately leading to a decrease in arterial pressure as well [171]. This decrease in total peripheral resistance is mainly achieved with an increase in endothelial-derived vasodilators, such as NO and prostacyclin [172-174]. In addition, there is a decrease in vascular reactivity to vasoconstrictors [171]. Together, these changes lead to a significant decrease in vascular resistance and thus mean arterial pressure.

In contrast to normal pregnancy, preeclampsia is associated with intense vasoconstriction, together with impaired endothelium-dependent vasodilation, resulting in the increase in arterial pressure that is observed clinically [171]. The intense vasoconstriction in preeclampsia is due to a significant increase in the circulatory levels of the vasoconstrictors, ET-1, thromboxane A_2 and isoprostanes in preeclamptic women [154-156, 175, 176]. Endothelin-1 is a major contributor to the hypertension observed in preeclampsia, given its potent vasoconstriction properties, and also because its production is stimulated by several key vasoactive factors involved in preeclampsia, including TNF-α, AT1-AAs and sFlt1 [79, 92, 114]. In addition to these vasoconstrictors, AT1 receptor activation by AT1-AAs also contributes to the intense vasoconstriction in preeclampsia (section 1.2.3.2).

The impairment of endothelial-controlled vasodilation in preeclampsia is due to the decrease in endothelial production of the vasodilators, NO and prostacyclin [177-179]. However, the status of NO levels in preeclampsia remains controversial with variable results reported [165, 175, 180-182]. This could be due to the difficulties associated with accurate assessment of NO levels. Nevertheless, eNOS expression is significantly lower in endothelial cells from preeclamptic pregnancies compared with those from healthy pregnancies, indicating that NO production could be impaired in preeclampsia [183]. VEGF and TGF-β are key stimulators of endothelial NO and prostacyclin production, and thus the inhibition of VEGF and TGF-β by sFlt1 and sEng, respectively, is one the key reasons suggested for the decrease in endothelial NO and prostacyclin (section 1.2.3.3). In addition, Activin A is also capable of decreasing eNOS expression and contributing to reduced endothelial NO levels (section 1.2.3.4) [146]. NO levels can also decrease as a result of endothelial oxidative stress since increased endothelial ROS can lead to the uncoupling of eNOS, a state in which eNOS forms superoxide instead of NO [83]. The superoxide can react with any remaining NO to form peroxynitrite, further decreasing NO levels and increasing ROS levels [83]. As previously mentioned, both Activin A and TNF-α have been shown to increase endothelial ROS levels, and there is evidence to suggest that sFlt1 can also stimulate vascular oxidative stress and thus contribute to the depletion of NO. The pathways by which the vasoactive factors produced by the placenta trigger systemic hypertension are represented in figure 1.4.

1.2.4.2 Development of renal insufficiency in preeclampsia

The renal changes associated with preeclampsia include proteinuria, an increase in serum creatinine levels, as well as the distinct glomerular lesion known as glomerular endotheliosis.

The exact mechanisms underlying the development of proteinuria in preeclampsia are still not very well understood. Elevated intracellular ROS levels in endothelial cells potentially contribute to the development of proteinuria by increasing vascular permeability via the dissociation of endothelial cell junctions. Phosphorylation of VE-cadherin, a major component of endothelial adherens junctions, by endothelial ROS results in junctional dissociation and thus an increase in intercellular gaps in the endothelial monolayer, which increases vascular permeability [84]. Both TNF-α and Activin A have been shown to stimulate endothelial ROS production, and can thus cause proteinuria via this mechanism. Furthermore, eNOS-derived NO also plays a key role in the maintenance of endothelial cell junctions [184]. Hence, the decrease in NO bioavailability induced by all of the key vasoactive factors could contribute to the dissociation of endothelial cell junctions and a subsequent increase in vascular permeability. Another mechanism that contributes to the development of proteinuria in preeclampsia is the decrease in the expression of nephrin, which is mainly due to the inhibition of VEGF by sFlt1, but also due to the inhibitory actions of ET-1 on nephrin expression [113, 185]. Hence, the increase in circulatory ET-1 levels in preeclampsia, stimulated by TNF-α, AT1-AAs and sFlt1, can also contribute to the development of proteinuria by reducing nephrin expression. The pathways by which the placental-derived vasoactive factors give rise to proteinuria in preeclampsia are summarized in figure 1.4.

Glomerular endotheliosis, characterized by the swelling of glomerular endothelial cells and loss of endothelial fenestrations, is also most likely caused by lack of VEGF signaling due to increased circulatory sFlt1. This is demonstrated by a study which found that VEGF knockdown in mouse kidneys led to the development of glomerular endotheliosis, and eventually to the disappearance of endothelial cells from the glomerular tuft [107].

The increase in serum creatinine levels observed in preeclamptic women is an indicator that renal filtration capacity is significantly impaired. Loss of endothelial fenestrations, due to a lack of VEGF can contribute to the reduction in renal filtration capacity, as they are vital for

the filtration of low-molecular waste from the circulation [111]. Renal biopsies from preeclamptic women have shown a 72% reduction in the fraction of the glomerular basement membrane occupied by endothelial fenestrae compared with controls [110]. Furthermore, electron microscopy revealed accumulation of fibrinoid and granular deposits beneath the endothelial monolayer in these glomeruli [110]. Collectively, these changes significantly impair renal filtration, and give rise to the increase in serum creatinine levels observed in women with preeclampsia. Hypertension and the presence of proteinuria can also contribute to reducing renal filtration [110].

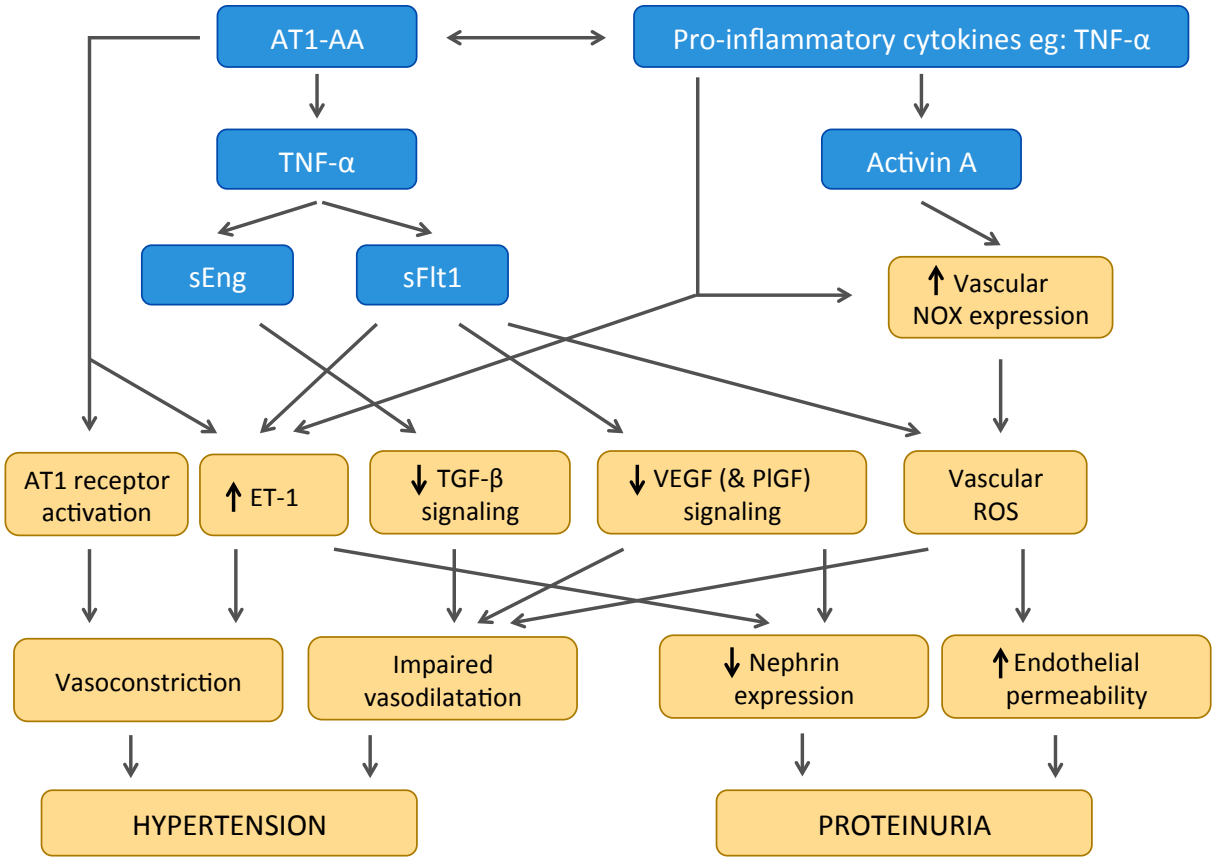

Figure 1.4: A schematic representation of the pathways by which the placental-derived vasoactive factors (shown in blue) lead to the development of hypertension and proteinuria in preeclampsia.

1.2.4.3 Development of liver, hematological and neurological dysfunction in preeclampsia

The clinical features that indicate hematological and liver dysfunction in preeclampsia are hemolysis, elevated liver enzymes and low platelet count. As previously mentioned in section 1.1, these features are collectively known as HELLP syndrome, and develop in approximately 10% of women with severe preeclampsia [15, 109]. All of these clinical features arise due to the fact that the dysfunctional endothelium is pro-thrombotic and promotes intravascular coagulation and fibrin deposition. This is corroborated by the increase in serum levels of pro-coagulant factors, such as von Willebrand factor, fibronectin, PAI-1 and soluble tissue factor in women with preeclampsia [159-161, 186, 187]. As a result of the thrombus formation and fibrin deposition, the vascular intima becomes extremely coarse and causes the lysis of red blood cells passing through the vessel [109, 188]. In addition, hepatic sinusoids can become obstructed leading to the elevation of liver enzymes [109]. Furthermore, the pro-thrombotic state of the dysfunctional endothelium leads to platelet activation, aggregation and adherence to sites of endothelial damage, thus reducing platelet count [109].

Some women with severe preeclampsia develop headaches and impaired vision. These neurological abnormalities can herald the onset of eclampsia, characterized by generalized seizures [15]. Even though the exact cause of these clinical features are not well-known, cerebral edema and vasospasm have been suggested as potential mechanisms [15, 109]. Using magnetic resonance imaging (MRI), studies have found seizures to be much more common in patients with cerebral edema than in those with normal MRI findings [189]. Cerebral edema could result from increased permeability of the blood-brain barrier caused by endothelial dysfunction [190]. Indeed, the presence of cerebral edema in patients with preeclampsia was found to correlate with fairly indirect markers of endothelial damage, such as lactate dehydrogenase and abnormal red blood cell morphology, rather than with the severity of hypertension [189, 190]. This suggests that the development of edema is due to endothelial dysfunction rather than the elevated blood pressure.

HELLP syndrome and the neurological clinical features arise as a result of severe endothelial dysfunction and as such, these features are characteristic of the more severe form of the disease. The appearances of these features warrant immediate treatment to prevent greater morbidity or mortality for the mother and the fetus.

1.3 Treatment of preeclampsia

The current clinical approach towards preeclampsia is to prolong the pregnancy as far as the maternal clinical features would allow in order to minimise fetal mortality/morbidity. Maternal hypertension is controlled with the use of anti-hypertensive agents and magnesium sulfate is used to prevent seizure development. Whilst these therapies may stabilize some of the maternal clinical features, they do not resolve the features and treat the disease. This strategy may improve disease outcome for patients that develop preeclampsia late in gestation (34 weeks or later), but it is not always feasible for those that develop early-onset preeclampsia (before 34 weeks). This is because the mechanisms that contribute to disease development continue to progress unhindered, and therefore the maternal clinical features can worsen anytime if pregnancy continues [3]. To date delivery of the placenta is the only known method of preventing disease progression and resolving the maternal clinical features. However, as previously mentioned in section 1.1, at early gestations, this is a treatment option that can result in severe consequences for the baby. Hence, an alternative treatment option that directly targets and resolves the mechanisms underlying disease development is very much needed, as it would resolve the maternal clinical features, whilst allowing the pregnancy to continue till term.

A few compounds have been tested, or are currently being tested, for their efficacy in impeding the underlying mechanisms that give rise to preeclampsia. As previously described in section 1.2, oxidative stress in both the placenta and the endothelium plays a large role in the development of systemic endothelial dysfunction and the subsequent clinical features of preeclampsia. This led to an investigation of whether vitamin C and vitamin E supplements given prophylactically from second trimester would reduce the incidence of preeclampsia in women deemed to be at high risk of developing the disease. While the smaller trials showed vitamin C and E supplementation reduced the risk of preeclampsia, this data was not supported by the much larger randomised trials [191-193]. In fact, one large-scale trial found that along with being ineffective at reducing the risk of preeclampsia, vitamin C and E supplementation actually increased the risk of a low birth weight baby [193]. These outcomes could be due to several reasons. Firstly, at higher doses and under certain conditions vitamin C and E can exert toxic pro-oxidant effects [194]. Furthermore, starting supplementation in

second trimester could have been too late, as regular periconceptual intake of multivitamins was found to be associated with a 45% reduction in the risk of preeclampsia [195].

Along with oxidative stress, inflammation is also a hallmark of preeclampsia. Inflammatory cytokines, such as TNF-α and IL-6 play essential roles in the production of almost all of the vasoactive factors, as well as in disrupting endothelial function. Due to this reason, anti-inflammatory agents offer great promise as potential treatment options for preeclampsia. Indeed, use of low doses of the non-steroidal anti-inflammatory agent, aspirin, as a prophylaxis has been shown to reduce the incidence of preeclampsia in high-risk women by 10-24% [196, 197]. However, its efficiency is limited, as over 70 women would need to be treated to prevent one case of preeclampsia [196]. Furthermore, there is conflicting evidence on the time of administration onset and the corresponding efficacy of aspirin, with some studies noting that treatment needs to begin before 16 - 17 weeks to have any effect, while others found no difference in commencing treatment before or after 20 weeks of gestation [197-199]. Hence, further investigations are required to determine when aspirin treatment should commence, at what dosage and which women are most likely to benefit from it.

In the last decade, a popular strategy for halting the pathophysiology of preeclampsia has been to identify and inhibit the vasoactive factors produced by the placenta. Inhibition of sFlt1 and sEng has been the most extensively investigated due to the considerable clinical and experimental evidence indicating their importance in the development of many of the maternal clinical features of preeclampsia. This has been validated by a small pilot study, which demonstrated that extracorporeal removal of sFlt1 by apheresis stabilized blood pressure, reduced proteinuria and prolonged pregnancy [200]. However, the effects of each apheresis treatment lasted only a few days before the circulatory sFlt1 levels began to increase again, closely followed by maternal blood pressure and proteinuria. This demonstrates that while apheresis might be clinically unfavorable as a routine treatment, reduction of circulatory sFlt1 levels can contribute to improving the maternal clinical condition and prolonging pregnancy. However, the challenge is to find a safer and more effective way of permanently reducing or antagonizing the effects of sFlt1 and sEng.

In addition, several compounds known to improve endothelial function have also been tested for their efficacy in prevention or treatment of preeclampsia. L-arginine, the precursor for endogenous NO production, has been examined due its ability to promote vasodilation by

increasing systemic NO levels. Several small trials have found L-arginine supplementation to reduce the incidence of preeclampsia in high-risk women, however larger adequately powered trials are required to confirm these findings [201]. The efficacy of calcium supplementation as a prophylaxis for preeclampsia has also been investigated. Calcium supplementation has been suggested to reduce the risk of preeclampsia by reducing smooth muscle contractility in blood vessels, especially in those of the uterus, by decreasing parathyroid hormone release and intracellular calcium levels [202]. Even though its exact mechanisms of action are unclear, randomised control trials have shown calcium supplementation to almost halve the risk of developing preeclampsia with the effect even greater in high-risk women [203]. This appears to be the most effective prophylaxis for preeclampsia to-date. Several small trials have also tested sildenalfil citrate, which induces vasodilatation via vascular smooth muscle relaxation, as well as the coagulation inhibitor, antithrombin as potential treatments for established preeclampsia. Whilst antithrombin was found to prolong pregnancy and reduce the number of low birth weight babies when given to severe preeclamptic patients, sildenalfil citrate was shown to have no beneficial effect [204, 205]. Larger trials are currently underway to confirm the potential beneficial effects of antithrombin treatment in preeclampsia [206].

All of these above-mentioned compounds target one particular aspect of the pathophysiology of preeclampsia. In contrast, statins have been proposed as a promising treatment option for preeclampsia as they can potentially target multiple aspects of the pathophysiology of preeclampsia. Apart from their lipid-lowering effects, statins have been shown to have anti-oxidant and anti-thrombotic properties [207, 208]. They are also anti-inflammatory due to their ability to decrease pro-inflammatory cytokine production, as well as decrease the expression of endothelial cell and leukocyte adhesion molecules [208, 209]. Furthermore, statins have been shown to improve endothelial function via two mechanisms that would be beneficial in preeclampsia as well. The first mechanism is by decreasing ET-1 production, and the second is by increasing the expression and activity of eNOS and therefore the bioavailability of NO [210-212]. Further confirming the potential of statins as a treatment for preeclampsia, pravastatin treatment was shown to completely abolish hypertension, proteinuria, glomerular endotheliosis and fetal growth restriction in mice with lentiviral mediated placental-specific expression of sFlt1 [213]. Interestingly, these effects seem to be at least partially mediated by the ability of statins to shift the angiogenic balance towards pro-angiogenic factors, as they have been shown to inhibit sFlt1 expression and stimulate production of both VEGF and PlGF [211, 213, 214]. Clinical trials are currently ongoing to

test the efficacy of pravastatin as a prophylaxis for high-risk women, and also as a treatment for women with early-onset preeclampsia (StAmP Trial) [215].

1.3.1 Therapeutic potential of Nrf2 activation in preeclampsia

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that mediates adaptive responses to a variety of cellular stresses by increasing the expression of a range of cytoprotective genes [216]. Pharmacological activation of Nrf2 has been shown to be protective against several disorders due to the ability of Nrf2 to form a defence against the effects of ischemia-reperfusion injury, oxidative stress and inflammation [217-220]. Furthermore, there is some evidence to suggest that Nrf2 activation can improve vascular endothelial dysfunction [217, 221-223]. Hence, it appears that similar to the statins, activation of the Nrf2 pathway could also target multiple aspects of the pathophysiology of preeclampsia and could therefore be a promising treatment option for preeclampsia.

Under normal cellular conditions, Nrf2 is found in the cytosol bound to Kelch-like ECH-associating protein 1 (Keap1) [216]. Keap 1 acts as the negative regulator of Nrf2 by targeting it for proteosomal degradation [224]. Reactive oxygen species and other molecules present under conditions of cellular stress cause the dissociation of Keap1 from Nrf2, thus stabilizing Nrf2 and allowing it to translocate to the nucleus [216]. Once inside the nucleus, Nrf2 binds to the antioxidant response element (ARE) contained within the promoter regions of a variety of genes, including those that encode for the Phase II detoxification enzymes and anti-oxidant proteins, such as glutathione S-transferases (GST), NADPH quinone oxidoreductase (NQO1) and heme oxygenase-1 (HO-1) [216, 225]. A schematic representation of the Nrf2 transcription factor under basal cellular conditions and under conditions of cellular stress is shown in Figure 1.5.

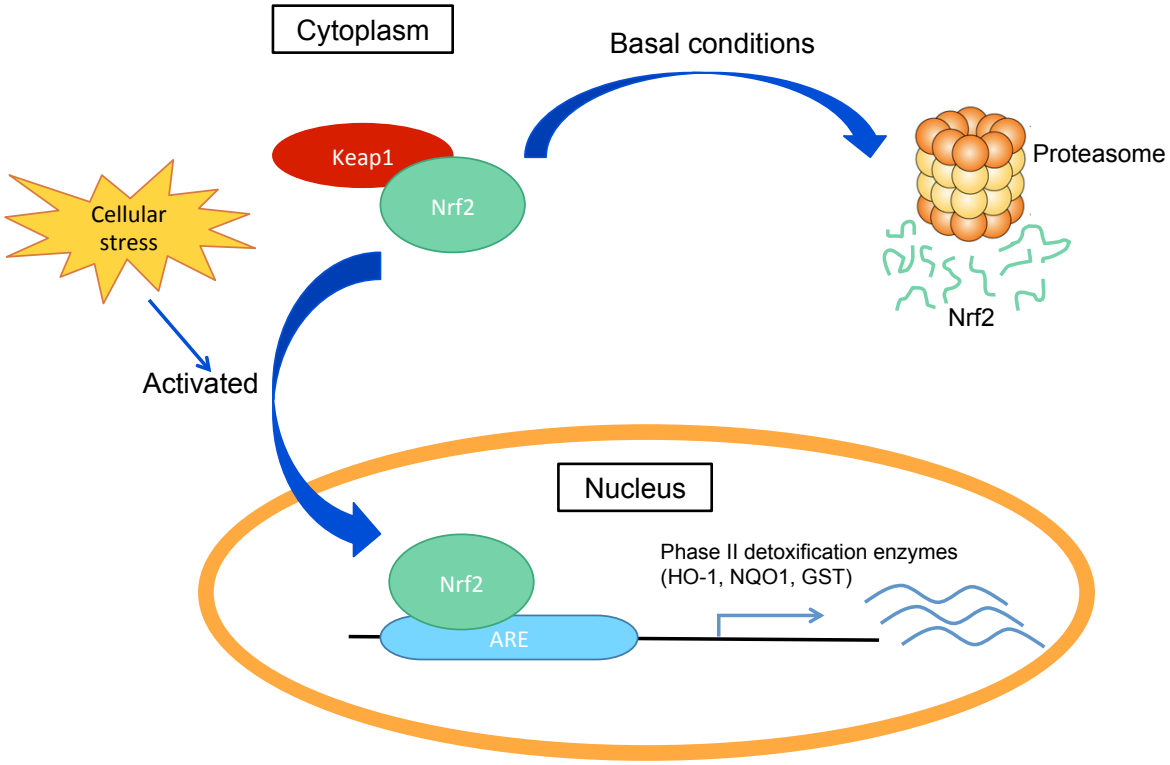

Figure 1.5: The Nrf2 transcription factor under basal cellular conditions and under conditions of cellular stress. In normal cellular conditions, Nrf2 is found in the cytosol attached to its repressor protein, Keap1, which targets Nrf2 for proteosomal degradation. In conditions of cellular stress, Nrf2 dissociates from Keap1 and translocate to the nucleus, where it binds to the antioxidant response element (ARE) promoter to activate the transcription of phase II detoxification enzymes, such as heme-oxygenase (HO-1), NADPH quinone oxidoreductase (NQO1) and glutathione S-transferases (GST).

Nrf2 activity in the placentas of preeclamptic patients has been examined with contradictory outcomes. Some studies have shown Nrf2 activity to be elevated in EVT and cytotrophoblast cells of preeclamptic placentas compared with normal placentas, indicating an increase in placental oxidative stress in preeclampsia [226, 227]. However, Chigusa *et al*. (2012) have shown a decrease in Nrf2 expression in preeclamptic placentas, indicating a failure of the placental tissue to upregulate the anti-oxidant enzymes and combat the placental oxidative stress in preeclampsia [228]. One possible reason for this discrepancy could be due to local differences in Nrf2 expression in the placentas of preeclamptic women. Studies reporting an increase in Nrf2 examined regional expression in the placenta using immunohistochemistry, whilst the study that noted a decrease in Nrf2 analysed whole placental tissue by western blot. Hence, further studies are needed to elucidate the changes in Nrf2 in women with preeclampsia.

Irrespective of the changes in Nrf2 in preeclampsia, there is evidence to suggest that pharmacological activation of Nrf2 could have a therapeutic benefit for women with preeclampsia. Nrf2 activation has been demonstrated to protect a wide range of cell types from oxidative stress via the stimulation of the cellular repertoire of endogenous anti-oxidant enzymes, which include GST, NQO1, HO-1, superoxide dismutase, catalase and glutathione reductase [229]. Furthermore, studies have shown Nrf2 activators to protect a wide range of organs and tissues against ischemia-reperfusion injury, including the heart, intestines, kidneys, retina and the brain [218, 230-233]. Interestingly, the anti-oxidant enzyme HO-1, usually activated by Nrf2, has been shown to mitigate placental oxidative stress and attenuate the placental ischemia-induced increase in blood pressure in pregnant rats subjected to reduced uterine perfusion pressure [234]. HO-1 has recently gained considerable interest as a therapeutic target for cardiovascular diseases, as it has been demonstrated to have anti-inflammatory properties and the ability to improve vascular function, apart from its role as an anti-oxidant enzyme [234, 235]. Hence, it is possible that many of the beneficial effects of Nrf2 activation are mediated mainly via HO-1 expression.

The anti-inflammatory properties of Nrf2 appear to be mainly due to its ability to decrease cellular ROS and therefore the expression of ROS-mediated inflammatory mediators, such as ICAM-1, VCAM-1, monocyte chemoattractant protein-1 (MCP-1) and potentially cyclooxygenase 2 [236]. There is also some evidence to suggest that the Nrf2 pathway may interfere with Nuclear factor- κB (NF-κB) signaling, with several Nrf2 activators shown to reduce NF-κB signaling [237]. However, a direct relationship between Nrf2 and NF-κB has not been established. In addition, HO-1 upregulation by Nrf2 could also significantly contribute to suppressing inflammation, as it is known to attenuate the production of pro-inflammatory cytokines, including TNF-α and IL-6, while also increasing the secretion of anti-inflammatory cytokines, such as IL-10 [235, 238, 239].

Another significant feature of Nrf2 that makes it a promising target for preeclampsia is its ability to improve vascular endothelial function. Nrf2 activators have been shown to improve endothelium-dependent relaxation in mice fed a high fat diet, as well as in rats with chronic kidney disease [217, 223]. Furthermore, HO-1 activation has also been demonstrated to improve vessel relaxation [221, 240]. Along with the anti-oxidant and anti-inflammatory properties of Nrf2 and HO-1, their ability to increase NO bioavailability and decrease ET-1

production has been suggested to be responsible for their capacity to improve endothelial function [234, 240, 241].

In addition to the above-mentioned beneficial properties, Nrf2 activators could also impede the development of preeclampsia by inhibiting sFlt1 and sEng production via the induction of HO-1. Several *in vitro* studies have shown HO-1 stimulation to inhibit both placental and endothelial production of sFlt1 and sEng [242, 243]. Intriguingly, HO-1 knockout mice were found to have significantly higher levels of circulatory sFlt1 and sEng, indicating the importance of HO-1 in the negative regulation of both sFlt1 and sEng [242]. However, the exact mechanism by which HO-1 mediates these changes to sFlt1 and sEng levels is unknown.

In summary, activation of Nrf2 and its downstream mediator HO-1 could be a promising treatment option for preeclampsia due to their ability to mitigate oxidative stress, inflammation, and production of anti-angiogenic factors, while also improving vascular endothelial function. Indeed a recent study has shown the Nrf2 and HO-1 activator, sofalcone to significantly decrease placental sFlt1 production, as well as improve TNF-α induced endothelial dysfunction by reducing monocyte adhesion, VCAM1 expression and ET-1 expression [244]. There are many more Nrf2 activators that could prove to be an effective therapy for preeclampsia, and the efficacy of three such activators, melatonin, resveratrol and sulforaphane, will be examined in chapters 3, 4 and 5 respectively.

1.4 Rationale and aims of the studies

As mentioned in section 1.2.3.5, there is strong evidence to suggest that the key vasoactive factors: the AT1-AAs, pro-inflammatory cytokines, and anti-angiogenic factors, interact with each other along one pathway in the development of preeclampsia. Blocking either one of these vasoactive factors would impede this entire pathway, and could therefore improve endothelial function significantly. Recently, Activin A has also been identified as a vasoactive factor that significantly contributes to the development of systemic endothelial dysfunction in preeclampsia (Section 1.2.3.4). Unlike for the other vasoactive factors, there are several small-molecule inhibitors that can block Activin A signaling and could therefore be potential therapeutics for preeclampsia [245]. However, these Activin A inhibitors would be most effective as a therapy, only if Activin A is also involved in the same pathway as the other key vasoactive factors. Whilst it is known that pro-inflammatory cytokines can trigger placental production of Activin A, it is not yet known whether Activin A and the anti-angiogenic factors interact with each other or whether they contribute to the endothelial dysfunction in preeclampsia via independent pathways. Hence, the aim of the first study described in chapter 2 was to examine the relationship between Activin A and the anti-angiogenic factors in the development of preeclampsia.

In order to examine the therapeutic potential of Nrf2 activation, three compounds known to activate Nrf2 were tested in vitro to determine their efficacy in improving hallmarks of endothelial and placental dysfunction. Chapters 3, 4 and 5 describe the studies examining the Nrf2 activators, melatonin, resveratrol and sulforaphane respectively. Chapter 6 details the process undertaken when developing a rat model of preeclampsia via the adenoviral overexpression of sFlt1 and sEng, and includes preliminary data obtained from testing the efficacy of melatonin *in vivo*.

Chapter 2

The relationship between Activin A and anti-angiogenic factors in the development of preeclampsia

---

2.1 Introduction

A widely investigated treatment strategy for preeclampsia is the inhibition of one or more of the placental-derived vasoactive factors that trigger systemic endothelial dysfunction. This approach gained popularity as more and more studies demonstrated that there is considerable interaction between the key vasoactive factors involved in the development of preeclampsia, and thus it seemed possible that blocking just one factor may significantly attenuate endothelial dysfunction and the clinical features of preeclampsia [85, 92].

As described in detail in chapter 1.2.3, there is strong evidence indicating that the AT1-AAs, pro-inflammatory cytokines, and the anti-angiogenic factors, work together during disease development [85, 92]. It appears that the injured placenta initially produces pro-inflammatory cytokines, which in turn triggers placental production of AT1-AAs [87, 88]. These two factors then work together to stimulate placental release of the anti-angiogenic factors, sFlt1 and sEng [85, 92, 93]. Blocking either one of these vasoactive factors could disrupt this entire pathway and prevent significant endothelial damage. However, a method or drug that could inhibit one of these vasoactive factors while being safe for use in pregnancy is yet to be found.

As described in section 1.2.3.4, Activin A has also been identified as a vasoactive factor that significantly contributes to the development of systemic endothelial dysfunction in preeclampsia [146]. In contrast to the other vasoactive factors, there are several small-molecule inhibitors that can block Activin A signaling, such as follistatin and SB-431542, and could therefore be useful as potential therapeutics for preeclampsia [245, 246]. However,

these Activin A inhibitors would be most effective as a therapy, only if Activin A is also involved in the same pathway as the other key vasoactive factors. Otherwise, inhibition of Activin A alone may not be sufficient to rescue the endothelium, as the other vasoactive factors would continue their disruption of endothelial function. While it is known that pro-inflammatory cytokines can trigger placental production of Activin A, it is not yet known whether Activin A and the anti-angiogenic factors interact with each other or whether they act via independent pathways in the development of preeclampsia [86, 145]. Hence, the relationship between Activin A and the anti-angiogenic factors at the level of placental production, as well as at the level of triggering endothelial dysfunction was explored in Chapter 2.

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design, performing experiments, data analysis, drafting of manuscript	95%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Euan M Wallace	Intellectual input	NA
Rebecca Lim	Intellectual input and drafting of manuscript	NA

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		Date 30/03/16
Main Supervisor's Signature		Date 30/03/16

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The relationship between Activin A and anti-angiogenic factors in the development of pre-eclampsia

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ABSTRACT

Anti-angiogenic factors such as sFlt/sEng contribute to the pathology seen in preeclampsia. Activin A, which is released by the placenta following exposure to oxidative stress and elevated in preeclampsia, may interact with sFlt/sEng during the disease process. Using placental explant cultures, we determined that transcription of *sFLT1*, *ENG* and *INHBA* was upregulated following exposure to oxidative stress or IL-6. Explants treated with Activin A did not increase transcription of *sFLT1*, *ENG*. Conversely, treatment of placental explants with sFlt/sEng did not increase transcription of *INHBA*. These data may suggest that Activin A and sFlt/sEng contribute to preeclampsia via separate pathways.

Introduction

Pre-eclampsia is a leading cause of maternal and fetal morbidity and mortality worldwide. To date, placental delivery remains the only cure. The difficulty in finding a pharmacological treatment for pre-eclampsia is mainly due to the limited understanding of its pathophysiology.

The maternal symptoms are due to circulatory factors that induce endothelial dysfunction following their release from the placenta undergoing severe oxidative stress. Pro-inflammatory cytokines, such as TNF-α and IL-6, are significantly elevated in pre-eclamptic women [1]. However, two of the most well known circulatory factors are the anti-angiogenic factors, soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng) [2–5]. When exogenously delivered into pregnant rats, sFlt1and sEng induce the classical features of pre-eclampsia including hypertension, proteinuria and HELLP syndrome [3].

Activin A, a member of the transforming growth factor-β (TGF-β) superfamily, is another less-investigated circulatory factor linked with pre-eclampsia. It is comprised of dimerised *INHBA* subunits. During pregnancy, the placenta is the major source of circulating Activin A [6,7] and maternal levels are approximately 10-fold greater in pre-eclamptic women [8–14]. Furthermore, placental as well as endothelial oxidative stress has been shown to increase production of Activin A [15]. The relationship between the anti-angiogenic factors and Activin A in the placenta and endothelium remains unknown. Improving our understanding of these interactions may improve our ability to develop therapeutics that target specific pathways in the development of pre-eclampsia. Hence, our aim was to examine the interactions between Activin A and the anti-angiogenic factors.

Methods

Placental explants were isolated from the placentas of healthy pregnant women undergoing caesarian sections at term. After overnight equilibration, the explants were treated with X/XO (X-2.3 mM; XO-15 mU/mL), IL-6 (3 ng/mL), Activin A (50 ng/mL) or sFlt1 and sEng (100 ng/mL

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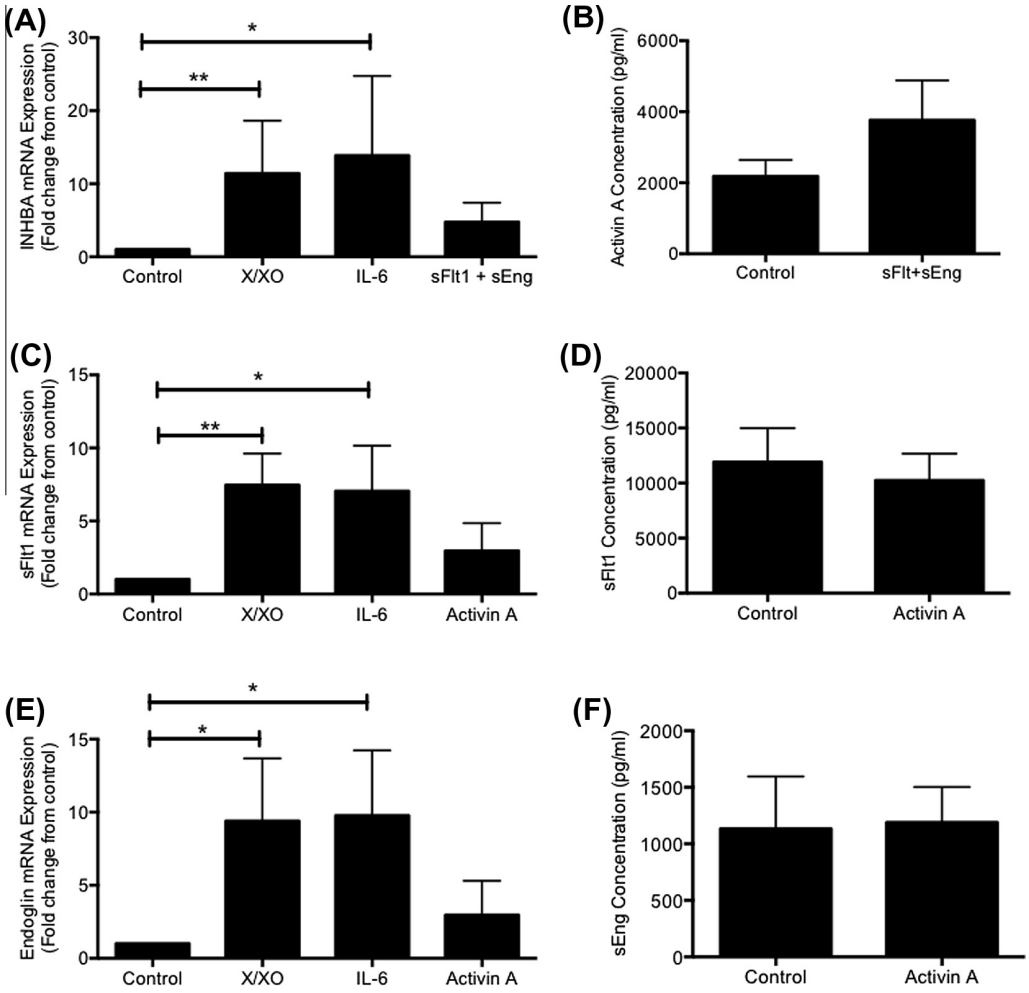

Fig. 1. Factors stimulating placental production of Activin A, sFlt1 and endoglin. (A) Oxidative stress and exposure to IL-6 significantly increased placental INHBA mRNA expression, however sFlt1 and sEng treatment had no effect. (B) sFlt1 and sEng also did not significantly affect placental Activin A protein production. (C) Placental sFlt1 mRNA levels were significantly elevated in response to oxidative stress and IL-6, but not in response to Activin A. (D) Activin A does not significantly alter placental sFlt1 protein production either. Similarly, (E) placental endoglin mRNA levels were increased by oxidative stress and IL-6 treatment, but Activin A treatment had no effect. (F) Placental sEng protein levels were also unaffected by Activin A treatment ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

each) for 24 h. *sFLT1*, *ENG* and *INHBA* mRNA levels in the explants were assessed by quantitative PCR, whilst sFlt1, sEng and Activin A protein levels in the culture supernatants were measured by ELISA.

For the endothelial tube formation assay, Matrigel (100 μL/well) was placed in a pre-chilled 48-well plate and incubated for 30 min at 37 °C. Human umbilical vein endothelial cells (HUVECs) in serum-free media were then plated onto the wells with or without Activin A (50 ng/ml) and incubated at 37 °C. Images were taken after 6 h and total tube length was analysed using Image J and Metamorph software.

To assess the production of reactive oxygen species (ROS), HUVECs were treated with Activin A or sFlt1 and sEng for 6 h. Cells were then stained with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), an indicator of cellular ROS, for 30 min and with Hoescht dye, which binds to DNA for 15 min. Percentage of DCFDA positive cells were detected and analysed using a Cellomics Arrayscan VTI HCS Reader (Thermo Scientific).

NOX2 mRNA levels were assessed in HUVECs treated with Activin A or sFlt1 and sEng for 6 h by qPCR.

Results and discussion

In this study, we demonstrated that oxidative stress induced by xanthine/xanthine oxidase (X/XO) and IL-6 increased placental transcription of *sFLT1*, *ENG* and *INHBA* in the placental explants. This supports previous findings that oxidative stress, as well as pro-inflammatory cytokines released in response to placental oxidative stress, stimulate the production of Activin A, sFlt-1 and sEng [15–17]. Notably, treatment with sFlt1 and sEng did not

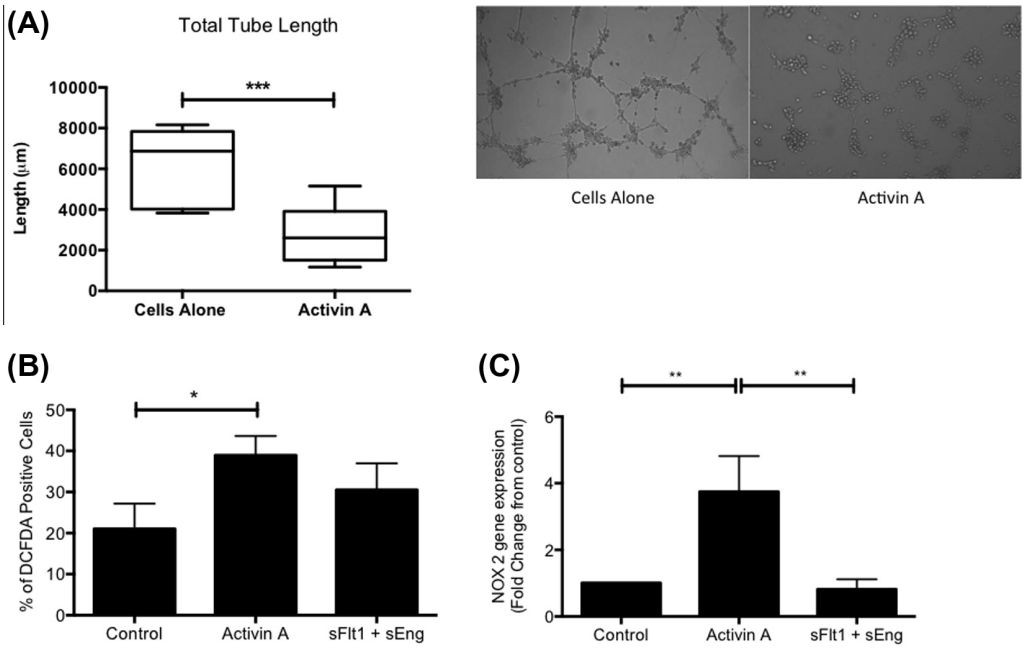

Fig. 2. Effects of Activin A and the anti-angiogenic factors on the endothelium. (A) Activin A significantly increases the production of reactive oxygen species in HUVECs, however sFlt1 and sEng did not. (B) Activin A significantly increased NOX2 mRNA levels in HUVECs, but sFlt1 and sEng had no effect on NOX2 levels in HUVECs. (C) Activin A significantly decreased tubule formation by HUVECs. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

affect *INHBA* transcription or Activin A protein levels (Fig. 1A and B), suggesting that they do not directly stimulate placental Activin A production. Conversely, placental explants treated with Activin A did not show any changes in sFlt1 mRNA and protein levels compared with the untreated explants (Fig. 1C and D). Likewise, endoglin mRNA levels and sEng protein levels were not significantly altered in placental explants treated with Activin A in comparison to the untreated explants (Fig. 1E and F). Thus, Activin A does not appear to be involved in the placental production of either of the anti-angiogenic factors.

We then examined whether the anti-angiogenic factors and Activin A induced endothelial dysfunction via similar mechanisms to determine their interaction on the endothelium. Previous studies have shown that sFlt1 and sEng impair endothelial tubule formation *in vitro* [3]. sFlt1 sequesters vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), thus reducing their beneficial effects on the endothelium [2]. Similarly, sEng binds to and neutralises circulatory transforming growth factor-β (TGF-β) impairing its pro-angiogenic actions [3].

We found that Activin A also impaired endothelial tubule formation *in vitro* where total tubule length formed by HUVECs was 55% shorter in the presence of Activin A (Fig. 2A). Thus, Activin A is also capable of significantly impairing endothelial function. Interestingly, Activin A also increased transcription of endothelial NADPH Oxidase 2 (*NOX2*) (Fig. 2C), which is a key molecule in the production of reactive oxygen species (ROS). This may contribute to the observed increase in endothelial oxidative stress (Fig. 2B). However, treatment of HUVECs with sFlt-1 and sEng did not change NOX2 expression or ROS production (Fig. 2B and C) indicating that these anti-angiogenic factors do not directly contribute to endothelial oxidative stress.

In conclusion, there appears to be no interaction between anti-angiogenic factors, sFlt-1 and sEng, and Activin A at the level of the placenta and endothelium. Placental production of Activin A and the anti-angiogenic factors occurs independently to each other. Similarly, Activin A and the anti-angiogenic factors appear to trigger endothelial dysfunction independently.

The findings from this study have significant implications when considering treatment strategies for pre-eclampsia since targeting therapeutics towards singular circulatory factors is highly unlikely to completely ameliorate all features of pre-eclampsia. Targeting placental oxidative stress and inflammation, whilst concurrently supporting maternal endothelial defence may develop better treatment strategies.

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Chapter 3

Melatonin as a potential treatment for preeclampsia: examining its effects on in vitro hallmarks of endothelial and placental dysfunction

---

3.1 Introduction

A therapy for preeclampsia that effectively targets the mechanisms underlying disease progression is very much needed. As mentioned in section 1.3, such a treatment would ideally be one that can improve maternal endothelial function, while also reducing placental oxidative stress and production of the vasoactive circulatory factors. Melatonin is a well-known anti-oxidant that has also been shown to improve vascular endothelial function, making it a promising treatment option for preeclampsia.

Melatonin is predominantly produced by the pineal gland for regulation of circadian rhythm [247]. In normal pregnancy, maternal melatonin levels gradually increase throughout gestation, mainly due to placental production of melatonin [248]. Furthermore, it rapidly crosses the placenta to enter the fetal circulation [249]. Correspondingly, melatonin is known to promote fetal growth and brain development, while regulating placental homeostasis and hormone production [250-253]. Hence, most doses of melatonin are relatively safe during pregnancy. In preeclampsia; however, placental melatonin levels are significantly lower than gestation-matched controls and this correlates with a reduction in the activity of placental melatonin-synthesizing enzymes in preeclampsia [254].

Melatonin is also well known for its anti-oxidant properties, which arise from its ability to scavenge free radicals, as well as upregulate the cell's endogenous antioxidant enzymes via the activation of Nrf2 [255, 256]. In addition to their anti-oxidant capacity, pharmacological

activation of Nrf2 and the subsequent increase in HO-1 expression could have a therapeutic benefit in preeclampsia due to their anti-inflammatory properties, ability to inhibit sFlt1 and sEng production, and their capacity to improve vascular function (described in detail in section 1.3.1). As a result of these properties melatonin shows great promise as a potential therapeutic for preeclampsia. Indeed, a number of previous studies have shown melatonin to be protective against ischemia-reperfusion induced oxidative damage in various organ systems, including the placenta [257-261]. Furthermore, melatonin has demonstrated beneficial effects on vascular function, by protecting against endothelial dysfunction induced by angiotensin II or a high fat diet, as well as reducing blood pressure in spontaneously hypertensive rats and in patients with essential hypertension [262-266].

The aims of this study was to determine the efficacy of melatonin in improving endothelial function, while reducing placental oxidative stress and production of vasoactive factors, as well as its ability to activate Nrf2. To explore these aims, placental explant cultures were used to examine the effect of melatonin on placental oxidative stress and production of vasoactive factors, sFlt1, sEng and Activin A. Also, the impact of melatonin on several key indicators of endothelial function *in vitro*, such as endothelial permeability, as well as production of the potent vasoconstrictor ET-1 and the markers of endothelial activation were determined. Finally, the impact of melatonin on the activation of Nrf2 and HO-1 was examined.

3.2 Methods

3.2.1 Blood and tissue collection

Blood and placental samples were collected upon receiving informed written consent from each patient with approval from the Monash Health Human Research Ethics Committee (Reference number: 13357B). Placental samples were collected at the time of caesarean section from healthy term pregnancies and from pregnancies complicated by preeclampsia, as defined by the Society of Obstetric Medicine of Australia and New Zealand [3]. Umbilical cords were only collected from the healthy term pregnancies. Blood samples were collected from women diagnosed with established preeclampsia prior to delivery in the absence of labour. Blood samples collected from women with a healthy singleton pregnancy at matched gestations were used as controls.

3.2.2 Placental explant culture

Upon collection of the placenta several cotyledons were excised randomly and washed in ice-cold Hank's Balanced Salt Solution (HBSS) to remove as much blood as possible. Placental villous tissue was then dissected, weighed and approximately 50-70 mg of tissue placed in each well of a 24-well plate. The explants were cultured in Medium 199 (M199) supplemented with 1% antibiotics and 1% L-Glutamine (all from Life Technologies, Carlsbad, CA) containing different treatment conditions.

One group of placental explants was treated with the oxidative stress inducers, xanthine/ xanthine oxidase (X/XO) (Sigma-aldrich, St. Louis, MO; X – 2.3 mM, XO – 15 mU/ml), in the presence or absence of melatonin (Sigma-Aldrich; 1 mM, 500 μM, 100 μM and 1 μM). Placental explants treated with media only were used as negative controls. These explants were cultured in 5% O_2 at 37°C for 48 hours. Another group of placental explants were exposed to 1% O_2 in the presence or absence of melatonin (1 mM, 500 μM, 100 μM and 1 μM) at 37°C for 24 hours. Placental explants exposed to 5% O_2 were used as negative controls. Placental explants from women diagnosed with preeclampsia were cultured in the presence or absence of melatonin (1 mM, 500 μM, 100 μM and 1 μM) at 37°C in 1% O_2 for 24 hours.

3.2.3 8-isoprostane, sFlt1, sEng and Activin A measurement

The level of 8-isoprostane, a marker of oxidative stress, in placental explant culture supernatants was determined by enzyme immunoassay (Caymen Chemicals, Ann Arbor, MI), while the levels of sFlt, sEng and Activin A in the same supernatants were determined via ELISA (R&D systems, Minneapolis, MN). All assays were performed according to manufacturer's instructions.

3.2.4 Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from the umbilical cords collected from healthy term pregnancies as previously described [143]. All HUVEC cultures were plated at a density of 12, 000 cells per 100 mm^2 in M199 supplemented with 20% fetal bovine serum, 1% antibiotics and L-Glutamine (all from Life Technologies) and incubated at 37°C with 5% CO_2.

3.2.5 Markers of endothelial cell activation

Expression of endothelial cell activation markers was determined via flow cytometry in the HUVECs treated with melatonin (1μM, 100μM, 1mM) and/or TNFα (1ng/ml; R&D systems) for 6 hours. Briefly, HUVECs were mechanically scraped from their flasks prior to immunostaining with the following antibodies (antibody synonyms, antibody dilutions and catalogue numbers shown in parantheses): CD54-Pacific Blue (ICAM1, 1:100, 353109), CD106-PE (VCAM1, 1:50, 305805) CD62E-APC (E-selectin, 1:100, 336011). Immunostaining was compared with appropriate isotype controls. Antibodies and isotype controls were obtained from Biolegend (San Diego, CA). HUVECs were incubated on ice with either antibodies or isotype controls for 20 minutes prior to washing and fixing in 1% paraformaldehyde in FACS buffer. The markers of endothelial cell activation were then analysed on a BD FACS Canto II (BD Biosciences, San Jose, CA). Analysis was performed using FlowJo cytometric analysis software (Oakland, OR).

3.2.6 Endothelin-1 measurement

ET-1 levels in the supernatants of HUVEC cultures treated with melatonin (1μM, 100μM, 1mM) and/or TNFα (1ng/ml) for 24 hours were determined via ELISA (DET100, R&D systems, Minneapolis, MN), according to manufacturer's instructions.

3.2.7 Endothelial cell permeability

Integrity of the endothelial cell monolayer following TNFα treatment was assessed using a FITC-dextran based permeability assay. Firstly, HUVECs were plated in gelatinized polycarbonate transwell inserts (Corning, Oneonta, NY; 0.4μm, 6.5mm, 24 well plate) at a density of 50,000 cell/ insert, and incubated at 37°C for 3 days to form a monolayer. Cells were then treated with recombinant TNFα (100ng/ml) in the presence or absence of melatonin (1mM) for 24 hours. At the end of the treatment period, culture media was removed from both the upper and lower chambers. Culture media containing 1mg/ml FITC-dextran (Sigma-Aldrich; MW 40,000) was added to the upper chamber, while fresh culture media was added to the lower chamber. Cells were then incubated for 1 hour at 37°C. Media was removed from the lower chamber and diluted 1:20 with PBS in a black-walled 96-well plate before fluorescence readings were obtained at 485nm excitation and 535nm emission.

Endothelial cell permeability following treatment with serum from women with preeclampsia (5% serum in M199 media supplemented with 1% antibiotics and 1% L-Glutamine) was measured using the 24-well *in vitro* vascular permeability assay kit (Merck Millipore, Billerica, MA). The assay was performed according to manufacturer's instructions. A melatonin concentration of 1mM was used as preliminary studies showed this to be the most effective dose *in vitro*. Endothelial cells treated with serum from gestation matched healthy pregnant women were used as controls.

3.2.8 Nrf2 nuclear translocation and HO-1 production

Placental explants exposed to X/XO and 1% O_2, as well as the HUVEC cultures treated with TNFα (1ng/ml) in the presence or absence of melatonin (1mM) were assessed for Nrf2 activation and antioxidant response by determining Nrf2 nuclear translocation and total HO-1 protein levels. Nucleic and cytoplasmic protein fractions were separated and collected from the samples using the NE-PER nuclear and cytoplasmic reagents (Thermo Scientific,

Rockford, IL) according to manufacturer's instructions. Protein quantification was performed using the Pierce BCA protein assay kit (Life Technologies). For western blots, 40µg of protein was loaded for each sample. Membranes were then blocked with 5% (w/v) skim milk in PBS with 0.1% (v/v) Tween-20 (Sigma, St Louis, MO) for 1 hour prior to probing with antibodies. Membranes were stripped in a mild stripping buffer (1.5% (w/v) glycine, 0.1% (w/v) SDS, 1% (v/v) Tween-20 in distilled water) for 5 minutes between antibodies. The following primary antibodies and concentrations were used: Nrf2 (ab62352, 1:2000, Abcam, Cambridge, UK), and HO-1 (ab52947, 1:2000, Abcam). All antibodies were diluted in blocking buffer and incubated overnight at 4°C.

3.2.9 Statistical Analysis

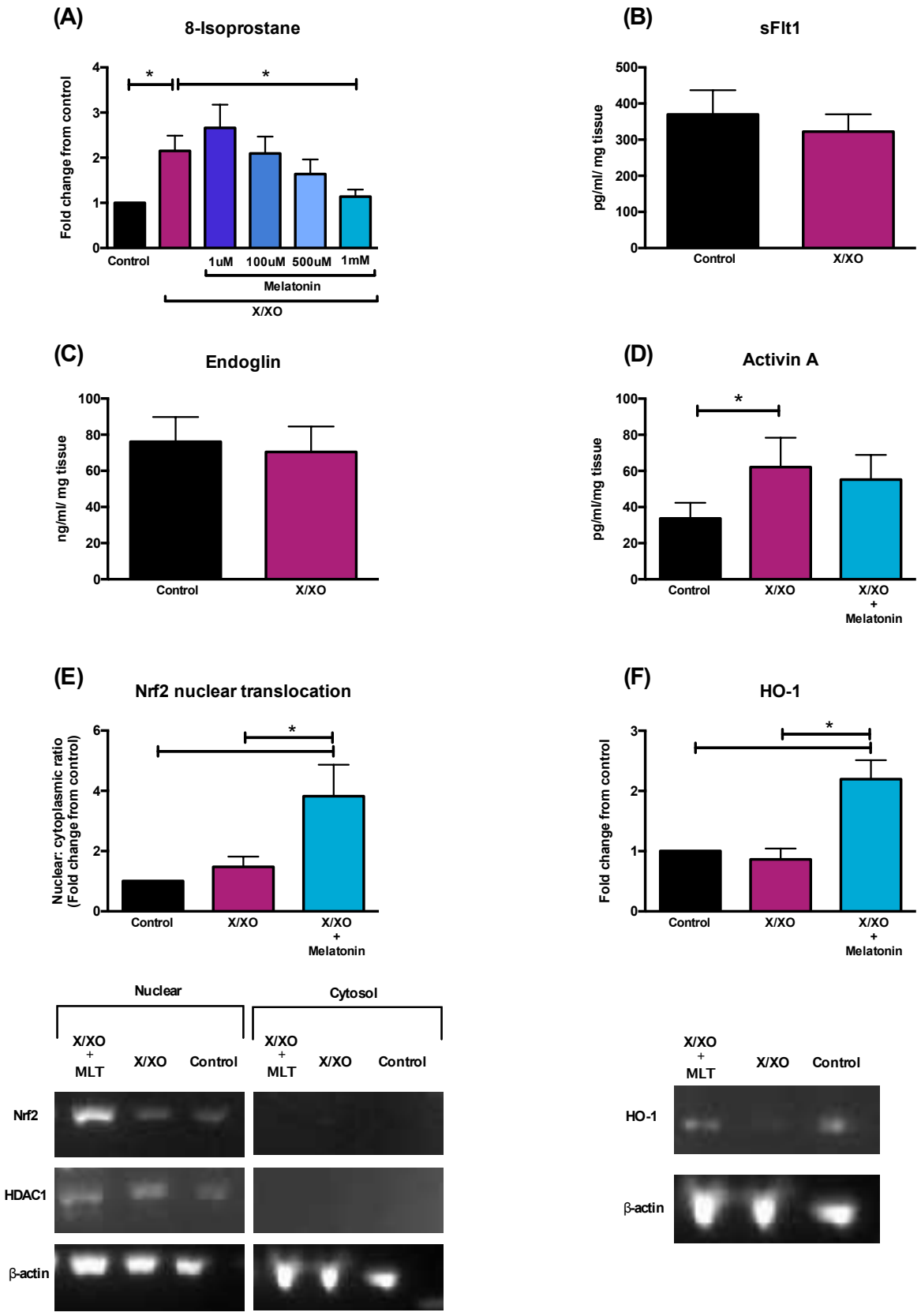
Statistical analysis was performed using one-way ANOVA followed by multiple comparisons with a Tukey ad-hoc test. Groups were considered to be significantly different if *P* values were <0.05. All data were analysed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

3.3 Results

3.3.1 Impact of melatonin on placental explants exposed to oxidative stress

In order to mimic the placental oxidative stress that develops during the reperfusion phase of placental ischemia-reperfusion in preeclampsia, term placental explants were treated with X/XO. As hypothesized, the X/XO treated placental explants produced significantly more of the oxidative stress marker 8-isoprostane than the untreated explants ($P<0.01$; Figure 3.1A). This X/XO-induced increase in 8-isoprostane was completely ameliorated by melatonin at a dosage of 1mM ($P<0.05$; Figure 3.1A). Lower doses of melatonin did not have a significant impact on 8-isoprostane production triggered by X/XO (All $P>0.05$; Figure 3.1A). Despite the increase in 8-isoprostane production following X/XO treatment, there was no difference in placental production of sFlt1 and sEng between the X/XO-treated and untreated groups (both $P>0.05$; Figure 3.1B & 3.1C respectively). However, placental explant activin A production was significantly increased by X/XO in comparison to the untreated explants ($P<0.05$), and treatment with 1mM melatonin did not ameliorate this effect ($P>0.05$; Figure 3.1D). However, melatonin treatment significantly increased the translocation of Nrf2 from the cytoplasm to the nucleus, compared with the untreated explants and explants treated with X/XO only ($P<0.05$; Figure 3.1E). Correspondingly, HO-1 protein levels were doubled in the X/XO and melatonin treated placental explants compared with the untreated and X/XO only treated placental explants ($P<0.05$; Figure 3.1F).

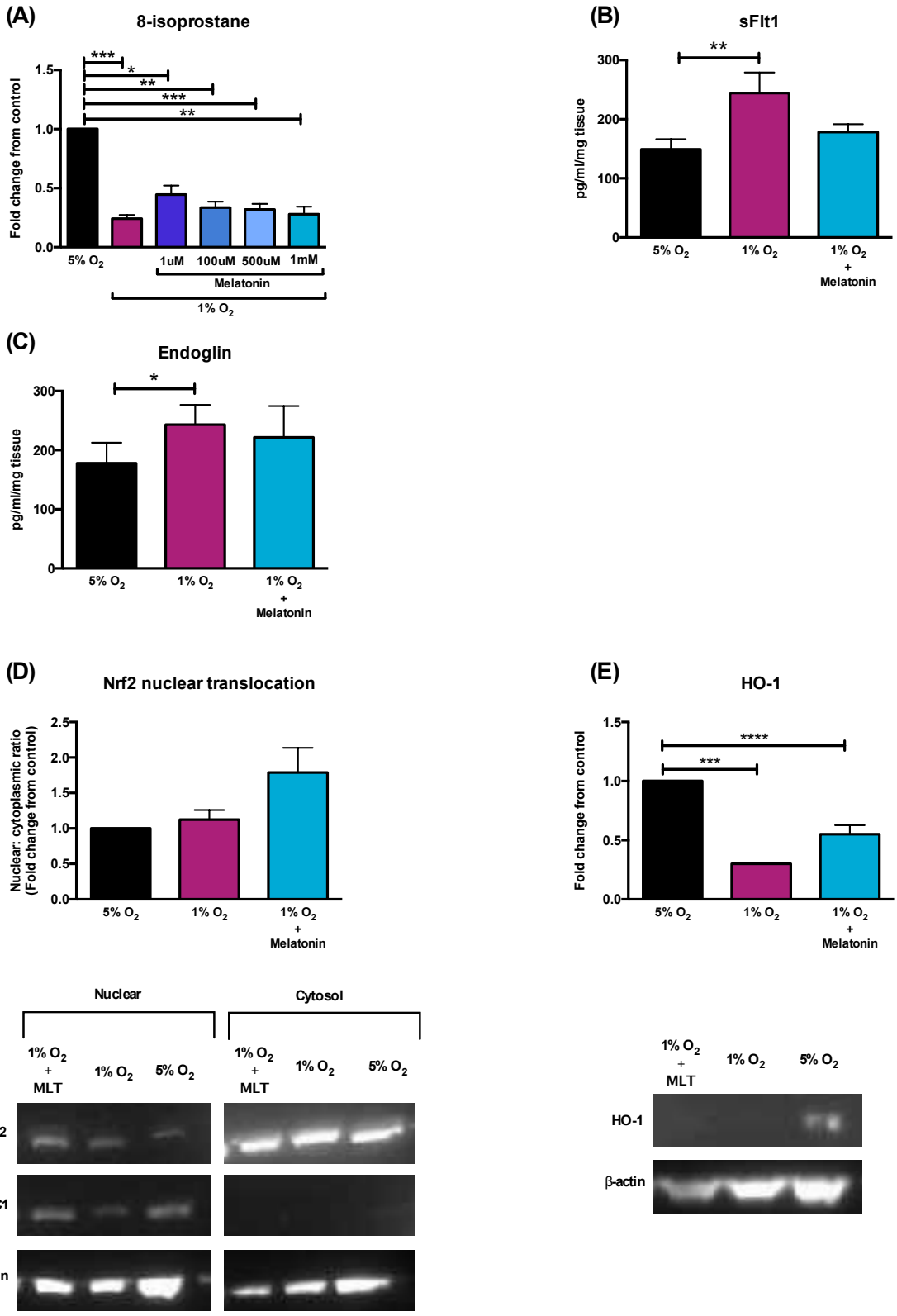
Figure 3.1: Effect of melatonin on placental explants exposed to oxidative stress. Term placental explants were cultured with either control media, X/XO only or X/XO with 4 different doses of melatonin (MLT; 1μM, 100μM, 500μM, 1mM) for 48 hours. Concentration of (A) 8-isoprostane in the culture supernatants of all groups was measured via enzyme immunoassay. Concentration of (B) sFlt1 and (C) endoglin in the culture supernatants of the media only and X/XO only groups were measured via ELISA. In the media only, X/XO only and X/XO with 1mM melatonin groups (D) Activin A concentration in the culture supernatants were measured via ELISA, while (E) Nrf2 nuclear translocation and (F) HO-1 protein expression was measured in the corresponding explants by western blot. Representative western blot images are shown below each graph. The white space indicates noncontiguous lanes from the same blot. Data are expressed as mean ± SEM. **P*<0.05. n = 6 independent placental donors.


(A)
8-Isoprostane
Fold change from control
Control
1uM
100uM
500uM
1mM
Melatonin
X/XO
(B)
sFlt1
pg/ml/ mg tissue
Control
X/XO
(C)
Endoglin
ng/ml/ mg tissue
Control
X/XO
(D)
Activin A
pg/ml/mg tissue
Control
X/XO
X/XO + Melatonin
(E)
Nrf2 nuclear translocation
Nuclear: cytoplasmic ratio (Fold change from control)
Control
X/XO
X/XO + Melatonin
Nuclear
Cytosol
X/XO + MLT
X/XO
Control
Nrf2
HDAC1
β-actin
(F)
HO-1
Fold change from control
Control
X/XO
X/XO + Melatonin
X/XO + MLT
X/XO
Control
HO-1
β-actin

3.3.2 Effect of melatonin on placental explants exposed to hypoxia (1%O_2)

In order to more closely mimic the low oxygen environment of the preeclamptic placenta that exists during the ischemia phase of placental ischemia-reperfusion in preeclampsia, we then exposed term placental explants to 1% O_2. Placental explants exposed to 5% O_2 produced nearly 4-fold more 8-isoprostane than those exposed to 1% O_2 (all $P<0.05$; Figure 3.2A). There was no difference in 8-isoprostane production by 1% O_2 exposed placental explants, regardless of melatonin (all $P>0.05$; Figure 3.2A). sFlt1 and sEng production was significantly higher in the 1% O_2 exposed placental explants compared with those exposed to 5% O_2 (both $P<0.05$; Figure 3.2B & 3.2C respectively). However, 1mM melatonin treatment did not significantly change this hypoxia-induced increase in sFlt1 and sEng (both $P>0.05$; Figure 3.2B & 3.2C respectively). Nrf2 translocation to the nucleus was similar between the explants exposed to 5% O_2, 1% O_2, and 1% O_2 + 1mM melatonin (all $P>0.05$; Figure 3.2D). In contrast, HO-1 protein levels were significantly higher in the placental explants exposed to 5% O_2 compared to those exposed to 1% O_2 (all $P<0.01$; Figure 3.2E). There were no differences in HO-1 protein levels between the 1% O_2 exposed explants regardless of 1mM melatonin treatment (all $P>0.05$; Figure 3.2E).

Figure 3.2: Impact of melatonin on placental explants exposed to 1% O_2 conditions. Term placental explants were cultured in control media at 5% O_2, 1% O_2 or in 1% O_2 with 4 different doses of melatonin (MLT; 1μM, 100μM, 500μM, 1mM) for 24 hours. Concentration of (A) 8-isoprostane in the culture supernatants of all groups was measured via enzyme immunoassay. Concentration of (B) sFlt1 and (C) endoglin in the culture supernatants of the 5% O_2, 1% O_2 and 1% O_2 with 1mM melatonin groups were measured via ELISA. (D) Nrf2 nuclear translocation and (E) HO-1 protein expression was measured in the corresponding explants by western blot. The white space indicates noncontiguous lanes from the same blot. Representative western blot images are shown below each graph. Data are expressed as mean ± SEM. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. n = 6 independent placental donors.


(A)
8-isoprostane
Fold change from control
1.5
1.0
0.5
0.0
***
*
**
***
**
5% O2
1uM
100uM
500uM
1mM
Melatonin
1% O2
(B)
sFlt1
pg/ml/mg tissue
300
200
100
0
**
5% O2
1% O2
1% O2
+
Melatonin
(C)
Endoglin
pg/ml/mg tissue
300
200
100
0
*
5% O2
1% O2
1% O2
+
Melatonin
(D)
Nrf2 nuclear translocation
Nuclear: cytoplasmic ratio
(Fold change from control)
2.5
2.0
1.5
1.0
0.5
0.0
5% O2
1% O2
1% O2
+
Melatonin
(E)
HO-1
Fold change from control
1.5
1.0
0.5
0.0
****
***
5% O2
1% O2
1% O2
+
Melatonin
Nuclear
Cytosol
1% O2
+
MLT
1% O2
5% O2
1% O2
+
MLT
1% O2
5% O2
Nrf2
HDAC1
β-actin
1% O2
+
MLT
1% O2
5% O2
HO-1
β-actin

3.3.3 Impact of melatonin on placental explants from women with preeclampsia

Treatment of placental explants collected from preeclamptic women with melatonin did not significantly change 8-isoprostane production in these explants when compared with the untreated group (all $P>0.05$; Figure 3.3A). Likewise, sFlt1, sEng and activin A production was also similar between the preeclamptic placental explants that were treated with 1mM melatonin and those that were untreated (all $P>0.05$; Figure 3.3B, 3.3C & 3.3D respectively).

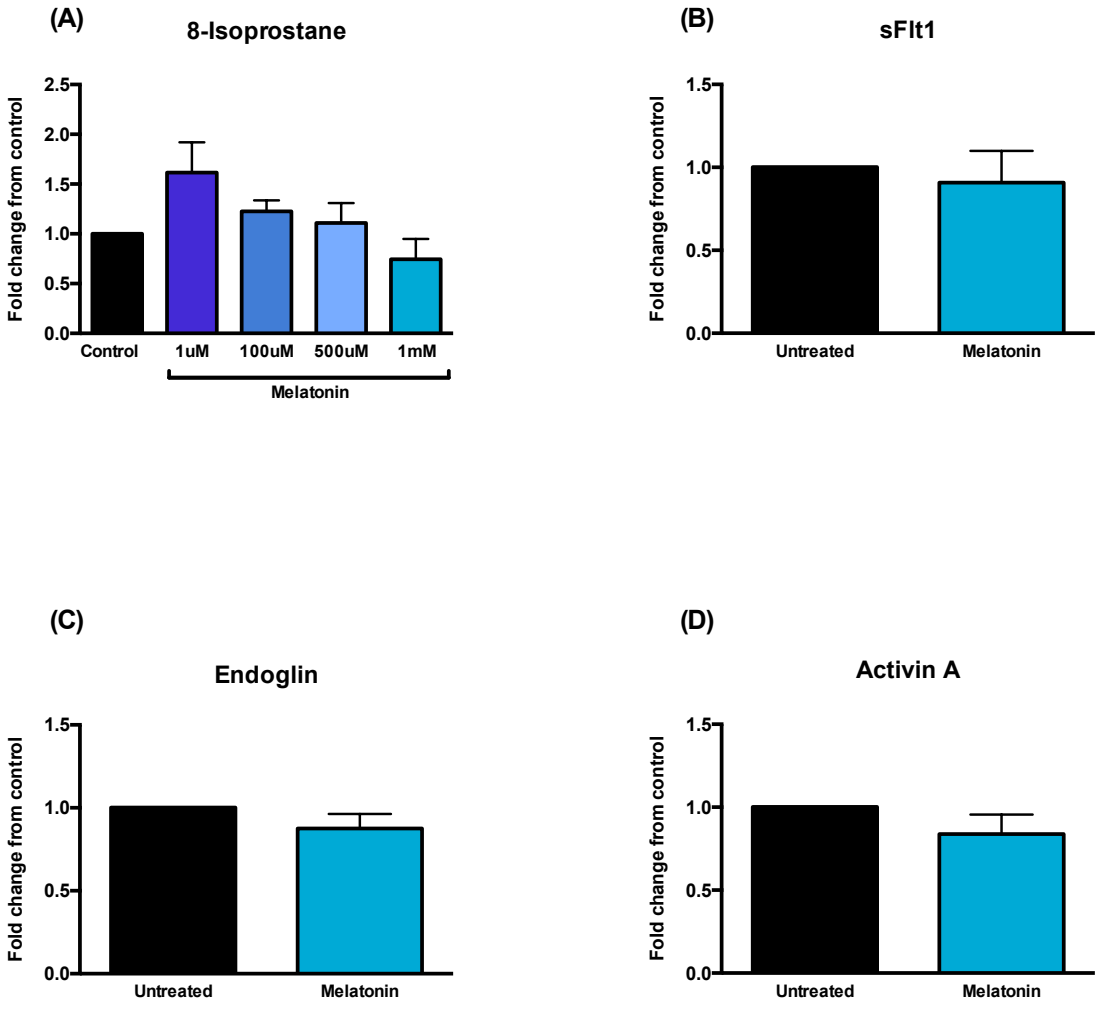


Figure 3.3: Effect of melatonin treatment on placental explants from women with preeclampsia. Placental explants from women with preeclampsia were cultured at 1% O_2 with or without melatonin (1µM, 100µM, 500µM, 1mM) for 24 hours. The concentrations of (A) 8-isoprostane, (B) sFlt1, (C) endoglin and (D) activin A in the culture supernatants were measured via enzyme immunoassay or ELISA. sFlt1, endoglin and activin A levels were assessed only in the untreated explants and the explants treated with 1mM melatonin. Data are expressed as mean ± SEM. n = 3 independent placental donors.

3.3.4 Effect of melatonin on endothelial cell dysfunction *in vitro*

Treatment of HUVECs with 1ng/ml TNFα significantly increased the protein expression of the endothelial activation markers ICAM1, VCAM1 and E-selectin ($P<0.001$; Figure 3.4A, 3.4B & 3.4C respectively). Melatonin decreased this TNFα-induced increase in VCAM1 by nearly 3-fold ($P<0.001$; Figure 3.4B), but had no significant effect on the TNFα-induced increase in ICAM1 and E-selectin expression ($P>0.05$; Figure 3.4A & 3.4C respectively). TNFα (1ng/ml) also significantly increased the production of the potent vasoconstrictor ET-1 by the HUVECs ($P<0.01$; Figure 3.4D) and this effect was not significantly altered by treatment with melatonin ($P>0.05$; Figure 3.4D). However, melatonin mitigated the TNFα (100ng/ml) stimulated increase in endothelial monolayer permeability by nearly 35%, and completely rescued the disruption to the endothelial monolayer integrity induced by serum from women with preeclampsia ($P<0.05$; Figure 3.4E & 3.4F respectively).

Figure 3.4: Effect of melatonin treatment on the expression of endothelial dysfunction markers and on endothelial monolayer permeability. HUVECs were treated with control media, TNF-α (1ng/ml or 100ng/ml) only or TNF-α together with 3 different doses of melatonin (1μM, 10μM, 1mM). Expression of (A) ICAM1, (B) VCAM1 and (C) E-selectin in the HUVECs were measured 6 hours after treatment via flow cytometry, whilst the concentration of (D) endothelin-1 was measured in HUVEC culture supernatants collected 24 hours after treatment. (E) FITC-dextran permeability through HUVEC monolayers treated with either control media, TNF-α (100ng/ml) only or TNF-α with 1mM melatonin was measured 24 hours after treatment. (F) FITC-dextran permeability was also measured in HUVEC monolayers treated with serum from normal pregnant women (NP serum), serum from preeclamptic women (PE Serum) or PE serum with 1mM melatonin for 24 hours. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01, *****P*<0.0001. n = 6 cell lines from independent placental donors.

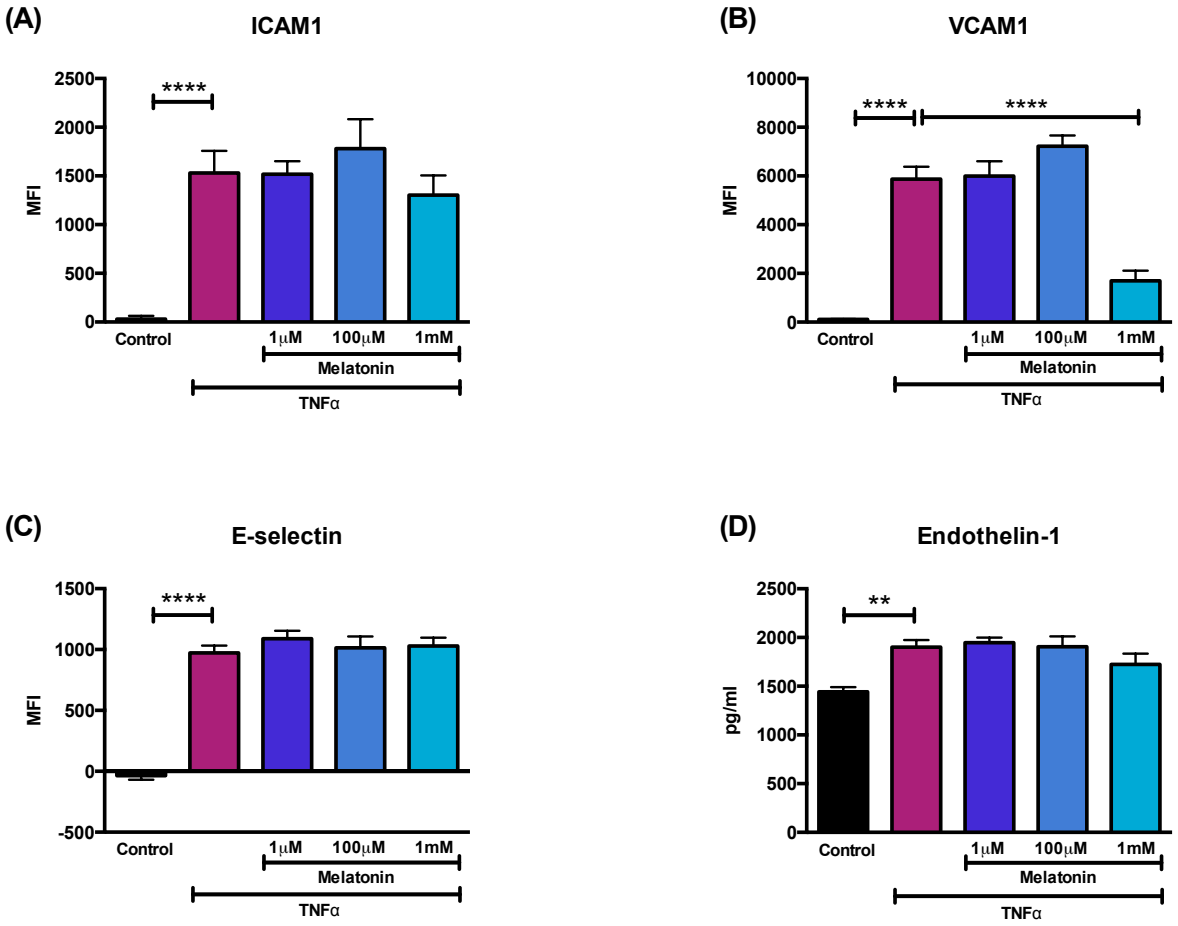
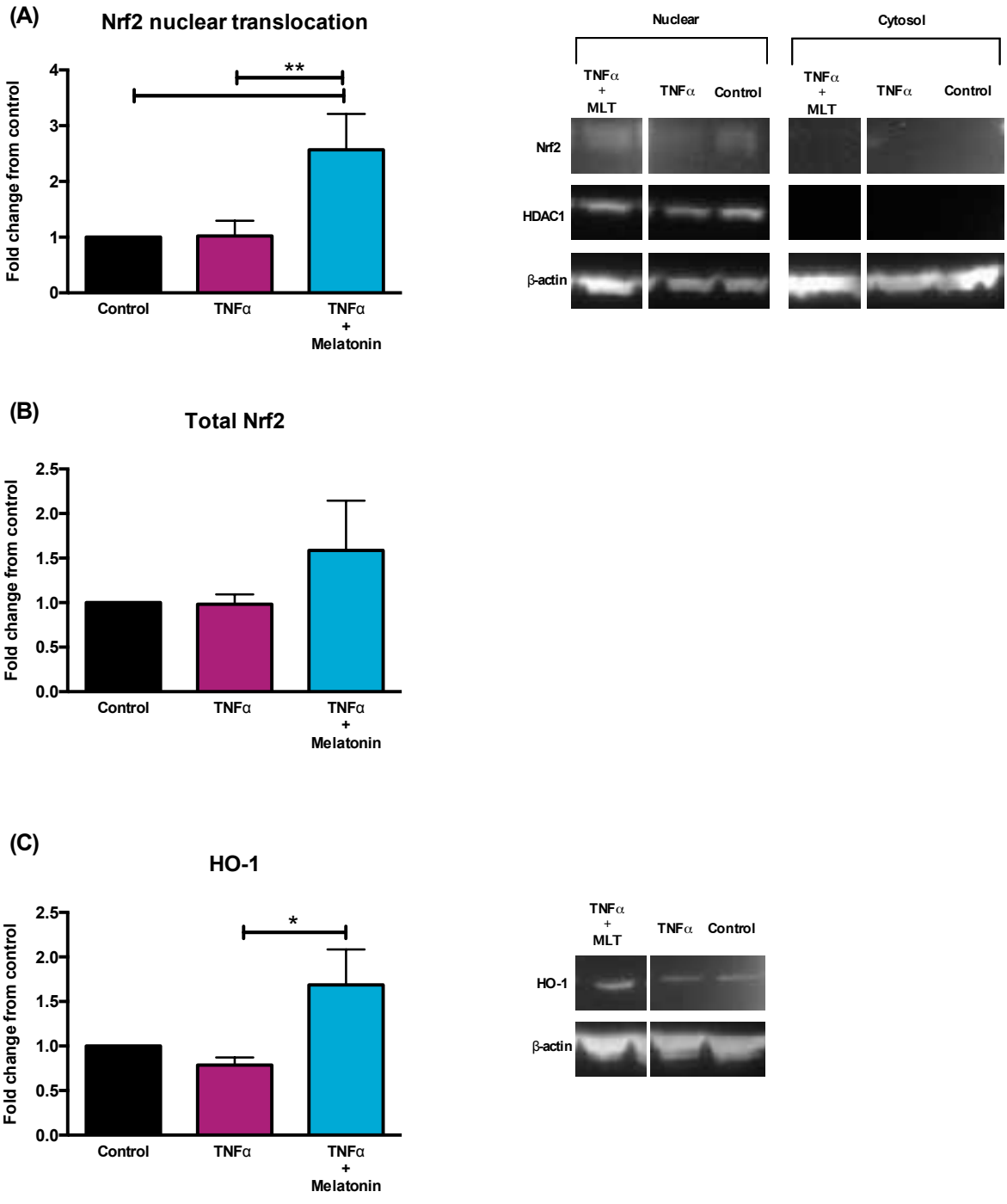

(A)
ICAM1
MFI
2500
2000
1500
1000
500
0
****
Control
1μM
100μM
1mM
Melatonin
TNFα
(B)
VCAM1
MFI
10000
8000
6000
4000
2000
0
****
****
Control
1μM
100μM
1mM
Melatonin
TNFα
(C)
E-selectin
MFI
1500
1000
500
0
-500
****
Control
1μM
100μM
1mM
Melatonin
TNFα
(D)
Endothelin-1
pg/ml
2500
2000
1500
1000
500
0
**
Control
1μM
100μM
1mM
Melatonin
TNFα
(E)
HUVEC monolayer permeability
Fold change from control
200
150
100
50
0
*
*
Control
TNFα
TNFα + Melatonin
(F)
HUVEC monolayer permeability
Fold change from control
200
150
100
50
0
**
*
NP Serum
PE Serum
PE Serum + Melatonin

Figure 3.5: Nrf2 and HO-1 expression in HUVECs treated with melatonin. HUVECs were treated with control media, TNF-α (100ng/ml) only or TNF-α with 1mM melatonin (MLT). (A) Nuclear translocation of Nrf2 and (B) total Nrf2 protein expression was measured 6 hours after treatment, whilst (C) total HO-1 protein expression was measured 24 hours after treatment, all via western blot. Representative western blot images are shown next to each graph. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01. n = 6 cell lines from independent placental donors.

Nuclear translocation of Nrf2 was significantly increased in the TNFα + 1mM melatonin treated HUVECs, when compared with HUVECs that were untreated and treated with TNFα only (all $P<0.01$; Figure 3.5A). However, total Nrf2 levels were not significantly different between the 3 groups ($P>0.05$; Figure 3.5B). HO-1 protein levels were significantly higher in the TNFα and melatonin treated HUVECs in comparison to the HUVECs treated with TNFα only ($P<0.05$; Figure 3.5C).



3.4 Discussion

The aim of this study was to determine the ability of melatonin to mitigate placental oxidative stress, placental production of vasoactive factors and endothelial dysfunction *in vitro*, and therefore assess its therapeutic potential for treatment of preeclampsia. In term placental explants, a 1mM dose of melatonin reduced X/XO-induced oxidative stress, but not activin A, sFlt1 or sEng production. In preeclamptic placental explants, melatonin did not affect oxidative stress levels or the production of sFlt1, sEng and activin A. In endothelial cells, melatonin decreased TNFα-induced VCAM1 expression, but did not affect the expression of the other markers for endothelial activation and dysfunction. Importantly, melatonin rescued the TNFα, as well as preeclamptic serum, induced disruption to endothelial monolayer integrity. These data demonstrate that while melatonin may not show promise as a prophylaxis for preeclampsia as it did not resolve placental dysfunction, it may prove to be an effective adjuvant therapy for established preeclampsia due to its capacity to resolve endothelial function.

Numerous studies have provided evidence that the placenta undergoes severe oxidative stress in preeclampsia. As described in section 1.2, the origin of the placental oxidative stress are the periods of hypoxia and reoxygenation resulting from failed spiral artery remodeling during placentation [28]. A known effect of ischemia-reperfusion is the conversion of xanthine dehydrogenase to xanthine oxidase, a potent producer of the free radical, superoxide [267]. Correspondingly, xanthine oxidase activity is elevated in the placentae of women suffering from preeclampsia [268]. It is for this reason that we used xanthine oxidase (XO) and its substrate xanthine (X), to induce oxidative stress in term placental explants, and thus mimic conditions of the preeclamptic placentae. Predictably, treatment with X/XO induced oxidative stress in the placental explant cultures as evidenced by an increase in 8-isoprostane levels in the X/XO-treated group compared with the untreated group. However, in terms of vasoactive factors, X/XO treatment only induced production of activin A by the placental explants and not sFlt1 or sEng. In contrast, exposure of placental explants to a hypoxic (1% O_2) environment to mimic the ischemia phase of the placental ischemia-reperfusion that occurs in preeclampsia, led to an increase in sFlt1 and sEng. Previous studies by our lab have shown that exposure to hypoxic conditions does not stimulate placental production of activin A [142]. All of this data clearly demonstrate that while the placental production of activin A

is stimulated by oxidative stress, an independent hypoxia-regulated pathway drives the production of sFlt1 and sEng. This is supported by previous observations that sFlt1 and sEng production are regulated by HIF-1α, while oxidative stress is the key driver for the production of inflammatory cytokines, such as activin A and TNFα by the placenta [42, 43, 143, 269]. Therefore, it appears that in preeclampsia placental hypoxia resulting from the ischemia phase gives rise to sFlt1 and sEng, while the oxidative stress subsequent to the reperfusion phase triggers the production of inflammatory cytokines, such as activin A and TNFα.

Another interesting observation in this study was the reduced oxidative stress levels in the placental explants exposed to hypoxic (1% O_2) conditions as opposed to those exposed to 5% O_2. In contrast, previous studies have shown exposure to hypoxia (1% O_2) to result in placental oxidative stress [243, 270]. However, this difference could be due to the fact that in these previous studies, the placental explants were cultured in hypoxia (1% O_2) for 48-72 hours, whilst in this study the treatment period was only 24 hours. Chronic exposure to a low oxygen environment may be required to build up sufficient reactive oxygen species to induce oxidative stress. Nevertheless, exposure to hypoxia (1% O_2) for 24 hours did increase placental production of sFlt1 and sEng.

Melatonin significantly decreased X/XO-induced oxidative stress in the placental explants, which was accompanied by an increase in Nrf2 translocation to the nucleus, as well as an increase in HO-1 expression in the same explants. Hence, the decrease in placental oxidative stress by melatonin was most likely mediated by the Nrf2 mediated up-regulation of the endogenous antioxidant enzymes, such as HO-1. However, the ability of melatonin to scavenge free radicals cannot be ruled out as a contributing factor to the reduction in placental oxidative stress following melatonin treatment. Even though melatonin decreased placental oxidative stress, it failed to significantly decrease the oxidative stress-triggered increase in placental activin A production. It is possible that a longer treatment period was required to observe the decrease in placental oxidative stress leading to a decrease in placental activin A production. Alternatively, the decrease in oxidative stress induced by melatonin could be insufficient to significantly quench placental activin A production as well.

Apart from its strong antioxidant properties, HO-1 was also hypothesised to be beneficial in preeclampsia due to its inhibitory effects on the release of sFlt1 and sEng [242]. Accordingly, the ability of melatonin to attenuate hypoxia-induced placental production of sFlt1 and sEng

by activating Nrf2 and HO-1 was tested, and found this not to be the case. Nuclear translocation of Nrf2, HO-1 protein levels, as well as sFlt1 and sEng production were similar between the melatonin-treated and untreated explants exposed to hypoxia. It is interesting to note that melatonin was able to increase Nrf2 nuclear translocation and HO-1 protein levels in the X/XO treated explants, but not in the explants exposed to 1% O_2. Studies have shown that electrophilic and oxidative stress trigger nuclear accumulation and activation of Nrf2 by preventing its suppression by Keap-1, as well as its proteasomal degradation [271, 272]. Therefore, it is possible that in the X/XO treated explants the reactive oxygen species generated by X/XO triggered Nrf2 nuclear translocation and activation, whilst in the explants exposed to 1% O_2, the lack of an oxidative stress stimulus, as seen in figure 2A, led to failure of Nrf2 activation.

The data from the preeclamptic explants confirm those from the hypoxia and X/XO- treated placental explants to a certain extent. Melatonin did not significantly change oxidative stress levels or the production of sFlt1, sEng and Activin A by the preeclamptic explants. The failure of melatonin to have an effect on the preeclamptic explants could be due to the significantly reduced expression of MT1 and MT2 melatonin receptors in the preeclamptic placentae compared with placentae from healthy pregnancies [254]. It is therefore possible that this limits the efficacy of melatonin treatment *in vitro*.

Systemic maternal endothelial dysfunction is the underlying causative factor for all of the maternal clinical features of preeclampsia [14]. TNF-α is one of the factors that significantly contribute to the development of endothelial dysfunction in preeclampsia, as it is significantly elevated in the serum of women with preeclampsia and infusion of TNF-α into pregnant animals causes hypertension and proteinuria, two of the major clinical features of preeclampsia [61, 273]. Furthermore, it is well known to stimulate endothelial dysfunction *in vitro* [274]. It is for these reasons that TNF-α was used to mimic endothelial cell dysfunction in these *in vitro* conditions.

The main feature of melatonin that makes it an attractive therapeutic option for preeclampsia is its well-known ability to improve endothelial function, mainly by preventing vasoconstriction, platelet aggregation and leuckocyte infiltration [262, 275, 276]. ICAM-1, VCAM-1 and E-selectin are markers of endothelial activation that are significantly elevated in women diagnosed with preeclampsia [152]. They promote leukocyte infiltration of tissues

leading to inflammation. ET-1 is a potent vasoconstrictor produced by endothelial cells [277]. Preeclamptic women have significantly higher serum levels of ET-1 compared with healthy pregnant women and it is considered to be a key contributor to the development of hypertension in these women [277, 278]. In this study, melatonin was found to decrease TNFα-induced VCAM1 expression, but not the expression of ICAM1, E-selectin and endothelin-1. Furthermore, melatonin increased Nrf2 nuclear translocation and HO-1 protein levels in the HUVECs. The expression of ICAM1, VCAM1 and E-selectin is mainly induced by NF-κB, following activation by reactive oxygen species [279-281]. Hence, it is possible that melatonin mediated the reduction in VCAM1 expression by reducing cellular oxidative stress and inhibiting the NF-κB pathway, by activating Nrf2 and triggering the subsequent increase in HO-1. This is supported by previous studies that have shown melatonin treatment to mitigate NF-κB activity, as well as by studies that have shown Nrf2 activation to negatively regulate the NF-κB pathway, possibly via the reduction of reactive oxygen species [279, 282-284]. However, if melatonin did exert its actions via the inhibition of NF-κB and a reduction in reactive oxygen species, a subsequent decrease in the expression of ICAM1 and E-selectin would have been expected. Since this was not the case, it is possible that melatonin inhibited VCAM1 expression via a mechanism downstream of NF-κB, as there is evidence to suggest that the molecular mechanisms involved in the expression of VCAM1, ICAM-1 and E-selectin vary downstream of NF-κB activation [285, 286]. The role of melatonin in modulating these molecular pathways has not been extensively examined yet. However, it must be noted that animal studies involving prolonged treatment of melatonin have reported a decrease in ICAM-1 and E-selectin expression, in addition to a decrease in VCAM1, following treatment [276, 287]. Hence, more beneficial effects of melatonin in reducing the expression of endothelial adhesion molecules, and thus reducing leukocyte infiltration, following *in vivo* treatment cannot be ruled out.

Furthermore, melatonin rescued the increase in endothelial permeability induced by both TNFα and serum from women with preeclampsia. Melatonin is known to improve endothelial integrity by protecting the endothelial adherens and tight junctional proteins, such as VE-cadherin, occludin, claudin-5 and zonula occludens-1, from disruption by cytokines and reactive oxygen species, as well as by increasing their protein expression [288, 289]. Enhanced endothelial permeability is responsible for many of the clinical features of preeclampsia and eclampsia, such as proteinuria, pulmonary oedema, increase in serum liver transaminases and the development of seizures. Thus, the ability of melatonin to restore

endothelial integrity, even in the presence of all the vasoactive factors present in the serum of women with preeclampsia, highlight its potential to be an adjuvant therapy for preeclampsia.

To our knowledge, this is the first study that has examined the potential of melatonin as a therapeutic for preeclampsia. Animal and clinical studies have shown that melatonin is safe in pregnancy and clinical trials are underway to determine its efficacy in reducing pregnancy conditions involving oxidative stress, such as intrauterine growth restriction [290]. Data from this study demonstrate that whilst melatonin may not be effective in resolving placental dysfunction, it does have potential to resolve maternal endothelial dysfunction, and thus could prove to be an effective adjuvant therapy for established preeclampsia. Further studies to determine the effectiveness of melatonin in ameliorating endothelial function *in vivo* would be beneficial.

Chapter 4

Resveratrol activation of nuclear factor erythroid 2-related factor-2 (nrf2): potential therapy for preeclampsia

---

4.1 Introduction

As described in section 1.2, the pathophysiology of preeclampsia is characterized by systemic maternal endothelial dysfunction, which in turn is triggered by several anti-angiogenic factors derived from the placenta following exposure to hypoxia and oxidative stress. A therapy that resolves these underlying features of the disease could mitigate the clinical features and prolong pregnancy in women with preeclampsia. Activators of the Nrf2 transcription factor have the potential to be such a therapy. As described in detail in section 1.3.1, studies have shown activation of Nrf2 and its downstream endogenous anti-oxidant enzymes, particularly HO-1 to mitigate placental oxidative stress and production of vasoactive factors, such as sFlt1 and sEng, as well as improve vascular function [221, 223, 234, 242, 243].

Data from chapter 3 demonstrated that melatonin, an Nrf2 activator, could improve certain *in vitro* hallmarks of endothelial dysfunction. Hence, chapter 4 examines the effectiveness of another more well-known Nrf2 activator, resveratrol, in mitigating the placental and endothelial pathologies characteristic of preeclampsia, *in vitro*.

Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

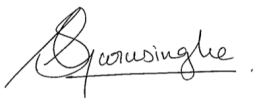
In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experimental design, optimization of protocols, performing experiments, data analysis, drafting of manuscript	90%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Rahana Rahman	Assistance with experiments	3%
Siow T Chan	Assistance with experiments	NA
Ruth Muljadi	Optimization of protocols	NA
Harmeet Singh	Assistance with experiments	NA
Bryan Leaw	Assistance with experiments	NA
Joanne Mockler	Sample collection	2%
Padma Murthi	Intellectual input	NA
Rebecca Lim	Intellectual input and drafting of manuscript	NA
Euan M Wallace	Intellectual input and drafting of manuscript	NA

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date** 4/1/2017
Main Supervisor's Signature		**Date** 6th Jan 2017

TITLE: Resveratrol activation of nuclear factor erythroid 2-related factor-2 (nrf2): potential therapy for preeclampsia

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Condensation

Nuclear factor erythroid 2-related factor-2 activation improves endothelial and placental dysfunction *in vitro*, offering promise as an adjuvant therapy for preeclampsia.

Short title

Nrf2 as a therapeutic target in preeclampsia

Abstract

Background

The maternal endothelial dysfunction underlying preeclampsia is thought to arise from excessive placental release of anti-angiogenic factors, such as soluble fms-like tyrosine kinase-1, soluble endoglin and activin A. The nuclear factor erythroid 2-related factor-2 transcription factor mediates the gene expression of antioxidant and vasoprotective factors that may counter the endothelial damage imposed by these anti-angiogenic factors, offering the possibility of a targeted adjuvant therapy for preeclampsia.

Objective

To assess whether resveratrol, a nuclear factor erythroid 2-related factor-2 activator, could reduce placental oxidative stress and production of soluble fms-like tyrosine kinase-1, soluble endoglin and activin A *in vitro*, while also improving *in vitro* markers of endothelial dysfunction.

Study Design

We used *in vitro* term placental explants to assess the effects of resveratrol on the placental production of the oxidative stress marker, 8-isoprostane, and of soluble fms-like tyrosine kinase-1, soluble endoglin and activin A. Using human umbilical vein endothelial cells we investigated the effects of resveratrol on markers of *in vitro* endothelial dysfunction, including the expression of intercellular adhesion molecule 1, vascular cell adhesion molecule 1, E-selectin and endothelin-1, and endothelial permeability. To confirm that resveratrol mediated its effects via nuclear factor erythroid 2-related factor-2, we examined the impact of resveratrol on the same *in vitro* markers of endothelial dysfunction following the knockdown of nuclear factor erythroid 2-related factor-2.

Results

Resveratrol significantly decreased placental oxidative stress *in vitro* and the production of soluble fms-like tyrosine kinase-1 and activin A. Resveratrol significantly mitigated tumor necrosis factor–α stimulated endothelial expression of intercellular adhesion molecule 1, vascular cell adhesion molecule 1, E-selectin and endothelin-1 and prevented an increase in endothelial monolayer permeability. Knockdown of nuclear factor erythroid 2-related factor-2 abolished most of the protective effects of resveratrol on endothelial cells.

Conclusions

Modulation of the nuclear factor erythroid 2-related factor-2 pathway may be a potential therapeutic target for preeclampsia.

Key words: activin A, fms-like tyrosine kinase 1, heme oxygenase-1, nuclear factor erythroid 2-related factor-2 (Nrf2), preeclampsia, resveratrol

Introduction

Preeclampsia remains a leading cause of maternal and perinatal morbidity and mortality worldwide.[1] While the use of antihypertensives to control maternal blood pressure and allow pregnancy prolongation has greatly improved clinical outcomes it is widely accepted that a more targeted therapy, focused on more than simply controlling hypertension, is needed.[2-5] Two insights have heralded opportunities to develop such a therapy. First, the recognition that systemic maternal endothelial dysfunction underlies many of the maternal features of preeclampsia turned attention to exploring mechanisms of endothelial injury.[6,7] This then led to the observation that the endothelial dysfunction is likely secondary, at least in part, to excessive placental release of several anti-angiogenic factors, such as tumor necrosis factor–α (TNF-α), soluble fms-like tyrosine kinase-1 (sFlt1), soluble endoglin (sEng) and activin A.[8-16] These factors induce endothelial injury by up regulation of pro-oxidant enzymes, such as NADPH oxidase (NOX), and the induction of oxidative stress.[8,17,18] Together, these insights have afforded the possibility of novel approaches to the treatment of established preeclampsia by either targeting the anti-angiogenic factors or their downstream endothelial effects including NOX activation and oxidative stress.

In that regard, activation of the nuclear factor erythroid 2-related factor-2 (Nrf2) transcription factor offers much promise as an approach to reduce both maternal endothelial dysfunction and, perhaps, the underlying placental injury in pregnancies complicated by preeclampsia. Nrf2 mediates the response to cellular stresses by activating the gene transcription of a variety of cytoprotective enzymes, including the phase II detoxification and anti-oxidant enzymes, glutathione S-transferases, NADPH quinone oxidoreductase and heme oxygenase-1 (HO-1).[19] To date, studies on placental expression of Nrf2 in women with preeclampsia have revealed conflicting results. Some investigatos have reported increased placental Nrf2

expression in preeclampsia while others have reported a decrease.[20-22] Nonetheless, the targeted activation of Nrf2 in preeclampsia could be beneficial. Activation of a repertoire of endogenous anti-oxidant enzymes by Nrf2 protects against the oxidative stress that results from ischemia-reperfusion injury. Theoretically, this should be useful in resolving the underlying placental injury in preeclampsia.[23-25] Indeed, activation of HO-1 has been shown to ameliorate placental ischemia in a rodent model of reduced placental perfusion, which in turn reduced the production of sFlt1 and sEng.[26-28] In addition, Nrf2 activation has been shown to rescue endothelial function in mice fed a high fat diet and in a rat model of chronic renal disease. [29,30] These beneficial effects of Nrf2 activation on the endothelium arose mainly to the downstream anti-oxidant and anti-inflammatory gene targets.[29,30]

Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a polyphenolic compound found in a variety of plant foods including grapes, peanuts and berries.[31] It is an Nrf2 activator with both anti-oxidant and anti-inflammatory effects.[30,32] Therefore, not surprisingly, resveratrol has been shown to improve endothelial function and prevent end-organ injury in diverse models of ischemia-reperfusion injury.[33-35] We wished to explore whether resveratrol might afford anti-oxidant protection to both trophoblast and endothelial cells with a future view to using it as an adjuvant therapy in preeclampsia.

Materials and Methods

All human samples were collected with informed written consent and following approval from the Monash Health Human Research Ethics Committee.

Placental tissue collection and explant culture

Human placental samples were collected at the time of elective cesarean section from women with a healthy term singleton pregnancy. Upon collection of the placenta several cotyledons were excised randomly and washed in Hank's balanced salt solution (HBSS) to remove blood. Placental villous tissue was then dissected and weighed. About 50-70mg of tissue was placed in each well of a 24-well plate and cultured in Medium 199 supplemented with 1% antibiotics and L-Glutamine (all from Life Technologies, Carlsbad, CA), containing the different treatment conditions.

A group of placental explants were treated with 2.3mmol/Lxanthine/ 15mU/mLxanthine oxidase (X/XO; Sigma-Aldrich, St. Louis, MO) to induce oxidative stress, in the presence or absence of 50µmol/L, 100µmol/L or 200µmol/L resveratrol (Sigma-Aldrich). Placental explants treated with media only were used as negative controls. Explants were cultured in 5% O_2 at 37°C for 48 hours. A seperate group of placental explants were exposed to 1% O_2 in the presence or absence of resveratrol at 37°C for 24 hours. Placental explants exposed to 5% O_2 were used as controls.

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords collected from healthy term pregnancies at elective caesarean section, as previously described[36]. All HUVEC cultures were plated at a density of 12,000 cells per 100mm^2 in Medium 199 supplemented with 20% fetal bovine serum, 1% antibiotics and L-Glutamine (all from Life Technologies), and incubated at 37°C in 5% CO_2. HUVECs were then treated with recombinant 1-100ng/ml TNFα (R&D systems, Minneapolis, MN) in the presence or

absence of 50µmol/L, 100µmol/L or 200µmol/L resveratrol for 6 hours prior to measurement of Nrf2 nuclear translocation and expression of the endothelial activation markers intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VACM1) and E-selectin. Endothelin-1 and HO-1 protein levels were assessed following a 24-hour treatment period.

8-isoprostane, sFlt1, sEng, activin A and endothelin-1 measurement

8-isoprostane in placental explant and HUVEC culture supernatants was determined by enzyme immunoassay (Caymen Chemicals, Ann Arbor, MI). Levels of sFlt, sEng and activin A in placental explant culture supernatants and levels of endothelin-1 in HUVEC culture supernatants were all determined via enzyme-linked immunosorbent assay (ELISA; R&D systems). All assays were performed according to manufacturer's instructions.

Markers of endothelial cell activation

The markers of endothelial cell activation were determined in the HUVECs via flow cytometry (FACS). Briefly, HUVECs were mechanically scraped from their flasks prior to immunostaining with the following antibodies: (antibody synonyms, antibody dilutions and catalogue numbers shown in parentheses). CD54-Pacific Blue (ICAM1, 1:100, 353109), CD106-PE (VCAM1, 1:50, 305805) CD62E-APC (E-selectin, 1:100, 336011). Immunostaining was compared with appropriate isotype controls. Antibodies and isotype controls were obtained from Biolegend (San Diego, CA). HUVECs were incubated on ice with either antibodies or isotype controls for 20 minutes prior to washing and fixing in 1% paraformaldehyde in FACS buffer. The markers of endothelial cell activation were then analyzed on a BD FACS Canto II (BD Biosciences, San Jose, CA). Analyses were performed using FlowJo cytometric analysis software (Oakland, OR).

Endothelial cell permeability

Integrity of the endothelial cell monolayer following TNFα treatment was assessed using a FITC-dextran based permeability assay. HUVECs were plated in gelatinized polycarbonate transwell inserts (Corning, Oneonta, NY; 0.4μm, 6.5mm, 24 well plate) at a density of 50,000 cell/insert and incubated at 37°C for 3 days to form a monolayer. Cells were then treated with recombinant 100ng/mLTNFα in the presence or absence of 100μmol/L resveratrol for 24 hours. At the end of the treatment period the culture media was replaced in both chambers with fresh media containing 1mg/ml FITC-dextran (Sigma-aldrich; MW 40,000) added to the upper chamber. Cells were then incubated for 1 hour at 37°C. Media was removed from the lower chamber and diluted 1:20 with HBSS in a black-walled 96-well plate before fluorescence readings were obtained at 485nm excitation and 535nm emission.

Endothelial cell permeability following treatment with serum from women with preeclampsia (5% serum in Medium 199 supplemented with 1% antibiotics and L-Glutamine) was measured using the 24-well *in vitro* vascular permeability assay kit (Merck Millipore, Billerica, MA) according to manufacturer's instructions. Endothelial cells treated with serum from gestation matched healthy pregnant women were used as controls.

Nrf2 nuclear translocation and HO-1 protein levels

Placental explants and HUVEC cultures were assessed for Nrf2 activation and antioxidant response by determining nuclear Nrf2 translocation and total HO-1 protein levels. Protein extracts of nucleic and cytoplasmic fractions were obtained using the NE-PER nuclear and cytoplasmic reagents (Thermo Scientific, Rockford, IL) according to manufacturer's instructions. Protein quantification was performed using the Pierce BCA kit (Life

Technologies). For western blots, 40μg protein was loaded for each sample. Membranes were then blocked with 5% (w/v) skim milk in phosphate buffered saline with 0.1% (v/v) Tween-20 for 1 hour prior to probing with antibodies. Membranes were stripped in a mild stripping buffer (1.5% w/v glycine, 0.1% w/v sodium dodecyl sulfate, 1% v/v Tween-20 in distilled water) for 5 minutes between antibodies. The primary antibodies and concentrations used were: Nrf2 (ab62352, 1:2000, Abcam, Cambridge, UK) and HO-1 (ab52947, 1:2000, Abcam). Antibodies were diluted in blocking buffer and incubated overnight at 4°C.

Small interfering RNA transfection

Using Lipofectamine RNAimax Reagent (Life Technologies), according to manufacturer's instructions, HUVECs were reverse transfected with either a single stranded small interfering RNA directed towards Nrf2, a scrambled sequence (negative control), or Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH; positive control). All experiments were completed within 4 days of transfection.

Statistical Analysis

Statistical analysis was performed using one-way ANOVA followed by multiple comparisons with a Tukey ad-hoc test. Differences were considered significant where $P < 0.05$. All data were analyzed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

Results

Resveratrol reduces oxidative stress and secretion of sFlt1 and activin A by term placental explants but not the secretion of endoglin.

To assess the effects of resveratrol on placental ischemia-reperfusion induced injury, we treated one set of placental explants with X/XO to mimic the oxidative stress that

characterizes the reperfusion phase, while another set of placental explants were exposed to 1% O_2 to mimic the ischemia phase.[36] Treatment with X/XO significantly increased the levels of 8-isoprostane, an effect mitigated by resveratrol in a dose dependent manner (Figure 1A; $p<0.05$). Resveratrol also prevented the X/XO-induced increase in placental activin A (Figure 1B; $p<0.05$). Compared to control and X/XO-only treated explants, Nrf2 nuclear translocation and HO-1 protein levels were significantly increased in resveratrol treated explants (Figure 1C & Figure 1D; $p<0.05$ both).

Resveratrol mitigated the increased sFlt1, but not sEng, production by placental explants exposed to 1% O_2, (Figure 2A; $p=0.03$ & Figure 2B respectively). Resveratrol also significantly increased explant Nrf2 nuclear translocation (Figure 2C; $p<0.05$) and HO-1 production (Figure 2D; $p<0.05$) compared to those exposed to hypoxia alone.

Resveratrol improves endothelial cell dysfunction in vitro

TNF-α is a vasoactive factor that contributes to the maternal endothelial dysfunction in preeclampsia.[12,37] We have used it here to simulate endothelial dysfunction *in vitro*, as previously described by others.[3,38,39] Resveratrol attenuated the TNF-α induced increase in ICAM-1, VCAM-1, E-selectin and endothelin-1 in a dose-dependent manner (Figure 3A-D; $p<0.01$). Resveratrol also mitigated TNF-α and preeclamptic serum induced increases in endothelial cell permeability compared to controls (Figure 3E; $p=0.02$ & Figure 3F; $p=0.01$, respectively).

Resveratrol increases Nrf2 nuclear translocation and HO-1 protein levels in HUVECs

We then examined whether resveratrol could activate Nrf2 in endothelial cells by increasing Nrf2 protein expression and inducing nuclear translocation. Resveratrol increased Nrf2

nuclear translocation two-fold compared with untreated and TNF-α treated HUVECs (Figure 4A & 4C, p=0.005 both) but did not affect total Nrf2 protein levels (Figure 4B & 4C). Resveratrol significantly increased HO-1 protein levels in the HUVECs compared to untreated or TNF-α alone treated HUVECs (Figure 4D & 4E, $p<0.01$ both).

Nrf2 knockdown abolishes some of the effects of resveratrol on endothelial cells

In order to determine whether Nrf2 is the mediator of the effects of resveratrol on endothelial cells we assessed resveratrol effects on HUVECs following the siRNA knockdown of Nrf2. Knockdown of Nrf2 was confirmed by western blot (Figure 5B). Resveratrol failed to increase HO-1 protein levels in the HUVECs without Nrf2 (Figure 5A & 5B). Furthermore, in the absence of Nrf2, resveratrol failed to rescue the TNF-α induced increase in endothelial permeability and ICAM1 expression (Figure 5C & 5D respectively). However, despite Nrf2 knockdown resveratrol still significantly reduced TNF-α stimulated increase in VCAM1 (Figure 5E; $p<0.05$). The increase in E-selectin expression following TNF-α treatment was significantly reduced by resveratrol in the HUVECs treated with the scrambled siRNA (Figure 5F; $p<0.01$) but not in the Nrf2 knocked down HUVECs (Figure 5F). Endothelin-1 production following TNF-α treatment was significantly decreased by resveratrol in both the HUVECs with and without Nrf2 (Figure 5G; $p<0.001$). Similarly, resveratrol significantly mitigated TNF-α induced 8-isoprostane production in the HUVECs treated with scrambled siRNA and siRNA against Nrf2 (Figure 5H; $p<0.05$).

Comment

Here we report that the Nrf2 activator, resveratrol, can mitigate the increase in placental production of sFlt-1 and activin A that occurs in response to placental injury *in vitro* and

ameliorate endothelial dysfunction induced by TNF-α and preeclamptic serum *in vitro*. The effects on endothelial function were principally mediated via Nrf2, as evidenced by loss of effect when Nrf2 was knocked down. These observations suggest that resveratrol and other Nrf2 activators have the potential to be both useful adjuvant therapies in women with established preeclampsia, by reversing the endothelial dysfunction, and preventative therapies in women at risk of preeclampsia by protecting against placental damage.

Systemic maternal endothelial dysfunction underlies the key clinical features of preeclampsia.[6] Specifically, the maternal features of preeclampsia are characterized by increased vasoreactivity, endothelial permeability and expression of a variety of cell adhesion molecules that, in turn, activate immune cells and platelets.[40-42] Much of these effects are mediated by NOX derived oxidative stress, itself triggered by placental derived vasoactive factors such as TNF-α, sFlt1 and activin A.[8,18,43-46] The resulting endothelial oxidative stress leads to systemic hypertension by triggering the release of vasoconstrictors (endothelin-1) and decreasing the availability of vasodilators (nitric oxide).[47,48] Oxidative stress further disrupts endothelial cell junctional proteins resulting in enhanced endothelial monolayer permeability, contributing to the development of proteinuria, pulmonary edema, hepatic dysfunction and the development of cerebral edema.[49] In addition, reactive oxygen species (ROS)-sensitive NF-κB activation increases leuckocyte adhesion molecules leading to the systemic inflammation characteristic of preeclampsia.[50] Given the central role of oxidative stress in maternal endothelial dysfunction it was most disheartening that trials of the anti-oxidants vitamin C and E proved ineffective at the prevention of preeclampsia.[51,52] Indeed, so disappointing were those results that anti-oxidant approaches as adjuvant therapies in preeclampsia have all but been abandoned.[53] However, rather than providing exogenous antioxidants, decreasing maternal endothelial oxidative stress via the up regulation of

endogenous cellular anti-oxidant enzymes, such as HO-1, secondary to Nrf2 activation, could be a more effective approach. Indeed, the observations in the current study that resveratrol can alleviate endothelial dysfunction via this exact mechanism should, we hope, re-open the field to exploring anti-oxidant therapies as adjuvant treatments for women with established preeclampsia. Certainly, there are several other Nrf2 and HO-1 activators, such as statins and sofalcone that have shown promise as potential therapies for preeclampsia.[4,39,54]

Interestingly, Nrf2 knockdown did not abolish all the beneficial effects of resveratrol. Even without Nrf2, resveratrol diminished endothelial oxidative stress and VCAM1 and endothelin-1 expression. It is not currently clear how those actions were effected but resveratrol could be acting via the sirtuin 1 (SIRT1) pathway. SIRT1 is a protein deacetylase that mediates cell survival during cellular stress, such as oxidative stress.[55,56] Resveratrol is a strong activator of SIRT1 and is known to decrease ROS levels and inhibit NF-κB via SIRT1.[55-57] Certainly it would be worth exploring the SIRT1 pathway for other future therapeutic compounds. It is important to note that despite resveratrol decreasing endothelial oxidative stress without Nrf2, it failed to reduce the TNF-α triggered increase in endothelial permeability and expression of ICAM1 and E-selectin in the absence of Nrf2. This highlights the importance of Nrf2 and its downstream antioxidant enzymes in mediating most of the beneficial effects of resveratrol on endothelial function.

The prospect of treating established preeclampsia by improving endothelial health is certainly exciting. Perhaps even more enticing is the potential to mitigate or reverse placental oxidative stress and the resultant placental production of excess anti-angiogenic factors such as sFlt1 and activin A. In this regard it was promising that resveratrol was able to quench both placental oxidative stress and the excess production of sFlt1 and activin A. This was an

intriguing observation because the triggers for increased placental production of sFlt-1 and activin A are quite different. Specifically, activin A production by the placenta is induced by oxidative stress while sFlt-1 is induced by hypoxia and/or inflammation.[36,58-60] Indeed, activin A and sFlt1 appear to act via independent pathways at the level of the placenta, as well as the endothelium, in the development of preeclampsia.[61] The ability of resveratrol to mitigate the increase in both of these vasoactive agents is likely due to the Nrf2 induction of antioxidant systems reducing activin A, and HO-1 reducing sFlt-1.[8,27,28] In this way resveratrol and possibly other Nrf2 activators could target both the placenta and the maternal endothelium at the same time.

Despite these potential benefits of resveratrol in resolving the endothelial and placental pathologies of preeclampsia, others have raised concern regarding its safety for use during pregnancy. In a study exploring whether resveratrol could reverse metabolic dysfunction following a maternal high fat diet in Japanese macaques the investigators showed that while resveratrol improved metabolic function in both mother and fetus, it led to an increase in fetal pancreatic mass.[62] However, the dams in this study were given a resveratrol-supplemented diet for 3 months prior to breeding and throughout pregnancy. Nonetheless, any progress to clinical trial will need to take these findings into account.

We believe that the observations we have made here justify further assessment of Nrf2 activation as an adjuvant treatment for preeclampsia. In that regard there are other potent Nrf2 activators with a better bioavailability and specificity than resveratrol, such as the synthetic triterpenoids, that would be worth assessing.[63,64] We also suggest that non-Nrf2 dependent pathways, such as SIRT1, may be worth exploring for novel therapeutics.

In conclusion, while the rather non-specific anti-oxidant approach of vitamin C and E were not successful in the prevention or treatment of preeclampsia, our findings suggest that therapies targeting specific anti-oxidant pathways, such as Nrf2 induced enzymes may well prove successful.

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Figure Legends

Figure 1: Effect of resveratrol treatment on placental explants exposed to oxidative stress. Term placental explants were cultured with media only, X/XO only or X/XO with 3 different doses of resveratrol (Res; 50μM, 100μM, 200μM) for 48 hours. The level of (A) 8-isoprostane in the supernatant was measured in all groups. However, the level of (B) Activin A in the supernatant was measured only in the media only, X/XO only and X/XO with 100μM resveratrol groups. In these same explants, (C) Nrf2 nuclear translocation and (D) HO-1 protein expression was also measured via western blot. Representative western blot images for Nrf2 and HO-1 are shown below the respective graph. The white space indicates noncontiguous lanes from the same blot. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001. n = 4-8 independent placental donors.

Figure 2: Impact of resveratrol on placental explants exposed to 1% O_2 conditions. Term placental explants were cultured with media only at 5% O_2 , media only at 1% O_2 and at 1% O_2 with 100μM resveratrol (RES) for 24 hours. (A) sFlt1 and (B) endoglin concentrations in the supernatants were measured via ELISA, while (C) Nrf2 nuclear translocation and (D) HO-1 protein expression was measured in the corresponding explants by western blot. Representative western blot images for Nrf2 and HO-1 are shown below the respective graph. The white space indicates noncontiguous lanes from the same blot. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01, *****P*<0.0001. n = 4-7 independent placental donors.

Figure 3: Impact of resveratrol treatment on the expression of endothelial dysfunction markers and on endothelial monolayer permeability *in vitro*. HUVECs were treated with either media only, TNF-α (1ng/ml or 100ng/ml) only or TNF-α with 3 different doses of

resveratrol (50μM, 100μM, 200μM). Expression of (A) ICAM1, (B) VCAM1 and (C) E-selectin was measured 6 hours after treatment via flow cytometry. Protein levels of (D) endothelin-1 was measured in the culture supernatants collected 24-hours after treatment via ELISA. (E) FITC-dextran permeability through HUVEC monolayers treated with either media only, TNF-α only (100ng/ml) or TNF-α with 100μM resveratrol was measured following 24 hours of treatment. (F) FITC-dextran permeability was also measured in HUVEC monolayers treated with either serum from normal pregnant women (NP serum), serum from preeclamptic women (PE Serum) or with PE serum and 100μM resveratrol, 24 hours after treatment. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01, *****P*<0.0001. n = 5 cell lines from independent placental donors.

Figure 4: Nrf2 and HO-1 protein expression in HUVECs treated with resveratrol. HUVECs were treated with either media only, TNF-α (100ng/ml) only or TNF-α with 100μM resveratrol (Res). (A) Total Nrf2 protein expression and (B) Nrf2 nuclear translocation was assessed 6 hours after treatment via western blot. (C) Representative western blot images for Nrf2 are shown. The white space indicates noncontiguous lanes from the same blot. (D) Total HO-1 expression was measured 24-hours after treatment by western blot as well. (E) Representative western blot images for HO-1 are shown. The white space indicates noncontiguous lanes from the same blot. Data are expressed as mean ± SEM. ***P*<0.01, ****P*<0.001. n = 6 cell lines from independent placental donors.

Figure 5: Effect of resveratrol on markers of endothelial dysfunction, endothelial monolayer permeability and HO-1 protein expression in HUVECs following Nrf2 knockdown. HUVECs were transfected with small interfering RNA directed towards Nrf2 (siNrf2) and then treated with either media only, TNF-α (1ng/ml or 100ng/ml) only or TNF-α with 100μM resveratrol

(RES). HUVECs transfected with small interfering RNA containing a scrambled sequence (scrambled siRNA) and then exposed to the treatments were used as controls. (A) Total HO-1 protein expression in the HUVECs was measured via western blot 24-hours after treatment. (B) Representative images for HO-1 western blot are shown. The white space indicates noncontiguous lanes from the same blot. (C) FITC-dextran permeability though HUVEC monolayers was also measured after 24-hours. Expression of (D) ICAM1, (E) VCAM1 and (F) E-selectin was measured 6 hours after treatment via flow cytometry. Levels of (G) endothelin-1 and (H) 8-isoprostane in HUVEC culture supernatants were measured 24 hours after treatment via ELISA and enzyme immunoassay respectively. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. n = 5-6 cell lines from independent placental donors.

Figure 1

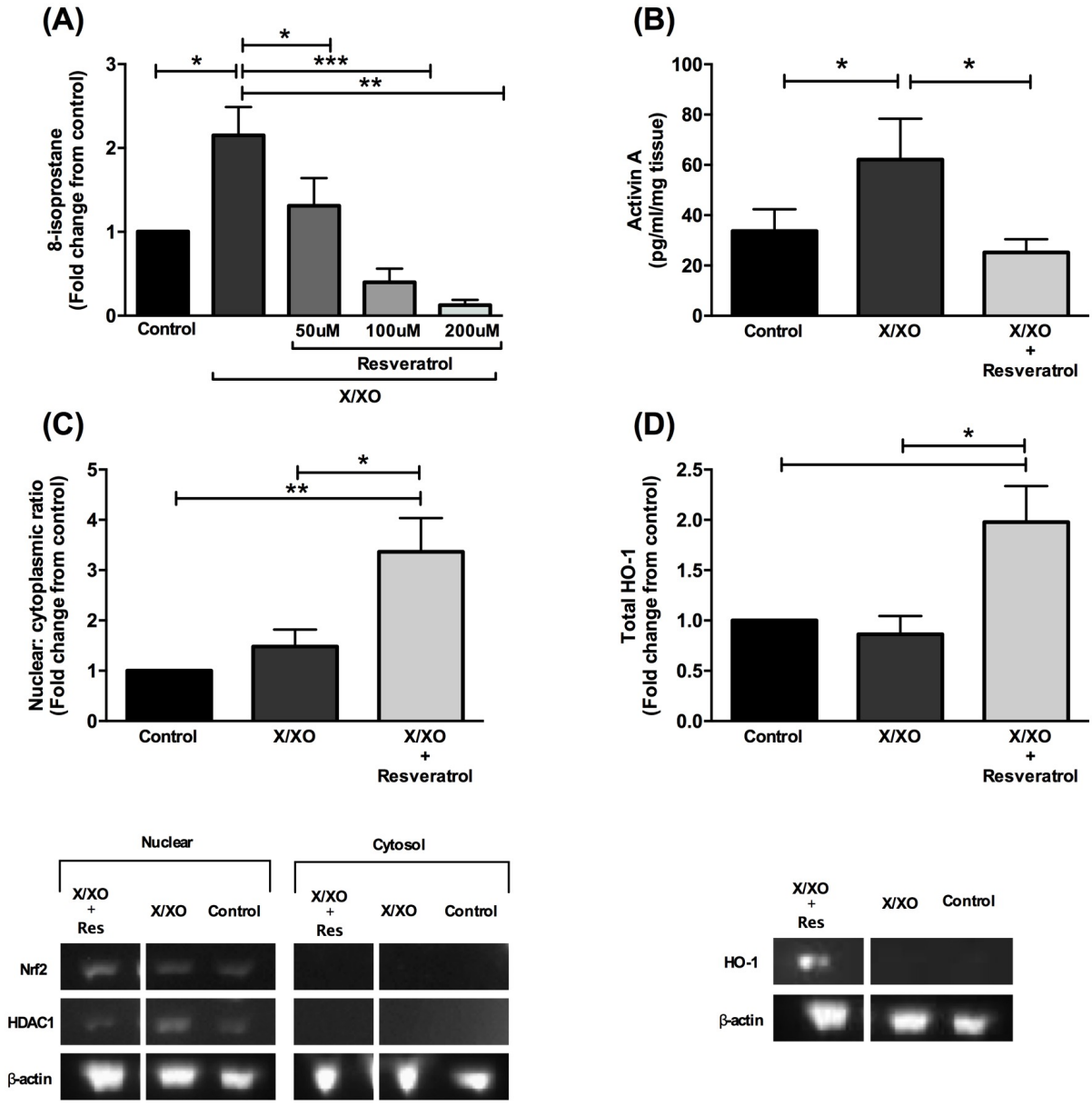

(A)
8-isoprostane (Fold change from control)
Control
50uM
100uM
200uM
Resveratrol
X/XO
(B)
Activin A (pg/ml/mg tissue)
Control
X/XO
X/XO + Resveratrol
(C)
Nuclear: cytoplasmic ratio (Fold change from control)
Control
X/XO
X/XO + Resveratrol
(D)
Total HO-1 (Fold change from control)
Control
X/XO
X/XO + Resveratrol
Nuclear
Cytosol
X/XO + Res
X/XO
Control
Nrf2
HDAC1
β-actin
HO-1
β-actin

Figure 2

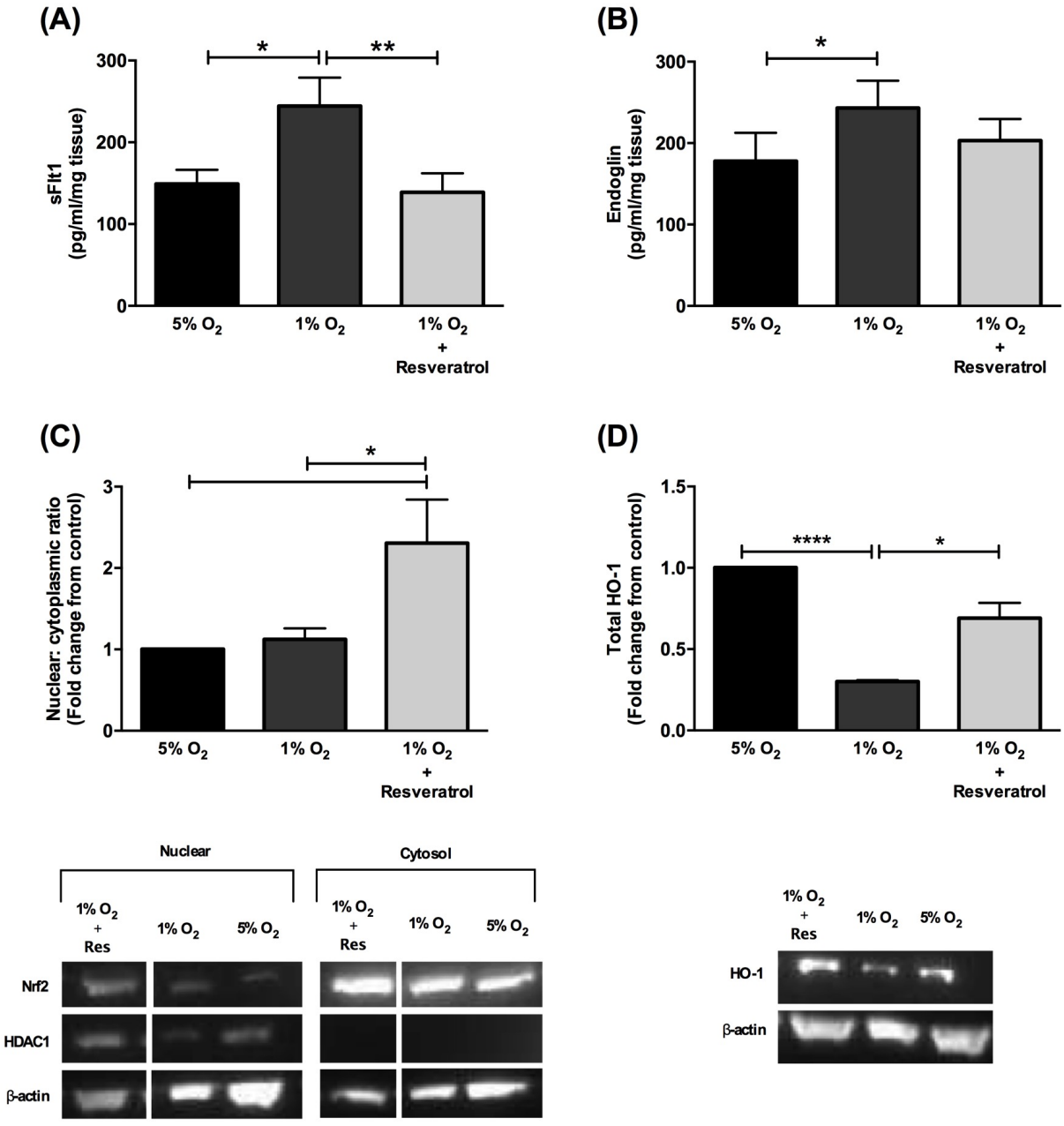

(A)
sFlt1 (pg/ml/mg tissue)
300
200
100
0
*
**
5% O2
1% O2
1% O2 + Resveratrol
(B)
Endoglin (pg/ml/mg tissue)
300
200
100
0
*
5% O2
1% O2
1% O2 + Resveratrol
(C)
Nuclear: cytoplasmic ratio (Fold change from control)
3
2
1
0
*
5% O2
1% O2
1% O2 + Resveratrol
(D)
Total HO-1 (Fold change from control)
1.5
1.0
0.5
0.0
****
*
5% O2
1% O2
1% O2 + Resveratrol
Nuclear
Cytosol
1% O2 + Res
1% O2
5% O2
Nrf2
HDAC1
β-actin
HO-1
β-actin

Figure 3

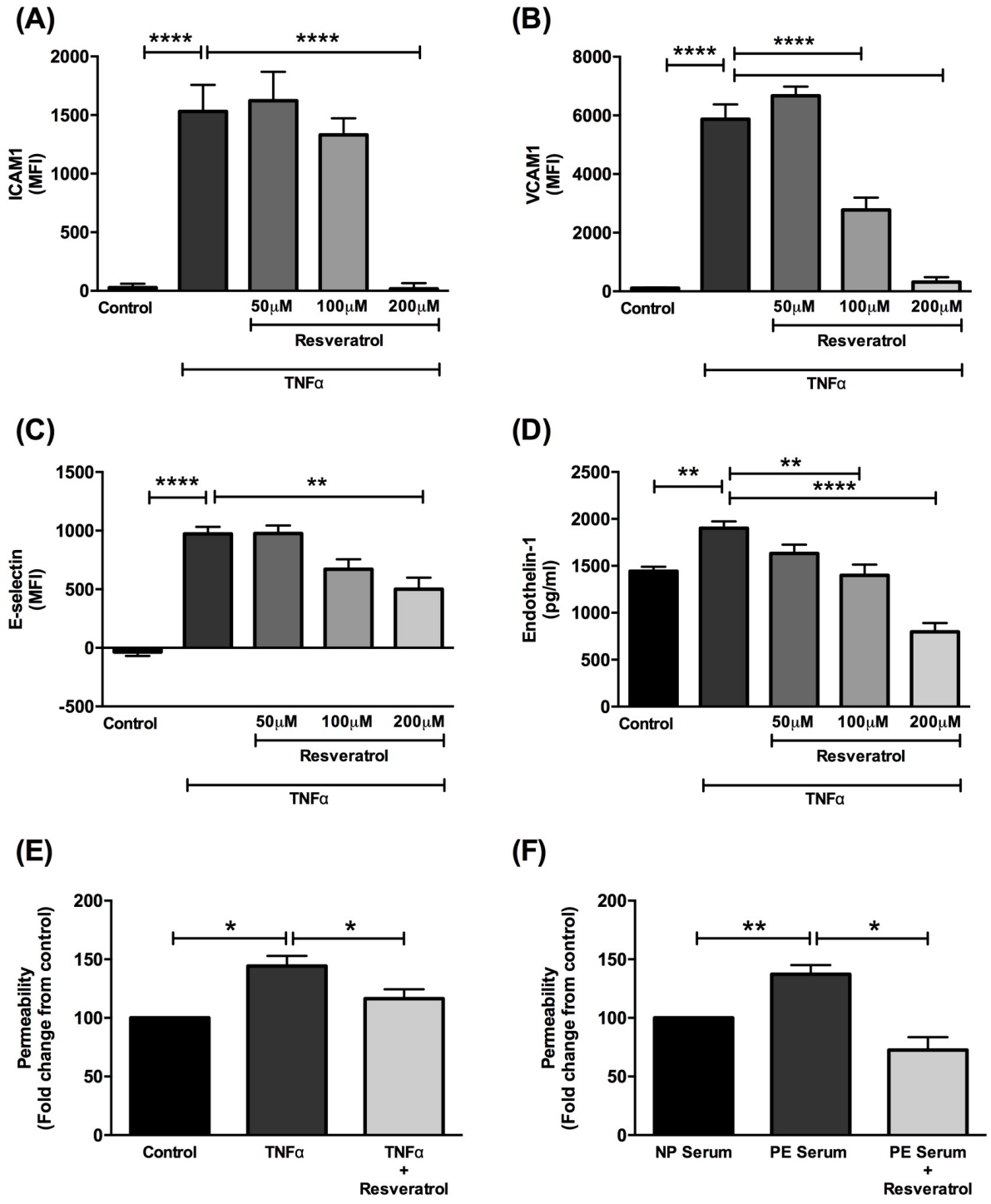

(A)
ICAM1 (MFI)
2000
1500
1000
500
0
****
****
Control
50μM
100μM
200μM
Resveratrol
TNFα
(B)
VCAM1 (MFI)
8000
6000
4000
2000
0
****
****
Control
50μM
100μM
200μM
Resveratrol
TNFα
(C)
E-selectin (MFI)
1500
1000
500
0
-500
****
**
Control
50μM
100μM
200μM
Resveratrol
TNFα
(D)
Endothelin-1 (pg/ml)
2500
2000
1500
1000
500
0
**
**
****
Control
50μM
100μM
200μM
Resveratrol
TNFα
(E)
Permeability (Fold change from control)
200
150
100
50
0
*
*
Control
TNFα
TNFα + Resveratrol
(F)
Permeability (Fold change from control)
200
150
100
50
0
**
*
NP Serum
PE Serum
PE Serum + Resveratrol

Figure 4

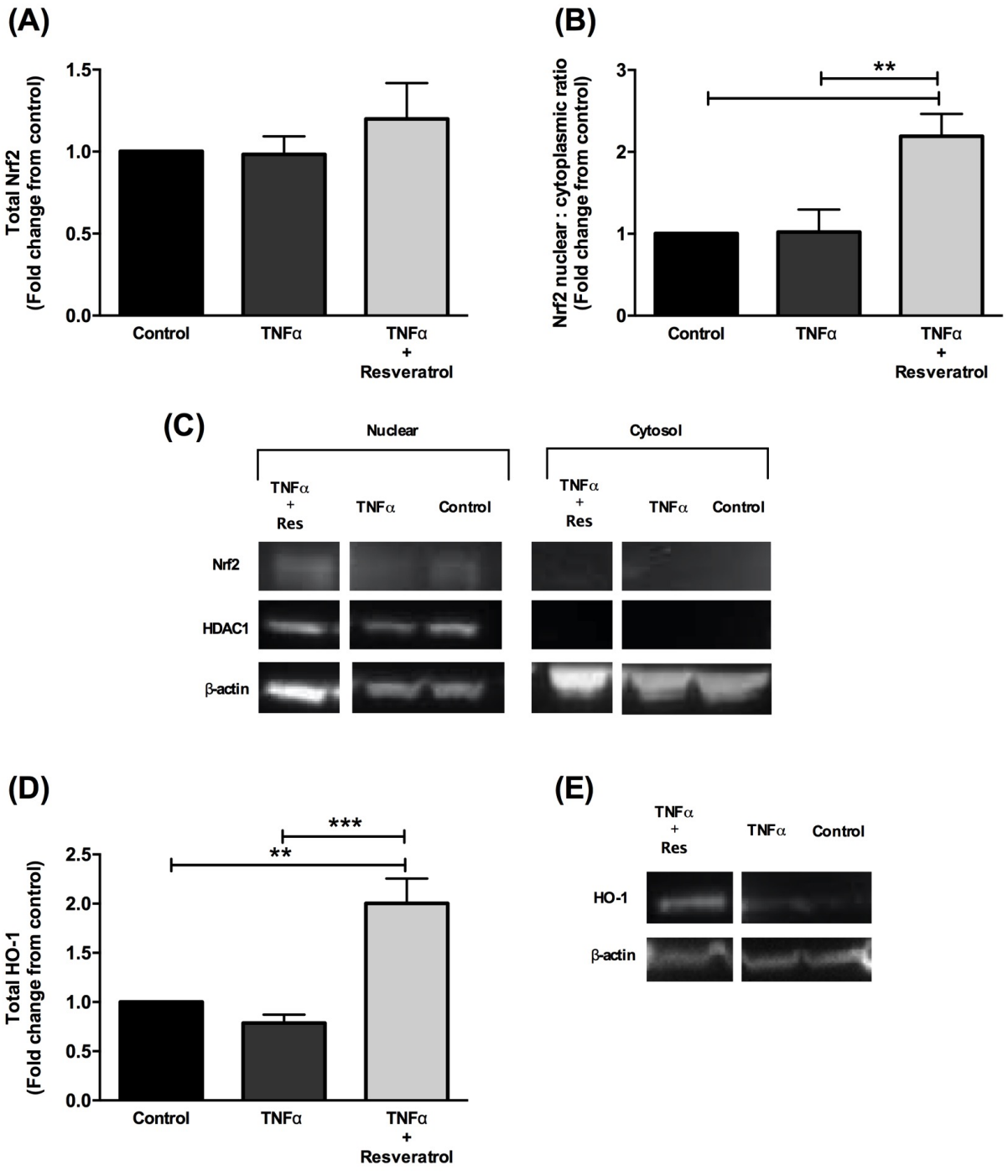
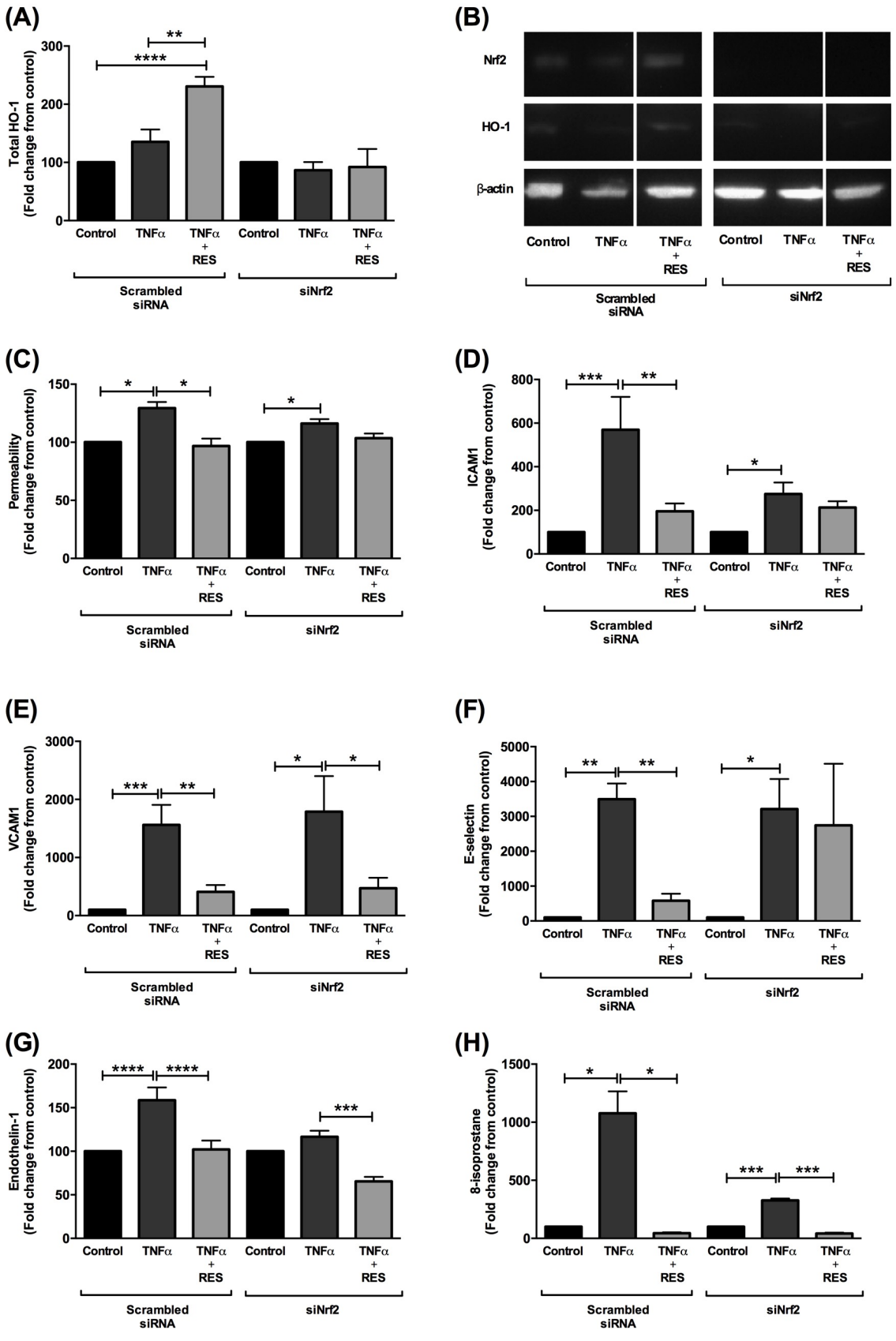

(A)
Total Nrf2
(Fold change from control)
1.5
1.0
0.5
0.0
Control
TNFα
TNFα + Resveratrol
(B)
Nrf2 nuclear : cytoplasmic ratio
(Fold change from control)
3
2
1
0
**
Control
TNFα
TNFα + Resveratrol
(C)
Nuclear
Cytosol
TNFα + Res
TNFα
Control
TNFα + Res
TNFα
Control
Nrf2
HDAC1
β-actin
(D)
Total HO-1
(Fold change from control)
2.5
2.0
1.5
1.0
0.5
0.0
***
**
Control
TNFα
TNFα + Resveratrol
(E)
TNFα + Res
TNFα
Control
HO-1
β-actin

Figure 5


(A)
Total HO-1 (Fold change from control)
300
200
100
0
**
****
Control
TNFα
TNFα + RES
Control
TNFα
TNFα + RES
Scrambled siRNA
siNrf2
(B)
Nrf2
HO-1
β-actin
Control
TNFα
TNFα + RES
Control
TNFα
TNFα + RES
Scrambled siRNA
siNrf2
(C)
Permeability (Fold change from control)
150
100
50
0
*
*
*
Control
TNFα
TNFα + RES
Control
TNFα
TNFα + RES
Scrambled siRNA
siNrf2
(D)
ICAM1 (Fold change from control)
800
600
400
200
0
***
**
*
Control
TNFα
TNFα + RES
Control
TNFα
TNFα + RES
Scrambled siRNA
siNrf2
(E)
VCAM1 (Fold change from control)
3000
2000
1000
0
***
**
*
*
Control
TNFα
TNFα + RES
Control
TNFα
TNFα + RES
Scrambled siRNA
siNrf2
(F)
E-selectin (Fold change from control)
5000
4000
3000
2000
1000
0
**
**
*
Control
TNFα
TNFα + RES
Control
TNFα
TNFα + RES
Scrambled siRNA
siNrf2
(G)
Endothelin-1 (Fold change from control)
200
150
100
50
0
****
****
***
Control
TNFα
TNFα + RES
Control
TNFα
TNFα + RES
Scrambled siRNA
siNrf2
(H)
8-isoprostane (Fold change from control)
1500
1000
500
0
*
*
***
***
Control
TNFα
TNFα + RES
Control
TNFα
TNFα + RES
Scrambled siRNA
siNrf2

Chapter 5

Sulforaphane activates nuclear factor erythroid 2-related factor-2 activator and improves endothelial dysfunction in vitro: potential therapeutic for preeclampsia

---

5.1 Introduction

Current management of preeclampsia mainly relies on the use of anti-hypertensives to control maternal blood pressure and allow pregnancy prolongation. A more targeted therapy that addresses the underlying disease pathology is needed, particularly for the women who develop preeclampsia at early gestations [291]. Our findings in chapter 4 suggest that the Nrf2 pathway could be an effective therapeutic target for both the prevention and treatment of preeclampsia. There we showed that the Nrf2 activator, resveratrol improves endothelial dysfunction, while also reducing placental oxidative stress and production of sFlt1 and activin A. Therefore, investigations into safe and effective Nrf2 activators are warranted.

Sulforaphane is a naturally occurring compound found in cruciferous vegetables, with broccoli sprouts being the richest source [292]. Studies have shown that sulforaphane treatment protects against endothelial dysfunction induced by a variety of conditions, including diabetes and hypercholesterolemia [293-295]. Furthermore, sulforaphane treatment has prevented end-organ injury in several models of ischemia-reperfusion [232, 233, 296]. These beneficial actions of sulforaphane have been attributed to its anti-oxidant and anti-inflammatory properties derived from its ability to activate Nrf2 and its downstream anti-oxidant enzymes [222, 232, 233, 294-296]. In fact, sulforaphane is one of the most potent inducers of Nrf2 [297-299]. Furthermore, no adverse maternal or fetal effects were noted in pregnant mice treated with sulforaphane for 30 days, however maternal anti-oxidant enzyme

levels were significantly increased [300]. In fact, the numbers of resorptions were significantly lower among the sulforaphane treated dams, compared with control dams [300]. For these reasons sulforaphane appears to be a promising therapy for preeclampsia. Therefore, the aim of this study was to examine whether sulforaphane treatment could prevent hallmarks of endothelial dysfunction characteristic of preeclampsia *in vitro* via the activation of Nrf2.

5.2 Methods

5.2.1 Endothelial cell culture

Umbilical cords were collected from women with a healthy term pregnancy at the time of caesarean section. Samples were collected upon receiving informed written consent from each patient with approval from the Monash Health Human Research Ethics Committee. HUVECs were isolated and cultured for each experiment as described in section 3.2.4.

5.2.2 Small interfering RNA transfection

HUVECs were reverse transfected with a single stranded small interfering RNA (siRNA) directed towards Nrf2, a scrambled sequence (negative control) or GAPDH (positive control) with the use of Lipofectamine RNAimax Reagent (all from Life Technologies, Carlsbad, CA), according to manufacturer's instructions. All experiments were completed within 4 days of transfection as described below.

5.2.3 Markers of endothelial cell activation

Expression of endothelial cell activation markers was determined in the HUVECs via flow cytometry 6 hours after treatment with sulforaphane (5μM, 10μM, 20μM) and/or TNFα (1ng/ml) as described in section 3.2.5.

5.2.4 Endothelin-1 and 8-isoprostane production by HUVECs

ET-1 levels in the supernatants of HUVEC cultures treated with sulforaphane (5μM, 10μM, 20μM) and/or TNFα (1ng/ml) for 24 hours were determined via ELISA (DET100, R&D systems, Minneapolis, MN). Level of 8-isoprostane, a marker of oxidative stress, was measured in the same culture supernatants by enzyme immunoassay (Caymen Chemicals, Ann Arbor, MI). All assays were performed according to manufacturer's instructions.

5.2.5 Endothelial cell permeability

Integrity of the endothelial cell monolayer was assessed 24-hours after treatment with either recombinant TNFα (100ng/ml) or serum from women with preeclampsia (5% serum in

Medium 199 supplemented with 1% antibiotics and 1% L-glutamine) in the presence or absence of sulforaphane (20μM), as described in detail in section 3.2.7.

5.2.6 Nrf2 nuclear translocation and HO-1 protein levels

HUVEC cultures treated with TNFα (1ng/ml) in the presence or absence of sulforaphane (20μM) were assessed for Nrf2 activation and antioxidant response by determining Nrf2 nuclear translocation and total HO-1 protein levels. Nuclear and cytoplasmic Nrf2 protein levels were determined 6 hours after treatment, while total HO-1 protein levels were assessed 24 hours after treatment, both via western blot as described in detail in section 3.2.8.

5.2.7 Statistical Analysis

Statistical analysis was performed using one-way ANOVA followed by multiple comparisons with a Tukey ad-hoc test. Groups were considered to be significantly different if *P* values were <0.05. All data were analysed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

5.3 Results

5.3.1 Sulforaphane increases Nrf2 protein levels, Nrf2 nuclear translocation and HO-1 protein levels

Firstly, the ability of sulforaphane to activate Nrf2 in the HUVECs was tested and sulforaphane was found to increase nuclear translocation of Nrf2 by 3-fold and total Nrf2 protein levels by 4-fold compared with untreated and TNF-α alone treated HUVECs (Figure 5.1A & 5.1C; $p<0.01$, Figure 5.1B & 5.1C; $p<0.0001$ respectively). Sulforaphane also increased HO-1 protein levels in HUVECs by nearly 4-fold compared with the TNF-α treated HUVECs (Figure 5.1D & 5.1E; $p=0.04$).

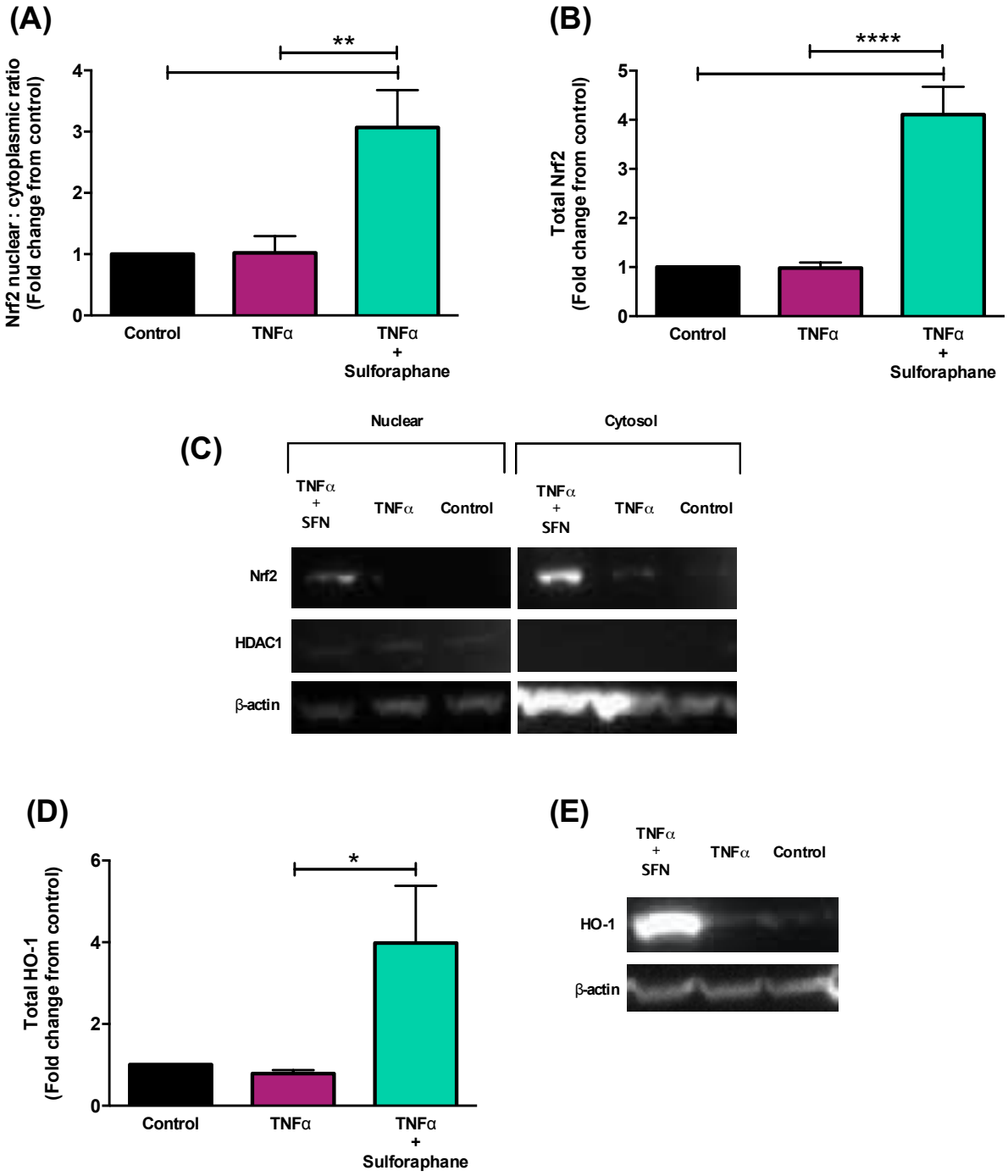


Figure 5.1: Nrf2 and HO-1 protein expression in HUVECs treated with sulforaphane. HUVECs were treated with either media only, TNF-α (100ng/ml) only or TNF-α with 20μM sulforaphane (SFN). (A) Nrf2 nuclear translocation and (B) total Nrf2 protein expression was measured 6 hours after treatment via western blot. (C) Representative western blot images for Nrf2 are shown. The white space indicates noncontiguous lanes from the same blot. (D) Total HO-1 expression was measured 24-hours after treatment also by western blot. (E) Representative western blot images for HO-1 are shown. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01, *****P*<0.0001. n = 6 cell lines from independent placental donors.

5.3.2 Sulforaphane improves endothelial cell dysfunction in vitro

Sulforaphane significantly mitigated the TNF-α stimulated increase in ICAM-1, VCAM-1, and E-selectin in a dose-dependent manner (Figure 5.2A-C; $p<0.01$). Only the highest tested dose of sulforaphane (20μM) significantly decreased ET-1 production by the HUVECs when compared with untreated HUVECs (Figure 5.2D; $p=0.04$). At this concentration sulforaphane also significantly attenuated both TNF- α and preeclamptic serum stimulated increase in endothelial cell monolayer permeability (Figure 5.2E; $p=0.001$ & Figure 5.2F; $p=0.04$, respectively).

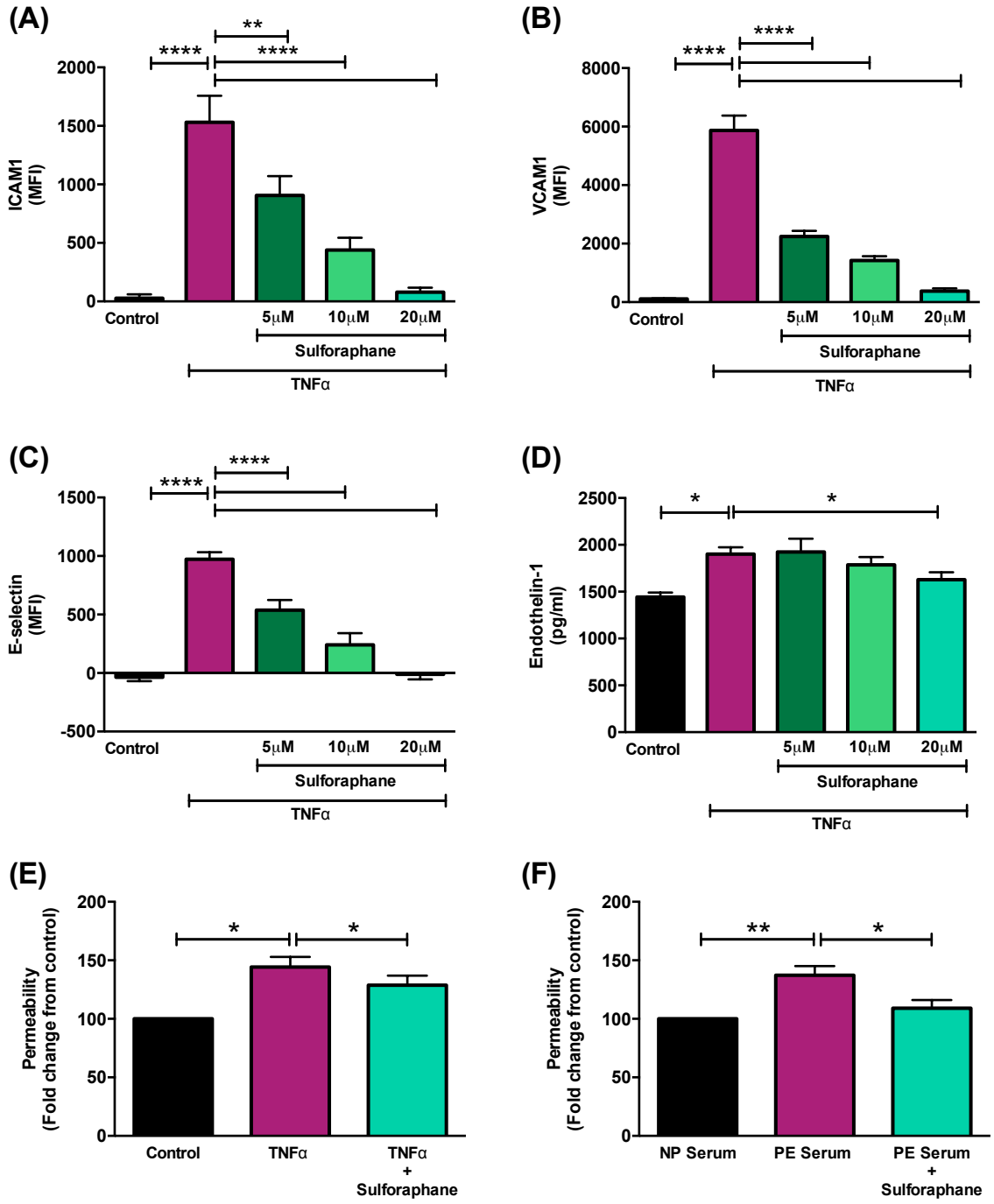
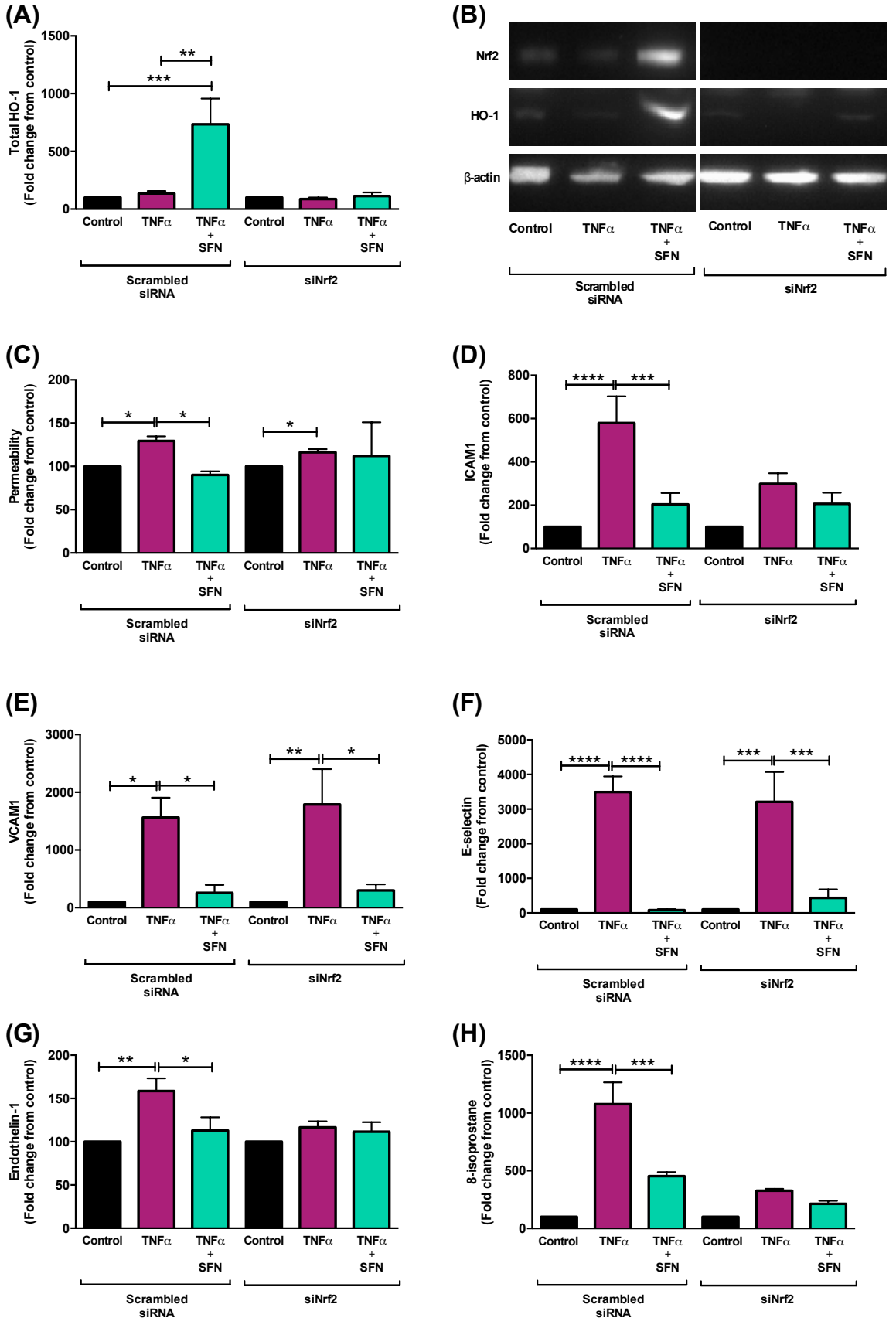


Figure 5.2: Effect of sulforaphane treatment on hallmarks of endothelial dysfunction *in vitro*. HUVECs were treated with either media only, TNF-α (1ng/ml or 100ng/ml) only or TNF-α with 3 different doses of sulforaphane (5μM, 10μM, 20μM). Expression of (A) ICAM1, (B) VCAM1 and (C) E-selectin was measured 6 hours after treatment via flow cytometry. Protein levels of (D) endothelin-1 was measured via ELISA in the culture supernatants collected 24-hours after treatment. (E) FITC-dextran permeability through HUVEC monolayers treated with either media only, TNF-α only (100ng/ml) or TNF-α with 20μM sulforaphane was measured following 24 hours of treatment. (F) FITC-dextran permeability was also measured in HUVEC monolayers treated with either serum from normal pregnant women (NP serum), serum from preeclamptic women (PE Serum) or with PE serum and 20μM sulforaphane, 24 hours after treatment. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01, *****P*<0.0001. n = 5-7 cell lines from independent placental donors.

5.3.3 Nrf2 knockdown abolishes some of the effects of sulforaphane on endothelial cells

In order to determine the importance of Nrf2 for sulforaphane to mediate its effects on the *in vitro* hallmarks of endothelial function, a series of Nrf2 gene silencing studies were performed. Knockdown of Nrf2 was confirmed by western blot (Figure 5.3A & 5.3B). Sulforaphane did not increase HO-1 protein levels in the HUVECs with Nrf2 knockdown (Figure 5.3A & 5.3B). Furthermore, sulforaphane failed to improve endothelial monolayer permeability in the HUVECs treated with siRNA against Nrf2 (Figure 5.3C). Similarly, TNF-α induced increase in ICAM1 was not significantly affected by sulforaphane in the HUVECs without Nrf2 (Figure 5.3D). In contrast, sulforaphane significantly reduced TNF-α stimulated increase in VCAM1 and E-selectin in both the HUVECs regardless of Nrf2 gene expression (Figure 5.3E & 5.3F respectively; all $p<0.05$). However, sulforaphane treatment did not significantly alter the production of ET-1 and oxidative stress marker, 8-isoprostane, following Nrf2 gene silencing in HUVECs (Figure 5.3G & 5.3H respectively).

Figure 5.3: Impact of sulforaphane on markers of endothelial dysfunction, endothelial monolayer permeability and HO-1 protein expression in HUVECs following Nrf2 knockdown. HUVECs were transfected with small interfering RNA directed towards Nrf2 (siNrf2) and then treated with either media only, TNF-α (1ng/ml or 100ng/ml) only, TNF-α with 20μM sulforaphane (SFN). HUVECs transfected with small interfering RNA containing a scrambled sequence (scrambled siRNA) and then exposed to the treatments were used as controls. (A) Total HO-1 protein expression in the HUVECs was measured via western blot 24-hours after treatment. (B) Representative images for HO-1 western blot are shown. The white space indicates noncontiguous lanes from the same blot. (C) FITC-dextran permeability though HUVEC monolayers was also measured after 24-hours. Expression of (D) ICAM1, (E) VCAM1 and (F) E-selectin was measured 6 hours after treatment via flow cytometry. Levels of (G) endothelin-1 and (H) 8-isoprostane in HUVEC culture supernatants were measured 24 hours after treatment via ELISA and EIA respectively. Data are expressed as mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. n = 5-6 cell lines from independent placental donors.


(A)
Total HO-1 (Fold change from control)
1500
1000
500
0
**
***
Control
TNFα
TNFα + SFN
Control
TNFα
TNFα + SFN
Scrambled siRNA
siNrf2
(B)
Nrf2
HO-1
β-actin
Control
TNFα
TNFα + SFN
Control
TNFα
TNFα + SFN
Scrambled siRNA
siNrf2
(C)
Permeability (Fold change from control)
200
150
100
50
0
*
*
*
Control
TNFα
TNFα + SFN
Control
TNFα
TNFα + SFN
Scrambled siRNA
siNrf2
(D)
ICAM1 (Fold change from control)
800
600
400
200
0
****
***
Control
TNFα
TNFα + SFN
Control
TNFα
TNFα + SFN
Scrambled siRNA
siNrf2
(E)
VCAM1 (Fold change from control)
3000
2000
1000
0
*
*
**
*
Control
TNFα
TNFα + SFN
Control
TNFα
TNFα + SFN
Scrambled siRNA
siNrf2
(F)
E-selectin (Fold change from control)
5000
4000
3000
2000
1000
0
****
****
***
***
Control
TNFα
TNFα + SFN
Control
TNFα
TNFα + SFN
Scrambled siRNA
siNrf2
(G)
Endothelin-1 (Fold change from control)
200
150
100
50
0
**
*
Control
TNFα
TNFα + SFN
Control
TNFα
TNFα + SFN
Scrambled siRNA
siNrf2
(H)
8-isoprostane (Fold change from control)
1500
1000
500
0
****
***
Control
TNFα
TNFα + SFN
Control
TNFα
TNFα + SFN
Scrambled siRNA
siNrf2

5.4 Discussion

Data from this study demonstrate the capacity for sulforaphane to mitigate several hallmarks of endothelial dysfunction *in vitro* mainly via the activation of Nrf2. Sulforaphane reduced the TNF-α triggered increase in all three markers of endothelial activation namely ICAM1, VCAM1 and E-selectin, as well as the vasoconstrictor, endothelin-1. Furthermore, sulforaphane mitigated both the TNF-α and preeclamptic serum induced increase in endothelial monolayer permeability. However, Nrf2 knockdown abrogated most of these effects of sulforaphane on endothelial cells. These observations suggest that Nrf2 activators, such as sulforaphane could reverse endothelial dysfunction and be effective as adjuvant therapies in women with established preeclampsia.

Since the clinical features of preeclampsia are the result of systemic maternal endothelial dysfunction, a therapy that mitigates endothelial dysfunction could have the potential to alleviate the maternal clinical features and prolong pregnancy in women with preeclampsia. Endothelial oxidative stress triggered by placental derived vasoactive factors, such as TNF-α, sFlt1 and activin A, play a central role in the development of maternal endothelial dysfunction in preeclampsia [115, 146, 301]. Systemic hypertension is triggered by endothelial oxidative stress increasing the release of vasoconstrictors, such as endothelin-1, and decreasing the availability of vasodilators, such as nitric oxide [302, 303]. The disruption of endothelial cell junctional proteins by ROS contributes to the development of proteinuria, pulmonary oedema, increase in serum liver transaminases and the development of seizures in women with preeclampsia and eclampsia [304]. Furthermore, ROS-sensitive NF-κB activation increases endothelial activation markers leading to leuckocyte adhesion and thus the systemic inflammation characteristic of preeclampsia [305].

Given the contribution by endothelial oxidative stress to preeclampsia, upregulation of endogenous cellular anti-oxidant enzymes via the activation of Nrf2 could be an effective therapeutic option. The findings of the current study confirm this hypothesis. Sulforaphane alleviated all of the TNF-α triggered hallmarks of endothelial dysfunction *in vitro*, mostly by reducing endothelial oxidative stress via the activation of Nrf2 and the subsequent increase in endogenous anti-oxidant enzymes, such as HO-1. TNF-α was chosen to stimulate the features of endothelial dysfunction characteristic of preeclampsia *in vitro* for several reasons, which

are detailed in section 3.4. That sulforaphane can mitigate TNF-α triggered endothelial dysfunction *in vitro*, highlights its potential to resolve endothelial function and be an effective adjuvant therapy for established preeclampsia.

In the absence of Nrf2 sulforaphane failed to reduce the TNF-α triggered increase in ICAM1 and endothelin-1 expression, as well as endothelial monolayer permeability. This was most likely due to the inability of sulforaphane to upregulate endogenous phase II anti-oxidant enzymes, such as HO-1 in the absence of Nrf2. This was supported by the fact that sulforaphane treatment had no effect on the levels of the oxidative stress marker, 8-isoprostane or HO-1 protein levels in the HUVECs without Nrf2. Interestingly, knockdown of Nrf2 did not abolish all of the beneficial effects of sulforaphane on endothelial cells. Sulforaphane decreased the TNF-α stimulated increase in endothelial VCAM1 and E-selectin expression even after the knock down of Nrf2. However, ICAM1 expression was not regulated by sulforaphane when Nrf2 was silenced. Studies have shown the endothelial activation markers to be differentially regulated under certain circumstances, however the detailed mechanisms are still under investigation [306, 307]. Sulforaphane has been shown to decrease NF-κB activity, which could be the potential mechanism by which it down regulates VCAM1 and E-selectin expression in the absence of Nrf2 [308-310]. Further investigations are needed to confirm this mechanism of action of sulforaphane.

Another important aspect of sulforaphane that needs to be explored is its effect on placental oxidative stress and production of vasoactive factors, such as sFlt1, sEng and activin A. It was not possible to explore the impact of sulforaphane on these characteristic placental features of preeclampsia due to time constraints associated with my PhD. Data from chapter 4 demonstrated that the Nrf2 activator, resveratrol, significantly decreased placental oxidative stress, as well as placental production of sFlt1 and activin A. Sulforaphane is known to be a more potent activator of Nrf2, which is confirmed by data from this study and chapter 4 that show sulforaphane inducing Nrf2 nuclear translocation to a greater extent than resveratrol [297-299]. In light of this data, sulforaphane shows great promise in resolving at least some of the placental features characteristic of preeclampsia and further investigations are warranted.

Testing the efficacy of sulforaphane on an animal model of preeclampsia would also be beneficial to ensure that the improvements in TNF-α stimulated hallmarks of endothelial dysfunction seen *in vitro* can be translated *in vivo*. A major concern associated with the use of sulforaphane as an adjuvent therapy for preeclampsia is its relatively low bioavailability in some individuals. The bioavailability of sulforaphane ranges from 2% to 40%. This wide range in bioavailability is due to the fact that most dietary sources and capsules contain its more stable precursor, glucoraphanin, which is hydrolysed to sulforaphane by an enzyme found only in plants and intestinal microbiota, and thus variations in intestinal microbiota is a key determinant of sulforaphane bioavailability [311]. Despite the relatively low oral bioavailability of sulforaphane, one particular study has reported at least a 3-fold increase in the expression of phase II detoxification enzymes, including HO-1, in nasal lavage cells of healthy humans given just 3 doses equivalent to 102 μmol sulforaphane, with each dose expected to give a peak serum concentration of only 1 μM [298]. This data, along with the studies indicating the safety of sulforaphane in pregnant animals demonstrate its potential to be effective *in vivo*. Therefore further examinations of the efficacy of sulforaphane in an animal model of preeclampsia are important to affirm its feasibility for clinical use.

In conclusion, the findings from this study highlight the potential for sulforaphane to be an effective adjuvant treatment for preeclampsia, as well as the need for further assessment of Nrf2 activation as a therapy for women with preeclampsia.

Chapter 6

Testing the therapeutic potential of melatonin in an animal model of preeclampsia

6.1 Introduction

Melatonin is a well-known anti-oxidant that is also known to improve vascular function. As previously described in chapter 3, melatonin improved *in vitro* hallmarks of endothelial dysfunction characteristic of preeclampsia, demonstrating its potential to be an effective adjuvant therapy for established preeclampsia. Furthermore, melatonin is safe to be used in pregnancy [290, 312]. Therefore, our laboratory aimed to confirm the therapeutic potential of melatonin in an animal model of preeclampsia.

Preeclampsia is predominantly a human disease, and as such, animal models for the purpose of testing therapeutics have been developed mainly by mimicking different stages of the human disease [313, 314]. The reduced uterine perfusion pressure (RUPP) model mimics the early placental stages of preeclampsia development in humans when blood flow to the utero-placental unit is significantly reduced [37, 315]. Both, primates and rodents subjected to RUPP present with hypertension, as well as proteinuria [37, 315]. These animals also develop downstream disease pathologies, such as increased levels of circulatory vasoactive factors (TNF-α, sFlt1, sEng) and impairment of endothelial function [37, 102, 125, 316]. Another model to mimic the early placental phase of preeclampsia pathophysiology is the HIF-1α overexpression model, which replicates the constitutive placental activation of HIF-1α, characteristic of early-onset preeclampsia [44]. The HIF-1α overexpression model is representative of severe preeclampsia, as these animals develop HELLP syndrome, in addition to hypertension, proteinuria and fetal growth restriction [44]. Similar to the RUPP model, they too demonstrate downstream disease pathologies, such as elevated levels of serum sFlt1 and sEng levels [44].

Preeclamptic animal models have also been developed by treating pregnant animals, mainly rodents, with the vasoactive factors associated with the development of preeclampsia. These vasoactive factors include AT1-AA, TNF-α, Activin A, as well as the anti-angiogenic factors, sFlt1 and sEng [91, 100, 121, 146, 316]. Each one of these vasoactive factors induces hypertension and altered renal function in pregnant rodents, with most factors also inducing fetal growth restriction. Treatment of pregnant rodents with both sFlt1 and sEng is the most well known animal model for preeclampsia, as it was the first model to present the spectrum of features characteristic of severe preeclampsia, that is hypertension, proteinuria, fetal growth restriction and HELLP syndrome [121]. Animal models that replicate the systemic maternal endothelial dysfunction to a certain extent have also been developed, such as by the inhibition of nitric oxide synthase [317, 318]. Also, there are several genetically manipulated mouse strains that display a preeclampsia phenotype during pregnancy, such as the catechol-O-methyltransferase deficient ($COMT^{-/-}$) mice or the BPH/5 mice [319, 320]. However, as the underlying causes of the preeclamptic features in these mice differ from that of the human disease, the opportunities to interpret data from these models are limited.

In our laboratory, we chose to establish the sFlt1 and sEng animal model, as it was a well-published model that also presented features of severe preeclampsia. Hence, the aim of this study was to establish the sFlt1 and sEng animal model of preeclampsia and test the efficacy of melatonin in ameliorating endothelial dysfunction, and thus the features of preeclampsia.

6.2 Methods

6.2.1 Animals

All animal experiments were approved by the Monash Medical Centre Animal Ethics Committee (Project number: MMCA/2012/03) and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2006). All rats were housed under standard laboratory conditions with a 12-hour light-dark cycle. An outline of the rat experimental procedure is shown in figure 6.1. Firstly, 10- to -12 week old female Sprague-Dawley rats were implanted with a radio telemetry transmitter, in order to obtain 24-hour blood pressure recordings. Once they recovered from the surgery, they were mated and the day a vaginal plug was observed was considered as day 0 of gestation. 24-hour blood pressure recordings were also commenced from this day. Based on previous publications on the sFlt1 and sEng model of preeclampsia, on gestation day 8 the rats were injected intravenously with adenoviruses expressing sFlt1 and sEng, at an initial dose of 1 x 10^9 IFU per adenovirus per animal [100, 121]. The control group was injected with adenoviruses expressing β-galactosidase, a protein commonly found in all cell types, at an initial dose of 2 x 10^9 IFU. On day 17 of gestation, the rats were culled via CO_2 asphyxiation. A blood sample was collected immediately afterwards via cardiac puncture. A bladder aspirate sample was also collected. Samples of the maternal brain, liver, kidney and placenta were collected for both RNA and protein extractions. The weight and crown-to-rump length of each fetus was recorded as well.

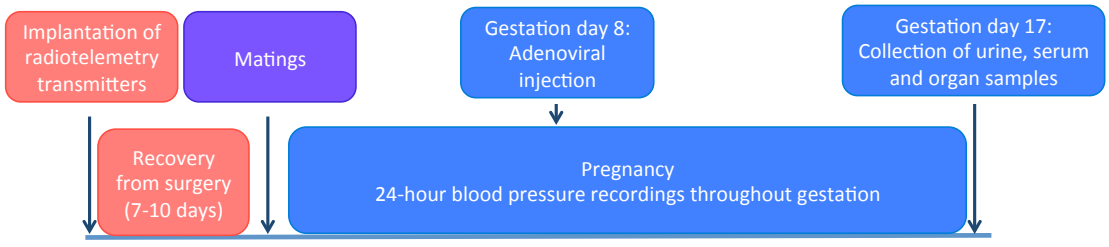


Figure 6.1: Outline of the rat experimental procedure.

6.2.2 Melatonin treatment for the rats

The rats were given melatonin (Sigma-Aldrich, St. Louis, MO) in their drinking water from gestation day 0 and were continued throughout their gestation. The volume of drinking water was measured daily to ensure that the minimum dosage of melatonin received by each rat was 50mg/kg/day. This dosage was based on a previous study that noted an aversion to treatment in pregnant rats when given melatonin at a dosage greater than 50mg/kg/day [312]. Furthermore, this dosage was well below the maternal toxicity and developmental toxicity thresholds for melatonin, which was noted to be 100mg/kg/day and 200mg/kg/day, respectively [312].

6.2.3 Adenoviruses

Sample vials of the sFlt1 and sEng adenoviruses were obtained from Professor Karumanchi's laboratory at the Beth Israel Deaconess Medical Center in Massachusetts, USA. The β-galactosidase adenovirus was purchased from Viraquest Inc. (North Liberty, IA). At first, the sFlt1 adenovirus was amplified in our laboratory in HEK293A cells and purified on a cesium chloride density gradient as previously described [321]. Since recombinant adenoviruses are replication deficient, they need to be propagated in cell lines that express the adenoviral gene products that are required for viral replication [321]. HEK293A is such as cell line. Firstly, the HEK293A cells were expanded in a large-scale culture and plated in a 150mm dish (14.6 x 10^6 cells/ dish) along with the sFlt1 adenovirus at a multiplicity of infection (MOI) of 30. MOI is the ratio of adenoviruses to cells in the culture dish. After 3 days of culture, the transfected cells were collected, centrifuged and the cell pellet was frozen at -80°C. For sufficient amplification of the sFlt1 adenovirus, cell pellets from 10-12 dishes were collected. Thereafter, the sFlt1 adenoviruses were purified on a cesium chloride density gradient. The adenoviral titer was determined with the use of the QuickTiter Adenovirus Titer ELISA kit from Cell Biolabs Inc. (San Diego, CA). The sEng adenovirus was also amplified and purified similarly in our laboratory, however the resulting yield was too low for *in vivo* use. It was then sent to Viraquest Inc. (North Liberty, IA), a company specializing in adenovirus production services, for amplification and purification. These sFlt1 and sEng adenoviral stocks were used initially. During the optimization process, a new stock of each of the adenoviruses was generated using one of the original vials received from Professor Karumanchi's laboratory. This second stock of adenoviruses were amplified and purified by Vector Biolabs (Malvern, PA).

6.2.4 Delivery of recombinant sFlt1 and sEng to rats

Recombinant sFlt1 and sEng proteins (R&D Systems, Minneapolis, MN) were delivered into pregnant rats at a dose of 5.5 μg/kg/day each, with the use of subcutaneously implanted osmotic pumps (Model No. 2001, ALZET, Cupertino, CA). The pumps were implanted on day 10 of gestation. The dosage was based on two previous studies that used recombinant sFlt1 to trigger preeclampsia-like features in pregnant rats [114, 115]. The dosages of sFlt1 used by these studies were 1ng/kg/hr and 3.7μg/kg/day. Therefore, an initial testing dosage of 5.5μg/kg/day for each recombinant protein was chosen.

6.2.5 Measurement of blood pressure in the rats

Mean arterial pressure (MAP) in the rats was measured with the use of radio telemetry. It is currently the best available technique for the measurement of blood pressure, because, unlike the tail cuff or intra-carotid methods, radio telemetry allows continuous 24-hour recordings to be obtained from conscious unrestrained animals.

The radio telemetry system consists of an implantable radio telemetry probe transmitter (PA-C40, Data Sciences International, Minnesota U.S.A.), a receiver plate on which the rat box is placed, an ambient pressure reference monitor, a data exchange matrix and a computer-based data acquisition system (Figure 6.2; Data Sciences International, Minnesota, U.S.A). The transmitter consists of a probe body attached to a fluid filled catheter that is inserted into the abdominal aorta of the rat (Figure 6.3). The transmitter is then able to directly measure arterial pressure and transmit the readings digitally from within the animal [322]. This radiofrequency signal from the transmitter is detected by the receiver plate, which in turn transmits the signal that is consolidated by the matrix, and is stored and analyzed by the computer-based data acquisition system [322]. The ambient pressure reference monitor measures atmospheric pressure, and this information is used by the data acquisition system to correct the signal from the receiver for atmospheric pressure [322].

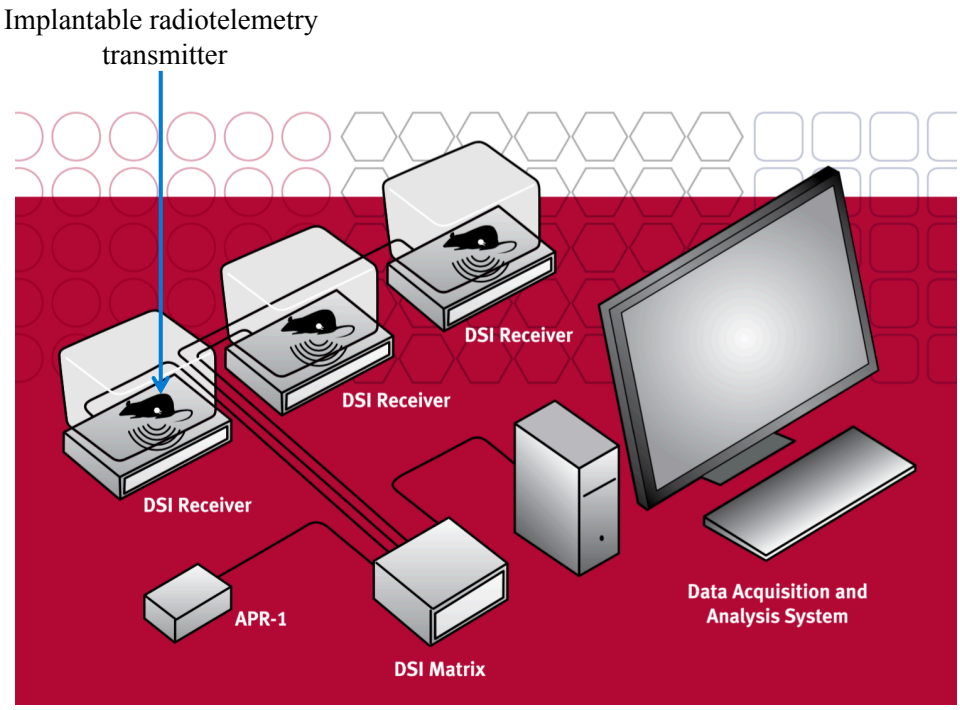


Figure 6.2: The radiotelemetry system used to obtain 24-hour blood pressure recordings. The system consists of a radiotelemetry transmitter implanted into the rat aorta, a receiver plate, an ambient pressure reference monitor (APR-1), a data exchange matrix (DSI Matrix) and a computer-based data acquisition system. (Image modified from "A guide to the DSI System". Data Sciences International. 2008)

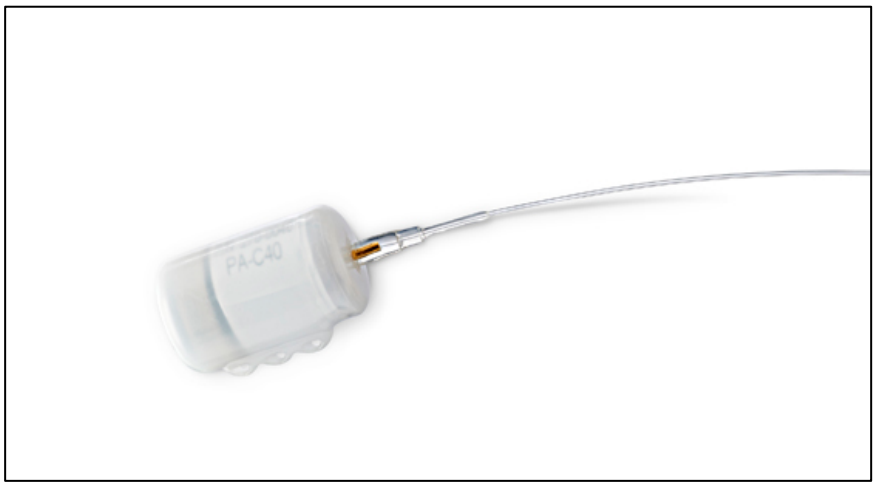


Figure 6.3: A radio telemetry transmitter for rats. The transmitter consists of a probe body attached to a fluid filled catheter. (Image from Data Sciences International, https://www.datasci.com/products/implantable-telemetry/small-animal/pa-c40)

For surgical implantation of the radio telemetry transmitters, the rat was first anaesthetized with isoflurane (Isoflo, Abbott Australasia Pty Ltd, New South Wales, Australia). Once unconscious, the rat was placed on its back on a heating pad, and continuous anesthesia was maintained by administering isoflurane through a nose cone. Once the rat was fully under anesthesia, as determined by monitoring of breathing rate and pedal reflexes, a midline incision was made in the lower abdomen of the rat. The abdominal aorta was then located and separated from the vena cava at two sites. The first site was just below the branching of the renal arteries and the second was just above the aortic bifurcation. Thereafter, vessel occlusion clips (Fine Science Tools Inc., British Columbia, Canada) were placed at these two sites to prevent blood flow through that part of the vessel. A 23-gauge needle with its beveled end bent at a 90° angle was used to make an opening in the aortic wall in the region between the vessel occlusion clips. The catheter tip of the radio telemetry probe (approximately 0.5cm in length) was then inserted into the aorta, and a small piece of sterile fiber and a few drops of vet bond (3M, New South Wales, Australia) were used to secure the catheter in place. Once the vet bond dried, the vessel occlusion clips were released. Afterwards, the radio telemetry probe transmitter was secured to the abdominal muscle wall approximately 1cm distal to the abdominal incision with the use of PDS sutures (Ethicon, Somerville, NJ). Approximately 5ml of sterile saline and 300µl of Tribactral (Provet, Victoria, Australia), an antibacterial solution, was placed in the abdominal cavity. The purpose of the sterile saline solution was to replenish any moisture lost during surgery and to facilitate the intestines to float back into position. The abdominal incision was then secured with a blanket stitch, and the skin incision was secured using a subcuticular stitch, both done with PDS sutures. Another 1ml of sterile saline was injected to the rat subcutaneously, to prevent dehydration.

Recovery of the rat from anesthesia was closely monitored. The rat was then placed in its home box, which in turn was placed on a heating pad for the next hour, and the rat was closely monitored for this duration. The rats were placed in the procedure rooms after they recovered well. An antibacterial solution, Baytril (Provet, Victoria, Australia), was placed in the drinking water (500µl in 300ml water) for 3 days, to provide the rat with continuous protection from infection in the first few days of recovery. The rats were monitored daily for 7 days after surgery to ensure they recovered well. At any point during this period if the health of the rat reached a clinical score of 2, they were culled immediately.

On day 0 of gestation the implanted radio telemetry probe was switched on magnetically and 24-hour MAP and heart rate recordings were obtained throughout gestation from each rat till they were culled on day 17 of gestation. The average MAP for each day of gestation was then calculated. The change in the average MAP from that recorded on day 8 of gestation, the day the adenoviruses were injected, was also calculated.

6.2.6 Assessment of proteinuria in the rats

The bladder aspirate samples were sent to the Pathology Department at Monash Medical Centre, Clayton for analysis of urinary albumin and creatinine levels. The results were used to calculate the urinary albumin/ creatinine ratio for each rat, as a measure of the level of proteinuria.

6.2.7 HUVEC culture and testing of adenoviruses

HUVECs were isolated and cultured as previously described in section 3.2.4. When the HUVECs reached 60% confluency, the media was replaced with fresh M199 media (Life Technologies, Carlsbad, CA) also containing the relevant concentration of either the sFlt1 or sEng adenovirus. The adenoviral concentrations used were MOI 10, 30 and 50. HUVECs treated with the β-galactosidase adenovirus at a concentration of MOI 30 were used as controls. After 3 days of culture, the cell culture supernatants were collected and stored at -80°C till further analysis.

6.2.8 Assessment of sFlt1 and sEng levels

The levels of sFlt and sEng in HUVEC culture supernatants, as well as the levels of sFlt1 in rat serum samples were determined via ELISA (both from R&D systems, Minneapolis, MN). All assays were performed according to the manufacturer's instructions.

6.2.9 Statistical Analysis

All graphs were created using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). There were insufficient animal numbers and replicates in certain groups for statistical analysis.

6.3 Results

6.3.1 Effects of melatonin on a rat model of preeclampsia

Administration of sFlt1 and sEng adenoviruses on day 8 of gestation lead to the rats developing two major features of preeclampsia, that is, an increase in MAP, as well as kidney dysfunction, as indicated by an elevated urinary albumin/ creatinine ratio (Figure 6.4A-D). The rats injected with sFlt1 and sEng demonstrated an average increase in MAP of approximately 8mmHg from day 8 to day 15 of gestation, compared with only a 2.4 mmHg increase in the control rats (Figure 6.4C). The urinary albumin/ creatinine ratio was also approximately 4 times higher in the sFlt1 and sEng treated rats, compared with the control rats (Figure 6.4D).

The rat treated with melatonin throughout gestation did not develop a considerable increase in MAP following adenoviral injection (Figure 6.4A-C). Similar to the controls, the MAP of the melatonin treated rat increased only by about 1 mmHg from day 8 to day 15 of gestation (Figure 6.4C). Furthermore, the melatonin treated rat did not develop a considerable increase in the urinary albumin/creatinine ratio, with its urinary albumin/creatinine ratio level remaining even below that of the control rats (Figure 6.4D).

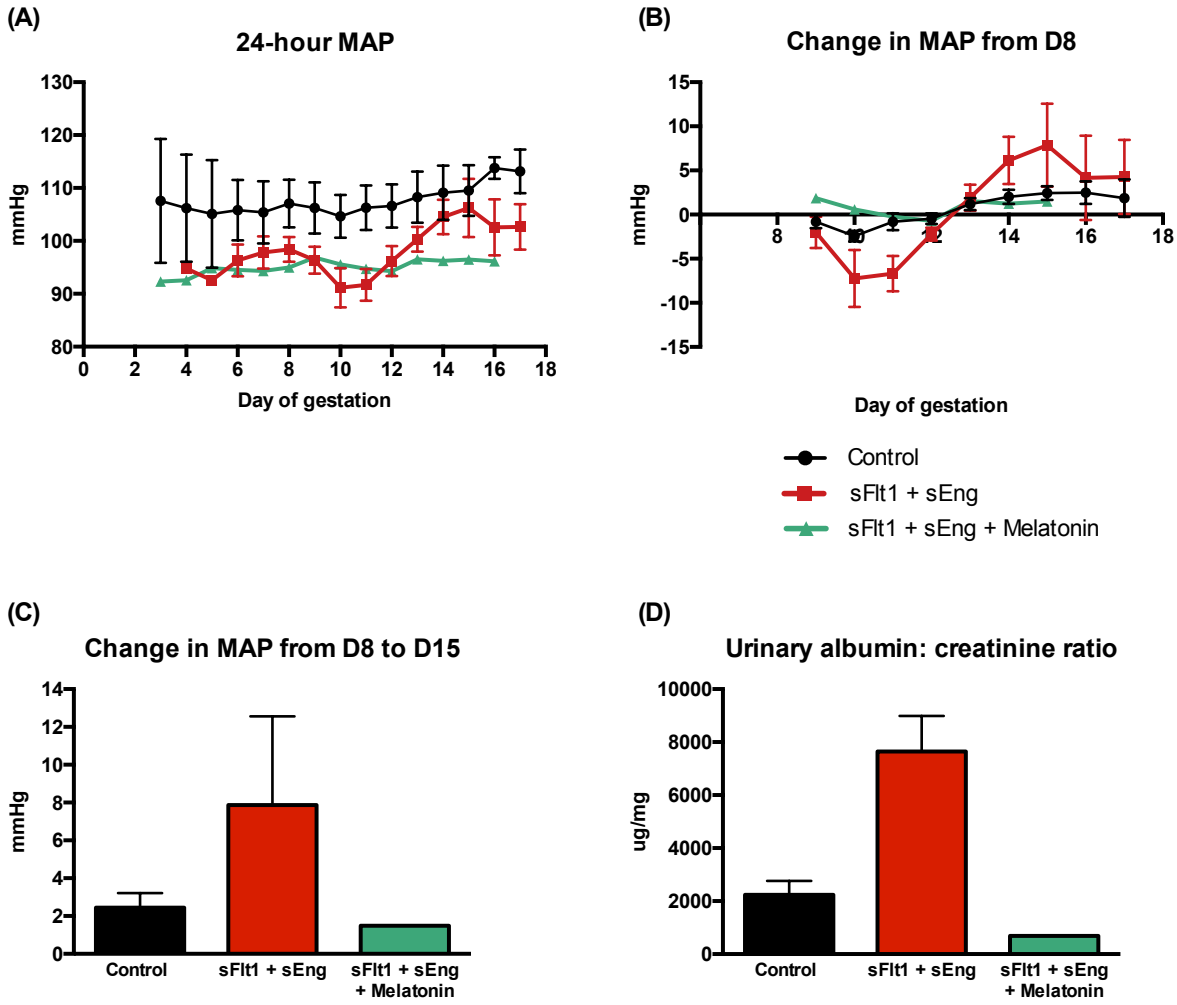


Figure 6.4: Effect of melatonin treatment on blood pressure and renal function in a rat model of preeclampsia. Pregnant rats received either the β-galactosidase adenovirus (control), sFlt1 and sEng adenoviruses or the sFlt1 and sEng adenoviruses with melatonin. 24-hour MAP was recorded throughout gestation and a bladder aspirate sample was collected on gestation day 17. (A) 24-hour MAP throughout gestation, (B) change in MAP compared with that of gestation day 8 (D8), (C) difference in MAP between gestation day 8 (D8) and 15 (D15), along with the (D) urinary albumin/ creatinine ratio is shown. Data are presented as mean ± SEM. Controls n=4, sFlt1 and sEng n=4, sFlt1 and sEng and melatonin n=1.

6.3.2 Optimization of the clinical features of preeclampsia in the rat model

Approximately two months after the above-mentioned first cohort of rats were given sFlt1 and sEng adenoviruses with one rat receiving melatonin treatment, another group of rats were injected with the same adenoviruses. However, unlike the first cohort, this second cohort did not display a considerable increase in MAP or in urinary albumin/ creatinine ratio, compared with the controls, following the injection of the sFlt1 and sEng adenoviruses (Figure 6.5A-D). In fact, the average increase in MAP from gestation day 8 to 15 in this second cohort of rats given sFlt1 and sEng was only 0.8 mmHg, which is lower than the 2.4 mmHg increase observed in the controls, and considerably lower than the nearly 8 mmHg increase observed in the first cohort of rats given the sFlt1 and sEng adenoviruses (Figure 6.5C). Likewise, the urinary albumin/creatinine ratio in this second cohort of sFlt1 and sEng treated rats was similar to the controls and substantially lower than the levels observed in the first cohort of rats given sFlt1 and sEng (Figure 6.5D).

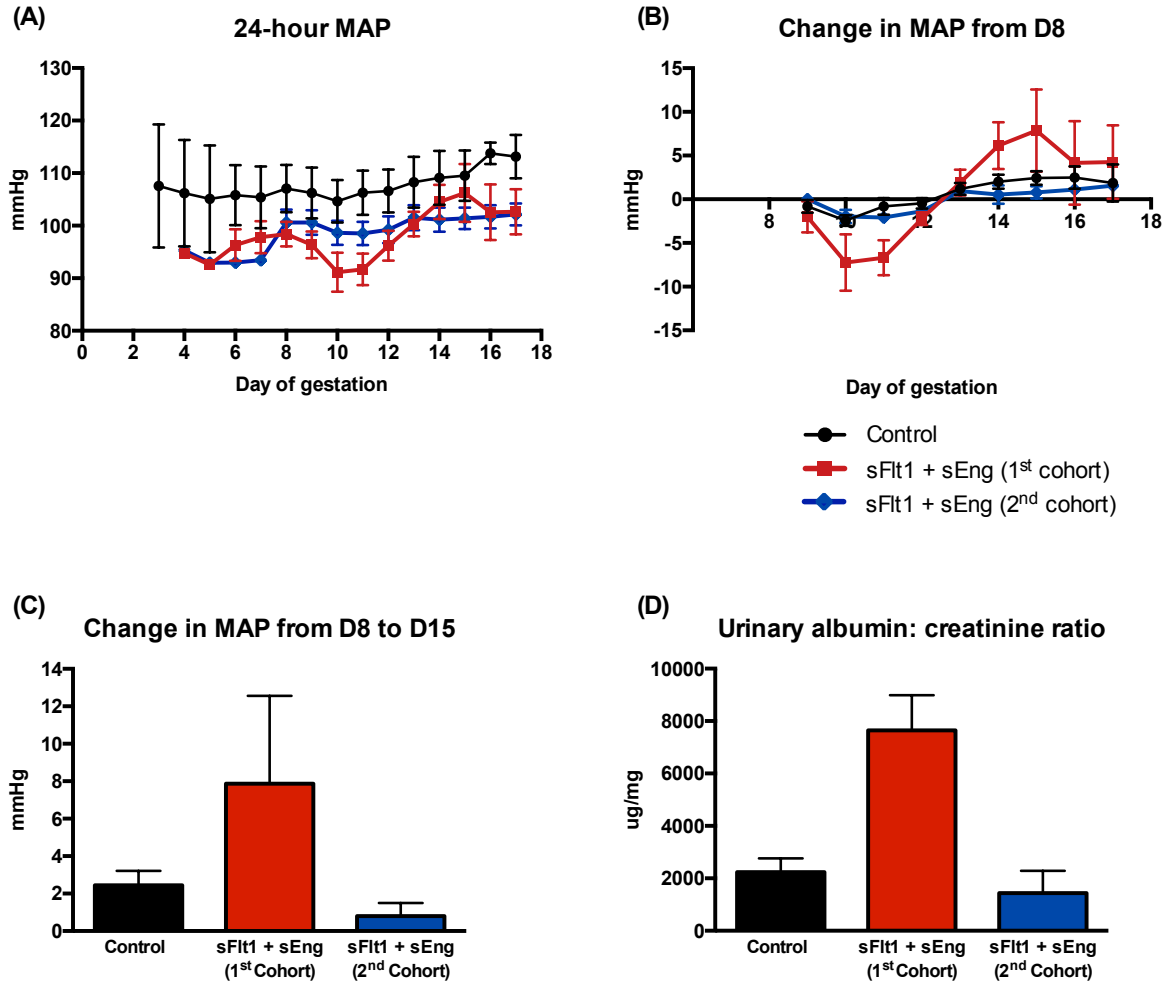


Figure 6.5: Mean arterial pressure and urinary albumin/ creatinine ratio in rats given sFlt1 and sEng adenoviruses. The first cohort of pregnant rats that received the sFlt1 and sEng adenoviruses were compared with the second cohort of pregnant rats injected with the same sFlt1 and sEng adenoviruses. Pregnant rats given the β-galactosidase adenovirus were used as controls. 24-hour MAP was recorded throughout gestation and a bladder aspirate sample was collected on gestation day 17. (A) 24-hour MAP throughout gestation, (B) change in MAP compared with that of gestation day 8 (D8), (C) difference in MAP between gestation day 8 (D8) and 15 (D15), along with the (D) urinary albumin/ creatinine ratio is shown. Data are presented as mean ± SEM. Controls n=4, sFlt1 and sEng (1st cohort) n=4, sFlt1 and sEng (2nd cohort) n=4.

At first these differences between the cohorts were thought to be due to a change in the titer of the adenoviruses over time. Therefore, the titer of both the sFlt1 and sEng adenoviruses were re-determined, and the sFlt1 adenoviral titer was found to be 200 times less than the original titer determined 3 years earlier, while the sEng was 3 times lower than that determined about 1 year earlier. When the sFlt1 and sEng adenoviruses were injected into a pregnant rat according to these new titer values, there was still no considerable increase in MAP or in the urinary albumin/ creatinine ratio, compared with the controls (Figure 6.6A-D).

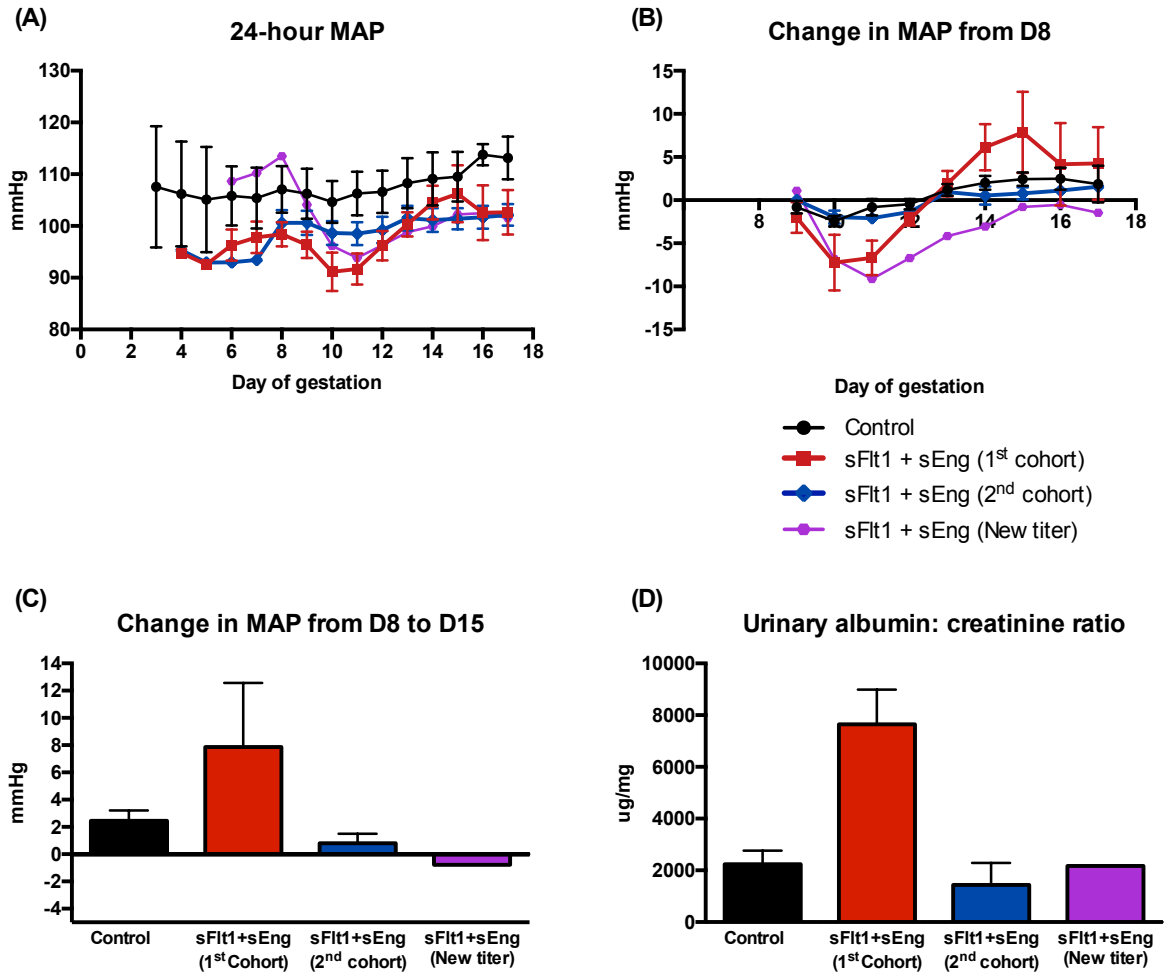


Figure 6.6: Mean arterial pressure and urinary albumin/ creatinine ratio in rats given sFlt1 and sEng adenoviruses after re-determining the adenovirus titer. The first and second cohort of pregnant rats that received the sFlt1 and sEng adenoviruses were compared with the pregnant rats injected with the same adenoviruses, but according to the new titer values. Pregnant rats given the β-galactosidase adenovirus were used as controls. 24-hour MAP was recorded throughout gestation and a bladder aspirate sample was collected on gestation day 17. (A) 24-hour MAP throughout gestation, (B) change in MAP compared with that of gestation day 8 (D8), (C) difference in MAP between gestation day 8 (D8) and 15 (D15), along with the (D) urinary albumin/ creatinine ratio is shown. Data are presented as mean ± SEM. Controls n=4, sFlt1 and sEng (1st cohort) n=4, sFlt1 and sEng (2nd cohort) n=4, sFlt1 and sEng (New titer) n=1.

There after, the amount of each adenovirus injected into the rats were doubled, and 2 x 10^9 IFU of each adenovirus was injected into the next cohort of rats to determine if that could trigger the features of preeclampsia in the rats. Following this increase in the adenoviral dosage, MAP was not significantly altered by the double dosage of the adenoviruses (Figure 6.7A-C), however two-thirds of the rats demonstrated a considerable increase in the urinary albumin/ creatinine ratio, compared with controls (Figure 6.7D).

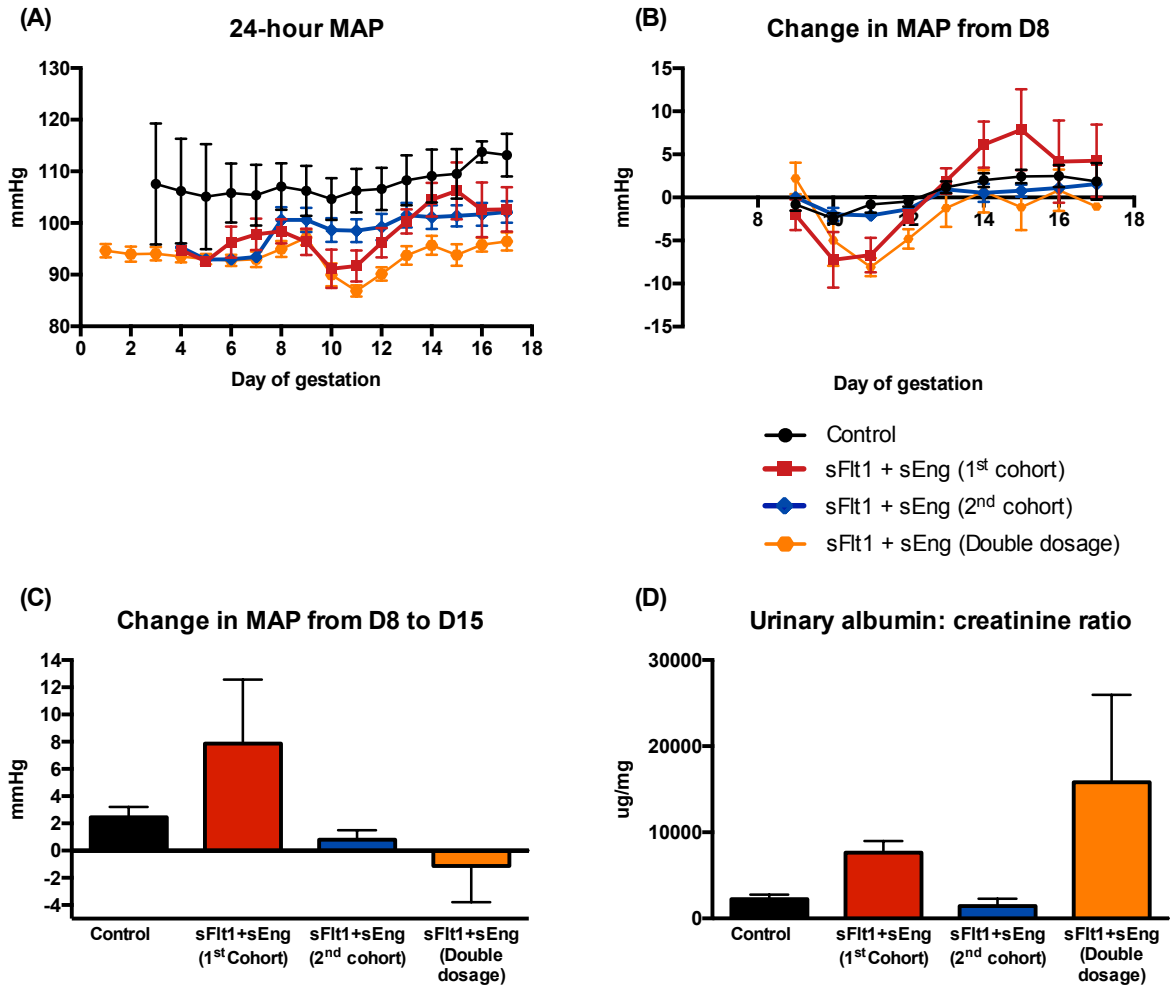


Figure 6.7: Mean arterial pressure and urinary albumin/ creatinine ratio in rats given a double dosage of the sFlt1 and sEng adenoviruses. The first and second cohort of pregnant rats injected with 1 x 10^9 IFU/each of the sFlt1 and sEng adenoviruses, were compared with pregnant rats injected 2 x 10^9 IFU/each of the same adenoviruses. Pregnant rats given the β-galactosidase adenovirus were used as controls. 24-hour MAP was recorded throughout gestation and a bladder aspirate sample was collected on gestation day 17. (A) 24-hour MAP throughout gestation, (B) change in MAP compared with that of gestation day 8 (D8), (C) difference in MAP between gestation day 8 (D8) and 15 (D15), along with the (D) urinary albumin/ creatinine ratio is shown. Data are presented as mean ± SEM. Controls n=4, sFlt1 and sEng (1st cohort) n=4, sFlt1 and sEng (2nd cohort) n=4, sFlt1 and sEng (Double dosage) n=4.

A new stock of each adenovirus was then generated from one of the original vials of each adenovirus, as it was hypothesized that the last stock of adenoviruses could have undergone a sequence change over time or during the different stages of expansion. However, injection of these new stocks of the adenoviruses at a dosage of 2 x 10^9 IFU of each adenovirus lead to an outcome similar to that observed following the injection of the previous stock of adenoviruses. MAP did not increase considerably following the injection of the new stock of adenoviruses (Figure 6.8A-C), but there was a slight increase in the urinary albumin/ creatinine ratio compared with controls (Figure 6.8D).

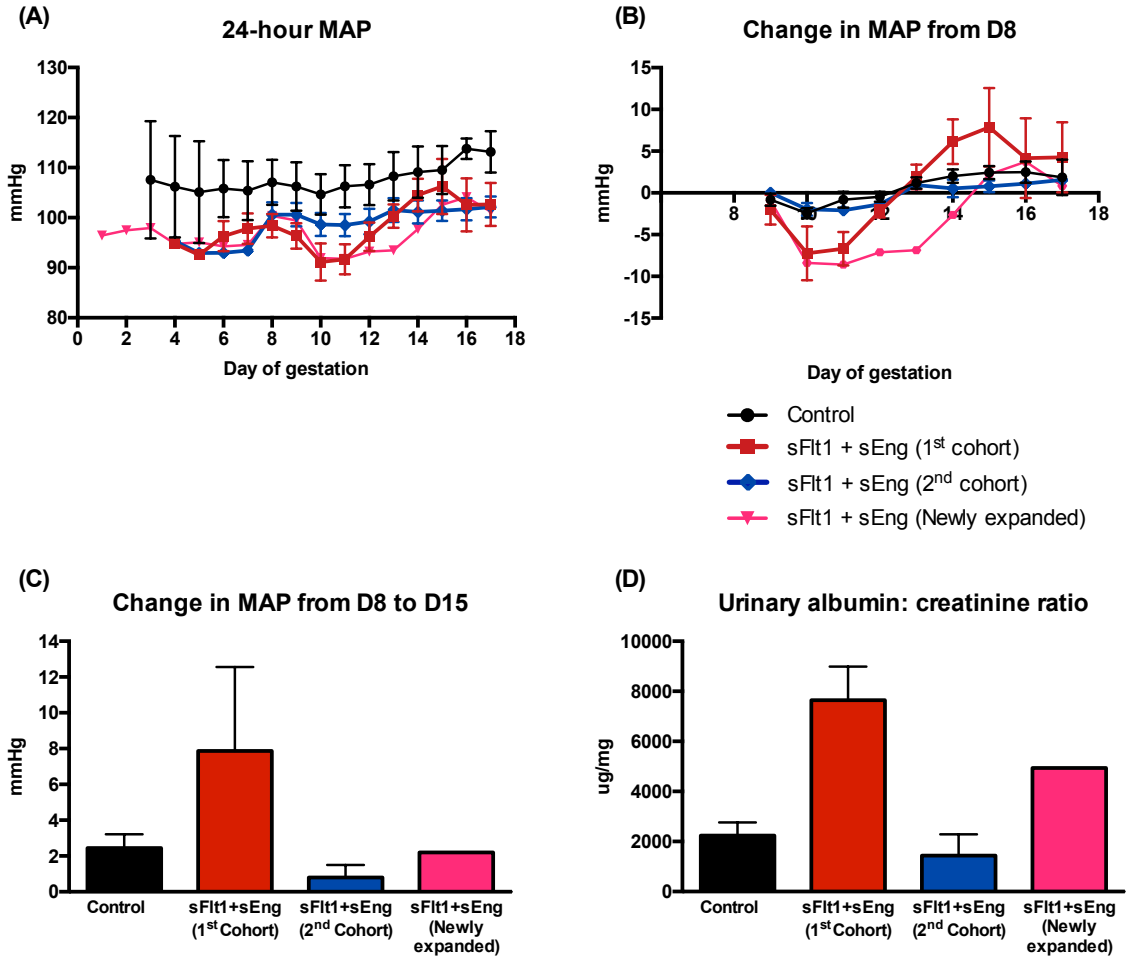


Figure 6.8: Mean arterial pressure and urinary albumin/ creatinine ratio in rats given a newly expanded stock of the sFlt1 and sEng adenoviruses. The first and second cohort of pregnant rats injected with the sFlt1 and sEng adenoviruses were compared with a pregnant rat injected with a newly expanded stock of the sFlt1 and sEng adenoviruses. Pregnant rats given the β-galactosidase adenovirus were used as controls. 24-hour MAP was recorded throughout gestation and a bladder aspirate sample was collected on gestation day 17. (A) 24-hour MAP throughout gestation, (B) change in MAP compared with that of gestation day 8 (D8), (C) difference in MAP between gestation day 8 (D8) and 15 (D15), along with the (D) urinary albumin/ creatinine ratio is shown. Data are presented as mean ± SEM. Controls n=4, sFlt1 and sEng (1st cohort) n=4, sFlt1 and sEng (2nd cohort) n=4, sFlt1 and sEng (Newly expanded) n=1.

The adenoviral dosage was then increased further to 6 x 10^9 IFU of each adenovirus, 6 times the original dosage, to determine if that could trigger an increase in MAP. This considerable increase in the adenoviral dosage lead to a 6 mmHg increase in MAP on day 14 of gestation, which is the closest increase in MAP to the original cohort that showed an average increase of 8 mmHg on day 15 of gestation (Figure 6.9A & 6.9B). However, MAP in these rats returned to control levels on day 15 of gestation (Figure 6.9C). A bladder aspirate sample could not be collected from this rat, in order to determine the urinary albumin/ creatinine ratio.

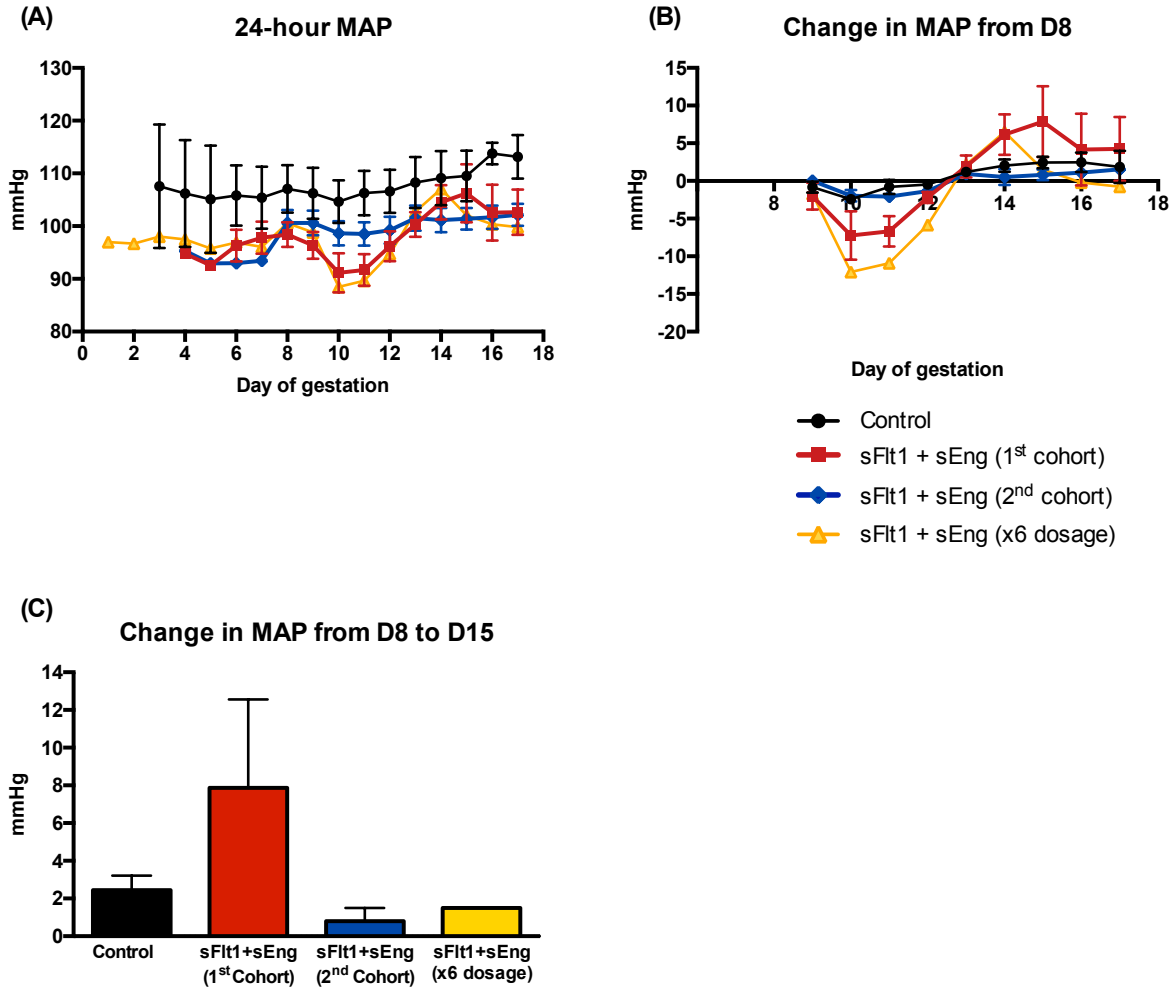


Figure 6.9: Mean arterial pressure and urinary albumin/creatinine ratio in rats given sFlt1 and sEng adenoviruses at a dose 6-fold higher than the initial dose. The first and second cohort of pregnant rats injected 1 x 10^9 IFU/each of the sFlt1 and sEng adenoviruses were compared with a pregnant rat injected with 6 x 10^9 IFU/each of the sFlt1 and sEng adenoviruses. Pregnant rats given the β-galactosidase adenovirus were used as controls. 24-hour MAP was recorded throughout gestation and a bladder aspirate sample was collected on gestation day 17. (A) 24-hour MAP throughout gestation, (B) change in MAP compared with that of gestation day 8 (D8), (C) difference in MAP between gestation day 8 (D8) and 15 (D15), along with the (D) urinary albumin/ creatinine ratio is shown. Data are presented as mean ± SEM. Controls n=4, sFlt1 and sEng (1st cohort) n=4, sFlt1 and sEng (2nd cohort) n=4, sFlt1 and sEng (x6 dosage) n=1.

It was then hypothesized that the stability of the adenoviruses could be compromised during thawing, transport to the animal facility and injection into the rats. Hence, following the thawing of the adenoviruses, they were re-suspended in x1 HBSS with 5% glycerol to improve the stability of the adenoviruses, instead of re-suspending them in sterile saline only as done previously. However, injection of these adenoviruses at a dosage of 2 x 10^9 IFU of each adenovirus, still did not lead to a considerable increase in either MAP or urinary albumin/creatinine ratio, when compared with controls (Figure 6.10A-D).

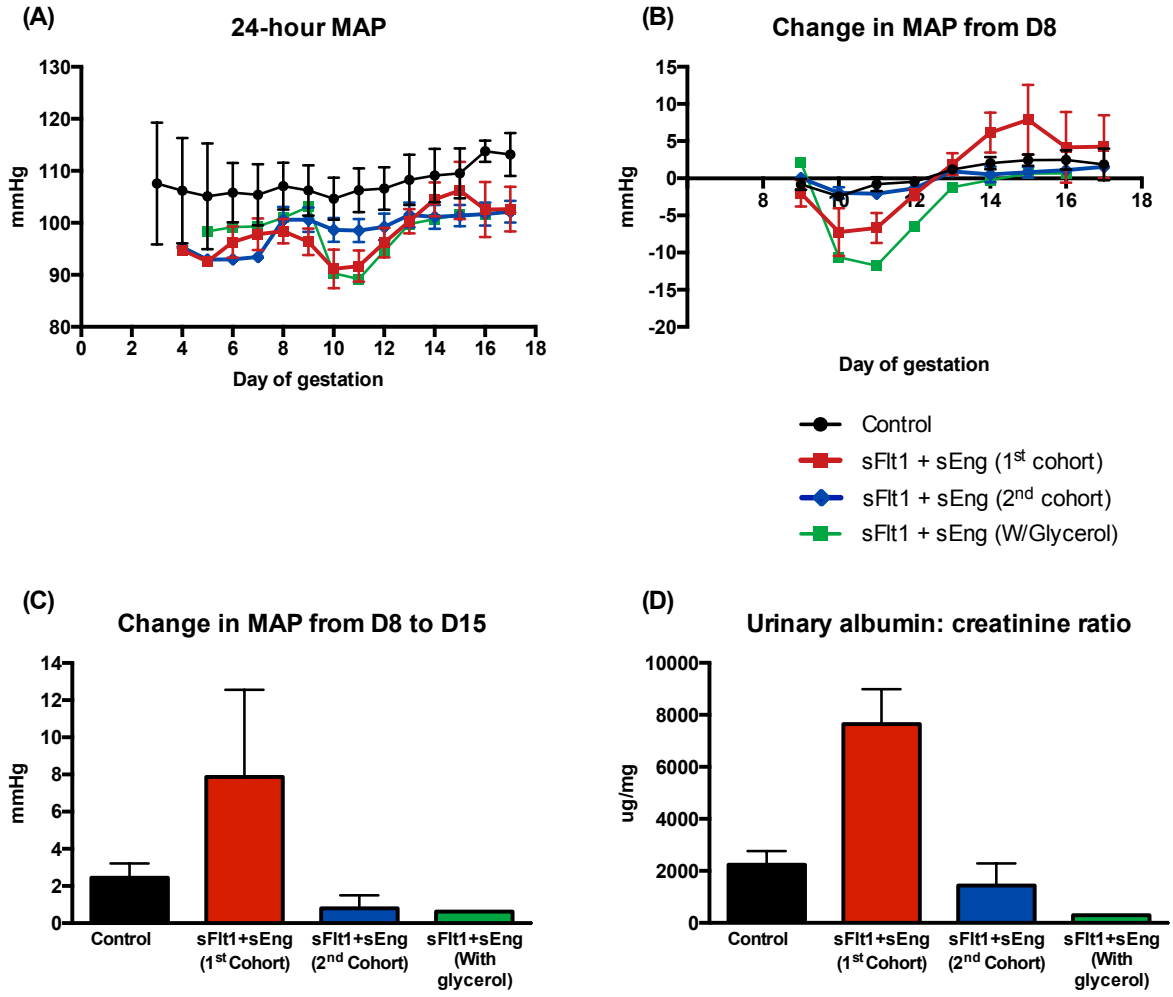


Figure 6.10: Mean arterial pressure and urinary albumin/creatinine ratio in rats given sFlt1 and sEng adenoviruses re-suspended in 5% glycerol. The first and second cohort of pregnant rats injected with the sFlt1 and sEng adenoviruses re-suspended only in sterile saline were compared with a pregnant rat injected with the same adenoviruses re-suspended in 5% glycerol. Pregnant rats given the β-galactosidase adenovirus were used as controls. 24-hour MAP was recorded throughout gestation and a bladder aspirate sample was collected on gestation day 17. (A) 24-hour MAP throughout gestation, (B) change in MAP compared with that of gestation day 8 (D8), (C) difference in MAP between gestation day 8 (D8) and 15 (D15), along with the (D) urinary albumin/ creatinine ratio is shown. Data are presented as mean ± SEM. Controls n=4, sFlt1 and sEng (1st cohort) n=4, sFlt1 and sEng (2nd cohort) n=4, sFlt1 and sEng (with glycerol) n=1.

The serum sFlt1 levels were examined in several groups of rats given adenoviruses in order to determine if injection of the sFlt1 adenovirus actually leads to a systemic increase in sFlt1 levels. Interestingly, the first cohort of rats given the sFlt1 adenovirus that demonstrated the features of preeclampsia actually had a lower serum sFlt1 level than the controls (Figure 6.11). The rat given the melatonin treatment also had a serum sFlt1 level similar to the first cohort of rats (Figure 6.11). In contrast to these groups, the second cohort of rats injected with the sFlt1 adenovirus that did not present the features of preeclampsia actually had an average serum sFlt1 level >3 times greater than the controls (Figure 6.11). The rat injected with the sFlt1 adenovirus following the re-determination of the adenoviral titer had an even higher systemic sFlt1 level (Figure 6.11). The serum sFlt1 levels of the group of rats given the double dosage (2 x 10^9 IFU) of the sFlt1 adenovirus were nearly 3 times greater than the controls, and thus similar to the second cohort of rats that were given 1 x 10^9 IFU and did not present the features of preeclampsia (Figure 6.11).

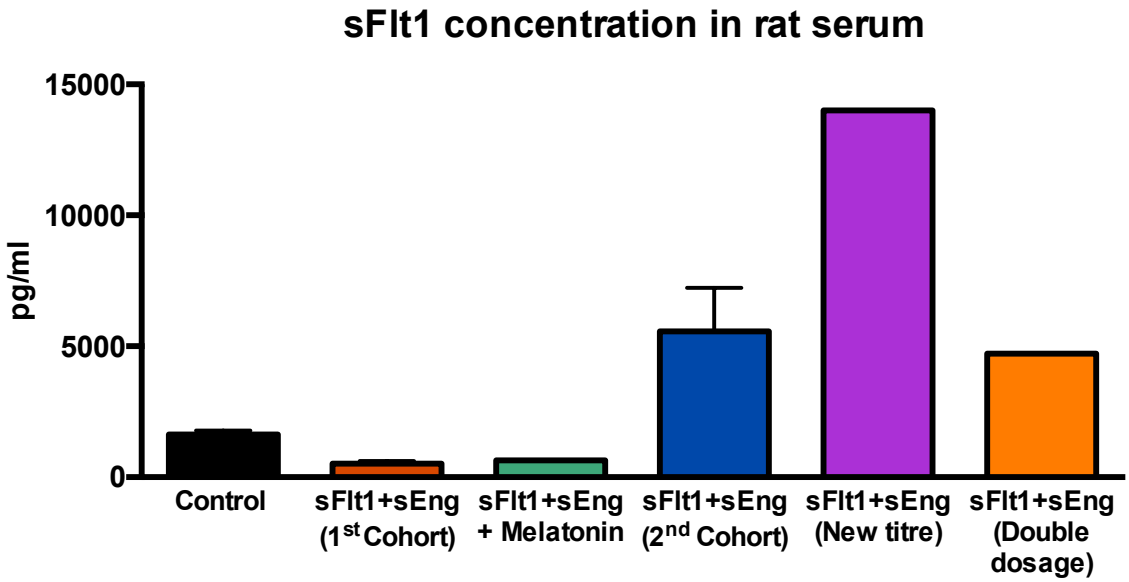


Figure 6.11: Serum sFlt1 concentration in the different groups of pregnant rats. Serum sFlt1 concentration was determined in the following groups of pregnant rats; control rats (n=4), sFlt1 and sEng adenoviruses (1st cohort; n=4), sFlt1 and sEng adenoviruses and melatonin (n=1), sFlt1 and sEng adenoviruses (2nd cohort; n=4), sFlt1 and sEng adenoviruses after re-determination of titer (n=1), double dosage of sFlt1 and sEng adenoviruses (n=1). Data are presented as mean ± SEM.

The efficacies of the sFlt1 and sEng adenoviruses was determined by exposing HUVECs to different doses of each adenovirus, and then assessing the level of sFlt1 or sEng in the HUVEC culture supernatants. Whilst, exposure to the sEng adenovirus lead to a dose dependent increase in the release of endoglin by the HUVECs, exposure to the sFlt1 adenovirus did not lead to an increase in sFlt1 release by the HUVECs (Figure 6.12A & 6.12B). The HUVECs treated with the 3 doses of the sFlt1 adenovirus produced a similar level of sFlt1 as the HUVECs exposed to the β-galactosidase adenovirus (Figure 6.12A).

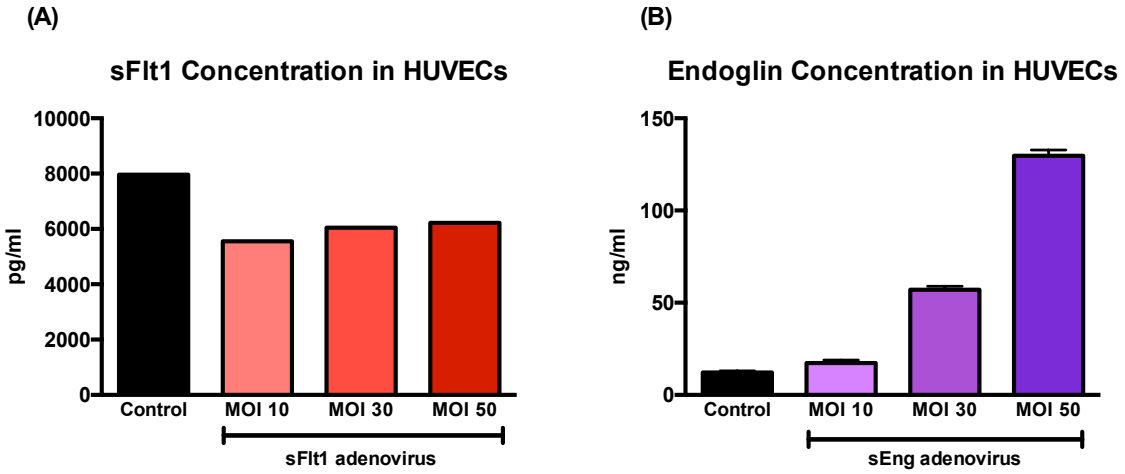


Figure 6.12: Levels of sFlt1 and endoglin protein released by HUVECs exposed to the sFlt1 or sEng adenovirus. (A) sFlt1 and (B) endoglin concentration in culture supernatants collected from HUVECs exposed to 3 different concentrations (MOI 10, 30 and 50) of either the sFlt1 or sEng adenovirus for 3 days. Culture supernatants from HUVECs exposed to the β-galactosidase adenovirus at an MOI of 30 was used as a control. MOI – Multiplicity of infection. Data are expressed as mean ± SEM. HUVECs exposed to sFlt1 adenovirus n=1, HUVECs exposed to sEng adenovirus n=2.

Since the sFlt1 and sEng adenoviruses failed to give rise to the features of preeclampsia even after several attempts, it was hypothesized that subcutaneous delivery of recombinant sFlt1 and sEng via an osmotic pump may prove to be more effective. Whilst there was a small increase in the urinary albumin/ creatinine ratio compared with controls following the implantation of the mini osmotic pump, there was no difference in MAP (Figure 6.13A-D).

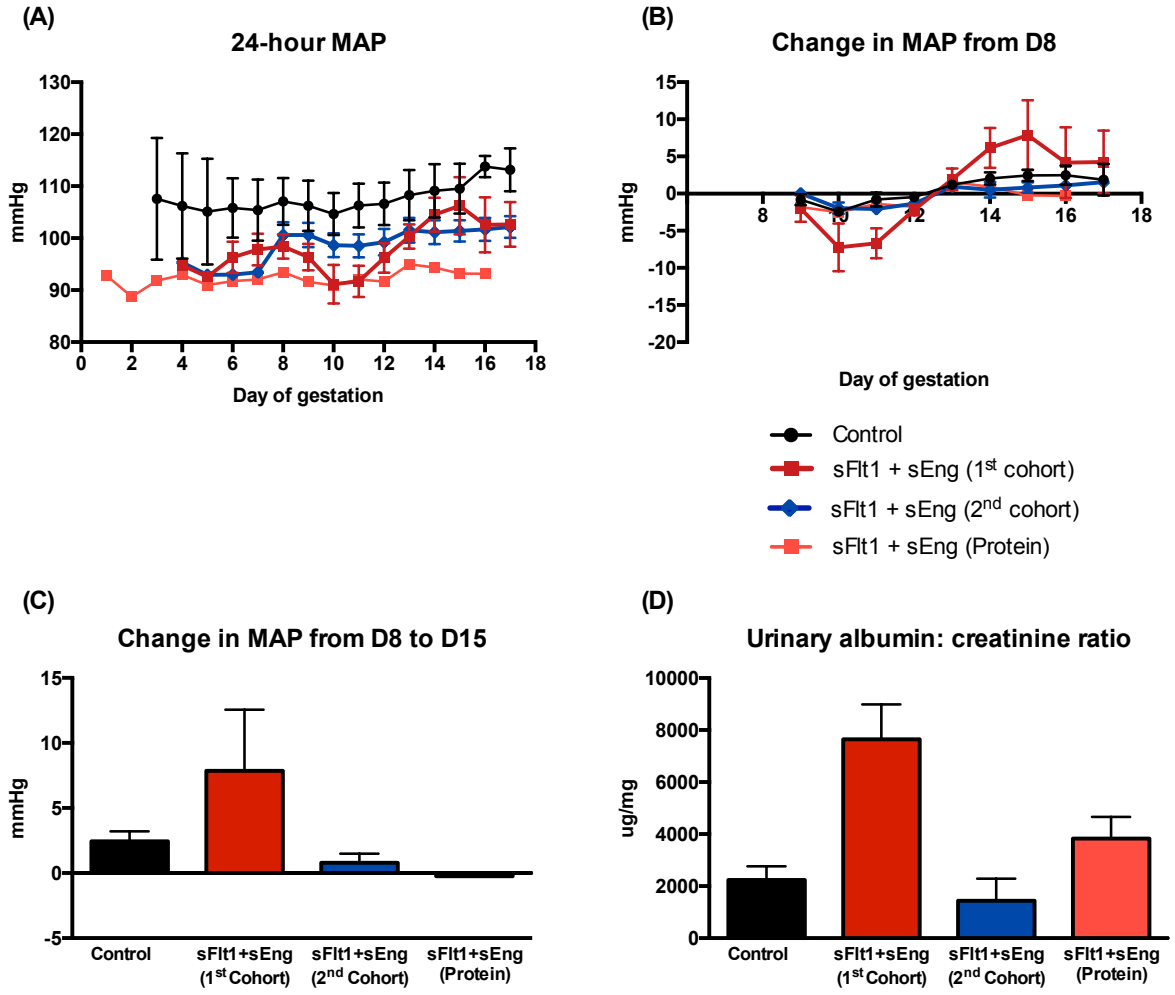


Figure 6.13: Mean arterial pressure and urinary albumin/creatinine ratio in rats treated with recombinant sFlt1 and sEng. The first and second cohort of pregnant rats injected with the sFlt1 and sEng adenoviruses were compared with pregnant rats treated with recombinant sFlt1 and sEng proteins via an osmotic pump from day 10 of gestation. Pregnant rats given the β-galactosidase adenovirus were used as controls. 24-hour MAP was recorded throughout gestation and a bladder aspirate sample was collected on gestation day 17. (A) 24-hour MAP throughout gestation, (B) change in MAP compared with that of gestation day 8 (D8), (C) difference in MAP between gestation day 8 (D8) and 15 (D15), along with the (D) urinary albumin/ creatinine ratio is shown. Data are presented as mean ± SEM. Controls n=4, sFlt1 and sEng (1st cohort) n=4, sFlt1 and sEng (2nd cohort) n=4, sFlt1 and sEng (Protein) n=1 for MAP and n=2 for urinary albumin/creatinine ratio.

6.4 Discussion

This project provides preliminary data on the potential for melatonin to mitigate the development of preeclampsia by preventing the development of the two major features of preeclampsia, hypertension and proteinuria, in an sFlt1 and sEng treated pregnant rat. However, it cannot be conclusively determined unless tested in a larger number of animals. I was unable to further explore the efficacy of melatonin due to technical difficulties with establishing the sFlt1 and sEng animal model of preeclampsia. Ultimately, the absence of a preeclampsia phenotype in the pregnant rats given sFlt1 and sEng adenoviruses appears to have been due to the sFlt1 adenovirus failing to trigger the cellular release of sFlt1 protein. Further optimization of the animal model, either with a newly synthesized sFlt1 adenovirus or with sFlt1 and sEng recombinant proteins is required. Alternatively, a different animal model of preeclampsia would need to be established in our laboratory.

Recombinant adenoviruses are replication-defective adenoviral vectors that can be used to achieve efficient expression of a gene of interest in most host cells, including in non-dividing cells [321]. Maynard *et al* (2003) were the first to demonstrate that adenoviral delivery of sFlt1 to pregnant rats, and the resulting systemic increase in sFlt1, leads to hypertension and proteinuria, two of the major features of preeclampsia [100]. The same group then demonstrated that a concomitant increase in systemic sEng, also achieved with the use of adenoviral vectors, leads to the pregnant rats developing fetal growth restriction and HELLP syndrome, in addition to hypertension and proteinuria [121]. There after, several other research groups have confirmed that adenoviral delivery of sFlt1 leads to the development of hypertension and proteinuria in pregnant rodents [210, 323, 324].

Our group obtained a small vial of each adenovirus from Professor Karumanchi, whose laboratory group originally published the two above-mentioned articles on the capacity of sFlt1 and sEng to induce preeclampsia features in pregnant rats. The sFlt1 adenovirus was expanded and purified in our laboratory. I attempted to expand and purify the sEng adenovirus as well, however the yield was consistently low. This could have been due to the fact that the sEng adenovirus that we received from Professor Karumanchi was several years older than the sFlt1 adenovirus. We then sent the sEng adenovirus to a specialist adenovirus production company in the US for expansion and purification.

The sFlt1 and sEng adenoviruses worked fairly well in the first cohort of pregnant rats, with the sFlt1 and sEng treated rats demonstrating an approximately 4-fold increase in both MAP and urinary albumin/ creatinine ratio, compared with the control rats. Mean arterial pressure in the sFlt1 and sEng treated rats showed an average increase of 8 mmHg 7 days after the injection of the adenoviruses, whilst the urinary albumin/ creatinine ratio increased by 6000 μg/mg on average, compared with the control rats. Whilst these are considerable increases, it must be noted that they are still significantly less than the changes reported in the original study by Venkatesha *et al.* [121]. This study reported a 38 mmHg average increase in MAP and a nearly 9000 μg/mg mean increase in urinary albumin/ creatinine ratio in the sFlt1 and sEng injected rats 8-10 days after injection, compared with control rats. When the sFlt1 and sEng adenoviruses were injected into the second cohort of pregnant rats 2 months later, they did not demonstrate even the small changes observed in the first cohort. Increasing the adenoviral dosage, using newly expanded adenoviruses or attempting to improve the stability of the adenoviruses with glycerol did not improve the outcome.

Whilst it is difficult to ascertain the exact cause for the sudden absence of preeclampsia features following sFlt1 and sEng adenoviral injection, the most probable cause is a complete loss in adenoviral efficacy. This is confirmed by the fact that the sFlt1 adenovirus was unable to trigger sFlt1 production by the HUVECs, and had thus completely lost its efficacy. The sEng adenovirus appeared to be effective, as the HUVECs exposed to it demonstrated a dose –dependent increase in the production of sEng. It was only the sFlt1 adenovirus that had lost efficacy and not the sEng adenovirus, when both adenoviruses were stored under the same conditions and were of a similar age. If anything, the sEng adenovirus was older by a few years. It is possible that the sFlt1 adenovirus is more sensitive and lost efficacy more rapidly. Indeed, the sFlt1 adenoviral titer decreased by nearly 200 times in 3 years, while the sEng titer reduced by 3-fold within a year. Very few studies have examined in detail the factors regulating adenoviral efficacy, with conflicting results [325, 326]. However, there is evidence to suggest that the adenoviral genome size can affect the stability of the adenovirus particles [327, 328]. It appears that a genome size smaller than that of the original wild type adenovirus, which is 30kb, can lead to destabilization of the adenoviral capsid at body temperature, resulting in reduced transfection efficiency [328]. The exact sizes of the sFlt1 and sEng adenoviruses are unknown, as this information has not been explicitly published.

However, this could potentially explain the sensitivity of the sFlt1 adenovirus and its rapid loss in transfection efficacy.

Interestingly, on day 17 of gestation the rats that did not develop a preeclampsia phenotype still had a serum sFlt1 level that was very much higher than the controls or the first cohort of rats that did develop preeclampsia features. It is possible that the loss in efficacy of the sFlt1 adenovirus results in it triggering a very slow increase in systemic sFlt1 levels. Thus the resulting increase in MAP and urinary albumin excretion could appear after a much longer time period than in the first cohort of rats.

It is also quite surprising to note that the first cohort of pregnant rats that developed features of preeclampsia had lower serum sFlt1 levels than controls. A possible explanation is that the increase in sFlt1 triggered by the adenovirus led to a counteractive decrease in the rat's own systemic production of sFlt1, thus once the adenoviral production ceased, the remaining systemic production of sFlt1 was lower than that of the controls.

The use of sFlt1 and sEng recombinant proteins, as an alternative to adenoviruses, was also unsuccessful in giving rise to the features of preeclampsia in the pregnant rats. Serum sFlt1 and sEng levels in these rats need to be analysed to confirm that the osmotic pumps were indeed functional, and the results could also guide further optimization of the dosage of the recombinant proteins that need to be delivered to achieve a sufficient systemic increase of the proteins.

I was unable to continue exploring further options and establish a reliable animal model of preeclampsia in our laboratory due to the time constraints of a 4-year PhD. In order to continue using the sFlt1 and sEng animal model of preeclampsia, an entirely new sFlt1 adenovirus will need to be generated, with attention being paid to the adenoviral genome size during viral construction. However, due to the temperamental nature of adenoviruses, as observed in this study, it is important to consider other options. The only other model to present the same features of severe preeclampsia as the sFlt1 and sEng animal model is the HIF-1α overexpression model. It also has the added advantage of presenting the placental pathologies and the natural increase in vasoactive factors that is characteristic of the human disease, unlike the sFlt1 and sEng model, which bypasses the placenta. However, replicating

the originally published HIF-1α overexpression model by Tal *et al.*, also involves the use of adenoviruses [44]. Pharmacological activation of HIF-1 is an option, but a certain degree of optimization might be needed to identify a HIF-1 activator that gives rise to the preeclampsia phenotype without any other maternal or fetal adverse effects. Another alternative, which would also be the most feasible and efficient, is to use the RUPP model to test therapeutics for preeclampsia. Even though the RUPP model does not present all the features of severe preeclampsia, it accurately replicates the pathophysiology of the human disease, making it a suitable model for the testing of therapeutics.

In conclusion, an alternative animal model to the sFlt1 and sEng model of preeclampsia will need to be established to complete the testing of melatonin as a therapeutic. Due to the temperamental nature of adenoviruses, the RUPP model could be the most feasible alternative. Preliminary data from this study suggest that melatonin may have the potential to mitigate endothelial dysfunction triggered by anti-angiogenic factors in vivo, however this needs to be confirmed by further testing in a larger sample size of a reliable animal model of preeclampsia.

Chapter 7

General Discussion

The overall aim of my PhD was to identify potential therapeutic targets and therapies for preeclampsia. In chapter 2, I demonstrated that there is no apparent relationship between Activin A and the anti-angiogenic factors in the development of preeclampsia, and therefore blocking Activin A alone is unlikely to be an effective treatment strategy for preeclampsia. Data from chapters 3, 4 and 5 revealed that Nrf2 activators, such as melatonin, resveratrol and sulforaphane have the potential to be effective therapies for preeclampsia, mainly due to their ability to mitigate endothelial dysfunction.

The studies detailed in chapter 2 demonstrated that whilst the placental production of activin A is triggered by pro-inflammatory cytokines, there appears to be no link between activin A and the anti-angiogenic factors, either at the level of placenta or at the level of the endothelium. The implications of these findings are that blocking either activin A or anti-angiogenic factors in isolation, may be ineffective as a therapy for preeclampsia. However, a limitation of the studies in chapter 2 is that the experiments were performed *in vitro*, and due to the systemic nature of preeclampsia the findings should be confirmed by examining the serum levels of activin A in pregnant animals treated with the anti-angiogenic factors and vice versa. However, the conclusion from this study is supported by several pilot studies that have attempted to reduce serum sFlt1 in women with very preterm preeclampsia using extracorporeal dextran sulfate apheresis [200, 329, 330]. The temporary reduction in serum sFlt1 achieved by regular apheresis stabilised the clinical features in the short-term and prolonged pregnancy by 2-4 weeks, but could not alleviate the clinical features completely or prevent the eventual deterioration of the clinical condition [200, 329, 330]. This lack of disease resolution following a significant reduction in sFlt1 is most likely due to the presence of other vasoactive factors, such as activin A, that continue to disrupt endothelial function. Whilst prolongation of pregnancy by 2-4 weeks in women with very preterm preeclampsia is

a significant achievement, a reduction in several more vasoactive factors, including activin A may be needed to further alleviate clinical features and prolong pregnancy more significantly.

Targeting the vasoactive factors that contribute to the development of preeclampsia is associated with several challenges, apart from having to target multiple factors. Complete inhibition of the vasoactive factors or their signaling could lead to adverse fetal effects, as some vasoactive factors, like Activin A, are required for placental function and fetal development [132, 331]. A recent study by our laboratory found that blocking activin A signaling in pregnant mice, with the use of inhibitors that target its receptors, result in fetal craniofacial and karyotype abnormalities even when treated late in gestation [332]. In this regard, the extracorporeal apheresis technique is an effective approach for targeting vasoactive factors, as it removes excess circulatory factors rather than completely inhibiting them. No adverse maternal or fetal effects were observed in the pilot studies that have tested the technique for removal of sFlt1, even after several apheresis sessions [200, 329, 330]. However, it is unlikely to be implemented as a routine treatment especially in low resource settings, due to the need for multiple sessions per patient and specialist equipment.

An alternative treatment strategy is to target the two sites of injury in preeclampsia – the maternal endothelium and the placenta. The main goal is to alleviate the clinical features of preeclampsia by enhancing maternal endothelial function, thus allowing the endothelium to maintain its function sufficiently even in the presence of the vasoactive factors. The secondary goal is to mitigate placental injury and the production of vasoactive factors from the source. In this regard activation of the Nrf2 transcription factor offers great promise as a potential treatment strategy, due to its anti-oxidant and anti-inflammatory properties that could target both the maternal endothelium and the placenta in women with preeclampsia. Indeed, in chapters 3, 4 and 5 it was demonstrated that the Nrf2 activators, melatonin, resveratrol and sulforaphane, could mitigate hallmarks of endothelial dysfunction *in vitro*, with resveratrol also reducing *in vitro* placental dysfunction. Together these studies support further investigations into Nrf2 as a potential treatment target for preeclampsia.

Among the three Nrf2 activators tested, sulforaphane was the most potent inducer of Nrf2 nuclear translocation. Furthermore, it effectively improved the *in vitro* hallmarks of endothelial dysfunction. Resveratrol also improved the same hallmarks of endothelial

dysfunction significantly, but at a dose 10 times higher. The Nrf2 gene-silencing studies showed that both compounds depend on Nrf2 to execute most of these beneficial effects on endothelial cells. However, both resveratrol and sulforaphane were able to resolve some *in vitro* hallmarks of endothelial dysfunction even after Nrf2 silencing. Resveratrol was able to regulate oxidative stress levels and the expression of VCAM1 and endothelin-1 even in the absence of Nrf2, while sulforaphane was able to decrease VCAM1 and E-selectin expression. In the absence of Nrf2, the SIRT1 pathway most likely mediated the effects of resveratrol, while sulforaphane decreased VCAM1 and E-selectin expression by inhibiting NF-κB [308-310, 333]. However, further investigations are required to confirm this. While melatonin was not as effective as sulforaphane and resveratrol, it did decrease endothelial monolayer permeability and VCAM1 expression. However, melatonin exerted these effects only at the very high concentration of 1mM. It would be impossible to achieve an equivalent serum concentration in humans. Normal serum melatonin levels in healthy subjects have been shown to be in the range of 30pM to 210pM, with oral administration of 100mg of melatonin shown to give a peak serum concentration of only about 435nM [334-336]. However, our laboratory observed extremely favorable maternal outcomes in a small number of women with preeclampsia originally given melatonin to improve fetal growth restriction outcomes [290]. In light of these preliminary findings, our laboratory began testing melatonin for its efficacy in prolonging the time interval between diagnosis and delivery in women with pre-term preeclampsia [337]. This pilot trial is currently ongoing. Whilst sulforaphane was the most effective of these compounds *in vitro*, its relatively low bioavailability in some individuals is a concern when considering clinical use [311]. However, further testing of sulforaphane in an animal model of preeclampsia would be beneficial. A major problem associated with the use of resveratrol as a therapy for preeclampsia is the recent concerns regarding its risk to the fetus [338]. It is important to note that there are many other Nrf2 activators that are yet to be tested for their therapeutic potential in preeclampsia, such as the synthetic triterpenoids, currently known to be the most potent activators of Nrf2 [339, 340].

Lending further support to Nrf2 being an effective treatment target for preeclampsia, two compounds that successfully improved both endothelial and placental injury in preclinical studies via the activation of the Nrf2/HO-1 pathway are currently being tested in double blind, randomized, placebo-controlled trials. That is pravastatin and esomeprazole [215, 341]. Preclinical studies have shown pravastatin to increase HO-1 expression, improve vascular

reactivity, and decrease oxidative stress as well as placental production of sFlt1 and pro-inflammatory cytokines [207, 209, 211, 242]. Further, pravastatin treatment completely abolished hypertension, proteinuria and fetal growth restriction in an sFlt1 treated mouse model of preeclampsia [213]. Esomeprazole has also been shown to reduce trophoblast sFlt1 and sEng production, and improve hallmarks of endothelial dysfunction *in vitro*. These effects have been attributed to its ability to increase Nrf2 nuclear translocation and expression of its downstream target genes, including HO-1 [342]. Preclinical studies have also shown another Nrf2 activator, sofalcone, to be effective in improving endothelial dysfunction and reducing placenta production of sFlt1 *in vitro* [244]. The success of these compounds further highlights the therapeutic potential of the Nrf2 pathway.

Apart from the compounds linked to the Nrf2/HO-1 pathway, there are several other potential therapies and therapeutic targets that show promise in being effective in preeclampsia. The efficacy of recombinant human anti-thrombin as a treatment for preterm preeclampsia is currently being tested in a phase III trial [206]. The rationale for testing anti-thrombin is mainly its anti-coagulant and anti-inflammatory properties [206]. Furthermore, several smaller clinical trials have shown anti-thrombin treatment to prolong pregnancy by almost 6 days and improve perinatal and maternal outcomes [204, 343].

Inhibition of the complement cascade is another pathway that has been examined for its therapeutic potential in preeclampsia. An extensive maternal inflammatory response is characteristic of preeclampsia and includes activation of the complement cascade [344]. Burwick and Feinberg reported significant clinical improvement and prolongation of pregnancy by 17 days in a patient with severe preeclampsia/HELLP syndrome treated with eculizumab, a monocolonal antibody targeting complement protein C5 [345]. Preclinical studies have also shown inhibition of complement C3a signaling to decrease circulatory sFlt1 levels and improve features of preeclampsia in an AT1-AA treated mouse model of preeclampsia [346]. These studies highlight the importance of further exploring antagonists of the complement cascade as potential therapies for preeclampsia.

Other drugs that have demonstrated potential to be an effective treatment for preeclampsia in preclinical studies are edaravone and ouabain that reduced placental sFlt1 production, along with metformin and YC-1 that improved hallmarks of endothelial dysfunction as well [347-

350]. Ouabain, YC-1 and metformin reduce sFlt1 production by inhibiting placental HIF-1α expression [347-349].

So far almost every potential therapeutic has been tested individually for its efficacy in preventing or treating preeclampsia. However, since preeclampsia is a systemic disease with a highly variable presentation between patients, the possibility of the need to use a combination of therapies to achieve disease resolution should be considered. A combination of drugs that, together, target all the critical aspects of disease development very potently may prove to be more effective than a single drug. The group of drugs could include compounds that collectively target placental injury and production of oxidative stress, endothelial dysfunction, inflammation and oxidative stress. In these studies Nrf2 activation was studied as a therapeutic target due to its potential to target all of these aspects of preeclampsia development, and data from chapter 4 demonstrate that it can resolve these aspects, at least *in vitro*. However, the possibility of greater effectiveness when combined with a complement cascade inhibitor, such as eculizumab, a HIF-1α inhibitor, such as ouabain and an anti-coagulant, such as anti-thrombin or even with other activators of the Nrf2/HO-1 pathway, such as statins and esomeprazole should be explored further.

An essential requirement for testing the efficacy and safety of individual or combinations of potential therapeutics for preeclampsia is a reliable animal model that closely mimics the human condition. The sFlt1 and sEng treated animal model is probably the most recognized animal model of preeclampsia as it was the first model to demonstrate a severe preeclampsia phenotype by developing HELLP syndrome in addition to hypertension, proteinuria and fetal growth restriction [121]. However, a major limitation of this model is that it only mimics the maternal endothelial dysfunction stage of preeclampsia, and does not replicate the placental injuries or the natural production of vasoactive factors associated with the human disease. Hence, the sFlt1 and sEng model allows us to determine only if candidate drugs can resolve endothelial dysfunction in the presence of the vasoactive factors. Whilst this is extremely useful, it would be more beneficial to utilize an animal model that allows us to determine the therapeutic potential of a drug on both the placental and endothelial stages of the pathophysiology of preeclampsia.

Among the currently available animal models for preeclampsia, the models that most closely replicate the human disease in terms of pathophysiology are the RUPP model and the HIF-1α overexpression model. These models are the closest to the human disease as they both mimic the placental hypoxia stage of disease development, which is the earliest stage in the pathophysiology of preeclampsia that we have yet been able to replicate. Both of these animal models show signs of placenta injury, have naturally elevated levels of serum vasoactive factors, such as sFlt1 and sEng, and demonstrate impaired endothelial dysfunction [38, 44, 351, 352]. As such, both animal models can provide information on how the candidate drugs would impact most stages of injury in preeclampsia, and would be the best suited for testing therapeutics for established preeclampsia. While both models develop hypertension, proteinuria and fetal growth restriction, the HIF-1α overexpression model also demonstrates features of HELLP syndrome [38, 44, 351]. Therefore, it could be debated that between these two models, the HIF-1α overexpression model would be more suited for testing therapeutics, as it is representative of severe preeclampsia.

It is interesting to note that the RUPP model does not develop HELLP syndrome, despite a significant increase in placental expression of HIF-1α in these animals [125, 353]. This could be due to the fact that the RUPP model is mostly performed in rats, resulting in a relatively short period of insult, as the uterine arteries are clipped on day 14 of gestation and delivery of pups occur around days 21-22. Nevertheless, both the HIF-1α overexpression model and the RUPP model of preeclampsia would be suitable for examining the therapeutic potential of drug candidates on both the placental and endothelial dysfunction that is characteristic of preeclampsia.

In summary, the findings outlined in this thesis have identified several avenues that could be explored further to find a safe and effective therapy for preeclampsia. One such key avenue is to explore the signaling pathways, other than Nrf2, that are activated by resveratrol, such as the SIRT1 pathway, which may also be involved in giving rise to the beneficial effects of resveratrol on the endothelium and placenta. Therapeutics that could activate these pathways, possibly in combination with the Nrf2 pathway, may prove to be effective therapies for preeclampsia. Another major avenue of future research is to explore the therapeutic potential of the synthetic triterpenoids in preeclampsia. They are currently known to be the most potent activators of Nrf2, and could thus prove to be more effective at mitigating endothelial

function than resveratrol or even sulforaphane [339, 340]. However, the safety profile of the synthetic triterpenoids in pregnancy also needs to be explored in animal studies. Finally, the safety profile, and if possible efficacy as well, of using a combination of therapies, such as a Nrf2 activator, complement cascade inhibitor, an anti-coagulant and/ or a HIF-1α inhibitor should be further explored in an animal model of preeclampsia.

In conclusion, the findings from this PhD thesis have highlighted that targeting a single vasoactive factor is unlikely to be sufficient as a treatment strategy for preeclampsia. Instead, it is postulated that improving endothelial function, as well as potentially mitigating placental injury may be a more effective strategy, possibly via the activation of Nrf2 and its downstream anti-oxidant enzymes. The Nrf2 activators, sulforaphane and melatonin, demonstrated significant efficacy as potential therapeutics for preeclampsia and warrant further investigation. Whilst resveratrol also proved to potently activate Nrf2 and efficacious in mitigating hallmarks of endothelial and placental dysfunction, the safety concerns associated with resveratrol limit its potential use as a therapeutic for preeclampsia. However, further investigation of its mechanisms of action may unveil new signaling pathways that could be targeted for treatment of preeclampsia.

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