The maternal brain in pregnancy and preeclampsia : physiologic adaptations and neurologic complications

The Maternal Brain in Pregnancy and Preeclampsia
Physiologic Adaptations and Neurologic Complications

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Contents

Chapter 1:

General Introduction

The brain: the blood-brain barrier and cerebral autoregulation.

The brain is one of the most highly perfused organs of the human body because of its high metabolic needs that are critical for normal functioning [1]. Therefore, its blood supply requires strict regulated transport of nutrients with exclusion of potentially harmful plasma constituents [1, 2]. To accomplish this strictly regulated transport, the cerebral vasculature has several unique features compared to the peripheral vascular system. First, the cerebral vessels contain an unique endothelial lining that forms the blood-brain barrier (BBB). For all other tissues in the human body, water and several ions and solutes such can move easily between the vascular endothelial cells and the surrounding tissues [2-4]. However, the BBB acts as a 'physical barrier' between the brain and other tissues and prevents extravasation of virtually all molecules except those that are small and liphophilic [3]. The BBB lacks fenestrations, has only few endocytotic vesicles and contains complex high electrical resistance tight junctions between adjacent endothelial cells all of which results in restricted transcellular and paracellular flux [2-4]. Consequently, under physiological conditions, these unique properties prevent proteins and ions to cross the BBB and thereby minimizing the effect of hydrostatic pressure on capillary filtration of water due the remaining osmotic pressure gradient in the vessels [2-4]. Therefore, the BBB is a strict regulator of transport of nutrients and a strong protective mechanism against formation of vasogenic brain edema, that is involved in numerous pathologic neurologic conditions.

Another important aspect of the cerebral vasculature is that it has the intrinsic ability to maintain a relatively constant cerebral blood flow (CBF) in the face of changing perfusion pressures [1, 5]. Where peripheral vasculature can vary their blood flow due to changes in perfusion pressure, the brain needs a constant supply of oxygen. Constant blood supply towards the cerebral tissues is therefore critical for its homeostasis [1]. This "cerebral autoregulation" of blood flow ensures that the blood flow through the brain is maintained at a relatively constant level despite changes in perfusion pressure or arterial blood pressure. Cerebral autoregulation is maintained between the arterial pressures of 50-60 mm Hg to 150-160 mm Hg and forms an autoregulatory plateau to provide the brain an relatively constant oxygen supply [5-7]. Blood flow is related to the fourth power of the vessel radius and thus even small changes in lumen diameter in cerebral vessels can significantly change cerebrovascular resistance (CVR) and thereby CBF [5]. Therefore, the occurence of the autoregulatory plateau is regulated through different intrinsic myogenic

mechanisms, and endothelial derived or metabolic factors that modulate CVR and thereby maintain a constant CBF to maintain optimal function of the brain [8, 9].

The effect of circulating factors on the brain during pregnancy

During normal pregnancy, the endometrium, decidua and placenta initiate a profound rise in cytokines and circulating permeability-increasing factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PLGF)[10]. These circulating factors released in the systemic circulation seem to be critical for the normal development and growth of the maternal-fetal unit and also direct the cardiovascular adaptation to pregnancy by initiating a high flow and low resistance circulation[10]. One growth factor that is important for a successful pregnancy is VEGF [11]. VEGF is produced in significantly higher levels during pregnancy by several cell types, including endothelial cells and is increased both locally in the maternal fetal unit and systemically in the maternal circulation [12, 13]. VEGF was initially discovered as a permeability factor, but it is known now to also have important roles in angiogenesis, vascular growth, endothelial cell survival and vasorelaxation, including in the brain and cerebral circulation [14-16]. Several studies have shown that VEGF can increase BBB permeability and thereby inducing vasogenic edema in neurologic conditions such as stroke [17, 18]. However, in normal pregnant women, increased VEGF increases vascular permeability locally in the uterine circulation without apparently affecting BBB permeability because in pregnant women vasogenic edema does not normally occur. Currently, there is still limited understanding of how the BBB is affected in pregnancy, especially regarding to the increased circulating permeability factors and the delicate balance between supply and drainage and with it the formation of vasogenic brain edema.

VEGF affects BBB permeability through a complex interaction between VEGF and its two VEGF receptors, known in rats as FMS-like tyrosine kinase1 (Flt1) and fetal liver kinase1 (Flk1), but also known as VEGFR1 and VEGFR2, respectively [19]. These two receptors can, after phosphorylation of the cytoplasmic tyrosine kinase domains of either Flt1 or Flk1, transphosphorylate adjacent domains ("cross-talk") and even form Flt1/Flk1 receptor-dimers [19-22]. VEGFR-mediated signaling to induce vascular permeability has been studied intensively in peripheral tissues. However, it still remains unclear whether Flt1, a receptor with low tyrosine kinase activity compared to Flk1, is involved in VEGF-induced permeability or if it functions as a non-signaling reservoir for VEGF [19-24]. Little is known about the VEGFR-mediated

signaling pathway in the cerebral endothelium to induce BBB permeability. In addition to VEGF, PLGF, another member of VEGF-family, is produced in high levels in the placenta and is highly present in the maternal blood circulation. PLGF is known only to bind to Flt1 and is also involved in the angiogenesis and endothelial viability through its own pathway and by amplifying VEGF-driven actions [19]. Although PLGF has shown to increase vascular permeability in peripheral tissues, it is unknown if PLGF affects the BBB during pregnancy [25, 26]. Nevertheless, it is important to understand the possible pathways that affect the BBB during normal pregnancy in the face of neurologic complications that occur due to BBB disruption in pregnancy and preeclampsia.

Preeclampsia and its neurologic complications

Preeclampsia is a complex heterogeneous disorder unique to the second half of pregnancy, that affects 3-8% of all pregnancies worldwide [27, 28]. Preeclampsia is defined by high blood pressure (blood pressure systolic equal or higher than 140 mm Hg and or diastolic equal or higher than 90 mm Hg) and proteinuria (higher than 300 mg/24 hours) in previously normotensive women [29]. Although the incidence of preeclampsia is rising and extensive research has been performed over the past years [30], the exact pathogenesis of preeclampsia remains to be elucidated. To date, the only current curative treatment is removal of the placenta and thus termination of pregnancy, often resulting into iatrogenic perinatal morbidity and mortality [31]. It is generally accepted that endothelial dysfunction plays a key role in the pathogenesis of preeclampsia, however, contributory causes remain to be determined [32-34]. Epidemiologic data show that women who suffered from preeclampsia, have increased risk of developing cardiovascular disease in later life, suggesting common factors to be involved [34, 35].

Preeclampsia is a systemic disorder that can affect multiple maternal organs including the liver, kidney and the brain. Neurologic complications are the most serious and life-threatening complications and are responsible for approximately 75% of those maternal deaths and is thereby and is responsible for the largest maternal death rate worldwide. Eclampsia is the best known neurologic complication and is defined by the new onset of seizures in a women with preeclampsia [36]. In the Netherlands, the incidence of eclampsia is 6.2 per 10.000 deliveries with a fatality rate of 1.4% [37]. Importantly, epidemiologic studies suggest that neurologic complications such as eclampsia, cerebral hemorrhage, cerebral

infarction or coma occur most often in severe early-onset preeclampsia (EPE; preeclampsia diagnosed before 34 weeks of gestation), suggesting EPE and late-onset preeclampsia (LPE; preeclampsia diagnosed after 34 weeks of gestation) have different etiologies [38-40]. Nevertheless, over the last century, the incidence of eclampsia has shifted from antepartum to the postpartum period. Recent studies suggest an incidence of 33-50% of all eclamptic cases in the postpartum period [39, 41, 42]. This is likely due to the fact that pregnant women receive more aggressive treatment when diagnosed with preeclampsia during pregnancy. This observation proposes new question about the care of women during the postpartum period. Only recently, late post-partum eclampsia was recognized as a defined entity [41]. Late-postpartum eclampsia is defined as eclampsia occurring later that 48 hour postpartum. Late postpartum eclampsia is serious problem due to lack of recognition of prodromes and symptoms that could result into eclampsia, when women have exceeded the 48 hour postpartum period.

The pathogenesis of neurologic complications in preeclampsia

Previous studies have shown that vasogenic brain edema may be the hallmark event in the development of neurologic complications in preeclamptic women [5, 43, 44]. In a recent retrospective study comparing 47 women with eclampsia, 46 of these women show evident vasogenic edema in the parietal-occipital lobes on MRI-imaging [45]. Vasogenic brain edema is the result of BBB disruption, suggesting that the BBB plays a central role in the development of neurologic complications in preeclampsia. However, the unique factors contributing to the development of BBB disruption in preeclampsia are currently not known.

One theory is that eclampsia represents as a form of PRES (posterior reversible encephalopathy syndrome), a syndrome that is a variant of hypertensive encephalopathy [46]. PRES is diagnosed by the clinical findings and imaging, most preferably on MRI that shows edema in the parietal-occipital lobe. PRES can have several causes including immunosuppressive therapy, systemic lupus erythematosus, kidney disease and pregnancy [47]. Both hypertensive encephalopathy and PRES can arise from an acute elevation in blood pressure. This acute rise in blood pressure can overcome myogenic constriction of the cerebral arteries and arterioles resulting in a loss of autoregulatory capacity. When autoregulation is lost, decreased CVR results into hyperperfusion that may lead to BBB disruption and vasogenic brain edema. However, it is important to realize why eclampsia represents

a form of PRES instead of a form of hypertensive encephalopathy. Previous reports showed that more than half of eclamptic women do not show blood pressures that reach the upper limit of the autoregulatory curve and even 17 % of the eclamptic women were normotensive [39, 45]. These numbers suggest that cerebral autoregulation is impaired during pregnancy or that autoregulatory breakthrough is not necessarily involved. Or it may be that the deltas of the rise of blood pressure is more important than the actual blood pressure and that women with low index blood pressure are more at risk for developing eclampsia at relatively low blood pressure. Previous studies in vitro have found that forced dilation occurred at lower pressure in cerebral arteries from late-pregnant animals compared to nonpregnant animals [48], however, in vivo the upper limit of the autoregulatory curve was not decreased in pregnancy [49]. Nevertheless, when autoregulatory breakthrough did occur in pregnant animals, cerebral edema formation was increased in pregnancy compared to the nonpregnant state [49]. These findings suggest that autoregulation breakthrough is not the only explanation in the occurrence of vasogenic brain edema. BBB disruption due to circulating factors that increase BBB permeability in pregnancy may be involved in the occurrence of vasogenic brain edema and neurologic complications in pregnancy and preeclampsia.

One of the many factors that is increased in pregnancy and preeclampsia is low-density lipoprotein (LDL). In normal pregnancy, levels of lipids such as LDL are increased and hormonally controlled to encourage lipogenesis and fat storage in preparation of rapid fetal growth in the late pregnancy but does not normally lead to endothelial dysfunction [50]. However, in preeclampsia the rise in lipids is often more dramatic and preeclamptic women show increased levels of circulating oxidized low-density lipoprotein (oxLDL) [51-53]. Increased oxidative stress in the placenta causes oxidative conversion of LDL to oxLDL [54, 55]. oxLDL has found to induce a wide variety of responses, such as the induction and expression of adhesion molecules, pro-inflammatory cytokines and reactive oxygen species, causing endothelial dysfunction in several cardiovascular diseases such as hypertension, diabetes, atherosclerosis and cerebral ischemic conditions [56]. In preeclampsia, decidual vessels of the placenta show fibrinoid necrosis of the vascular wall and focal accumulation of lipid-laden macrophages, similar as in atherosclerosis [57]. oxLDL binds to its receptor LOX-1 that is predominantly expressed on endothelial cells [58, 59]. Previous studies have shown that oxLDL is able to increase BBB permeability and peripheral vascular permeability [60, 61]. In addition, previous reports have associated increased LOX-1 expression in placental tissue and human

umbilical vein endothelial cultures cells with preeclampsia [62-64]. Further, women that suffered from preeclampsia, have a greater risk of developing atherosclerosis and other cardiovascular disease in later life, suggesting similar etiologies. Thus, it is tempting to speculate that oxLDL and LOX-1 activation are involved in BBB disruption, vasogenic brain edema and neurologic complications in preeclampsia. In addition, it is interesting to see if women that develop eclampsia, already have underlying metabolic disorder that is unmasked by the pregnancy itself.

Therapeutic options for protecting the maternal brain in preeclampsia.

Magnesium sulfate ($MgSO_4$) has been used throughout the 20th century in the prevention and treatment of eclampsia and other neurologic complications in preeclampsia [65]. Currently, $MgSO_4$ is still the primary and superior protective treatment for the prevention of neurologic complications in preeclampsia when termination of the pregnancy is not desired. $MgSO_4$ has been shown to be protective of the BBB and prevent cerebral edema during numerous conditions including traumatic brain injury, septic encephalopathy, hypoglycemia, and preeclampsia, however, its mechanism is poorly understood [66-68]. $MgSO_4$ is an unique calcium antagonist that can act on most type of calcium channels in vascular smooth muscle and decreases intracellular calcium, resulting in vasorelaxation in large systemic arteries [69]. However, other studies have shown that the unique cerebral arteries are less sensitive to the vasodilatory effects of $MgSO_4$ [66, 67]. Apparently, $MgSO_4$ elicits more protective effects than merely vasodilation.

Pressurized arteriograph system

For all animal studies performed in this thesis we will use a pressurized arteriograph system, specifically developed at the University of Vermont to measure hydraulic conductivity and vascular reactivity in both cerebral arteries and veins. A pressure-servo controller and a peristaltic pump maintain the intravascular pressure in isolated vessels and pressure can be adjusted manually. For the permeability measurements, the pressure-servo controller and peristaltic pump are disconnected from the transducer, and the intravascular pressure is allowed to decrease over time due to the volume flux across the vessel wall. The drop in intravascular pressure is recorded continuously. A leak in the pressurized system should influence the results because there will be a greater decrease in intravascular pressure not due to filtration. Therefore, the system is tested for leaks during set up for each experiment. If a leak is detected, all connections are

tightened until no leak is observed. For the reactivity experiments, the pressure-servo controller maintains pressure during the entire experiment and increases or decreases autormatically due to the servo mechanism when needed. Lumen diameter in response to changes in pressure or the addition of pharmacological agents are recorded continuously via video microscopy. Thus, this in vitro system is able to measure hydraulic conductivity of cerebral vessels providing important information about BBB permeability and to visualize myogenic responses that could influence cerebral vascular function and cerebral autoregulation.

Aims of this thesis.

This thesis will contain 5 separate studies, each of which were designed to determine the following aims:

1. To investigate the impact of increased angiogenic and anti-angiogenic factors on BBB permeability in pregnancy and the adaptation of the BBB to these factors. (**Chapter 2**)
2. To investigate if and how high levels of lipids impact the cerebral vasculature in pregnancy compared the nonpregnant state. (**Chapter 3**)
3. To investigate the effects and underlying mechanism of oxLDL on BBB permeability in preeclampsia. (**Chapter 4**)
4. To investigate the mechanism of oxLDL-induced BBB permeability and the effects of oxLDL on cerebral vascular reactivity. (**Chapter 4 and 5**)
5. To investigate the effect of $MgSO_4$ on oxLDL-induced cerebral dysfunction. (**Chapter 5**)
6. To investigate blood pressure and metabolic factors in a cohort of women that suffered from eclampsia compared to women that were diagnosed with preeclampsia only. **(Chapter 6)**

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Animal Studies:

Chapter 2:

The Adaptation of the Blood-brain Barrier to Vascular Endothelial Growth Factor and Placental Growth Factor during Pregnancy

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Abstract

Vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) are increased in the maternal circulation during pregnancy. These factors may increase blood-brain barrier (BBB) permeability, yet brain edema does not normally occur during pregnancy. We therefore hypothesized that in pregnancy, the BBB adapts to high levels of these permeability factors. We investigated the influence of pregnancy-related circulating factors on VEGF-induced BBB permeability by perfusing cerebral veins with plasma from non-pregnant (NP) or late-pregnant (LP) rats (n=6/group) and measuring permeability in response to VEGF. The effect of VEGF, PLGF and VEGF-receptor (VEGFR) activation on BBB permeability was also determined. Results showed that VEGF significantly increased permeability ($x10^7$ μm^3/min) from 9.7±3.5 to 21.0±1.5 ($p<0.05$) in NP veins exposed to NP plasma, that was prevented when LP veins were exposed to LP plasma; (9.7±3.8;$p>0.05$). Both LP plasma and soluble FMS-like tyrosine-kinase1 (sFlt1) in NP plasma abolished VEGF-induced BBB permeability in NP veins (9.5±2.9 and 12±2.6;$p>0.05$). PLGF significantly increased BBB permeability in NP plasma (18±1.4;$p<0.05$), and required only VEGFR1 activation whereas VEGF-induced BBB permeability required both VEGFR1 and VEGFR2. Our findings suggest that VEGF and PLGF enhance BBB permeability through different VEGFR-pathways and that circulating sFlt1 prevents VEGF- and PLGF-induced BBB permeability during pregnancy.

Introduction

The blood-brain barrier (BBB) comprises a unique endothelium compared with endothelium of peripheral organs in that it lacks fenestrations, contains high electrical resistance tight junctions, and has restricted paracellular flux of ions and proteins [1-3]. Under physiological conditions, these unique properties prevent entry of ions and large proteins into the brain from blood and minimize the effect of hydrostatic pressure on capillary filtration of water, all of which result in a strong protective mechanism against vasogenic brain edema [1-4]. During normal pregnancy, the endometrium, decidua and placenta initiate a large increase in release of cytokines and angiogenic growth factors into the circulation [5-8]. Many of these growth factors are essential for the increasing demand of the fetal-placental unit and normal intrauterine development of the fetus [6, 8, 9]. Several of these factors are vasoactive, can induce vascular permeability [6, 8], and may have the ability to affect BBB permeability. However, despite high levels of these permeability factors produced during pregnancy, vasogenic brain edema does not normally occur [10, 11]. Thus, the BBB appears to adapt during normal pregnancy to maintain its protective state in the face of these circulating permeability factors.

One growth factor important for a successful pregnancy is vascular endothelial growth factor (VEGF) [12]. It is produced in significantly higher levels during pregnancy by a variety of cells including endothelial cells and is increased both locally in the maternal-fetal unit and in the maternal circulation [13, 14]. VEGF was initially discovered as a vascular permeability factor, but it is now known to also have important roles in angiogenesis, vascular growth, endothelial cell survival and vasorelaxation, including in the brain and the cerebral circulation [15-19]. VEGF affects BBB permeability through a complex interaction between VEGF and its two VEGF receptors (VEGFRs) known in rats as FMS-like tyrosine kinase1 (Flt1) and fetal liver kinase1 (Flk1), but also known as VEGFR1 and VEGFR2, respectively. These two receptors can, after phosphorylation of the cytoplasmic tyrosine kinase domains of either Flt1 or Flk1, transphosphorylate adjacent domains ("cross-talk") and even form Flt1/Flk1 receptor-dimers [15, 19-21]. VEGFR-mediated signaling to induce vascular permeability has been studied intensely in peripheral tissues. However, it still remains unclear whether Flt1, a receptor with low tyrosine kinase activity compared to Flk1, is involved in VEGF-induced permeability or if it functions as a non-signaling reservoir for VEGF [15, 19-23]. To our knowledge, little is known about the

VEGFR-mediated signaling pathway in the cerebral endothelium to induce BBB permeability. Further, how the brain and BBB might adapt to high levels of the permeability factor VEGF during pregnancy is also not known.

In addition to VEGF, circulating levels of placental growth factor (PLGF), another member of the VEGF family, that is highly produced in the placenta during pregnancy, are also significantly elevated in the blood during normal pregnancy [24]. PLGF binds only to Flt1 and is involved in angiogenesis and endothelial cell viability, both by inducing its own signaling pathway and by amplifying VEGF-driven actions [20]. Although PLGF has been shown to increase vascular permeability in peripheral tissue, whether PLGF also affects the cerebral endothelium to enhance BBB permeability, or through which signaling pathway, are not known [21, 24, 25]. Thus, the first goal of this study was to determine if VEGF or PLGF could induce BBB permeability and by which VEGFR-mediated pathways. Because pregnancy is accompanied by increased levels of VEGF and PLGF in the circulation, our second goal was to investigate the adaptation during pregnancy that might diminish the influence of these permeability factors on BBB permeability.

The bioavailability of VEGF and PLGF is essential for successful pregnancy and is thought to be regulated by anti-angiogenic factors, such as soluble Flt1 (sFlt1) [12, 26], a soluble variant of Flt1 which lacks the cytoplasmic domains of Flt1 [27, 28]. A third trimester rise of sFlt1 reflects a physiologic anti-angiogenic shift of the placental milieu corresponding to the completion of placental growth [5, 6, 29]. Because sFlt1 is an important regulator of VEGF-mediated activity in the maternal-fetal unit, we hypothesized that an underlying mechanism by which the BBB is protected from the permeability effects of VEGF during normal pregnancy is through elevated levels of this soluble receptor. Therefore, we determined the influence of sFlt1 on VEGF-induced BBB permeability using levels found in normal pregnancy as well as in preeclampsia, a pregnancy-related hypertensive disorder with increased levels of sFlt1, beyond that of normal pregnancy that causes endothelial dysfunction in peripheral tissues. The influence of sFlt1 on VEGF signaling at the BBB is not known, but likely important because vasogenic brain edema is involved in neurological complications of preeclampsia [5, 30-33].

Materials and Methods

Animals. Female Sprague Dawley rats were used for all experiments. Female virgin nonpregnant (NP; 260-310g), or late-pregnant (LP; day 20; 300-340g) rats were purchased from Charles River, Canada. All of the procedures were approved by the University of Vermont Institutional Animal care and Use Committee and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were housed in the Animal Care facility, which is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. Animals had access to food and water ad libitum and maintained a 12-hour light/dark cycle.

Plasma samples. Plasma samples were obtained from trunk blood from female Sprague Dawley Rats, either virgin NP or LP (described as above). The plasma was collected in plasma separation tubes, pooled from approximately 20 rats to minimize biological diversity, and stored at -80°C until experimentation. For all experiments, plasma was diluted to 20% in HEPES buffer.

BBB permeability measurements. The effect of NP and LP rat plasma on BBB permeability and the role of VEGF, PLGF and sFlt1 were measured as previously described with slight modification [1]. Briefly, cerebral veins were carefully dissected out of the brain of either NP or LP rats and the proximal end mounted on one glass cannula in an arteriograph chamber. Veins were perfused intraluminally with NP (n=6) or LP (n=6) rat plasma in a HEPES buffer and equilibrated for 3 hours at 10±0.3 mmHg. After equilibration, intravascular pressure was increased to 25±0.1 mmHg and drop due to filtration was measured for 40 minutes. The decrease of intravascular pressure per minute (mmHg/min) was converted to volume flux through the vessel wall (μm^3) using a conversion curve, as previously described [1].

The first set of experiments tested the influence of VEGF and its receptor activation on BBB permeability by perfusing cerebral veins with NP plasma with or without the addition of 50 ng/ml VEGF (n=6/group). Receptor activation was determined by the addition of the selective antibodies against VEGFR1 (33 µg/ml for 75% neutralization rate; n=6) or VEGFR2 (4 µg/ml for 90% neutralization rate; n=6). A separate group of vessels was perfused with plasma plus a non-immune IgG (33 µg/ml; n=6) permeability measured as a control for the presence of the antibodies

in the lumen. A second set of experiments was performed to investigate the effect of PLGF on BBB permeability by the addition of 50 ng/ml PLGF to NP rat plasma perfused in a NP vein (n=6). Similarly, VEGFR activation in response to PLGF was determined by measuring permeability after the addition of selective VEGFR1 (33 µg/ml for 75% neutralization rate; n=6) or VEGFR2 (4 µg/ml for 90% neutralization rate; n=6) antibodies.

A third set of experiments was performed to examine the influence of pregnancy on VEGF-induced BBB permeability by measuring permeability after the addition of 50 ng/ml VEGF into NP veins perfused with NP plasma and a LP vein perfused with LP plasma (n=6). To isolate the effect of LP plasma on VEGF-induced BBB permeability, a separate group of NP veins was perfused with LP plasma and the permeability to VEGF measured (n=6). The last set of experiments was performed to determine the influence of sFlt1 on VEGF-induced BBB permeability during pregnancy by perfusing NP veins with 50 ng/ml (n=6) or 500 ng/ml (n=6) sFlt1 to NP plasma and measuring BBB permeability in response to 50ng/ml VEGF.

Determination of VEGF and VEGFR mRNA expression using real-time quantitative PCR. Total RNA was extracted from individual cerebral veins taken from NP (n=3-5) and LP rats (n=3-7) using the STAT-60 total RNA/mRNA isolation reagent (Tel-Test), as previously described [34, 35]. cDNA was synthesized from 1µg of total RNA per sample using SuperScript II reverse transcriptase and random hexamer primers with the SuperScript II Preamplification System (Invitrogen) in a 20-µl final reaction volume. All samples were reverse transcribed simultaneously to obviate reaction variability. The quantitative PCR standards for all transcripts were prepared with amplified rat VEGF, VEGFR1, VEGFR2, neuropilin-1, and 18S cDNA products ligated directly into pCR2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen); the fidelity of the cDNA inserts were verified by direct sequencing. The quantitative PCR primers for the rat transcripts were as follows: VEGF, (S) 5′-ATCATGCGGATCAAACCT-3′, (AS) 5′-ATTCACATCTGCTATGCT-3′; VEGFR1, (S) 5′-AAGACTCGGGCACCTATG-3′, (AS) 5′-TCGGCACCTATAGACACC-3′; VEGFR2, 5′-ACGGGGCAAGAGAAATGAAT-3′, (AS) 5′-GCAAAACAC-CAAAAGACCAC-3′; neuropilin-1, (S) 5′-CGCCTGGTGAGCCCTGTGGTCTATT-3′, (AS) 5′- TGTTCTTGTCGCCTTTCCCTTCTTC-3′. To estimate the relative expression of the receptor transcripts, 10-fold serial dilutions of stock plasmids were prepared as quantitative standards. For real-time quantitative PCR the reverse transcribed cDNA samples were diluted fivefold to minimize the

inhibitory effects of the RT reaction components and assayed using SYBR Green I JumpStart *Taq* ReadyMix (Sigma-Aldrich, St. Louis, MO) containing 5 mM $MgCl_2$; dATP, dGTP, dCTP, and dTTP (200 mM each); 0.64 U of *Taq* DNA polymerase; and primer (300 nM each) in a final 25-µl reaction volume. The real-time quantitative PCR was performed on an Applied Biosystems 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA) [34, 35] as follows: *1*) 94°C for 2 min and *2*) amplification over 40 cycles at 94°C for 15 s and at 58–62°C (depending on the primer set) for 30 s. SYBR Green I melting analysis of the amplified product from these amplification parameters was carried out by ramping the temperature of the reaction samples from 60°C to 95°C to verify unique product amplification in the quantitative PCR assays. Data analyses were performed with Sequence Detection Software version 1.4 (Applied Biosystems, Foster City, CA, USA) using default baseline settings. The standard curves were constructed by amplification of serially diluted plasmids containing the target sequence. The increase in SYBR Green I fluorescence intensity (ΔR_n) was plotted as a function of cycle number and the threshold cycle (C_T) was determined by the software as the amplification cycle at which the ΔR_n first intersects the established baseline. The transcript levels in each sample were calculated from the C_T by interpolation from the standard curve to yield the relative changes in expression. All data were normalized to 18S in the same samples; transcript levels in control NP samples were established as 100%.

Drugs and Solutions. HEPES physiological salt solution was made fresh daily and consisted of (mmol/L): 142.0 NaCl, 4.7 KCl, 1.71 $MgSO_4$, 0.50 EDTA, 2.8 $CaCl_2$, 10.0 HEPES, 1.2 KH_2PO_4, and 5.0 Dextrose. $VEGF_{165}$ and PLGF were purchased from Calbiochem, Gibbstown, NJ, USA (PF074 and 526610, respectively). VEGFR1 antibody, VEGFR2 antibody, sFlt1 and the goat-IgG antibody control were purchased at R&D systems, Minneapolis, MN, USA (321-FL, AF471, AF644 and AB-108-C, respectively). All drugs were diluted in sterile HEPES without glucose to prevent growth of bacteria and kept frozen at -80°C until use.

Statistical analysis. Data were presented as means ± standard error of the mean. Analyses were performed by one-way ANOVA with a post-hoc Student Newman Keuls test for multiple comparisons. Differences were considered statistical significant at $P < 0.05$.

Results

The effect of circulating VEGF and PLGF on BBB permeability and VEGFR activation. VEGF-induced BBB permeability involves complex signaling by its two receptors [15], however, little is known about VEGFR activation that induces BBB permeability. Thus, we determined BBB permeability in the cerebral vein of Galen from a NP rat perfused with plasma from a NP rat in which 50 ng/ml VEGF was added. The vein of Galen was used for all experiments because this vein has BBB properties and has been shown to be a major site of BBB disruption in pathological states, such as acute hypertension [36]. Permeability was also compared between veins that were perfused with selective Flt1 or Flk1 neutralizing antibodies to determine which receptors were involved in permeability. Figure 1 shows that VEGF significantly increased BBB permeability in NP veins. Neutralizing either Flt1 or Flk1 abolished VEGF-induced permeability, suggesting both receptors are necessary to induce BBB permeability in response to VEGF, with possible crosstalk between the two receptors. The IgG-antibody control group confirmed that the physical presence of the antibody itself in the plasma did not affect VEGF-induced BBB permeability. In addition to VEGF, PLGF has been shown to increase peripheral vascular permeability [24, 25], but little is known about the effect of PLGF on BBB permeability. Interestingly, after exposing a vein from a NP rat to NP plasma with 50 ng/ml PLGF, it also significantly increased BBB permeability similar to VEGF (Figure 2). To our knowledge, these results are the first showing the ability of PLGF to enhance BBB permeability. PLGF is known to interact only with Flt1, with higher affinity than VEGF [21]. As expected, selectively neutralizing Flt1, but not Flk1, inhibited PLGF-induced BBB permeability (Figure 2).

mRNA expression of VEGF and the VEGFRs in cerebral veins during pregnancy. During pregnancy, VEGF is highly expressed in peripheral tissue beyond that of the non-pregnant state [37]. However, whether VEGF expression is altered in cerebral vascular tissue during pregnancy that might affect BBB permeability is not known. Thus, we determined the expression of VEGF and its receptors in cerebral veins by qPCR from NP and LP rats. Figure 3A shows that VEGF mRNA expression was significantly increased in veins from LP versus NP rats. However, VEGFR1 and VEGFR2 mRNA expression was similar in veins from LP and NP rats (Figure 3B). Also, the expression of the neuropilin receptor, a receptor that enhances VEGFR activity [22], did not change. Thus, pregnancy caused increased VEGF expression in the cerebral circulation without an increase in VEGFR expression.

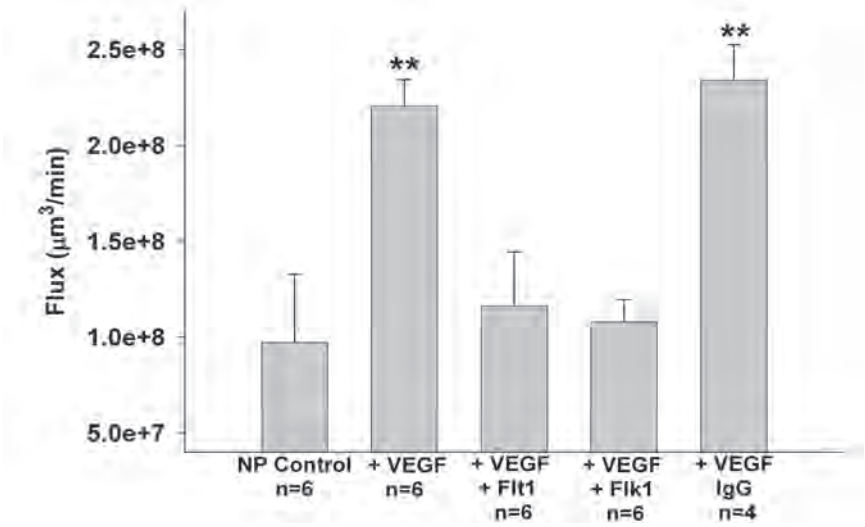

Figure 1: Effect of vascular endothelial growth factor (VEGF) on blood-brain barrier (BBB) permeability. Flux as a measure of BBB permeability of cerebral veins from nonpregnant (NP) rats perfused with plasma from NP rats in the absence or presence of 50 ng/ml VEGF. Selective neutralizing antibodies to VEGFR1 or VEGFR2 were also added to determine the role of each receptor in VEGF-induced BBB permeability. VEGF significantly increased BBB permeability. Neutralizing with specific antibodies (Ab) for either Flt1 or Flk1 prevented VEGF-induced BBB permeability. The IgG control group showed that the presence of antibody alone did not induce BBB permeability. (**p<0.01 vs. all)

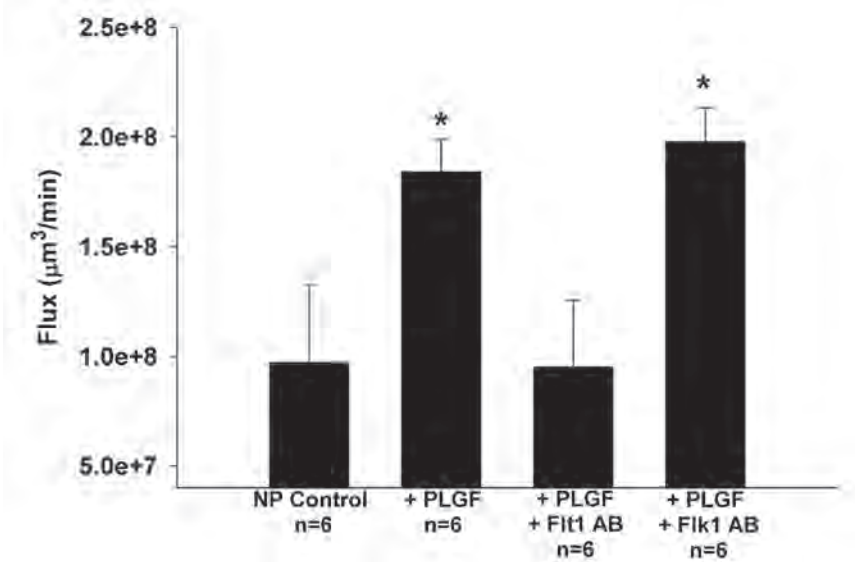

Figure2: **Effect of placental growth factor (PLGF) on blood-brain barrier (BBB) permeability.** Flux as a measure of BBB permeability of cerebral veins from nonpregnant (NP) rats perfused with plasma from NP rats in the absence or presence of 50 ng/ml PLGF. Selective neutralizing antibodies to VEGFR1 or VEGFR2 were also added to determine the role of each receptor in PLGF-induced BBB permeability. PLGF also significantly increased BBB permeability to a similar extent as VEGF. Neutralizing Flt-1, but not Flk1, abolished the PLGF-induced BBB permeability. (*p<0.05 vs. all).

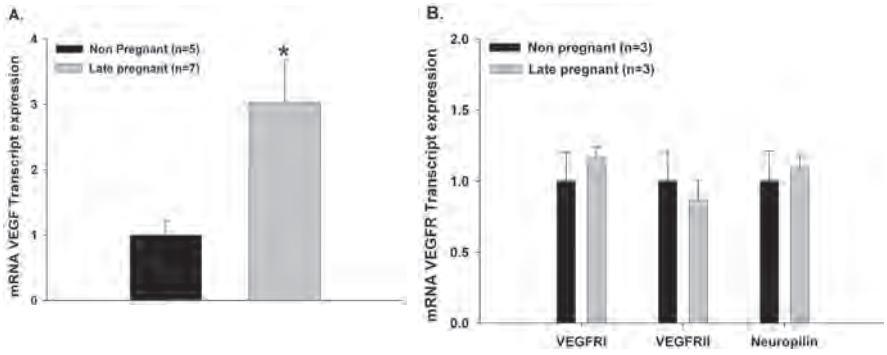

Figure 3: **Effect of pregnancy on vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) expression in cerebral veins using qPCR.** (A) mRNA expression of VEGF in cerebral veins from nonpregnant and late-pregnant rats. Expression of VEGF was significantly increased during pregnancy in cerebral veins. (B) mRNA expression of VEGFRs: VEGFR1 (Flt1), VEGFR2 (Flk1) and neuropilin in cerebral veins from nonpregnant and late-pregnant rats. There was no change in VEGFR expression with pregnancy. (*$p<0.05$ vs. nonpregnant)

The effect of plasma and sFlt1 on VEGF-induced BBB permeability in pregnancy. Circulating angiogenic factors during pregnancy may have an important role in protecting the BBB from permeability effects caused by factors such as VEGF and PLGF. Thus, we examined the role of circulating factors during pregnancy in mediating VEGF-induced BBB permeability. We choose VEGF as a well-known and widely accepted representative of all permeability factors in the VEGF family and thereby focused further experiments on examining the effect of VEGF. We compared permeability in veins from NP rats perfused with NP plasma and compared this to veins from LP rats perfused with LP plasma and measured BBB permeability in response to VEGF. Similar to the above result, Figure 4 shows that VEGF significantly increased BBB permeability in veins from NP animals perfused with NP plasma. However, VEGF had no effect on BBB permeability in veins from LP animals perfused with LP plasma. To determine if the lack of response to VEGF in veins from LP rats perfused with LP plasma was due to circulating factors in the LP plasma or to the vasculature being from LP animals, we perfused a cerebral vein from a NP animal with plasma from a LP animal and measured BBB permeability in response to VEGF. Importantly, LP plasma prevented the increase in BBB permeability in response to VEGF in NP veins, suggesting circulating factors in the LP plasma were responsible for the prevention of VEGF-induced BBB permeability during pregnancy.

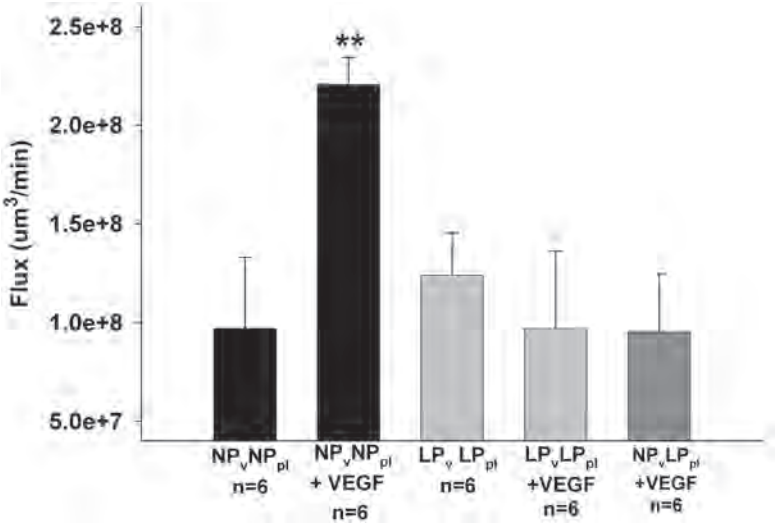

Figure 4: **Effect of pregnant plasma on vascular endothelial growth factor (VEGF)-induced blood-brain barrier (BBB) permeability.** Flux as a measure of BBB permeability in response to 50 ng/ml VEGF in cerebral veins from non-pregnant rats (NP_v) perfused with NP plasma (NP_{pl}) and veins from late-pregnant rats (LP_v) perfused with LP plasma (LP_{pl}). VEGF-induced permeability was prevented in LP vessels perfused with LP plasma. The lack of response to VEGF during pregnancy was due to circulating factors present in LP plasma since perfusing LP plasma in a cerebral vein from a NP rat also prevented VEGF-induced BBB permeability. (*p<0.05 vs. all).

Because elevated sFlt1 could be responsible for preventing VEGF-induced BBB permeability in LP plasma, we determined the specific effect of sFlt1 on VEGF-induced BBB permeability by perfusing NP plasma with 50 ng/ml sFlt1 into a vein from a NP rat and measuring BBB permeability in response to VEGF. We found that VEGF-induced BBB permeability was abolished by the presence of sFlt1, confirming the importance of sFlt1 in controlling VEGF actions at the BBB (Figure 5). Because excess levels of sFlt1 that lead to an angiogenic imbalance are thought to be important in the pathogenesis of preeclampsia, we created this angiogenic imbalance by increasing the sFlt1:VEGF ratio from 1 to 10, similar to preeclampsia [31, 38]. In this angiogenic imbalance, sFlt1 is thought to not only control VEGF-induced angiogenesis, but to also inhibit other VEGF-mediated actions which can result in vasoconstriction and endothelial cell damage [12, 32]. Interestingly, VEGF-induced BBB permeability was abolished similarly compared to the lower and more physiological concentration of sFlt1 (Figure5). Thus, sFlt1 also regulates VEGF-induced permeability at a high sFlt1:VEGF ratio, and does not appear to cause endothelial dysfunction in these cerebral veins.

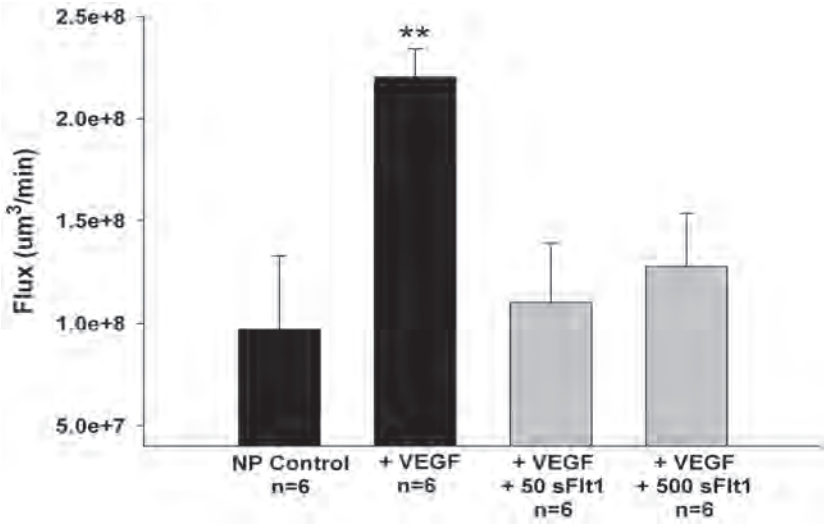

Figure 5: **Effect of soluble FMS-like tyrosine kinase-1 (sFlt1) on vascular endothelial growth factor (VEGF)-induced blood-brain barrier (BBB) permeability.** VEGF-induced permeability in cerebral veins from NP rats exposed to NP plasma was prevented by sFlt1 at both 50 ng/ml and 500 ng/ml, suggesting sFlt1 is an important circulating factor released during pregnancy that controls VEGF-induced permeability. (**p<0.01 vs. all).

Discussion

The major finding of this study was that circulating factors in LP plasma prevented VEGF-induced BBB permeability and preserved barrier function during pregnancy in the face of high circulating angiogenic factors. BBB permeability without exogenous VEGF was similar in both the NP and LP states, despite the finding that veins from LP rats showed increased VEGF mRNA expression. Exogenous VEGF initiated a significant increase in BBB permeability in the NP state, while it did not affect BBB permeability in LP. Interestingly, after exposing a vein from a NP rat to LP plasma with VEGF, increased BBB permeability was prevented, suggesting circulating factors in the LP plasma were responsible for this adaptation that limited the permeability effects of VEGF. Furthermore, sFlt1 regulated VEGF-induced permeability similar to LP plasma in that it abolished permeability in response to VEGF. Lastly, our results showed that PLGF had similar potential as VEGF to enhance BBB permeability. However, VEGF and PLGF appear to induce BBB permeability through distinct VEGFR activation, eg. PLGF-induced BBB permeability acted through Flt1, whereas VEGF-induced BBB permeability required activation from both receptors.

The BBB contains unique endothelium that provides a strong protective mechanism against edema in the brain [2, 3]. From our results here measuring transvascular water flux, VEGF can clearly increase BBB permeability. This finding is similar to what was found in previous studies measuring solute permeability of cerebral venules in vivo [16, 36]. In pregnancy, significant higher expression and levels of VEGF have been measured in the periphery and circulation, which is necessary for the development and maintenance of the maternal-fetal unit and uteroplacental circulation [5, 6, 12, 13]. Increased VEGF during pregnancy might also increase BBB permeability and promote edema formation. It is known that VEGF is expressed in the cerebral tissue regulating microvascular permeability [39], but the expression of VEGF in the brain during pregnancy, or the effect of VEGF on the BBB permeability in normal pregnancy have not been examined previously. Our results showed that similar to other tissues, there is increased expression of VEGF in cerebral veins during pregnancy, suggesting that the cerebral circulation may also be affected by higher levels of VEGF during pregnancy. Few studies examined the effects of VEGF on the BBB, and have shown increased expression of VEGF in the brain and in the cerebral circulation during brain injury that also caused vasogenic edema [16, 39, 40]. However, our results showed no increase in BBB permeability in pregnancy (Figure 4), despite increased expression of VEGF in the cerebral veins. There are 2 possible explanations for these findings. First, the BBB itself might adapt to the increased levels of VEGF during pregnancy through altered VEGFR expression in cerebral veins. Osol et al. examined VEGFR expression in uterine vessels and found increased VEGFR1 expression during pregnancy, accompanied by increased uterine venous permeability [41]. In contrast, our results showed no increased expression of VEGFRs in the cerebral veins and also no increase in BBB permeability. This could reflect a possible protective mechanism in the brain compared to the uterine circulation that requires increased vascular permeability for controlled extravasation of intravenous molecules in the maternal-fetal unit [42], while the BBB must maintain its protective state to prevent brain edema.

A second explanation for the apparent discrepancy between increased VEGF expression in the cerebral circulation and the lack of VEGF-induced BBB permeability during pregnancy is that there are circulating factors present in the LP plasma protecting the BBB from VEGF-induced BBB permeability. Our results showed that permeability in response to VEGF was prevented by LP plasma, supporting this concept. One important candidate is sFlt1 that is elevated during pregnancy

along with VEGF. It selectively binds VEGF and is an important regulator of the bioavailability of VEGF in both the nonpregnant and pregnant state. Our results here confirmed the importance of increased sFlt1 in normal pregnancy controlling the permeability actions of VEGF in the cerebral circulation as addition of sFlt1 in NP plasma abolished VEGF-induced BBB permeability. This finding is consistent with previous studies showing that sFlt1 controlled the actions VEGF during pregnancy in the maternal peripheral circulation [6, 12]. However, this is the first study that we are aware of showing sFlt1 critically regulates VEGF action at the BBB.

Although sFlt1 appeared to have an important role in limiting VEGF-induced BBB during normal pregnancy, it is also generally accepted that excess levels of sFlt1 are involved in the pathogenesis of preeclampsia and might cause endothelial damage [12, 31-33]. In this study, we measured VEGF-induced BBB permeability with a 10-fold higher concentration of sFlt1 than in the physiological condition of pregnancy. The VEGF-induced BBB permeability was abolished by higher concentrations of sFlt1 similarly as the physiological concentration. Thus, after acute exposure of increased sFlt1:VEGF ratio in cerebral veins, it did not appear to cause endothelial damage or have a greater effect on BBB permeability. However, this interpretation is limited because of the rather short exposure of the BBB to sFlt1 (3 hours). In addition, other cytokines present during pregnancy and preeclampsia were not examined in this study. However, previous studies also raised questions about the connection between sFlt1 with the etiology of neurological complications of preeclampsia. For example, Maynard et al. infused rats with high sFlt1 and found that these rats showed symptoms of preeclampsia, but none had complications of HELLP syndrome or eclampsia [27]. Karumanchi et al. also did not find BBB disruption in rats exposed to high sFlt1, but did find some BBB disruption in rat models exposed to both sFlt1 and soluble endoglin (sEng), suggesting an interaction of cytokines may be necessary for inducing vasogenic edema in preeclampsia [10]. It is worth noting that these previous studies lacked appropriate controls for both pregnancy and hypertension, ie., high sFlt1 likely causes similar effects in NP animals or the effects noted may be due to hypertension and not sFlt1. A recent study showed that sFlt1 did not cause endothelial damage itself, but sensitized VEGFRs to pro-inflammatory cytokines like TNF-α resulting in endothelial damage [43]. This finding could explain why in the present study exposure to excess sFlt1 did not increase BBB permeability and also why in previous studies rats treated with excess sFlt1 alone did not induce brain edema and neurological complications of

preeclampsia. Further, the association between sFlt1 and other pro-inflammatory cytokines present in preeclampsia such as TNF-α could explain why in normal pregnancy, a state with high levels of sFlt1, no apparent endothelial damage occurs.

In addition to VEGF, PLGF is also increased during pregnancy. This factor also has been shown to have permeability effects in the periphery [44], but to our knowledge no previous study has examined the effect of PLGF on BBB permeability. Previous studies showed that, similar to VEGF, PLGF is a strong vasodilator [41], however the role of PLGF in permeability during pregnancy was not investigated. Our results show, for the first time, that PLGF significantly increased BBB permeability and is a permeability factor in the cerebral endothelium. Little is known about the signaling pathway of PLGF that increases BBB permeability. In the present study we examined the receptor activation of both VEGF and PLGF and showed that PLGF-induced permeability was, as expected, abolished after neutralizing Flt1, but not after neutralizing Flk1. However, we also found that in contrast to PLGF, neutralizing either Flt1 or Flk1 abolished VEGF-induced BBB permeability, suggesting these two related factors need different receptor activation and possibly act through different signaling pathways to affect BBB permeability. Autiero et al. found using mass spectrometry that PLGF and VEGF interact with Flt1 with distinct patterns of tyrosine phosphorylation [21]. This result is consistent with our findings that VEGF and PLGF appear to act trough different receptor activation. Other studies also found that both VEGFRs are able to form heterodimers, especially after binding of VEGF, but not after binding PLGF [21, 45]. Further studies are needed to understand the distinct and complex signaling of VEGFRs after binding PLGF or VEGF in the cerebral endothelium that can affect BBB permeability.

Summary and perspectives

The present study found that VEGF and PLGF both induce BBB permeability through distinct VEGFR-mediated pathways. In addition, circulating sFlt1 controls the permeability actions of VEGF during pregnancy that may prevent vasogenic edema in normal gestation. To our knowledge, this is the first study showing effects of VEGF, PLGF and sFlt1 on the BBB during normal pregnancy. The finding that increased sFlt1 protects the BBB against VEGF-induced permeability raises

some new questions about the role of high sFlt1 in the onset of neurological complications in preeclampsia. As high sFlt1 appears to be protective of the BBB, there may be other pro-inflammatory factors released during preeclampsia, but not during normal pregnancy, that are responsible for increased BBB permeability and edema formation. Thus, these results may be important for understanding VEGF- and PLGF-induced BBB permeability during normal pregnancy when these factors are increased, but also for understanding the pathogenesis of neurological complications in preeclampsia.

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Chapter 3:

Pregnancy Enhances the Effects of Hypercholesterolemia on Posterior Cerebral Arteries

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Abstract

In preeclampsia, hyperlipidemia is enhanced compared to normal pregnancy that could adversely affect vascular function. In the cerebral vasculature, this could lead to dysregulation of cerebral blood flow and neurological complications. Here, we examined the effect of excessive hyperlipidemia, as seen in preeclampsia, on cerebral artery function and expression of inflammatory markers in pregnancy. Pregnant and nonpregnant rats received a 14-day high cholesterol diet or normal chow and posterior cerebral artery function was compared. High cholesterol significantly increased sensitivity of posterior cerebral arteries to the nitric oxide donor sodium nitroprusside that was accompanied by an ~12 fold increased mRNA expression of inducible nitric oxide synthase in late-pregnant rats only. Further, high cholesterol significantly increased peroxynitrite-induced dilation and decreased myogenic tone in cerebral arteries from late-pregnant compared to nonpregnant animals. These results suggest that pathologically high levels of cholesterol in pregnancy enhance inflammatory responses and peroxynitrite generation in cerebral arteries.

Introduction

Preeclampsia is a complex, hypertensive disorder that affects 5-8% of all pregnancies and is a main cause of maternal mortality in developed countries.[1,2] It is established that endothelial vascular dysfunction plays a central role in the pathogenesis of preeclampsia, however, contributory causes remain to be elucidated.[2, 3] Epidemiological data suggest that women with previous preeclampsia have an increased risk of atherosclerotic vascular disease in later life, suggesting common risk factors are involved.[4-6] Pregnancy might act as a "stress-test" that unmasks a woman's predisposition to metabolic syndrome that may re-emerge in later life, thus increasing the risk for atherosclerotic vascular disease.[7] Metabolic syndrome includes hyperlipidemia which is known to compromise endothelial function and contribute to atherosclerotic vascular disease, but may also be involved in endothelial dysfunction that occurs in preeclampsia.[8, 9]

During normal pregnancy, plasma lipid levels including cholesterol are increased due to elevated production of pregnancy-related hormones.[10, 11] This hormonally controlled "physiological hyperlipidemia" encourages lipogenesis and fat storage in preparation for rapid fetal growth in late-pregnancy, but does not normally cause endothelial dysfunction in the maternal vascular system.[10] However, women who develop preeclampsia might demonstrate poor adaptation to this stress-test in pregnancy because the increase in lipid levels is more dramatic compared to normal pregnant women.[8] This excessive hyperlipidemia in preeclampsia has an atherogenic profile, characterized by increased low-density lipoprotein (LDL) that may cause acute atherosclerosis in the maternal-fetal unit, vascular dysfunction and inflammation.[12, 13] The term "acute" reflects that the damaging effects induced by lipids in pregnancy develop over a shorter time compared to outside of pregnancy, suggesting that pregnancy is more susceptible to damaging effects of excessive high lipid levels.[12] If these acute events caused by excessive hyperlipidemia in pregnancy occur in the cerebral vasculature, it could promote dysregulation of cerebral blood flow and contribute to neurological complications seen in preeclampsia. We hypothesized that pathologically high levels of lipids during pregnancy will cause cerebral vascular dysfunction that may contribute to neurological complications as seen in preeclampsia. To our knowledge, no study has investigated the effects of hyperlipidemia on cerebral vascular function in pregnancy. Thus, the first aim of this study was to determine the effect of excessive hyperlipidemia on cerebral artery function in pregnancy compared to the nonpregnant state.

Hypercholesterolemia enhances oxidative stress by increasing the production of superoxide through enhanced NAPDH-oxidase activity.[14, 15] Superoxide rapidly binds nitric oxide (NO) which is elevated in pregnancy to form peroxynitrite ($ONOO^-$), a relatively stable reactive oxygen and nitrogen species.[16-18] $ONOO^-$ is known to cause endothelial and vascular dysfunction due to reduced bioavailability of NO.[19, 20] Increased $ONOO^-$ generation has been shown in the maternal-fetal unit and the systemic vasculature of women with preeclampsia.[21] In addition, NADPH-oxidase activity is greater in the cerebral circulation compared to the systemic vasculature, potentially making the production of superoxide through NADPH-oxidase induced by hypercholesterolemia greater in the cerebral vasculature.[22] Thus, we hypothesized that the cerebral vasculature would be particularly adversely affected by substantial $ONOO^-$ generation due to increased NADPH-oxidase expression stimulated by excessive hyperlipidemia in pregnancy. Therefore, the second aim of this study was to determine the effects of $ONOO^-$ generation due to pathological high levels of cholesterol on cerebral artery function in pregnancy. Additionally, we measured mRNA expression of inducible nitric oxide synthase (iNOS) and the NADPH-oxidase isoform NOX2 in the cerebral vasculature under conditions of high cholesterol as potential sources of NO and superoxide, respectively.

Methods

Animals. All animal procedures were approved by the University of Vermont Institutional Animal Care and Use Committee and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female Sprague Dawley rats aged 12-14 weeks were used for all experiments. Early pregnant rats (day 6 of gestation) and age-matched virgin nonpregnant rats were purchased (Charles River, Canada) and housed for 14 days at the University of Vermont Animal Care Facility, an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. Rats were divided into 4 groups: nonpregnant control (NP-CTL; n=7), nonpregnant high cholesterol (NP-HC; n=8), late-pregnant control (LP-CTL; n=6) and late-pregnant high cholesterol (LP-HC; n=7). NP-CTL and LP-CTL received Prolab® 3000 for 14 days with day 14 being the day of experimentation. Because our goal was to achieve a significant increase in cholesterol levels in NP-HC and LP-HC rats, these animals received a 14 day diet consisting of Prolab® 3000 rodent chow, including 2% cholesterol and 0.5% cholic

acid (added to lower hepatic clearance of cholesterol) to significantly increase total cholesterol and LDL-cholesterol, as shown in earlier reports.[23] All groups of animals had access to food and water ad libitum and maintained a 12-hour light/dark cycle.

Plasma cholesterol measurements. Plasma samples were obtained from trunk blood from all rats. Blood was collected in EDTA separation tubes and centrifuged for 10 minutes at 2500 revolutions per minute. Plasma was aliquoted and stored in -80ºC until needed for the lipid measurements. Lipid measurements, including total cholesterol and HDL cholesterol, were measured on the Roche Cobas Integra 400 Plus instrument using an enzymatic colorimetric method per manufacturer's protocol (Roche Integra®). LDL-c was calculated using the Friedewald's equation according to manufacturer's protocol.

Experimental protocols. On the day of experiment, animals were anesthetized with 3% isoflurane in oxygen and quickly decapitated. The brain was removed and placed in cold physiological salt solution (PSS). Two third-order branches of the posterior cerebral artery (PCA) were carefully dissected for isolated arteriograph experiments. Vessels were mounted on 2 glass cannulas within an arteriograph chamber and experimented separately. Arteries were perfused with PSS at a pH of 7.40 ± 0.05 and a temperature of 37.0 ± 0.5 °C. The proximal cannula of the arteriograph was connected to a pressure transducer and servo controller that maintained intravascular pressure at a set pressure or changed manually. The distal cannula was closed to avoid flow-mediated responses. Lumen diameter was continuously measured via video microscopy during the entire experiment.

The first experiment was started after an equilibration time of 1 hour at a pressure of 50 mmHg. Intravascular pressure was then increased in steps of 25 mmHg to 150 mmHg, which reflects the myogenic pressure range of these cerebral arteries. Active lumen diameter was recorded at each pressure once stabilized. Pressure was then returned to 75 mmHg and vessel diameter was compared to the vessel diameter at 75 mmHg prior to the pressure steps to confirm that tone was restored. For the remainder of the experiment, the pressure was kept on 75 mmHg. A single high concentration (0.1mmol/L) of the NOS inhibitor L-nitro-arginine (L-NNA) was added to the bath and constriction in response to NOS inhibition was measured. After approximately 20 minutes in the presence of L-NNA, the NO

donor sodium nitroprusside (SNP) was cumulatively added to the bath (10^{-8} M-10^{-5} M) and the dilation to SNP was measured at each concentration. At the end of the experiment, zero-Ca^{2+} PSS was added to the bath to obtain fully relaxed diameters. Passive diameters were then recorded at pressures from 5 to 175 mmHg using video microscopy.

The second experiment was performed to determine the influence of the $ONOO^-$ decomposition catalyst FeTMPyP on active myogenic responses in the high cholesterol-treated animals. This particular compound was chosen because previous studies have shown it is selective for blocking $ONOO^-$ effects without interfering with NO or superoxide. [24] The second third-order branch of PCA was mounted in the arteriograph and perfused with 50μM FeTPMyP. This concentration was used based on one of our earlier studies where FeTMPyP was used in isolated arteries to scavenge $ONOO^-$.[25] After equilibration, intravascular pressure was increased in steps of 25 mmHg to 150 mmHg. Active lumen diameter was recorded at each pressure once stabilized. Afterwards, zero-Ca^{2+} PSS was added to the bath and passive diameters were recorded at pressures from 5 to 175 mmHg.

Messenger RNA expression of iNOS and NOX 2. Because both left and right PCAs from each animal were used for the isolated artery experiments, the middle cerebral arteries (MCAs) from the same animals were used for PCR analysis of iNOS and NOX2. Total RNA was extracted from middle cerebral arteries from NP-CTL (n=6), NP-HC (n=6), LP-CTL (n=6) and LP-HC (n=7) by using Trizol reagent (Life Technologies) followed by purification using an Rneasy Micro Kit (Qiagen) per manufacturer's protocols. RNA concentrations and quality were determined using an Agilent Bioanalyzer (Agilent). Real-time PCR was performed in a two-step process. RNA was reverse transcribed using a mix of oligo dT primers and random primers using the iScript cDNA Synthesis Kit (Biorad). For each sample cDNA was used to amplify the target genes iNOS and NOX2 and two housekeeping genes, Hprt1 and Ywhaz. Primers were designed by the Obstetrics and Gynecology Departmental Molecular Core Facility at the University of Vermont using PrimerSelect (DNASTAR). The quantitative PCR primers for the rat transcripts were: iNOS (f): -GAGAAGTCCAGCCGCACCAC- and (r): -GAACAATCCACAACTCGCCCAAG-; NOX2 (f): -TTAGCATCCATATCCGCATTGT-TG- and (r): CTATCTTAGGTAGTTTCCAGGCATCTT; Hprt1 (f): -CTCATGGACTGTT-ATGGACAGGAC- and (r): -GCAGGTCAGCAAA-GAACTTATAGCC-; Ywhaz (f): -GATGAAGCCATTGCTGAACTTG- and (r): -

GTCTCCTTGGGTATCCGATGTC-. One microliter of cDNA was used per reaction with 150 nM of the forward and reverse primers and 12.5 µl of Power Sybrgreen Master mix (Life Technologies) in a 25 µl reaction. The reactions were performed using an initial denaturation of 3 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C, followed by a melt curve analysis to ensure only the correct product was amplified. One set of PCR products for each gene were checked for correct size on a 2% Agarose gel. Each sample was run in triplicate on the ABI 7000 Sequence Detection System (ABI). Negative water controls were run for each primer set in the real-time PCR reaction to ensure no contamination in the reagents as well as no secondary primer structures were amplified. In each primer set at least one primer was designed over an exon-exon junction or the amplicon was designed to span an exon-exon junction to make sure that genomic DNA was not amplified.

Data Calculations Percent tone was calculated as the percent decrease in diameter from the fully relaxed diameter in zero-Ca^{2+} PSS by the equation: $((Ø_{passive} - Ø_{active})/ Ø_{passive}) \times 100\%$, where $Ø_{passive}$ is the diameter in zero- Ca^{2+} PSS and $Ø_{active}$ is the diameter with tone. Percent constriction to L-NNA was calculated with the equation: $((Ø_{baseline}-Ø_{drug})/ Ø_{baseline}) \times 100\%$ where $Ø_{baseline}$ is diameter before giving L-NNA and $Ø_{drug}$ is diameter with presence of L-NNA. Percent sensitivity to the NO donor SNP using a concentration-response curve was calculated with the equation: $((Ø_{drug}-Ø_{minimum})/(Ø_{maximum} – Ø_{minimum})) \times 100\%$ where $Ø_{drug}$ is diameter at a specific concentration of SNP, $Ø_{minimum}$ is diameter at the lowest concentration of SNP and $Ø_{maximum}$ is the diameter at the highest concentration of SNP. To calculate the effective concentration that produced half maximal dilation (EC_{50}) of SNP, the concentration-response curve was log transformed and plotted for each experiment separately. The EC_{50} was then determined off the best-fit curve and averaged for each group. For PCR measurements, relative expression was determined using the $2^{-\Delta\Delta CT}$ method.[26]

Drugs and solutions. A bicarbonate-base PSS buffer was used for all experiments. The composition was (mmol/L) NaCl 119.0, $NaHCO_3$ 24.0, KCl 4.7, KH_2PO_4 1.18, $MgSO_4 7H_2O$ 1.17, $CaCl_2$ 1.6, EDTA 0.026, and glucose 5.5. PSS was aerated with 5% CO_2, 10% O_2 and 85% N_2 to maintain pH at 7.40 ± 0.05 and stored without glucose at 4°C. Glucose was added before each experiment. L-NNA and SNP were purchased from Sigma, FeTMPyP was purchased from Calbiochem.

Statistical analysis. Data from the isolated artery experiments are presented as mean ± standard error of the mean. Data from PCR measurements are presented as means only because values were averaged in each group due to limited samples. Data were analyzed using one-way ANOVA and post hoc Student-Newman Keuls test for multiple comparisons where appropriate or with a Student t-test. Differences were considered statistically significant when $P<0.05$.

Results

Plasma cholesterol levels in NP and LP rats. Total cholesterol levels were similar in NP and LP control animals, being 77 ± 3mg/dl and 79 ± 6 mg/dl respectively. High cholesterol diet caused a significant increase in total cholesterol levels in both LP-HC and NP-HC animals. In NP-HC rats, total cholesterol increased 550% to 500 ± 52 mg/dl and in LP-HC total cholesterol increased 740% to 669 ±81 mg/dl. The difference between total cholesterol levels in NP-HC and LP-HC animals was not statistically different (P=0.12). LDL-c levels could not be calculated in the control animals because levels of determinants needed for calculating LDL-c were too low. LDL-c levels in NP-HC animals were 400 ± 40 mg/dl and in LP-HC animals 547 ± 78 mg/dl (P= 0.13). HDL was increased only in LP-HC animals from 42 ± 4 mg/dl in LP-CTL rats to 77 ± 6 mg/dl in LP-HC rats ($P<0.05$). In NP-HC rats, HDL-cholesterol remained stable compared to NP-CTL rats and was 50 ± 5 mg/dl and 44 ± 5mg/dl respectively (P=0.27). We also calculated the HDL/LDL ratio and found no difference between LP-HC and NP-HC animals being 0.14 and 0.13 respectively, which confirms significant hypercholesterolemia in both NP and LP animals.

Effect of hypercholesterolemia on active myogenic responses in PCAs from NP and LP rats. A high cholesterol diet caused a trend towards decreased tone in PCAs from both goups of animals, although this decrease was not statistically different. In NP-CTL rats, percent tone of PCAs at the physiological pressure of 75 mmHg was 14 ± 6 % compared to 5 ± 2% in NP-HC rats (P=0.18). Percent tone in PCAs from LP-CTL rats was 11 ± 6 % compared to 4 ± 3 % in LP-HC rats (P=0.25).

Effect of hypercholesterolemia on constriction to NOS inhibition in PCAs from NP and LP animals. To assess the contribution of endothelium-derived NO to tone, constriction to NOS-inhibition was determined by the addition of a single high

concentration of L-NNA to the cerebral arteries from all 4 groups of animals. L-NNA caused constriction in PCAs from all animals, suggesting basal NO that inhibits tone was present in all vessels. However, there were no significant differences in the amount of constriction between the animals. In NP-CTL animals, the percent constriction was 20 ± 3 % compared to 18 ± 2 % in NP-HC animals ($P > 0.05$). In LP-CTL rats, the percent constriction to L-NNA was 22 ± 5 % compared to 18 ± 2% in LP-HC rats ($P > 0.05$).

Effect of hypercholesterolemia on NO-mediated vasodilation in the PCAs from NP and LP animals. To determine if the response of the smooth muscle to NO was different in PCAs from high cholesterol-treated animals, the sensitivity to the NO donor SNP was compared. A high cholesterol diet in NP rats did not cause significant changes in the response of cerebral arteries to SNP compared to NP-CTL animals (Figure 1A). However, PCAs from LP-HC rats displayed significantly greater sensitivity to SNP compared to LP-CTL animals (Figure 1B). Further, the EC_{50} of SNP showed no difference between NP-CTL and NP-HC animals (Figure 1C). However, the EC_{50} was significantly lower in the LP-HC rats compared to LP-CTL animals (Figure 1D). These results suggest that the vascular smooth muscle in PCAs from LP-HC animals was more sensitive to NO compared to the cerebral arteries from the other groups.

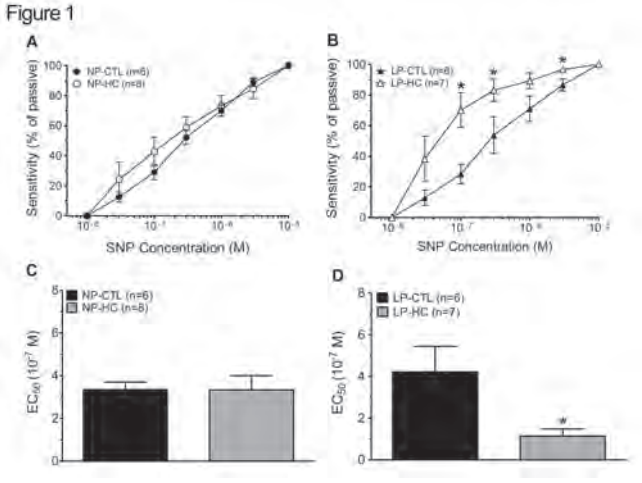

Figure 1: **Effect of hypercholesterolemia on SNP vasodilation in posterior cerebral arteries from nonpregnant and late-pregnant rats.** Graphs showing sensitivity to the NO donor SNP (A,B) and the EC_{50} of SNP (C,D) as percentage of the maximum dilation in the presence of NOS-inhibition in posterior cerebral arteries (PCAs). There was no difference in dilation to SNP in PCAs from NP-CTL or NP-HC rats. However, PCAs from LP-HC rats showed a significant increase in sensitivity to SNP compared to LP-CTL animals. NP-CTL: nonpregnant control animals; NP-HC: nonpregnant high cholesterol-treated animals; LP-CTL: late-pregnant control animals; LP-HC: late-pregnant high cholesterol-treated animals. *P<0.05 vs. LP-CTL.

The effect of FeTMPyP on active myogenic responses in PCAs from high cholesterol-treated NP and LP rats. We determined the effect of the $ONOO^-$ decomposition catalyst FeTMPyP on active myogenic responses in PCAs from high cholesterol-treated NP and LP rats only by perfusing the PCAs with 50 μM FeTMPyP. Treatment with FeTMPyP caused PCAs from both NP-HC and LP-HC rats to display significantly smaller diameters (Figure 2A). Interestingly, the decrease in diameter after perfusion with FeTMPyP was significantly greater in the PCAs from the LP-HC animals compared to the NP-HC animals. Further, PCAs from NP-HC and LP-HC animals perfused with FeTMPyP caused a significant increase in pressure-induced tone between 75 mmHg and 150 mmHg (Figure 2B). In addition, tone in PCAs from LP-HC animals perfused with FeTMPyP was significantly greater than the tone in FeTMPyP-treated PCAs from the NP-HC animals. Thus, FeTMPyP decreased vessel diameter and increased tone in PCAs from both NP-HC and LP-HC animals, an effect that was more pronounced in the LP-HC animals.

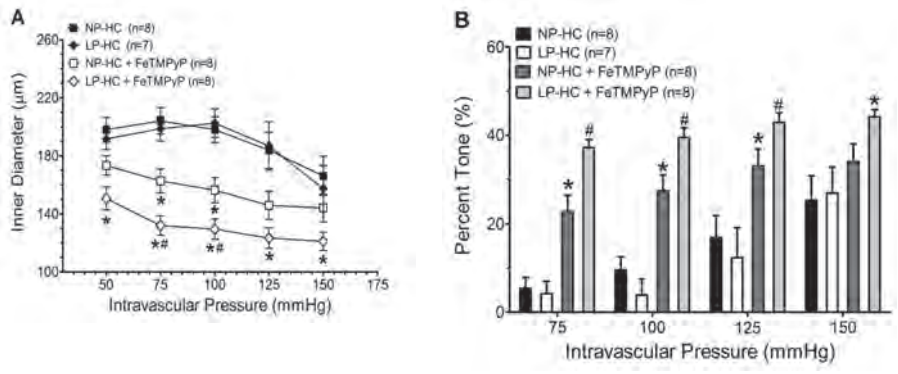

Figure 2: **Effect of FeTMPyP on the active lumen diameter and percent tone of posterior cerebral arteries from nonpregnant and late-pregnant high cholesterol-treated rats.** Graphs showing active lumen diameter (A) and percent tone (B) of posterior cerebral arteries (PCAs) as a function of intravascular pressure. There were no differences in active lumen diameter between NP-HC and LP-HC. However, treatment with the $ONOO^-$ scavenger FeTMPyP caused a significant decrease in active lumen diameter in PCAs from both NP-HC and LP-HC animals, that was significantly greater in LP-HC animals compared to NP-HC animals. Treatment with FeTMPyP caused a significant increase in tone in the PCAs from both NP-HC and LP-HC animals, that was significantly higher in FeTMPyP treated LP-HC animals compared to FeTMPyP treated NP-HC animal. NP-CTL: nonpregnant control animals; NP-HC: nonpregnant high cholesterol treated-animals; LP-CTL: late-pregnant control animals; LP-HC: late-pregnant high cholesterol-treated animals. *P<0.05 vs. NP-HC and LP-HC. #P<0.05 vs. all.

Effect of high cholesterol diet on iNOS and NOX-2 mRNA expression in middle cerebral arteries from NP and LP rats. We have previously shown that PCAs from normal pregnant animals have increased iNOS mRNA expression.[27] Therefore, we wanted to determine the effect of high cholesterol diet on iNOS mRNA expression that may contribute to increased sensitivity to SNP and increased $ONOO^-$ generation in PCAs from LP-HC rats. We measured the relative change in mRNA levels of iNOS in MCAs from NP-HC, LP-CTL and LP-HC animals compared to NP-CTL animals (Figure 3A). Because only 2 vessels in both the NP-CTL and NP-HC groups showed expression of iNOS within 40 treshold cycles (C_T), we averaged the ΔC_T's values of the vessels from these groups. This approach was then used for all other comparisons. Also, vessels were excluded if replicates were > 0.5 C_T apart. Treatment with high cholesterol in NP rats did not cause an increase in iNOS expression in cerebral arteries. However, expression of iNOS was increased in pregnancy compared to the nonpregnant state, as reported previously.[27] In addition, high cholesterol diet in pregnancy caused a ~12 fold increase in iNOS mRNA expression compared to NP-CTL rats which was a higher than the ~6 fold increase in LP-CTL rats.

NADPH-oxidase has 3 different isoforms expressed in the cerebral vasculature (NOX1, NOX2 and NOX4), however, NOX2 has been shown to be the major source for superoxide generation.[28] Thus, we wanted to determine the relative change in mRNA expression of NOX2 in MCAs from NP-HC, LP-CTL and LP-HC animals compared to NP-CTL animals (Figure 3B). Treatment with high cholesterol in NP rats caused an ~2 fold increase in mRNA expression of NOX2 compared to NP-CTL rats. However, there was no increase in mRNA expression of NOX2 in the cerebral vasculature from LP-CTL or LP-HC animals compared to NP-CTL animals.

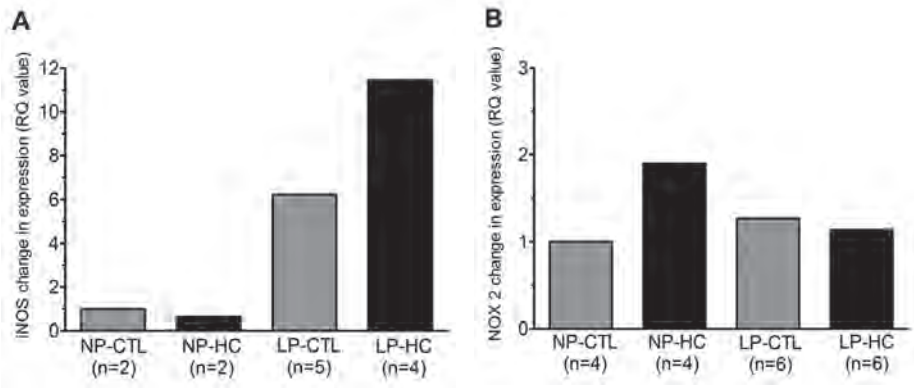

Figure 3: **Effect of high cholesterol on mRNA expression of iNOS and NOX2 in middle cerebral arteries from nonpregnant and late-pregnant animals.** Graphs showing the relative change in mRNA expression for iNOS (A) and NOX2 (B) in middle cerebral arteries from LP-CTL, LP-HC and NP-HC animals compared to NP-CTL animals. Cholesterol treatment in NP rats caused no change in iNOS mRNA expression. In contrast, pregnancy caused an ~6 fold increase in iNOS mRNA expression and an ~12 fold increase in iNOS mRNA expression when LP rats were treated with high cholesterol. High cholesterol caused an ~2 fold increase in NOX2 mRNA expression in NP rats. However, pregnancy did not appear to change the mRNA expression of NOX2 with or without treatment of high cholesterol. NP-CTL: nonpregnant control animals; NP-HC: nonpregnant high cholesterol-treated animals; LP-CTL: late-pregnant control animals; LP-HC: late-pregnant high cholesterol-treated animals.

Discussion

In the present study, we examined the effects of pathologically high levels of lipids, as seen in preeclampsia, in the cerebral vasculature of pregnant animals. Our results showed that an excessive hyperlipidemia caused PCAs to have less myogenic tone in both NP and LP animals. In addition, the $ONOO^-$ decomposition catalyst FeTMPyP caused significantly smaller vessel diameters and increased myogenic tone in both LP-HC and NP-HC animals, with the effect being greater in the LP-HC animals compared to the NP-HC animals. Further, treatment with high cholesterol sensitized PCAs from LP animals to the effects of the NO donor SNP and increased iNOS mRNA expression that was not seen in high cholesterol-treated NP animals. Taken together, excessive hyperlipidemia caused a greater inflammatory response and oxidative stress in cerebral arteries in pregnancy compared to the nonpregnant state.

Preeclampsia presents with an excessive hyperlipidemia compared to normal pregnancy that may adversely affect overall vascular function as seen in

atherogenic vascular disease.[13, 29, 30] In pregnancy, excessive hyperlipidemia causes acute atherosclerotic changes in the maternal-fetal unit over a shorter period of time than outside of pregnancy.[12] This suggests that in some women, pregnancy is more sensitive to the damaging effects of high cholesterol. In the present study, we sought to determine the effects of excessive hyperlipidemia in pregnancy compared to the nonpregnant state by using a high cholesterol rat model. Although we attempted to match the levels seen in preeclamptic women, the levels of cholesterol in the HC-treated rats were higher than those measured during preeclampsia and should be considered in the interpretation of the results. However, because the levels were pathologically high compared to control rats, we were able to determine the effects of high cholesterol in pregnancy on PCA function. Only a few studies measured active myogenic responses in the cerebral vasculature during hypercholesterolemia and to our knowledge no studies investigated the effects in pregnancy.[31-34] Myogenic responses describe the extent of constriction of the vessels in response to an increase or decrease in pressure and are important to maintain cerebral blood flow.[31] We found that hypercholesterolemia caused a trend to decrease myogenic tone in both LP-HC and NP-HC groups. This is in contrast with Ramirez et al, where they found an increase in myogenic constriction in mesenteric arteries in pregnancy due to hypercholesterolemia.[32] However, the cerebral vasculature differs from the systemic vasculature and may experience different effects from hypercholesterolemia. For example, McCalden et al. found a decrease in myogenic constriction in cerebral arteries due to hypercholesterolemia, however, this study was performed in rabbits instead of rats and pregnancy was not examined.[33] It is worth noting that changes in tone due to treatment with high cholesterol were mild and not statistically significant. In addition, responses to L-NNA were similar in both high cholesterol treated groups compared to the CTL animals. This is in contrast to a previous study that found that cerebral arteries in monkeys that received long-term atherogenic diet had reduced response to soluble guanylate cyclase inhibition, suggesting decreased NO production during atherosclerosis.[34] It is possible that this lack in significant differences could be explained by limitations of our rat model. First, rats are known to adapt to high cholesterol and exhibit little to no atherosclerotic changes because of the ability to carry most plasma cholesterol in HDL-particles and a high rate of hepatic clearance.[35] Also, in the present study, the high cholesterol diet was started at day 6 of pregnancy resulting in a 14-day high cholesterol diet. Thus, the duration of the diet may have been too short to unmask significant differences in

the active myogenic responses due to hypercholesterolemia in the pregnant and nonpregnant animals.

The mechanism of decreased myogenic tone in hypercholesterolemia may be explained with increased $ONOO^-$ generation. Previous reports have shown that hyperlipidemia causes increased $ONOO^-$ generation in the systemic vasculature and other organs.[20, 36] We found that by using the $ONOO^-$ decomposition catalyst FeTMPyP, vessel diameters significantly decreased and myogenic tone significantly increased in PCAs from both NP-HC and LP-HC animals. Because FeTMPyP is considered suitable to assess direct $ONOO^-$ contribution in functional rat studies, we suggest that presence of $ONOO^-$ significantly inhibited tone in these high cholesterol treated animals.[37] Our findings are consistent with multiple previous reports that $ONOO^-$ reduced tone in the cerebral vasculature. [38-40] However, these studies were not related with hypercholesterolemia. One other study showed in a rabbit model that hypercholesterolemia decreased basal vascular tone in systemic resistance arteries due to an increased production of NO.[41] These findings are similar to our findings since increased NO production could lead to increased $ONOO^-$ generation. Interestingly, in the present study, the effect of FeTMPyP was significantly greater in the LP-HC animals compared to the NP-HC animals, suggesting pathological high levels of lipids in pregnancy result in higher generation of $ONOO^-$ compared to NP-HC animals. This increased $ONOO^-$ generation due to high cholesterol in pregnancy would be of particular interest in the pathogenesis of preeclampsia, where increased levels of $ONOO^-$ have been found in the placental circulation in preeclampsia.[21]

Previous reports showed that hypercholesterolemia induces cerebral artery dysfunction through NADPH-oxidase activity, which is the major source of production of superoxide.[14, 15, 42] Superoxide rapidly binds to NO to form $ONOO^-$.[20] In addition, NADPH-oxidase activity is greater in the cerebral vasculature compared to the systemic vasculature.[42] The NADPH-oxidase isoform NOX2 appears to be more important than other isoforms in superoxide production in the cerebral vasculature, especially during pathological conditions including hypercholesterolemia.[14, 28] Interestingly, while the effects of FeTMPyP were the highest in LP-HC animals, our results showed that only the NP-HC, but not the LP-HC animals, had an ~2-fold increase in mRNA NOX2 expression. Thus, in LP-HC animals, high $ONOO^-$ generation was not likely due to high superoxide production

from increased NOX2 expression. However, our measurements were limited to mRNA expression of NOX2 only and it could be that NOX2 activity or other enzymes that generate superoxide are increased in pregnancy compared to the nonpregnant state.

Another explanation for the increased generation of $ONOO^-$ in pregnancy compared to the nonpregnant state could be the increased expression of iNOS. iNOS is known to produce excessive amounts of NO that is a critical factor for $ONOO^-$ generation.[20] Our results showed that high cholesterol treatment caused an increase in iNOS mRNA expression in pregnancy compared to the nonpregnant state. Although our data are limited to mRNA expression, these results showed an increase in iNOS mRNA and support our earlier work that pregnancy alone can be considered as a state of inflammation.[27] Interestingly, high cholesterol treatment further increased iNOS mRNA expression in the cerebral arteries from LP animals, which has also been found in placental tissue from preeclamptic women compared to normal pregnant women and in human umbilical vein cells after exposure to preeclamptic serum.[43-45] The increase in iNOS expression in the LP-HC animals may also be related to the increased sensitivity to the NO donor SNP. Because the enhanced dilation to SNP occurred with NOS inhibition, an increased sensitivity of the vascular smooth muscle to the effects of NO caused by iNOS is most likely. For example, Gunnett et al. showed that iNOS opposes contraction by activating soluble guanylate cyclase in the smooth muscle that inhibits contractile responses and sensitizes the vascular smooth muscle to the effects of NO.[46] In contrast, treatment with high cholesterol did not increase iNOS mRNA expression or sensitivity to dilation to SNP in the nonpregnant animals. This suggests that cerebral arteries are more sensitive to pathological high levels of lipids in pregnancy compared to the nonpregnant state, resulting in an excessive amount of NO and $ONOO^-$ generation.

In summary, this study was designed to determine if pathological levels of high cholesterol, similar to preeclampsia, would induce cerebral artery dysfunction during pregnancy. We found that oxidative stress in high cholesterol-treated LP animals was increased compared to high cholesterol-treated NP animals that may be explained by an enhanced inflammatory state, as suggested by increased iNOS expression. However, further studies are needed to determine what effect this may have in the pathogenesis of preeclampsia.

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Chapter 4:

Increased Oxidized Low-Density Lipoprotein causes Blood-Brain Barrier Disruption in Early-Onset Preeclampsia through LOX-1

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Abstract

Early-onset preeclampsia (EPE) is a severe form of preeclampsia that involves life-threatening neurologic complications. However, the underlying mechanism by which EPE affects the maternal brain is not known. We hypothesized that plasma from EPE women increases blood-brain barrier (BBB) permeability vs. late-onset preeclamptic (LPE) or normal pregnant (NP) women and investigated its underlying mechanism by perfusing cerebral veins from nonpregnant rats (n=6-7/group) with EPE, LPE or NP human plasma and measuring permeability. We show that plasma from EPE women significantly increased BBB permeability vs. LPE or NP women ($P<0.001$). BBB disruption in response to EPE plasma was due to a 260% increase of circulating oxidized LDL (oxLDL) binding to its receptor, LOX-1, and subsequent generation of peroxynitrite ($P<0.001$). A rat model with pathologically high lipid levels in pregnancy showed symptoms of preeclampsia, including elevated blood pressure, growth-restricted fetuses, and LOX-1-dependent BBB disruption, similar to EPE ($P<0.05$). Thus, we have identified LOX-1 activation by oxLDL and subsequent peroxynitrite generation as a novel mechanism by which disruption of the BBB occurs in EPE. As increased BBB permeability is a primary means by which seizure and other neurologic symptoms ensue, our findings highlight oxLDL, LOX-1 and peroxynitrite as important therapeutic targets in EPE.

Introduction

Neurologic symptoms are the most serious and life-threatening complication of preeclampsia [1]. The new onset of uncontrolled vomiting, cortical blindness, seizure and coma during preeclampsia represent a severe form of disease that accounts for ~75% of all maternal deaths worldwide [2, 3]. Epidemiologic studies have shown that neurologic complications occur more often in early-onset preeclampsia (EPE) where hypertension and proteinuria occur before 34 weeks of gestation, compared to late-onset preeclampsia (LPE) in which hypertension and proteinuria develop after 34 weeks of gestation [4-6]. This importantly suggests that EPE is a more severe form of preeclampsia that adversely affects the maternal brain, promoting neurologic complications. However, the unique factors contributing to neurologic involvement during EPE are largely unknown.

Vasogenic brain edema has been shown in preeclampsia and is the result of disruption of the blood-brain barrier (BBB) and increased cerebrovascular permeability [7-9]. In addition to brain edema, increased BBB permeability promotes the passage of damaging proteins and plasma constituents into the brain parenchyma that can activate microglia and promote seizure activity [8, 10, 11]. Thus, the BBB has a central role in promoting neurologic symptoms during preeclampsia. We previously showed that plasma from preeclamptic women but not from women with normal pregnancy (NP) increased BBB permeability of cerebral veins, demonstrating that circulating factors are an important means by which BBB disruption can occur in preeclampsia [12]. In that study, plasma was pooled from preeclamptic women with severe disease, however, EPE and LPE were not distinguished. The first aim of this study was to investigate the differential effect of circulating factors in plasma from EPE and LPE plasma on BBB permeability. We hypothesized that circulating factors in plasma from EPE women would have a greater impact on BBB disruption that underlies neurologic symptoms in that group.

One circulating factor that is increased in preeclampsia compared to normal pregnancy is oxidized low-density lipoprotein (oxLDL) [13-15]. Increased oxidative stress in the placental circulation during preeclampsia causes oxidative conversion of LDL to oxLDL [16, 17]. OxLDL has a greater negative charge compared to native LDL and therefore selectively binds lectin-like oxidized LDL receptor 1 (LOX-1), expressed predominantly on endothelial cells, to cause vascular dysfunction in

several disease states including hypertension, diabetes and atherosclerosis [18, 19]. Thus, our second aim of this study was to investigate oxLDL and LOX-1 activation as an underlying mechanism by which EPE plasma possibly selectively affects the BBB to be more permeable. To our knowledge, no study has shown that circulating oxLDL and LOX-1 activation is an underlying cause of BBB disruption in preeclampsia. However, understanding of this mechanism would be particularly important in the pathogenesis of preeclampsia considering recent reports of increased LOX-1 expression in the systemic vasculature from preeclamptic women and in a rat model of preeclampsia [20-22].

LOX-1 activation rapidly stimulates the production of superoxide in endothelial cells, mainly through activation of NADPH oxidase [23, 24]. Superoxide decreases the concentration of nitric oxide (NO) by binding NO to form peroxynitrite, a relatively stable reactive oxygen and nitrogen species that has deleterious effects on cell viability and endothelial function [25]. Peroxynitrite generation has been reported in the maternal systemic vasculature and is thought to contribute to endothelial dysfunction during preeclampsia. However, if peroxynitrite generation, secondary to LOX-1 activation, is also involved in disrupting the BBB during preeclampsia is not known. Thus, our last aim of the study was to determine if increased levels of circulating oxLDL in plasma from women diagnosed with EPE would activate LOX-1, resulting in subsequent peroxynitrite generation leading to BBB disruption in EPE women. In this study, we used an established method of measuring BBB permeability of cerebral veins perfused with plasma (12, 26, 27). Cerebral veins are used because they have BBB properties and are a major site of disruption during acute hypertension (26-28).

Material and Methods

Patients. Maternal plasma samples were obtained from an ongoing investigation of preeclampsia (Prenatal Exposures and Preeclampsia Prevention; PEPP) at the Magee-Womens Research Institute and Hospital, University of Pittsburgh, Pennsylvania. PEPP was approved by the University of Pittsburgh institutional review board and informed consent was obtained from all participants. The PEPP committee approved the use of these previously frozen samples by our institution and de-identified clinical data. Blood samples from all women were collected into

EDTA tubes. Plasma was centrifuged at 1600 revolutions per minute and aliquoted. Plasma was then pooled from 4 groups: NonP women who had never been pregnant (n=9), NP women (n=12), LPE women (n=10) and EPE women (n=5) and stored at -80ºC until experimentation. The women enrolled in the preeclamptic groups met the criteria according to the American College of Obstetricians and Gynecologists of blood pressure greater than or equal to 140 mmHg systolic and/or 90 mmHg diastolic plus an increase of greater than 30 mmHg systolic and/or 15 mmHg diastolic plus proteinuria greater than 300 mg/24hrs or at least equal to 2+ protein using a urine dipstick test. EPE women were diagnosed with preeclampsia and delivered before 34 weeks of gestation and LPE women were diagnosed and delivered after 34 weeks of gestation (see Table 1). Only non-smokers and nulliparous women were included.

Measurement of oxLDL in human plasma samples. The levels of oxLDL in the plasma from the NonP, NP, LPE and EPE women were determined using a sandwich Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) Kit (Immunodiagnostik, Bensheim, Germany) according to the manufacturers' instructions. Measurements were performed in duplicate and averaged.

Animals. Female Sprague Dawley virgin nonpregnant rats (12-14 weeks; 250-300 grams) or female Sprague Dawley pregnant rats (day 5; 12-14 weeks, 250-300 grams) were purchased from Charles River (Saint-Constant, QB, Canada). Animals were housed in the Animal Care facility, an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. Animals had access to food and water ad libitum and were maintained on a 12-hour light/dark cycle. The animal studies were approved by the IACUC of the University of Vermont and the Institutional Animal care and Use Committee and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

High-cholesterol rat model. On day 6 of pregnancy, rats were divided into a late-pregnant control group (LP-CTL; n=8) and a late-pregnant high-cholesterol treated group (LP-HC; n=8). The LP-CTL rats received Prolab® 3000 rodent chow for 14 days. The LP-HC animals received a 14 day diet consisting of Prolab® 3000 rodent chow, including 2% cholesterol and 0.5% cholic acid (added to lower hepatic clearance of cholesterol) to increase total and LDL-cholesterol. Experimentation was done on day 14 of the diet that equaled day 20 of pregnancy in all animals. Pups and placenta's were weight averaged per animal.

Blood pressure measurements. All LP-CTL and LP-HC animals had blood pressure measurements taken on day 2 and day 13 of their diet. Animals were trained for 2 days prior to the first day of blood pressure measurements to make the rats familiar with handling and restraint associated with the procedure and thereby avoid measuring artificially high blood pressures. Blood pressures were taken using a noninvasive tail cuff method (CODAS 8, Kent Scientific, Torrington, Connecticut). Briefly, animals were placed in individual holders on a heating plate and both an occlusion cuff and a volume pressure-recording cuff were placed on the tail close to the base. Animals were warmed to 30 °C for optimal volume pressure recording. Systolic, diastolic and mean blood pressure, heart pulse rate, tail blood volume, and tail blood flow were measured simultaneously.

Rat plasma samples. Plasma samples were obtained from trunk blood from LP-CTL and LP-HC rats. Plasma was collected in EDTA plasma separation tubes and centrifuged for 10 minutes at 2500 revolutions per minute. Plasma was then aliquoted and directly used for permeability experiments.

BBB permeability measurements. The first set of experiments was performed to determine the effects of circulating factors in plasma from EPE and LPE women on BBB permeability compared to NP women and NonP women. We measured L_p, the critical transport parameter that relates water flux to hydrostatic pressure, in isolated cerebral veins from nonpregnant female rats after perfusing with plasma from one of the 4 groups of women, as described previously [26]. This method of measuring BBB permeability was specifically developed to have a direct measure of water permeability and has been successfully used in several previous studies [12, 26, 27]. The vein of Galen was used for all permeability experiments as representation of the BBB because this vein has BBB properties and is where BBB disruption occurs first during acute hypertension [28]. Further, only veins from nonpregnant rats were used to isolate the possible effects of circulating factors in the plasma and our previous studies have shown no difference in BBB permeability comparing vessels from nonpregnant and pregnant rats [27]. Briefly, cerebral veins were carefully dissected out of the brain of nonpregnant rats and the proximal end mounted on one glass cannula in an arteriograph chamber. Veins were perfused intraluminally with 20% v/v plasma from either NonP (n=7), NP (n=7), LPE (n=6) and EPE (n=6) in a HEPES buffer for 3 hours at 10±0.3 mmHg and 37 ºC. The distal end of the vessel was tied off with a nylon suture. After this incubation period with plasma,

intravascular pressure was increased to 25±0.1 mmHg and the drop in pressure due to transvascular filtration of water out of the vessel in response to hydrostatic pressure was measured for 40 minutes. The decrease of intravascular pressure per minute (mmHg/min) was converted to volume flux across the vessel wall (μm^3) using a conversion curve as previously described [26]. After flux was determined, transvascular filtration per surface area (J_v/S) and L_p were calculated by normalizing flux to the surface area and oncotic pressure of the plasma perfusate that was determined by a commercially available oncometer.

In a separate set of experiments, we determined the involvement of LOX-1 activation on BBB permeability by adding a neutralizing antibody to LOX-1 (5µg/ml; n=6) to the plasma from EPE women before perfusing the plasma into the vein of Galen and the permeability experiment was repeated. Because we already showed that the presence of control goat IgG (also used as a control for this antibody) perfused with plasma in the cannula did not interfere with the permeability measurements [27], we did not repeat these control IgG experiments in the current study.

Another set of experiments was performed to determine the involvement of peroxynitrite generation in plasma from EPE women by adding the peroxynitrite decomposition catalyst FeTMPyP (50 µM; n=6) to the plasma from EPE women before perfusing the plasma in the cerebral veins. FeTMPyP is a ferric-porphyrin-complex that catalytically isomerizes peroxynitrite to nitrate in vitro and has been shown to be selective for blocking peroxynitrite effects without interfering with NO or superoxide [29]. Therefore, FeTMPyP serves as a selective peroxynitrite decomposition catalyst. In addition, the concentration of 50 µM was based on one of our earlier studies where FeTMPyP was used in isolated arteries to scavenge peroxynitrite [30].

A separate set of experiments was performed to determine the effects of exogenous oxLDL on BBB permeability by the addition of exogenous human oxLDL (3.5 µg/ml; n=7) to plasma from NonP women before perfusing the plasma in the cerebral veins. For these experiments, plasma from NonP women was chosen rather than plasma from NP women to eliminate other possible circulating factors present in pregnancy that could interact with oxLDL. To determine if there was a causal link between oxLDL increasing BBB permeability and peroxynitrite generation, we

repeated these experiments with the addition of FeTMPyP (50 μM; n=6) to the oxLDL-plasma mixture before perfusion in the vein of Galen and measured L_p.
The last set of experiments was performed to determine the involvement of high levels of cholesterol in pregnancy on pregnancy outcome and BBB disruption. The cerebral vein of Galen of LP rats that received either a control diet or a high-cholesterol diet was used for the permeability experiments as described above. For these experiments, 20% v/v plasma in HEPES buffer was taken and perfused in veins from the same animals. To determine the contribution of oxLDL in increasing BBB permeability in the high-cholesterol treated pregnant rats, the same neutralizing LOX-1 antibody (5μg/ml; n=8) was added to the plasma from the LP-HC animals before perfusion in the cerebral veins.

Measurement of mRNA expression of LOX-1 using real-time quantitative PCR. The middle cerebral artery (MCA) was used as a representative cerebral vessel to measure mRNA expression of LOX-1 in the LP-CTL (n=6) and LP-HC (n=7) animals, as the Vein of Galen was used for permeability experiments in these animals. Real-time quantitive PCR was performed as described earlier [31]. The quantitative PCR primer for the rat transcripts for LOX-1was: LOX-1 (f): -GATGATCTGAACTTCGTCTTACAAGC- and (r): - TCAGCAAACACAACTCCTCCTT.

Drugs and solutions. HEPES physiological salt solution was made fresh daily and consisted of (mmol/L): 142.00 NaCl, 4.70 KCl, 1.71 $MgSO_4$, 0.50 EDTA, 2.80 $CaCl_2$, 10.00 HEPES, 1.20 KH_2PO_4, and 5.00 dextrose. FeTMPyP was purchased from Calbiochem, (Gibbstown, NJ, USA; 341501). LOX-1 antibody was purchased from R&D systems (Minneapolis, MN, USA; AF1564). Highly oxLDL was purchased from Kalen Biomedical LLC (Montgomerey Village, Mn, USA; 770252-7).

Statistical analysis. Data are presented as mean ± standard error of the mean.
Analyses were performed by one-way ANOVA with a post-hoc Newman Keuls test for multiple comparisons where appropriate or with a Student's t-test. Differences were considered statistical significant at $P < 0.05$. mRNA expression of LOX-1 was determined by quantitative PCR and calculated using the $2^{-\Delta\Delta CT}$ method.

Results

Patients with EPE have worse pregnancy outcome compared to LPE and normal pregnancy. Although the pathogenesis of preeclampsia is not clear, it is accepted that the pathologic release of soluble factors from the ischemic placenta into the maternal circulation causes endothelial dysfunction, oxidative stress and the symptoms of hypertension and proteinuria [16]. Thus, it is reasonable to examine maternal blood as a source of circulating factors that might also be affecting the cerebral endothelium to cause BBB disruption and promote neurologic symptoms.

Table 1. Demographics

	NonP (n=9)	NP (n=12)	LPE (n=10)	EPE (n=5)
Age (years)	25.4 (± 4.6)	26 (± 4.5)	31 (± 3.6)	25 (± 5)
BMI	24.0 (± 3.2)	26.4 (± 3.7)	26.5 (± 7.4)	24.3 (± 3.0)
GA sample (weeks)	-	34.9 (± 1.8)	35.8 (± 1.6)	32.3 (± 1.6)
GA delivery (weeks)	-	40.1 (± 0.93)	36.0 (± 1.4)	32.5 (± 1.4)
SBP <20 wks gestation (mmHg)	107 (± 6.8)	110.1 (± 7.2)	119.5 (± 12.4)	122.0 (± 5.6)
DBP <20 weeks gestation (mmHg)	65.5 (± 5.5)	66.8 (± 3.9)	73.0 (± 7.3)	73 (± 4)
SBP at time delivery (mmHg)	-	119.4 (± 7.3)	152.40 (± 8.59)	160.0 (± 9.5)
DBP at time delivery (mmHg)	-	68.8 (± 11.7)	91.70 (± 6.43)	100.6 (± 11.0)
Birthweight (grams)	-	3601 (± 380)	2271.3 (± 143.2)	1462 (± 223)
Birthweight centile (%)	-	56.1 (± 7.1)	18.9 (± 4.9)	7.6 (± 1.8)

Abbreviations: NonP = nonpregnant; NP = normal pregnant; LPE = late-onset preeclampsia; EPE = early-onset preeclampsia; GA = gestational age; SBP = systolic blood pressure; DBP = diastolic blood pressure; BMI= body mass index.

Table 1 shows the patient characteristics from which plasma samples were taken. There were no significant differences between the 4 groups of women with respect to age or BMI. Blood pressure measured in early gestation before 20 weeks of pregnancy was similar in all pregnant groups and was comparable to the blood pressure measured in the group of women who had never been pregnant. These results are consistent with the symptoms of preeclampsia developing after 20 weeks of gestation, including hypertension.

At time of delivery, both systolic and diastolic blood pressure measured in NP women were similar to the blood pressure measured before 20 weeks of gestation. However, blood pressure of both preeclamptic groups was significantly increased and met the diagnostic criteria of preeclampsia. Although blood pressure at

time of delivery was higher in EPE women compared to LPE women, this was not significantly different. Further, all NP women delivered after 37 weeks of gestation. LPE women delivered after 34 weeks but before 37 weeks of gestation and EPE women were diagnosed and delivered before 34 weeks of gestation, in accordance with to the definition of EPE. Lastly, the infants born to EPE women had the lowest infant birth weight compared to the other groups of pregnant women. Importantly, only EPE women delivered babies with intrauterine growth restriction (< 10^{th} percentile) compared to the LPE and NP women (> 10^{th} percentile). Thus, EPE women that were diagnosed and delivered before 34 weeks of gestation from which plasma was taken had a more severe form of preeclampsia compared to LPE women.

Plasma from EPE women significantly increases BBB permeability compared to plasma from LPE and NP women. Epidemiologic studies suggest that women with EPE develop neurologic complications more often compared to women with LPE [4, 5]. Because the BBB has a central role in the development of neurologic symptoms such as seizure [8, 10, 32], we measured BBB permeability in cerebral veins perfused with 20% v/v plasma from NP, LPE or EPE women. Permeability in response to plasma from NonP women was used as a control. Several permeability parameters were measured in vitro in response to a 3 hour exposure to plasma, including filtration pressure drop, volume flux, transvascular filtration (J_v/S) and hydraulic conductivity (L_p). As shown in Figure 1, exposure to plasma from NP women caused an increase in intravascular pressure drop and J_v/S, but not in volume flux or L_p. However, in both preeclamptic groups, all permeability parameters were significantly increased compared to NonP. Importantly, the increase in permeability in response to LPE plasma was similar to NP plasma whereas EPE plasma caused a significant increase in permeability compared to all other groups, including LPE plasma. These results demonstrate that circulating factors present in plasma from EPE women disrupt the BBB and may relate to the propensity for neurologic complications to occur in that group of preeclamptic patients.

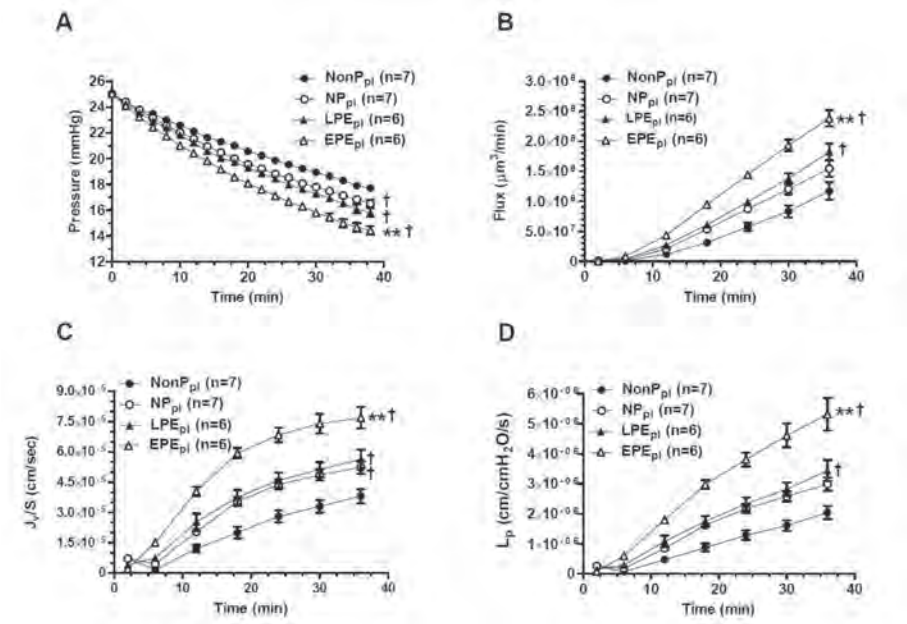

Figure 1. **Plasma from EPE women increases BBB permeability compared to plasma from LPE or NP women.** (A-D) The permeability parameters intravascular pressure drop, volume flux, J_v/S and hydraulic conductivity (L_p) were used to determine BBB permeability in cerebral veins from nonpregnant rats in response to plasma from EPE, LPE, NP and NonP women. (A) The decrease in intravascular pressure in response to plasma from EPE, LPE and NP women was significantly greater compared to NonP women. Importantly, the decrease in intravascular pressure was significantly greater in EPE women compared to all other groups, including LPE women. (B) Plasma from both preeclamptic groups showed a significant increase in flux compared to plasma from NonP women. Plasma from EPE women caused a significantly greater flux compared to all other groups. (C) Plasma from EPE, LPE and NP women caused a significant increase in J_v/S compared to plasma from NonP women and only plasma from EPE women significantly increased J_v/S compared to all other groups. (D) Plasma from both preeclamptic groups significantly increased L_p compared to NonP women and only plasma from EPE women significantly increased L_p compared to all other groups. (†$P < 0.01$ vs. NonP; **$P < 0.01$ vs. all. Abbreviations: NonP, nonpregnant; NP, normal pregnant; LPE, late-onset preeclampsia; EPE, early-onset preeclampsia.)

Inhibition of LOX-1 activation prevents increased BBB permeability induced by EPE plasma. Circulating oxLDL binds its receptor LOX-1 to cause oxidative stress and endothelial dysfunction in many pathological states such as atherosclerosis, diabetes and hypertension [18, 33]. To determine if LOX-1 activation is involved in increasing BBB permeability in response to acute exposure of plasma from EPE women, we neutralized LOX-1 with an antibody to inhibit its activation prior to perfusion with EPE plasma and measurement of permeability. Inhibition of LOX-1 abolished the increased BBB permeability induced by circulating factors in this plasma (Figure 2A). Thus, LOX-1 activation is involved in increasing BBB permeability in plasma from EPE women.

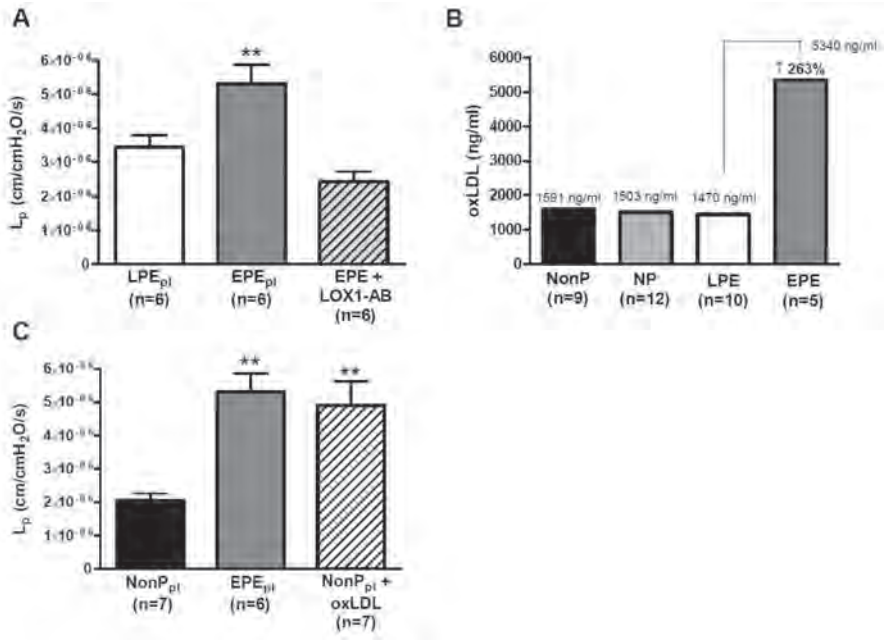

Figure 2. **Increased levels of oxLDL in plasma from EPE women are responsible for the significant increased BBB permeability.** (A) BBB permeability in response to plasma from LPE women, EPE women and from EPE women with the addition of 5µg/ml of a neutralizing LOX-1 antibody. The LOX-1 antibody inhibited the increased BBB permeability induced by plasma from EPE women. (B) OxLDL levels in plasma from women with EPE compared to LPE, NP and NonP women. Plasma from EPE women had a 260% increase in oxLDL compared to plasma from LPE women. (C) L_p as a measure of BBB permeability in cerebral veins from NonP rats in response to plasma from NonP and EPE women and plasma from NonP women with addition of 3.5µg/ml exogenous oxLDL. Exogenous oxLDL significantly increased BBB permeability to the same levels as plasma from EPE women. (**P < 0.01 vs. all; Abbreviations: NonP , nonpregnant; NP , normal pregnant; LPE , late-onset preeclampsia; EPE , early-onset preeclampsia.)

To determine if oxLDL was increased in plasma from EPE women that was responsible for the LOX-1-induced increase in BBB permeability, we measured oxLDL levels in the plasma from all 4 groups of women by ELISA. Plasma from EPE women had a 260% increase in oxLDL levels compared to plasma from LPE women (Figure 2B). The levels of oxLDL in plasma from NonP, NP and LPE women were comparable. We next confirmed that oxLDL was capable of increasing BBB permeability without the presence of other circulating factors present in the plasma from EPE women by adding 3.5 µg/ml of purified human oxLDL in plasma from NonP women and measuring permeability. This concentration of oxLDL was used based on the values in the EPE plasma measured by ELISA. Addition of exogenous oxLDL to plasma from NonP women caused a significant increase

in BBB permeability that was comparable to what was seen with plasma from women with EPE (Figure 2C). To our knowledge, this is the first report that suggest that high levels of oxLDL present in plasma from EPE women is the circulating factor responsible for the increase in BBB permeability in response to that plasma by activation of LOX-1.

Peroxynitrite decomposition with FeTMPyP prevents increased BBB permeability induced by EPE plasma and oxLDL. Activation of LOX-1 leads to increased production of superoxide through NADPH oxidase that rapidly binds NO to form peroxynitrite [23]. To determine if peroxynitrite generation in response to LOX-1 activation by oxLDL is responsible for increased BBB permeability, we perfused cerebral veins with plasma from EPE women plus 50 μM FeTMPyP, a selective peroxynitrite decomposition catalyst, prior to measuring permeability. FeTMPyP significantly inhibited the increase in BBB permeability caused by plasma from EPE women (Figure 3A). This result demonstrates that peroxynitrite generation is involved in BBB disruption with exposure to plasma from EPE women. We next confirmed that the increase in BBB permeability caused by exogenous oxLDL was due to increased generation of peroxynitrite by adding 50 μM FeTMPyP to plasma from NonP women with exogenous oxLDL. Peroxynitrite decomposition with FeTMPyP inhibited the increase in BBB permeability induced by oxLDL (Figure 3B). Together, these results demonstrate that peroxynitrite generation is induced by oxLDL and is the underlying mechanism by which BBB permeability is increased.

Pathologically high lipid levels during pregnancy in rats cause preeclamptic-like symptoms and LOX-1-dependent BBB disruption. Preeclampsia has been associated with high levels of LDL [13, 34]. In addition, preeclampsia is a state of increased oxidative stress in the placental circulation which causes oxidative conversion of LDL to oxLDL, resulting in increased circulating levels of oxLDL in preeclampsia [16, 17]. Because we found high levels of oxLDL in plasma from EPE women that caused disruption of the BBB, we wanted to determine its effects in vivo by creating a rat model with pathologically high levels of LDL during pregnancy. Rats treated with a high-cholesterol diet during pregnancy (LP-HC) had a 350% increase in cholesterol compared to pregnant rats who received a normal diet (LP-CTL), including a significant increase in LDL cholesterol (data not shown). Table 2 shows that LP-HC rats had a worse pregnancy outcome compared to LP-CTL rats with similar characteristics as seen in EPE women. LP-HC rats had significantly higher

systolic and diastolic blood pressures compared to LP-CTL rats and also had worse pregnancy outcome, including a significantly smaller number of pups, and a higher rate of reabsorptions. Further, LP-HC rats had growth restricted fetuses, with pup weights that were significantly lower compared to LP-CTL.

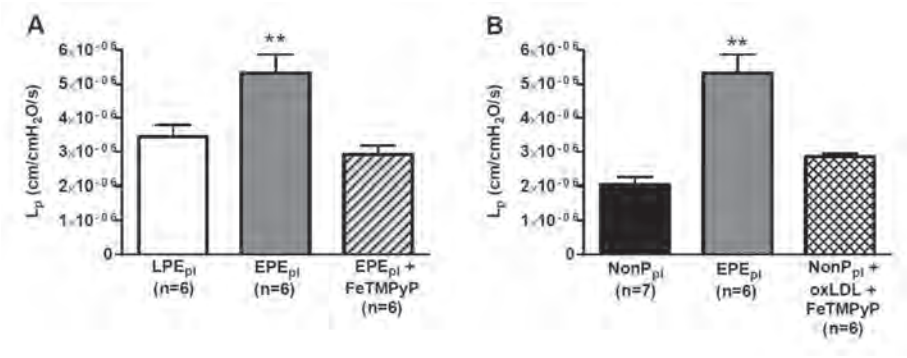

Figure 3. **oxLDL-induced increased BBB permeability in EPE women was prevented by the peroxynitrite scavenger FeTMPyP. (A)** Graph showing hydraulic conductivity (L_p) at 36 minutes as a measure of BBB permeability in cerebral veins of NonP rats in response to plasma from LPE and plasma from EPE women with and without the addition of 50μM FeTMPyP. FeTMPyP inhibited the BBB permeability induced by plasma from EPE women. (B) L_p as a measure of BBB permeability in response to untreated of plasma from NonP and EPE women, and in response to plasma from NonP women plus 3.5μg/ml oxLDL and 50μM FeTMPyP. FeTMPyP inhibited the BBB permeability induced by 3.5μg/ml exogenous oxLDL in plasma from NonP women. (**P< 0.01 vs. all; Abbreviations: LPE, late-onset preeclampsia; EPE, early-onset preeclampsia.)

Table 2. **Pregnancy outcome of LP-HC compared to LP-CTL rats**

	LP-CTL (n=8)	LP-HC (n=8)
Weight (grams)	408 (± 6)	403 (± 10)
SBP (day 13 of diet; mmHg)	118 (±2)	126 (±2) *
DBP (day 13 of diet; mmHg)	87 (±2)	94 (±2) *
Pups (#)	16 (0.5)	12 (±0.6) *
Resorptions (#)	0.7 (± 0.2)	1.4 (± 0.4)
Avg pup weight (grams)	2.42 (± 0.06)	2.12 (±0.04) *
Avg placenta weight (grams)	0.44 (± 0.01)	0.48 (± 0.01) *

*P <0.05 vs LP-CTL

Abbreviations: LP-CTL = late-pregnant control rats ; LP-HC = late-pregnant high cholesterol-treated rats; SBP = systolic blood pressure; DSP = diastolic blood pressure; Avg= average.

Next, we investigated if pathologically high levels of LDL in pregnant rats led to increased BBB permeability that was dependent on LOX-1 activation, as we have also shown in EPE women. Figure 4A shows that cerebral veins from LP-HC rats perfused with plasma from the same animals had increased BBB permeability compared to control animals that was abolished by addition of the LOX-1 antibody. These results suggest that oxLDL is responsible for increasing BBB permeability in the LP-HC animals, similar to EPE. Because oxLDL and not native LDL binds to LOX-1[24], these results also confirm that pathologically high levels of LDL in pregnancy lead to high levels of oxLDL.

Lastly, because the pregnant rats were exposed to high levels of cholesterol during their entire pregnancy, we wanted to determine if LOX-1 expression was increased in the cerebral circulation that could cause the increased BBB permeability. Recent studies showed increased LOX-1 expression HUVECs after 24 hour incubation with preeclamptic plasma [21] and in a rat model of preeclampsia [20]. To determine if LOX-1 mRNA expression was increased in the cerebral vasculature from LP-HC animals, we isolated the middle cerebral arteries (MCA) from the same animals used for permeability experiments and measured mRNA expression of LOX-1. mRNA expression of LOX-1 was similar in the LP-HC and LP-CTL animals (Figure 4B), suggesting that the mechanism of increased BBB permeability in the LP-HC animals was due to circulating oxLDL activating LOX-1 as opposed to upregulation of LOX-1 in the cerebral endothelium.

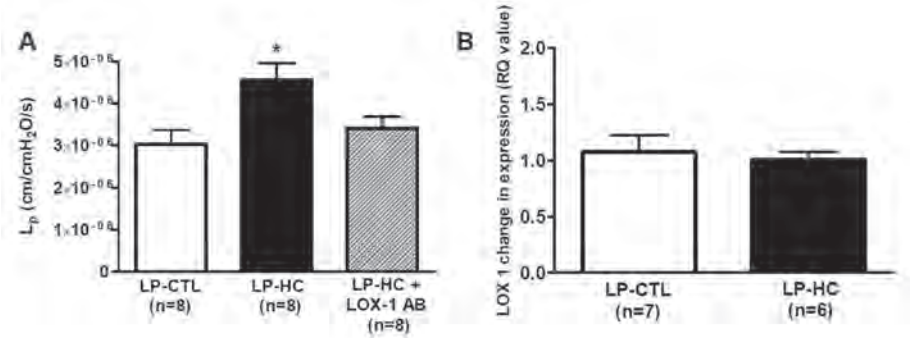

Figure 4. **Pathologically high levels of lipids in pregnant rats show symptoms of preeclampsia and also lead to LOX-1 mediated increased BBB permeability.** (A) Graph showing hydraulic conductivity (L_p) as a measure of BBB permeability at 36 minutes in cerebral veins from LP-CTL rats in response to LP-CTL plasma and from LP-HC rats in response to LP-HC plasma with and without the addition of 5µg/ml LOX-1 antibody. LP-HC rats showed a significant increase in BBB permeability that could be inhibited with the addition of LOX-1 antibody. (B) mRNA expression of LOX-1 in cerebral veins from LP-CTL and LP-HC animals. There was no difference in mRNA expression of LOX-1 in cerebral veins from LP-HC vs. LP-CL animals. (*P< 0.05 vs. all; Abbreviations: LP-CTL, late-pregnant control; LP-HC, late pregnant high-cholesterol treated.)

Discussion

In the present study, we provide new direct evidence for a different etiology between EPE and LPE and reveal a novel mechanism that is responsible for BBB disruption in EPE women. Circulating factors in plasma from EPE women significantly increased BBB permeability compared to LPE or NP women. The increased BBB permeability was prevented by inhibiting LOX-1 and was confirmed in vivo in pregnant rats with pathologically high levels of LDL that also showed LOX-1 dependent increased BBB permeability. Circulating oxLDL, a major ligand of LOX-1, was significantly increased in plasma from EPE women compared to LPE or NP women. In addition, exogenous oxLDL in plasma from NonP women increased BBB permeability comparable to EPE. Additionally, the selective peroxynitrite decomposition catalyst FeTMPyP inhibited the increased BBB permeability induced by plasma from EPE women or by exogenous oxLDL. Thus, our results show for the first time that increased circulating oxLDL present in plasma from EPE women significantly increase BBB permeability through LOX-1 activation and subsequent increased peroxynitrite generation (Figure 5).

The cerebral endothelium that comprises the BBB has unique features compared to endothelium outside the CNS, including high electrical resistance tight junctions, and a lack of fenestrations that results in low hydraulic conductivity [35]. Disruption of the BBB is accompanied by increased BBB permeability and subsequent vasogenic edema resulting into neurologic symptoms [7-9]. Preeclampsia, a hypertensive disorder unique to pregnancy, can be complicated with life-threatening neurologic symptoms, including cerebral edema, hemorrhage and seizure that is associated with increased BBB permeability [1, 7, 8]. As we have previously shown, increased BBB permeability in severe preeclampsia is caused by a pathologic release of circulating factors into the maternal circulation that can cause BBB breakdown [12]. Here, we demonstrated for the first time that intraluminal exposure of cerebral veins to plasma from EPE women for 3 hours significantly increased BBB permeability compared to plasma from LPE or NP women. These new findings point to the acute impact of circulating factors in plasma from EPE women on BBB permeability after a short exposure time. Also, these results directly strengthen the evidence that EPE and LPE have different etiologies, arising from greater placental underperfusion and subsequent release of circulating factors in EPE compared to LPE [4]. To our knowledge, no studies have differentiated effects of circulating factors

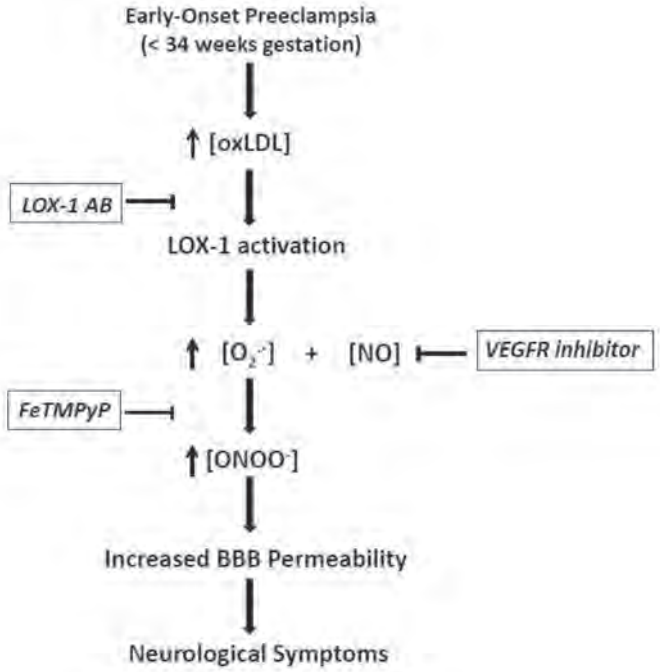

Figure 5. **Schematic showing the proposed mechanism leading to BBB disruption and neurological complications in EPE.** EPE women have a more severe form of disease that is associated with increased levels of circulating oxLDL. The increased circulating levels of oxLDL in plasma from EPE women bind to their receptor LOX-1 on the cerebral endothelium that comprises the BBB. LOX-1 activation leads to generation of peroxynitrite ($ONOO^-$), most likely through increased production of superoxide (O_2^-), causing BBB disruption and increased BBB permeability that is responsible for neurological complications in preeclampsia.

in LPE compared to EPE on BBB permeability. Only one report found increased permeability in mesenteric arteries from frogs in response to plasma from severe compared to mild preeclamptic women where the severity of preeclampsia was strongly correlated to EPE [36]. In addition, these findings are also of great clinical importance as they demonstrate that EPE women only show significant BBB disruption and are at highest risk for neurologic symptoms, thereby confirming epidemiologic studies [4, 5]. Although we did not examine the direct link between increased BBB permeability and neurologic symptoms, it is well-established that disruption of the BBB is a primary event in inducing neurologic complications and the key event for seizure activity [10, 32]. Thus, we show that specific circulating factors present in plasma from EPE women only significantly increase BBB permeability and thereby make EPE women most at risk for life-threatening neurologic symptoms.

Increased circulating levels of oxLDL in preeclampsia has been shown in several [15, 37-40], but not all [41], studies. In cardiovascular diseases such as atherosclerosis, oxidative modification of LDL increases its atherogenicity and induces a wide variety of cellular responses such as the induction and expression of adhesion molecules, proinflammatory cytokines and reactive oxygen species, resulting in endothelial dysfunction [42]. In preeclampsia, decidual vessels of the placenta show fibrinoid necrosis of the vascular wall and focal accumulation of lipid-laden macrophages, similar as in atherosclerosis, making oxLDL also a likely and important contributor in the pathogenesis of preeclampsia [43]. Here, we found that oxLDL levels were increased in plasma from EPE women but not in plasma from LPE women that was comparable to NP women. This increase in EPE only compared to LPE and NP may be related to the finding that oxidative stress was found only in placentas from EPE women [44], suggesting greater capacity for oxidative modification of LDL in EPE. In addition, we show that exogenous oxLDL to the levels as measured in plasma from EPE women significantly increased BBB permeability in cerebral veins, comparable to EPE women. Thus, increased oxLDL does not appear to be just a biomarker for neurological symptoms in EPE, but also the underlying cause of BBB disruption and a possible new target for understanding mechanisms of BBB disruption in several pathologic states.

LOX-1 is an endothelial receptor for circulating oxLDL that has been studied extensively in pathological states such as atherosclerosis, diabetes, coronary arterial heart disease and hypertension [18, 33], however, data regarding LOX-1 activation in preeclampsia are scarce. LOX-1 was associated with preeclampsia for the first time in 2005 in a report that showed up regulation of LOX-1 in preeclamptic hypoxic placentas [45]. Recently, it was found that upregulation of LOX-1 in HUVECs occurred in response to a 24 hour exposure to plasma from preeclamptic women and in a rat model of preeclampsia [20, 21]. Here, we show for the first time involvement of LOX-1 in increasing BBB permeability after acute exposure of plasma from EPE women for 3 hours that contained high levels of circulating oxLDL. Because of the 3 hour exposure time of the plasma, we did not determine possible upregulation of LOX-1. As shown previously, upregulation in HUVECs in response to plasma from preeclamptic women did not occur until 24 hours of exposure, while shorter exposure caused increased the production of ROS [21]. These findings are in support of increased circulating ligands such as circulating oxLDL activating LOX-1 to cause endothelial dysfunction. However, it remains to be determined in cerebral endothelial cells if longer incubation would cause upregulation of

LOX-1expression in response to plasma from EPE women as seen in HUVECs in response to preeclamptic plasma [21]. Importantly, the LOX-1-mediated increase in BBB permeability with EPE plasma was confirmed in vivo in pregnant rats with pathologically high levels of LDL, that also showed symptoms such as increased blood pressure and growth restricted fetuses. To our knowledge, few studies have investigated oxLDL/LOX-1 activation and vascular permeability. One study using mouse cultured cerebral endothelial cells showed that high concentrations of oxLDL were able to increase permeability [46]. In the systemic vasculature, Nakano et al. showed increased vascular permeability in mesenteric arteries from spontaneous hypertensive rats pretreated with oxLDL that was inhibited by a LOX-1 antibody [47]. In this study, we propose a similar, but novel mechanism that high levels of circulating oxLDL in EPE increase BBB permeability through LOX-1 activation.

LOX-1 activation has been shown to induce several intracellular signaling pathways, including increased expression of chemokines and adhesion molecules, triggering the CD40/CD40L pathway that activates the inflammatory cascade and increased production of reactive oxygen species such as superoxide in endothelial cells [19]. Increased levels of superoxide rapidly bind NO and form peroxynitrite, a toxic radical known to have deleterious effects on endothelial function [17, 25]. Also, peroxynitrite generation has been reported in the maternal systemic vasculature in preeclampsia [48] and in HUVECs after exposure to plasma from preeclamptic women [21]. Here, we show that BBB permeability induced by either plasma from EPE women or exogenous oxLDL to the levels of EPE women was inhibited with the specific peroxynitrite decomposition catalyst FeTMPyP, demonstrating that peroxynitrite generation caused the increased BBB permeability. Our findings that oxLDL/LOX-1 activation induces peroxynitrite generation are supported by earlier reports that showed in different cell culture models that binding of oxLDL to LOX-1 decreased the intracellular concentration of NO by inducing the production of superoxide through NADPH oxidase [23] and increased peroxynitrite generation [49]. Because NADPH oxidase expression and activity is greater in the cerebral vessels compared to systemic vasculature [50], the brain may be especially vulnerable for activation of NADPH oxidase and subsequent peroxynitrite generation. Importantly, peroxynitrite also stimulates LOX-1 activation and a self-perpetuating mechanism develops that could cause extensive endothelial damage with deleterious effects for the maternal cerebral vasculature [17]. Finally, these findings would explain the

results from our previous study in which increased BBB permeability in response to plasma from severe preeclamptic women was prevented by VEGF receptor inhibition without increased circulating levels of VEGF compared to plasma from NP women [12]. Phosphorylation of VEGF receptors results in a release of NO [51], thus, its inhibition would decrease the level of NO available for peroxynitrite generation, preventing BBB disruption (Figure 5).

In conclusion, this is the first report to identify oxLDL as a circulating factor in EPE that significantly increases BBB permeability compared to LPE and thereby provides direct evidence for EPE and LPE having 2 different etiologies. In addition, we reveal a new mechanism for BBB disruption in EPE by oxLDL/LOX-1 activation and subsequent peroxynitrite generation. These novel findings will contribute to improving the identification and treatment of neurological complications in women diagnosed with EPE and possibly other neurological diseases where oxidative stress-induced BBB disruption is involved. Further studies are required to determine how BBB disruption induced by oxLDL leads to specific neurological symptoms, which could lead to treatment or prevention of these complications in preeclampsia.

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Chapter 5:

Oxidized LDL induces Cerebrovascular Dysfunction and Blood-Brain Barrier Permeability in Female Rats that are Prevented by Apocynin and Magnesium Sulfate

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Abstract

Little is known about the effects of oxidized low-density lipoprotein (oxLDL) on the cerebral vasculature. However, understanding is important for many neurologic conditions involving cerebral edema such as neurologic symptoms in severe preeclampsia. We hypothesized that oxLDL induced blood-brain barrier (BBB) disruption and cerebrovascular dysfunction through NADPH oxidase-derived superoxide. Further, we investigated the effect of magnesium sulfate ($MgSO_4$) on oxLDL-induced changes in the cerebral vasculature, as this is a commonly used treatment in preventing cerebral edema formation. Posterior cerebral arteries (PCA) from female rats were perfused with 5µg/mL oxLDL in rat serum with or without 50µM apocynin or 16mM $MgSO_4$ and BBB permeability and vascular reactivity were compared. oxLDL increased BBB permeability and decreased myogenic tone that were prevented by apocynin. oxLDL increased constriction to the nitric oxide synthase inhibitor L-NNA that was unaffected by apocynin. oxLDL enhanced dilation to the NO donor sodium nitroprusside (SNP) that was prevented by apocynin. $MgSO_4$ prevented oxLDL-induced BBB permeablity without affecting oxLDL-induced changes in myogenic tone. Thus, oxLDL causes BBB disruption and vascular tone dysregulation through NADPH oxidase-derived superoxide, that was partially prevented by $MgSO_4$. These results highlight oxLDL and NADPH oxidase as important therapeutic targets in neurologic conditions that involve circulating oxLDL.

Introduction

It has become recognized that oxidized low-density lipoprotein (oxLDL) is one of the key factors in the pathogenesis of many cardiovascular diseases [1]. Oxidative modification of physiological native LDL (nLDL) into oxLDL occurs in numerous disease states as a result of oxidative stress and the presence of reactive oxygen species [2]. The formation of oxLDL initiates multiple pathways in both endothelial and vascular smooth muscle cells, mostly through binding to its receptor lectin-like oxLDL receptor (LOX-1) [3]. oxLDL binding to LOX-1 generates complex signaling cascades leading to induction of the inflammatory pathway and increased production of superoxide that can further promote vascular dysfunction [1-3]. Although less understood than in peripheral or cardiovascular disease, oxLDL also appears to contribute to cerebrovascular disease and stroke. Previous studies showed a significant association between increased circulating levels of oxLDL and cerebral ischemic lesions in stroke patients that may be related to the presence of oxidative stress [4, 5]. In addition, high doses of oxLDL increases BBB permeability in cultured cerebral endothelial cells [6] and in isolated cerebral arteries that was prevented by LOX-1 inhibition and scavenging peroxynitrite [7]. Thus, oxLDL may be an important therapeutic target for cerebrovascular disease and stroke, especially under conditions of oxidative stress and cerebral edema formation.

Oxidative and nitrosative stress are known to have detrimental effects on cerebrovascular reactivity and BBB permeability, however, the role of oxLDL in these events is largely unknown. oxLDL activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to produce superoxide and may have an important role in cerebrovascular dysregulation [8, 9]. Superoxide and peroxynitrite are known to affect vascular tone and myogenic reactivity that are important for control of cerebral blood flow (CBF) and cerebrovascular resistance (CVR)[10-12]. In addition, increased BBB permeability caused by oxLDL may also promote vasogenic edema formation and contribute to life-threatening neurologic symptoms associated with conditions such as ischemic stroke, seizure, and severe preeclampsia. In fact, our previous study showed that increased circulating levels of oxLDL in severe preeclamptic women induced BBB disruption, that was prevented by scavenging peroxynitrite [13]. However, the mechanism by which oxLDL causes peroxynitrite generation to induce BBB disruption, vasogenic edema and neurologic complication in preeclampsia is not known. In addition, data concerning the

effect of oxLDL on cerebrovascular reactivity and myogenic responses are very limited, however potentially important in neurologic symptoms in preeclampsia where impaired cerebral autoregulation is an important contributor to formation of cerebral edema and neurologic complications in preeclampsia. In the present study, we hypothesize that oxLDL to the level of women with severe preeclampsia would decrease myogenic tone and reactivity of cerebral arteries that could contribute to vascular dysfunction and the formation of cerebral edema. We further hypothesized that NADPH oxidase-derived superoxide is involved in oxLDL-induced changes in the cerebral vasculature.

Magnesium sulfate ($MgSO_4$) has been shown to be protective of the BBB and prevent cerebral edema during numerous conditions including traumatic brain injury, septic encephalopathy, hypoglycemia, and preeclampsia [14-16]. In fact, $MgSO_4$ is one of the primary treatments for prevention of vasogenic brain edema in severe preeclampsia [15, 16]. However, if $MgSO_4$ is also specifically protective of oxLDL-induced BBB permeability or vascular tone dysregulation remains unknown. Thus, our last aim was to investigate the effect of $MgSO_4$ on oxLDL-induced BBB permeability and cerebral vascular dysfunction.

Methods

Animals. All animal procedures were approved by the University of Vermont Institutional Animal Care and Use Committee and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female virgin nonpregnant Sprague Dawley rats (12-14 weeks; 250-300 grams) were used for all experiments. The animals were purchased from Charles River (Saint-Constant, QB, Canada) and housed until experimentation at the University of Vermont Animal Care Facility, an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. Animals had access to food and water ad libitum and maintained a 12-hour light/dark cycle.

Rat serum samples. Serum samples were obtained from trunk blood from female Sprague Dawley rats and collected in serum separator tubes. After a waiting period of 30 minutes, the tubes were centrifuged for 10 minutes at 2500 revolutions per minute, and the serum aliquoted and pooled from 10 animals. The pooled serum

was then stored at -80 ºC until experimentation. For every experiment, one tube of serum from the same pool of animals was used.

BBB permeability studies. To investigate the effect of oxLDL on BBB permeability and determine the mechanism by which oxLDL increases permeability, hydraulic conductivity (L_p), the critical transport parameter that relates water flux to hydrostatic pressure, was measured in isolated PCAs from female nonpregnant rats, as described previously [17]. This method of measuring BBB permeability has been successfully used in several previous studies [17-19]. Briefly, a third-order branch of the PCA was carefully dissected out of the brain and the proximal end mounted on a glass microcannula in an arteriograph chamber. PCAs were perfused intraluminally with 20% v/v serum from female rats with (n=8) or without (n=8) the presence of 5 µg/ml oxLDL or µg/ml native LDL (nLDL; n=4) in HEPES buffer for 1 hour at 75 ± 0.3 mmHg and 37 ºC ± 0.2 ºC. The concentration of oxLDL used for these experiments was based on a previous study that determined the concentration of oxLDL in plasma of severe preeclamptic women to be approximately 5 µg/ml [13]. To determine the involvement of NADPH oxidase activity in oxLDL-induced BBB permeability, a separate set of PCAs were perfused with 50 µM of the NADPH oxidase inhibitor apocynin (n=8) in addition to the oxLDL-serum mixture. To determine the effect of $MgSO_4$ on oxLDL-induced BBB permeability, another set of PCAs was perfused with 16 mM $MgSO_4$ in addition to the oxLDL-serum mixture. This dose of $MgSO_4$ was chosen because it is within the therapautic range for $MgSO_4$ for seizure profylaxis [16, 20]. After the equilibration period, intravascular pressure was increased to 80 mmHg ± 0.1 mmHg and the drop in pressure due to transvascular filtration across the vascular wall in response to hydrostatic pressure was measured for 40 minutes. The decrease of intravascular pressure per minute (mmHg/min) was converted to volume flux across the vessel wall (μm^3) using a conversion curve, as previously described [17]. L_p was then determined by normalizing flux to surface area and oncotic pressure of the serum perfusate.

Reactivity studies. Another set of experiments was performed to determine the effect of oxLDL on reactivity of PCAs. A third-order branch of the PCA was carefully dissected from the brain of female rats and and the proximal end mounted on one glass cannula in an arteriograph chamber. PCAs were perfused intraluminally with 20% v/v serum from female rats with (n=8) or without (n=8) the presence of 5 µg/ml oxLDL or 5 µg/ml nLDL (n=4) in a HEPES buffer for 2 hours at 80 ± 0.3

mmHg and 37 ºC ± 0.2 ºC. The distal end of the vessel was tied off to avoid flow-mediated responses. The proximal cannula of the arteriograph was connected to a pressure transducer and servo controller that maintained intravascular pressure at a set pressure or changed manually. Lumen diameter of the PCA was measured via video microscopy during the experiment. After the equilibration period, intravascular pressure was increased in steps of 20 mmHg to 140 mmHg and lumen diameter was recorded at each pressure once stable. Pressure was then returned to 80 mmHg for the remainder of the experiment. A single concentration (0.1 mmol/L) of the nitric oxide synthase (NOS) inhibitor nitro-L-arginine (L-NNA) was added to the bath and constriction in response to NOS inhibition was measured after approximately 20 minutes. In the presence of L-NNA, the NO donor sodium nitroprusside (SNP) was cumulatively added to the bath (10^{-8} M-10^{-5} M) and the dilation to SNP was measured at each concentration. Finally, zero-Ca^{2+} HEPES was added to the bath to obtain fully relaxed diameters. Passive diameters were then recorded at pressures from 140 to 5 mmHg.

Data Calculations. Percent tone was calculated as the percent decrease in diameter from the fully relaxed diameter in zero-Ca^{2+} HEPES by the equation: $((Ø_{passive} - Ø_{active})/ Ø_{passive}) \times 100\%$, where $Ø_{passive}$ is the diameter in zero- Ca^{2+} HEPES and $Ø_{active}$ is the diameter when the vessels had tone. Percent constriction to L-NNA was calculated with the equation: $((Ø_{baseline}-Ø_{drug})/ Ø_{baseline}) \times 100\%$ where $Ø_{baseline}$ is diameter before adding the L-NNA and $Ø_{drug}$ is diameter with presence of L-NNA. Percent sensitivity to the NO donor SNP using a concentration-response curve was calculated with the equation: $((Ø_{drug}-Ø_{minimum})/(Ø_{maximum} – Ø_{minimum})) \times 100\%$ where $Ø_{drug}$ is diameter at a specific concentration of SNP, $Ø_{minimum}$ is diameter at the lowest concentration of SNP and $Ø_{maximum}$ is the diameter at the highest concentration of SNP.

Drugs and solutions. HEPES physiological salt solution was made fresh daily and consisted of (mmol/L): 142.0 NaCl, 4.7 KCl, 1.71 $MgSO_4$, 0.50 EDTA, 2.8 $CaCl_2$, 10.0 HEPES, 1.2 KH_2PO_4, and 5.0 dextrose. HEPES without Ca^{2+} was also made daily but Ca^{2+} was left out of the solution. Apocynin, L-NNA and SNP were purchased from Sigma (St Louis, MO, USA). The concentrated apocynin was diluted in double distilled H_2O to reach the concentration of 50 µM and was stored in -20 ºC until used. L-NNA and SNP were made as stock solutions and diluted. Highly oxLDL was purchased from Kalen Biomedical (LLC, Montgomerey Village, Mn, USA; 770252-7) and stored at 4 ºC until experimentation.

*Statistical analysis.*All data are expressed as mean ± standard error of the mean. Data were analyzed using one-way ANOVA and post hoc Student-Newman Keuls test for multiple comparisons. Differences were considered statistically significant when $P < 0.05$.

Results

oxLDL increased BBB permeability that was prevented by apocynin

We recently demonstrated that increased levels of oxLDL increased BBB permeability [13]. Here, we determined if oxLDL-induced BBB permeability involved NADPH oxidase-derived superoxide. Figure 1 shows that oxLDL significantly increased BBB permeability in PCAs compared to controls without oxLDL ($P< 0.05$). Importantly, the presence of apocynin abolished the oxLDL-induced BBB permeability ($P< 0.05$), suggesting that increased NADPH oxidase activity was involved in oxLDL-induced BBB disruption. To confirm that BBB permeability was increased due to the oxidative state of LDL, the effect of nLDL on BBB permeability was compared to oxLDL-induced BBB permeability. nLDL did not increase BBB permeability compared to controls, demonstrating that the increased BBB permeability was a result of the oxidative modification of LDL.

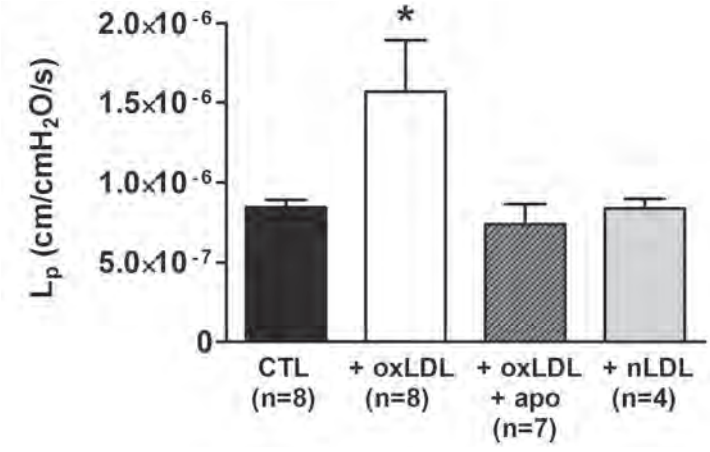

Figure 1. **Effect of apocynin on oxLDL-induced BBB permeability.** Graph showing hydraulic conductivity (L_p) as a measure of BBB permeability in response to perfusion of 20% v/v rat serum only compared to rat serum plus 5 µg/ml oxLDL (n=8), +/- the addition of 50 µM apocynin (n=8). oxLDL significantly increased BBB permeability in PCAs from female animals compared to controls. The addition of apocynin significantly abolished the oxLDL-induced BBB permeability. nLDL did not change BBB permeability compared to controls. (* $P< 0.05$ vs. all other groups by one-way ANOVA. Abbreviations: CTL, control without oxLDL; oxLDL, oxidized low-density lipoprotein; apo, apocynin; nLDL, native LDL)

oxLDL decreased myogenic tone of PCAs that was prevented by apocynin

Next, we determined the effects of circulating oxLDL on cerebral vascular function in PCAs and the possible involvement of NADPH oxidase. Figure 2A shows active diameter changes in response to increased intravascular pressure. All groups had modest constriction in response to increased pressure, demonstrating myogenic reactivity was present in all vessels. PCAs perfused with oxLDL had larger diameters at all pressures measured, but this was not statistically significant (P = 0.17).

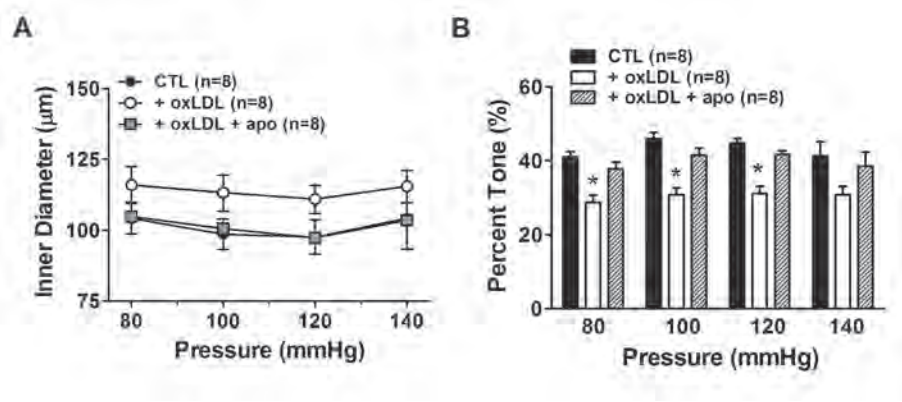

Figure 2. **Effect of oxLDL on myogenic tone of posterior cerebral arteries (PCA).** Graphs showing (A) active lumen diameter and (B) percent myogenic tone in response to increased intravascular pressure in PCA from female rats that were perfused with 20% v/v rat serum only (n=8) compared to serum plus 5 μg/ml oxLDL (n=8) +/- 50 μM apocynin (n=8). oxLDL caused larger lumen diameters compared to controls at all pressures that was abolished by apocynin, however, this was not statistically significant. oxLDL significantly decreased percent myogenic tone of PCA compared to control serum without oxLDL. The effect of oxLDL was prevented by the presence of apocynin. (* P< 0.05 vs. all other groups by one-way ANOVA. Abbreviations: CTL, control without oxLDL; oxLDL, oxidized low-density lipoprotein; apo, apocynin)

However, Figure 2B shows that oxLDL significantly decreased myogenic tone at all pressures compared to the control group (P<0.05), suggesting that the larger diameters were due to diminished basal tone. Importantly, apocynin prevented the oxLDL-induced decrease in myogenic tone in PCAs (P <0.05), suggesting activation of NADPH oxidase was involved in oxLDL-induced decreased myogenic tone. Also for these experiments, measurements were repeated with the presence of nLDL instead of oxLDL. nLDL did not cause any changes in myogenic responses or myogenic tone compared to the control group without oxLDL (data not shown), suggesting that the effects were due to the oxidative state of LDL.

oxLDL increased contriction to NOS inhibition and enhanced NO-mediated vasodilation

To determine the effect of oxLDL on basal endothelium-derived NO, a single high concentration of L-NNA was added to PCA from all 3 groups and changes in diameter measured (Figure 3A). All vessels constricted in response to L-NNA, suggesting basal NO was present and inhibited tone. The presence of oxLDL caused a significant increase in constriction to L-NNA compared to the controls ($P< 0.01$).

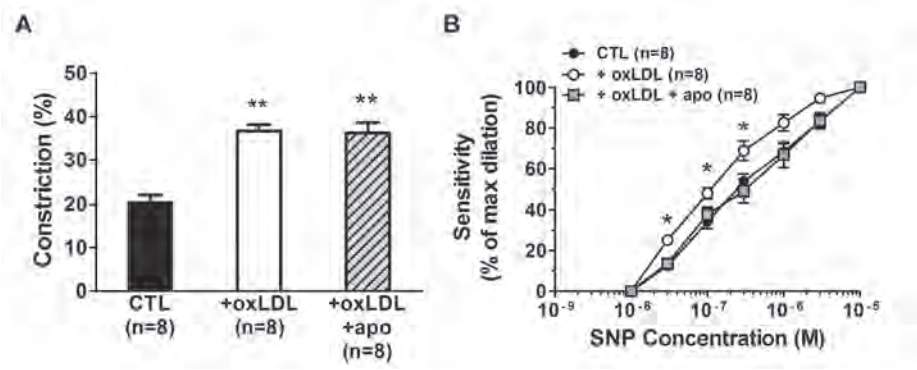

Figure 3. **Effect of oxLDL on constriction to NOS inhibition and dilation to sodium nitroprusside (SNP) in posterior cerebral arteries (PCA).** Graphs showing (A) constriction to NOS inhibition with L-NNA and (B) sensitivity to the NO donor sodium nitroprusside (SNP) in PCA from female rats in response to perfusion of 20% v/v rat serum (n=8) compared to rat serum plus 5 µg/ml oxLDL (n=8), +/- 50 µM apocynin (n=8). Perfusion of oxLDL caused a significant increase in constriction to L-NNA in PCA compared to control arteries without oxLDL. Apocynin did not affect the oxLDL-induced increase in contriction to L-NNA. oxLDL significantly increased sensitivity to SNP in PCA that was abolished by apocynin. (** $P< 0.01$ vs. all other groups by one-way ANOVA; * $P< 0.05$ vs. all other groups by one-way ANOVA. Abbreviations: CTL, control without oxLDL; oxLDL, oxidized LDL; apo, apocynin; SNP, sodium nitroprusside; L-NNA, nitro-L- arginine.)

However, there was no effect of apocynin on the increased constriction to NOS inhibition induced by oxLDL. Figure 3B shows the effect of circulating oxLDL on dilation to SNP in PCA. oxLDL caused PCAs to have increased sensitivity to SNP compared to the control group that was abolished by the presence of apocynin, suggesting involvement of NADPH oxidase activity in this response ($P< 0.05$).

$MgSO_4$ prevented oxLDL-induced BBB permeability

$MgSO_4$ has shown to be effective in preventing edema formation in several neurologic conditions, however, if $MgSO_4$ can prevent oxLDL-induced BBB permeability is not known. [16, 21]. Figure 4 shows that $MgSO_4$ prevented the oxLDL-

induced BBB disruption (P< 0.05), suggesting a direct protective effect on oxLDL-induced BBB permeability.

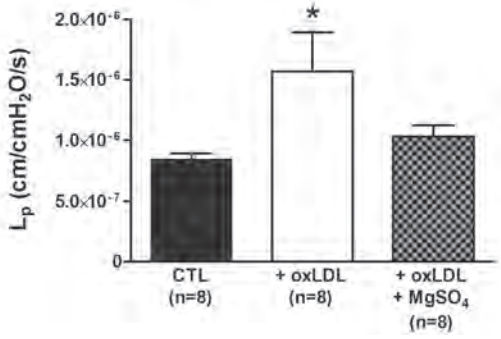

Figure 4. **Effect of $MgSO_4$ on oxLDL-induced BBB permeability.** Graph showing hydraulic conductivity (L_p) at 36 minutes as a measure of BBB permeability in response to perfusion of 20% v/v rat serum (n=8) compared to serum plus 5 µg/ml oxLDL (n=8) +/- 16 mM $MgSO_4$ (n=8). oxLDL caused an increase in BBB permeability that was prevented by $MgSO_4$. (*P< 0.05 vs. all other groups by one-way ANOVA. Abbreviations: CTL, control without oxLDL; oxLDL, oxidized low-density lipoprotein; $MgSO_4$, magnesium sulfate)

$MgSO_4$ did not affect oxLDL-induced decrease in myogenic tone

Although the data above suggest that $MgSO_4$ prevents BBB disruption caused by oxLDL, if $MgSO_4$ affects the oxLDL- induced changes in myogenic tone of PCA is not known. Figure 5A and B show that despite a protective effect on the BBB preventing oxLDL BBB permeability, $MgSO_4$ did not affect the active myogenic response in the PCAs or prevent the oxLDL-induced decrease in myogenic tone.

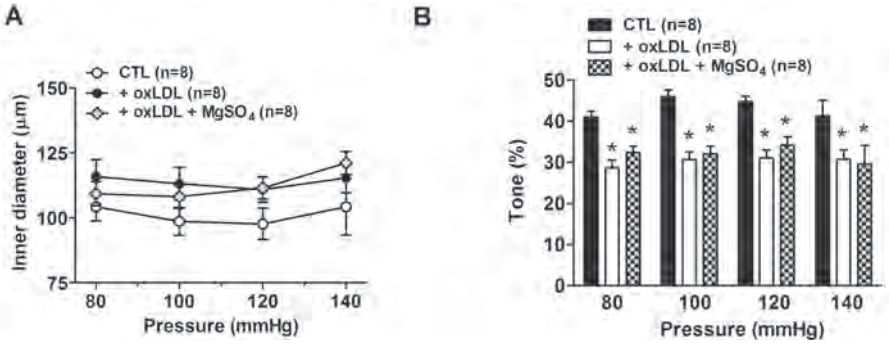

Figure 5. **Effect of $MgSO_4$ on oxLDL-induced changes in myogenic tone of posterior cerebral arteries (PCA).** Graphs showing (A) active myogenic responses and (B) myogenic tone in PCA in response to increased intravascular pressure after perfusion with 20% v/v rat serum (n=8) compared to serum plus 5 µg/ml oxLDL (n=8) +/- 16 mM $MgSO_4$ (n=8). There was no difference in active myogenic responses between groups nor did $MgSO_4$ affect the oxLDL-induced decrease in myogenic tone. (* P< 0.05 vs. all other groups by one-way ANOVA. Abbreviations: CTL, control without oxLDL; oxLDL, oxidized low-density lipoprotein; $MgSO_4$, magnesium sulfate)

$MgSO_4$ decreased oxLDL-induced dilation to SNP without affecting oxLDL-induced constriction to NOS inhibition Figure 6A and B show the constriction to NOS inhibition and dilation to the NO-donor SNP of PCAs due to oxLDL with or without the presence of $MgSO_4$. $MgSO_4$ treatment with oxLDL did not significantly affect the constriction to L-NNA, however, it did prevent the oxLDL-induced enhanced dilation to SNP in PCAs ($P< 0.05$).

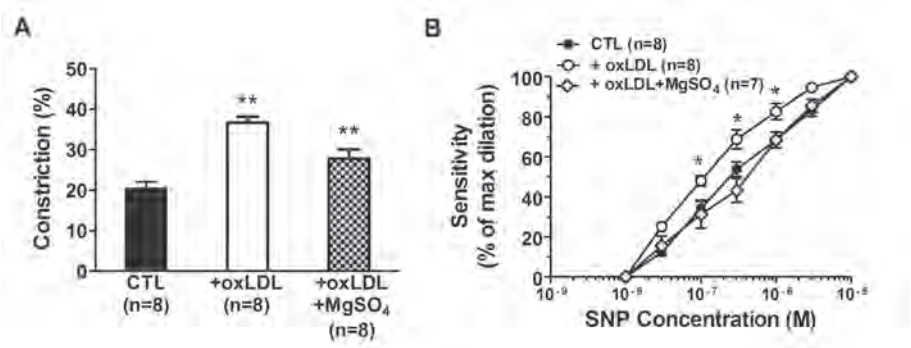

Figure 6. **Effect of $MgSO_4$ on oxLDL-induced constriction to NOS inhibition and dilation to sodium nitroprusside (SNP) in posterior cerebral arteries (PCA).** Graphs showing (A) constriction to NOS inhibition with L-NNA and (B) sensitivity to the NO donor SNP in PCA in response to perfusion of 20% v/v rat serum (n=8) compared to 5 µg/ml oxLDL +/- 16mM $MgSO_4$ (n=8). The presence of $MgSO_4$ did not significantly affect the oxLDL-induced increase in contriction to L-NNA. However, $MgSO_4$ prevented the increase in dilation to SNP in response to oxLDL in PCA. (** $P< 0.01$ vs. all other groups by one-way ANOVA; * $P< 0.05$ vs. all other groups by one-way ANOVA. Abbreviations: CTL, control without oxLDL; oxLDL, oxidized LDL; $MgSO_4$, magnesium sulfate; SNP, sodium nitroprusside; L-NNA, nitro-L-arginine.)

Discussion

In the present study, we determined the effect of oxLDL on BBB permeability and cerebral vascular function and investigated the mechanim by which it acts to increase permeability and diminsh myogenic tone. In addition, we examined the possible beneficial effects of $MgSO_4$ on oxLDL-induced changes in BBB permeability and cerebral reactivity, as it has been shown to be beneficial in treating several neurologic conditions including severe preeclampsia. Here, we demonstrate that oxLDL, but not native LDL, significantly increased BBB permeability that was prevented by apocynin, suggesting a role for NADPH oxidase activity in this response. In addition, acute exposure to oxLDL significantly decreased myogenic tone in PCAs that was also prevented by apocynin. Further, oxLDL increased constriction to NOS inhibition and

induced an enhanced dilation to the NO-donor SNP. Finally, treatment with $MgSO_4$ protected the BBB and prevented oxLDL-induced BBB disruption without affecting the oxLDL-induced changes in myogenic tone. Taken together, these results suggest that oxLDL causes BBB disruption and cerebrovascular tone dysregulation through activation of NADPH oxidase whereas the effect of $MgSO_4$ treatment on oxLDL-induced vascular dysfunction appears more limited to preventing BBB disruption and possibly vascular smooth muscle function.

oxLDL has become a known key factor in the pathogenesis of many cardiovascular diseases, such as hypertension, atherosclerosis and coronary heart disease that may also have consequences for the brain [1-3]. Extensive research of oxLDL in the systemic and coronary vasculature have revealed complex signaling cascades induced by oxLDL leading to increased expression of chemokines and adhesion molecules, trans-endothelial migration of monocytes, triggering of the inflammatory cascade, and increased production of reactive oxygen species leading to endothelial dysfunction [1-3]. However, information regarding the effects or mechanism of oxLDL in the cerebral vasculature remain limited. The cerebral endothelium that comprises the BBB differs from the peripheral vasculature in that it lacks fenestrations, and contains high electrical resistance tight junctions that limit paracellular permeability and L_p. BBB disruption induced by oxLDL could lead to vasogenic brain edema and contribute to neurologic complications by allowing damaging proteins, toxins and plasma constituents to enter the brain parenchyma [10, 22]. Thus, understanding the underlying mechanisms by which oxLDL affects the cerebral circulation and treating its damaging effects could potentially benefit several cardiovascular and cerebrovascular diseases.

One condition that involves increased levels of oxLDL and can be complicated with neurologic symptoms induced by vasogenic brain edema is preeclampsia [13, 23, 24]. In a previous study, we revealed a novel mechanism of BBB disruption in response to increased circulating oxLDL in severe preeclampsia through generation of peroxynitrite [13]. Here, we demonstrate for the first time that increased levels of oxLDL significantly increased BBB permeability in PCAs that was prevented by apocynin, suggesting the effect was mediated by NADPH oxidase-derived superoxide. We also show that this effect was specifically due to the oxidative modification of LDL, as nLDL did not affect BBB permeability. It is generally accepted that increased levels of superoxide rapidly bind NO to generate peroxynitrite, a toxic radical, that

can result in endothelial dysfunction [25]. Thus, we speculate that oxLDL increases superoxide production through NADPH oxidase that leads to subsequent generation of peroxynitrite resulting in BBB disruption. Although data investigating the mechanism of BBB disruption in preeclampsia or other neurologic conditions are scarce, our proposed mechanism is supported by several previous reports using cell-culture models that demonstrated that increased levels of oxLDL activate superoxide production through NADPH oxidase leading to subsequent increased generation of peroxynitrite [8, 26]. Importantly, because NADPH oxidase activity is greater in the cerebral vasculature compared to the systemic vasculature [27], oxLDL-induced increased activity of NADPH oxidase can result in excessive production of superoxide in the cerebral vasculature leading to extensive damage of the BBB and vasogenic brain edema. Thus, oxLDL and NADPH oxidase may be important therapeutic targets for treatment and/or prevention of neurologic symptoms in preeclampsia and possibly other neurologic conditions where BBB disruption and edema formation is involved.

Another unique aspect of the cerebral vasculature is that it has the intrinsic ability to maintain a relatively constant CBF despite changes in perfusion pressure partly due to intrinsic myogenic mechanisms that modulate CVR. Maintenance of cerebral hemodynamics, including autoregulation of CBF and CVR is important in conditions such as preeclampsia in which women often have sustained high blood pressure. Here, we found that acute intraluminal exposure of oxLDL caused PCAs to have larger diameters and significantly decreased myogenic tone, an effect that could lead to hyperperfusion, cerebral endothelial damage and BBB disruption. To our knowledge, this is the first report to show the detrimental effects of 5μg/ml oxLDL on PCA function. A previous study investigated the effect of 10 μg/ml oxLDL on PCAs from rabbits and found that oxLDL increased myogenic tone, which is in contrast to the results of the present study [28]. However, the concentration used in the previous study was higher than in the present study. Previous reports have described different effects from oxLDL on the systemic vasculature using different concentrations [29] such that concentrations of oxLDL higher than 10μg/ml increased myogenic tone due to decreased NO production, while concentrations of oxLDL between 1μg/ml and 10μg/ml increased NO production [29, 30]. Our results show a significant increase in constriction to NOS inhibition in response to oxLDL, suggesting increased basal NO production in the PCAs. Thus, although the previous studies focused on the effects of oxLDL on the systemic vasculature, it

may be possible that 5µg/ml oxLDL also increased endothelium-derived NO in the cerebral vasculature that could contribute to decreased myogenic tone.

The present study also found that the presence of the NADPH oxidase inhibitor apocynin prevented the oxLDL-induced decrease in myogenic tone, suggesting superoxide production may be involved in this response. In a previous study, we demonstrated that high cholesterol-treated rats had PCAs with decreased myogenic tone that was due to generation of peroxynitrite [7, 11]. Together with the finding that oxLDL caused increased constriction to NOS inhibition, we speculate that oxLDL-induced superoxide production combines with increased NO to generate peroxynitrite. This hypothesis is supported by previous reports that found that peroxynitrite is a powerful vasodilator at high concentrations and decreases myogenic tone in the cerebral vasculature [11, 31, 32]. Thus, next to the detrimental effects of oxLDL-induced generation of peroxynitrite in disrupting the BBB, it may also decrease myogenic tone in PCAs.

In addition, we determined the effect of $MgSO_4$ on oxLDL-induced BBB disruption and vascular tone dysregulation. $MgSO_4$ is widely used and effective in the management of severe preeclampsia for prevention of neurologic complications such as seizures[21]. In additon, several studies examing brain injury and acute hypertension have shown $MgSO_4$ to be protective of the BBB, however, the exact mechanism remains unclear [14, 15]. $MgSO_4$ is a calcium antagonist and a potent vasodilator by inhibiting vascular smooth muscle contraction, however, these effects are considerabely less effective in the cerebral vasculature compared to the systemic arteries, suggesting $MgSO_4$ is not likely acting as a vasodilator in the cerebral vasculature [16, 20]. Here, we found that $MgSO_4$ abolished oxLDL-induced BBB permeability without affecting oxLDL-induced changes in myogenic tone. These results confirm earlier reports that $MgSO_4$ is protective of the BBB without causing significant dilation in the cerebral vasculature. However, it is not clear from this study if $MgSO_4$ directly antagonizes the mechanism of oxLDL that leads to BBB disruption or if $MgSO_4$ had an overall protective effect from the damaging effects of oxLDL. It is possible that the mechanism by which $MgSO_4$ acts is through its ability to scavenge free radicals such as superoxide and decrease lipid-peroxidation [33]. Although this may explain the protective effect of $MgSO_4$ on BBB disruption, oxLDL-induced decrease in myogenic tone was not prevented, suggesting $MgSO_4$ may act primarily to protect the cerebral endothelium.

Finally, we found that oxLDL increased sensitivity to SNP that was prevented by both apocynin and $MgSO_4$. A previous study examining effects of oxLDL on smooth muscle function found that oxLDL did not influence the dilation to SNP in the abdominal aorta [34]. However, another study found that oxLDL decreased dilation to SNP in carotid arteries without affecting dilation to SNP in basilar arteries, suggesting oxLDL may affect smooth muscle function differently in different vascular beds [35]. Another study found that oxLDL enhances smooth muscle contraction in cultured cells when exposed to contractile agents, suggesting that oxLDL sensitizes smooth muscle after interaction with factors that influence smooth muscle vasomotion [36]. Importantly, all these previous studies used concentrations that were at least 10-fold higher in concentration than that used in this study. Nevertheless, we also found that oxLDL influences smooth muscle vasomotion in that oxLDL sensitizes the smooth muscle to the NO donor SNP. However, our data are too limited to fully explain how oxLDL increased sensitivity to SNP, or how apocynin and $MgSO_4$ prevented this effect. Future studies are necessary to examine how 5 µg/ml oxLDL enhances dilation to SNP in PCAs and how apocynin and $MgSO_4$ are involved in this response.

Conclusion

In summary, the present study demonstrates for the first time that oxLDL causes increased BBB permeability and cerebral vascular tone dysregulation that was prevented by apocynin, suggesting involvement of NADPH oxidase derived superoxide in these oxLDL-induced damaging effects. In addition, treatment with $MgSO_4$ prevented oxLDL-induced BBB disruption without affecting active cerebral myogenic responses. These results demonstrate the significance of oxLDL in the occurence of cerebral vascular dysfunction in conditions such as severe preeclampsia and may be an important marker in the pathogenesis of many neurologic conditions.

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Human studies:

Chapter 6:

Formerly Eclamptic Women have Lower Nonpregnant Blood Pressure Compared to Formerly Preeclamptic Women: a Retrospective Cohort Study.

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Abstract

Objective: To compare nonpregnant blood pressure and circulating metabolic factors between formerly preeclamptic women who did and did not deteriorate to eclampsia.

Design: Retrospective observational cohort study.

Setting: Tertiary referral centre.

Population: Formerly preeclamptic women with (n=88) and without superimposed eclampsia (n=638).

Methods: Women who experienced preeclampsia with or without superimposed eclampsia during their pregnancy or puerperium were tested for possible underlying cardiovascular risk factors at least 6 months postpartum. We measured blood pressure and determined cardiovascular and metabolic risk markers in a fasting blood sample. Groups were compared using Mann-Whitney U test, Spearman's Rho test or Fisher's Exact test (odds ratios).

Main outcome measures: Differences in postpartum blood pressures and features of the metabolic syndrome between formerly preeclamptic and formerly eclamptic women.

Results: Formerly preeclamptic women who developed eclampsia differed from their counterparts without eclampsia by a lower blood pressure ($p<0.01$) with blood pressure correlating inversely with the likelihood of having experienced eclampsia. In addition, formerly eclamptic women had higher circulating C-reactive protein levels than formerly preeclamptic women ($p< 0.05$). All other circulating metabolic factors were comparable.

Conclusions: Formerly preeclamptic women with superimposed eclampsia have lower nonpregnant blood pressure compared to their counterparts without neurological sequellae with blood pressure negatively correlated to the occurrence of eclampsia.

Introduction

Preeclampsia is a complex vascular disease unique to pregnancy defined by hypertension (blood pressure ≥ 140/90 mmHg) combined with proteinuria (>0.3g/l) [1, 2]. A serious, life-threatening complication in preeclamptic women is eclampsia, defined as the new onset of generalized seizures [3, 4]. Although relatively rare (3-6 per 10,000 births), eclampsia carries a major burden on maternal and perinatal morbidity and mortality worldwide [5]. In the brain, eclampsia relates to edema, haemorrhage and stroke, but also impairment of cognitive function in later life [3, 4].

Eclampsia is generally accepted to be a form of posterior reversible encephalopathy syndrome (PRES) [6]. PRES is characterized by vasogenic brain edema notably in the white matter of the parietal and occipital lobes, as seen in nearly all eclamptic patients [7-10]. Blood-brain barrier (BBB) disruption is the hallmark event in the development of vasogenic brain edema by promoting the passage of damaging plasma constituents into the brain parenchyma potentially evoking seizure activity[8, 11]. BBB disruption can occur when blood pressure rises above the upper cerebral autoregulatory limit. However, during pregnancy, a large proportion of eclamptic women had no precedence of hypertension [4, 12, 13]. Although animal studies suggest the cerebral blood flow autoregulatory curve is shifted to higher pressures during pregnancy, it is not known whether biological variations in this curve exist in healthy young women outside of pregnancy [14, 15]. It is possible that eclamptic women without significant hypertension had even lower nonpregnant blood pressure resulting in a lower limit of the cerebral autoregulatory curve.

In addition to the rise in blood pressure, circulating factors capable of initiating BBB disruption may be involved increasing the susceptibility to eclampsia in preeclamptic women. Previous studies have shown that increased plasma levels of oxidized low density lipoprotein (oxLDL), a known key factor in vascular dysfunction in severe preeclamptic women, also increase BBB permeability [16, 17,18]. Elevated levels of oxLDL may be the result of enhanced oxidation of LDL during preeclampsia. Alternatively, women with preeclampsia developing eclampsia may differ from their non-eclamptic counterparts by a preexistent abnormal metabolic profile, such as dyslipidemia.

In this study, we hypothesized that formerly eclamptic women differed from women that suffered from preeclampsia only by a lower blood pressure outside of pregnancy. In addition, we explored whether the peripheral levels of metabolic factors related to the metabolic syndrome are more prevalent in the nonpregnant state in formerly eclamptic women than in formerly preeclamptic women.

Methods

Study population. In the period between 1996 and 2010, we screened women with a previous hypertensive pregnancy disorder (such as preeclampsia or eclampsia) several months postpartum for possible underlying cardiovascular risk factors at two tertiary care centers. The study was approved by the Hospital Medical Ethical Committees (Maastricht University Medical Center and Radboud University Medical Center; MEC 0-4-049, 2007/252). In the present study, we analyzed the data of all primiparous women enrolled in the screening program with a history of preeclampsia complicated by eclampsia (n=92) versus preeclampsia without eclampsia (n=867). Preeclampsia was defined according to the criteria of the Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy [2]. All patients discontinued breastfeeding or hormonal contraceptive use at least 4 weeks before screening. Also, women diagnosed with pre-existing hypertension (hypertension diagnosed before 20 weeks of pregnancy or at the time of screening) were excluded (eclampsia group: n=4; preeclampsia group: n= 169). Hypertension was diagnosed as systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg, or when women were taking antihypertensive drugs. On the evening before the day of screening all patients refrained from eating, drinking and smoking until screening completion.

Blood pressure measurements. Measurements took place at 8:00 AM in a quiet and temperature-controlled room. All women were positioned in a sitting position with the upper part of the measurement arm at heart level, while the whole arm was relaxed. After an acclimatization period of 30 minutes, arterial blood pressure was recorded at 3-minute intervals for a period of 30 minutes using a semiautomatic oscillometric device (Dinamap Vital Signs Monitor 1846; Critikon, Tampa, FL) with a cuff size of 13.5×30.7 cm if upper arm circumference ranged from 27.5 to 36.5 cm

or a cuff size of 17×38.6 cm if upper arm circumference ranged from 35.5 to 46 cm. After accumulating 9 measurements, the median systolic, diastolic, mean arterial pressures and median heart rate was calculated and registered.

Measurements of metabolic factors. All blood samples were collected in the morning after an overnight fast. Blood samples were taken for plasma lipid profile, glucose, insulin and renal function. Triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL), uric acid, C-reactive protein (CRP) and glucose were determined using standard automated laboratory techniques. Low-density lipoprotein (LDL) cholesterol was calculated by the Friedewald equation [19]. Plasma insulin concentrations were measured using AutoDelfia time-resolved fluoroimmunoassay. Finally, all women had collected a 24 hour urine sample to determine the presence of proteinuria normalized for creatinine output using normal lab standards.

Statistical analysis. All data are presented as means ± standard deviation. All data were analyzed with Mann-Whitney U test. Sub-analysis of the blood pressure data was performed using trend analysis and odd ratios. Inter-group differences were considered statistically significant when the probability (P) was below 0.05.

Results

Table 1 lists the demographics and clinical characteristics of the women with pregnancy being complicated by preeclampsia, without (PE; n=698) and with superimposed eclampsia (E; n=88). Both subgroups were comparable with respect to age, height, weight, BMI, percentage smokers, and also with respect to time elapsed between delivery and screening. In contrast, both subgroups differed from one another with respect to clinical characteristics of the index pregnancy. Compared to the E-subgroup, we noticed that women in the PE-subgroup had delivered at an earlier gestational age. In addition, the birth weight and birth weight centile were lower in the PE-subgroup compared to the E-subgroup, which was not only due to more severe prematurity but also because of a higher incidence of fetal growth restriction.

Table 1. Demographics of formerly preeclamptic and eclamptic women.

Indices	PE (n=698)	E (n=88)	*P*
Age (yrs)	31.0 ± 4.1	30.3 ± 4.5	0.19
BMI (kg/m^2)	25.2 ± 4.9	24.9 ± 4.8	0.67
Height (cm)	168.3 ± 6.5	167.6 ± 6.6	0.51
Weight (kg)	71.4 ± 14.7	70.3 ± 15.1	0.46
Smoking (%)	12	15	0.25
Time between delivery and screening (months)	11 ± 8	11 ± 9	0.81
Index pregnancy			
SGA (<p10;%)	33	20	0.02
Gestational days birth	232 ± 38	242 ± 31	0.01
Gestational weeks birth	$33^{1/7} \pm 5^{2/7}$	$34^{6/7} \pm 4^{3/7}$	0.01
Birth weight (grams)	1737 ± 864	2212.9 ± 1000	<0.01
Birth weight centile	28 ± 14	35 ± 19	0.01

BMI = body mass index; SGA = small for gestational age. PE = preeclampsia; E= eclampsia.

At the time of screening, we compared both subgroups with respect to blood pressures and heart rates (Table 2). Importantly, the systolic, diastolic and mean arterial pressure were lower outside of pregnancy in the E-subgroup compared to the PE-subgroup. Also, heart rate was lower in the E-subgroup compared to the PE-subgroup.

Table 2. Nonpregnant blood pressure of formerly preeclamptic vs. eclamptic women.

Indices	PE (n=698)	E (n=88)	*P*
BP systolic	119 ± 12	111 ± 9	< 0.01
BP diastolic	72 ± 9	67 ± 8	< 0.01
MAP	88 ± 10	83 ± 8	< 0.01
Heart rate (beats per minute)	71 ± 10	68 ± 10	0.03

BP = Blood Pressure; MAP = Mean Arterial Pressure; PE= preeclampsia; E= eclampsia.

In addition, our results demonstrate that the blood pressure is negatively correlated with the likelihood of the development of eclampsia (Figure 1). When organizing both subgroups according to different blood pressure quintiles, trend analysis showed a significant negative correlation between blood pressure and the occurrence of eclampsia ($p < 0.001$). Also, odds ratio of the quintile subgroups compared to the 20% lowest blood pressure population showed that women in the 4th and highest quintile group had a significant lower incidence of eclampsia

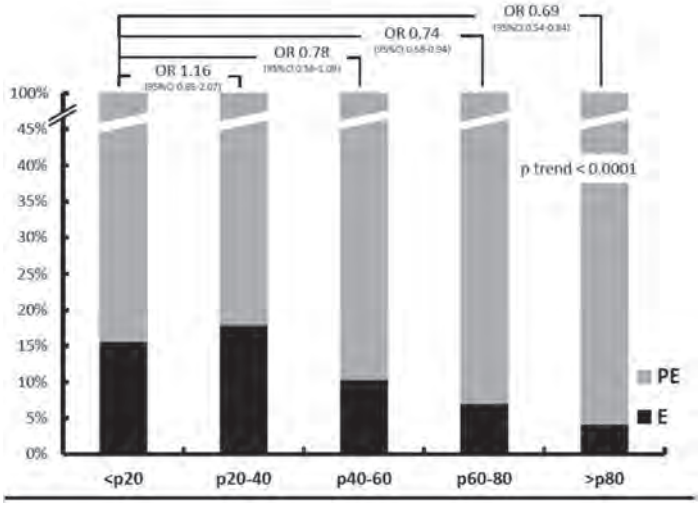

Figure 1. Percentages of the occurrence of eclampsia (E) versus preeclampsia (PE) arranged in quintile groups of the mean arterial blood pressure (MAP). Graph showing trend-analysis that demonstrates a significant negative correlation between MAP and the occurrence of eclampsia (E). In addition, graph showing odd ratio of 2nd to 5th quintile group compared to the lowest quintile group of the MAP. Odds ratio's show a decreased risk of the occurrence of eclampsia when increasing MAP in the different population quintiles that is reaching statistical difference in the 4th and 5th quintile group ($p < 0.05$). Population Quintiles and corresponding MAP: <p20: MAP < 79 mmHg; p20-40: MAP 79-84 mmHg; p40-60: MAP 84-89 mmHg; p60-80: MAP 89-97 mmHg; >P80: MAP > 97 mmHg.

We also analyzed if women diagnosed with eclampsia differed to their counterparts with respect to contributing signs of an underlying form of metabolic syndrome The difference in metabolic variables between the 2 subgroups was inconsistent, except for a marginally but significantly higher C-reactive protein (CRP) level in the E-subgroup (Table 3).

Table 3. Nonpregnant metabolic factors formerly preeclamptic vs. eclamptic women.

Indices	PE (n=698)	E (n=88)	*P*
Proteinuria (mg/mmol)	7.5 ± 3.3	7.3 ± 6.0	0.19
Uric acid (mmol/l)	4.2 ± 1.1	4.3 ± 1.0	0.56
Total cholesterol (mmol/l)	4.7 ± 0.9	4.8 ± 0.9	0.13
HDL (mmol/l)	1.4 ± 0.8	1.3 ± 0.3	0.87
LDL (mmol/l)	3.0 ± 0.8	2.9 ± 0.9	0.38
Triglycerides (mmol/l)	1.1 ± 0.7	1.2 ± 0.7	0.13
Glucose (mmol/l)	5.0 ± 0.7	5.1 ± 1.0	0.75
Insulin (mmol/l)	11.1 ± 5.7	10.5± 5.3	0.17
HbA1c	5.2 ± 0.4	5.4 ± 0.8	0.88
CRP (mmol/l)	3.6 ± 2.1	4.8 ± 3.1	0.02

PE = preeclampsia; E= eclampsia; HDL= high density lipoprotein; LDL= low density lipoprotein.; CRP= C-reactive protein.

To determine whether the occurrence of developing eclampsia relative to childbirth related to any of variables measured at screening, we subdivided all women that suffered from eclampsia Both antepartum and postpartum E-subgroups were similar with regard to age, height, weight, BMI and percentage of smokers (Table 4). The antepartum E-subgroup differed from the postpartum E-subgroup by delivery at an earlier gestational age, resulting more often in an infant with a lower birth weight and a lower birth weight centile (Table 4).

Finally, we investigated if there were any differences in blood pressure or metabolic syndrome constituents between antepartum or postpartum eclampsia.

Table 4. Demographics of formerly antepartum vs. postpartum eclampsia.

Indices	Antepartum (n=53)	Postpartum (n=35)	*P*
Age (yrs)	30.2 ± 4.1	30.5 ± 4.9	0.73
BMI (kg/m^2)	24.8 ± 4.5	25.0 ± 5.2	0.95
Height (cm)	168 ± 7	168 ± 7	0.94
Weight (kg)	70.0 ± 13.7	70.6 ± 13.4	0.81
Smoking (%)	19	14	0.26
Time between delivery and screening (months)	11 ± 8	12 ± 9	0.50
Index pregnancy			
SGA (<p10)	20	17	0.88
Gestational days birth	222 ± 22	273 ± 9	< 0.01
Gestational weeks birth	$31^{5/7} \pm 5^{2/7}$	$39^{0/7} \pm 4^{3/7}$	< 0.01
Birth weight (grams)	1588 ± 847	3123 ± 416	< 0.01
Birth weight centile	30 ± 28	47 ± 25	0.02

BMI = body mass index; SGA = small for gestational age.

Table 5A and 5B show that blood pressure, heart rate and metabolic indices were similar in both groups. Women in the antepartum E-subgroup showed a trend towards having higher LDL and total cholesterol compared to the postpartum E-subgroup, but those variables did not reach statistical significance. Triglycerides were significantly higher in the postpartum E-subgroup.

Table 5A. Nonpregnant blood pressure in formerly antepartum vs. postpartum eclampsia.

Indices	Antepartum (n=53)	Postpartum (n=35)	*P*
BP systolic	113 ± 9	111 ± 9	0.29
BP diastolic	68 ± 8	66 ± 6	0.25
MAP	84 ± 8	81 ± 8	0.63
Pulse rate	68 ± 9	68 ± 11	0.87

BP = Blood Pressure; MAP = Mean Arterial Blood Pressure.

Table 5B. Nonpregnant metabolic factors in formerly antepartum vs. postpartum eclampsia.

Indices	Antepartum (n=53)	Postpartum (n=35)	*P*
Proteinuria (mg/mmol)	7.3 (± 0.7)	7.4 (± 0.4)	0.19
Uric acid (mmol/l)	0.24 (± 0.06)	0.20 (± 0.07)	0.18
Total cholesterol (mmol/l)	4.7 (± 0.9)	4.4 (± 1.0)	0.08
HDL (mmol/l)	1.3 (± 0.4)	1.3 (± 0.6)	0.56
LDL (mmol/l)	2.9 (± 0.6)	2.6 (± 0.8)	0.09
Triglycerides (mmol/l)	1.09 (± 0.7)	1.13 (± 0.7)	0.02
Glucose (mmol/l)	5.2 (± 0.9)	4.9 (± 0.5)	0.89
Insulin (mmol/l)	11.0 (± 5.7)	9.8 (± 4.8)	0.29
HbA1c	5.3 (± 0.9)	5.3 (± 0.4)	0.23
CRP (mmol/l)	4.5 (± 2.9)	4.9 (± 3.0)	0.73

HDL= high density lipoprotein; LDL= low density lipoprotein.

Discussion

This study provides convincing evidence for formerly eclamptic women to have a lower blood pressure and heart rate in the nonpregnant state compared to women that suffered from preeclampsia only. Importantly, blood pressure is negatively correlated with the occurrence of eclampsia. In addition, formerly preeclamptic patients with or without superimposed eclampsia were comparable with respect to metabolic features, except for slightly higher peripheral levels of CRP after eclampsia.

Eclampsia remains a significant life-threatening complication of pregnancy and can be considered as a form of PRES [13]. A recent study found that nearly all eclamptic patients had MRI changes that could be interpreted radiologically as PRES [10]. In this study, we analyzed a cohort of eclamptic women and searched for clinical and metabolic features predisposing to superimposed eclampsia in

preeclamptic women. We demonstrated that formerly eclamptic women had lower nonpregnant blood pressure compared to formerly preeclamptics with a negative correlation between blood pressure and the occurrence of eclampsia. We can only speculate about the clinical relevance of this finding. Preeclampsia is partly defined by the development of hypertension after 20 weeks of pregnancy in a previously normotensive woman [20]. This definition does not consider the magnitude of the rise in blood pressure throughout the course of gestation. However, we speculate that the change in blood pressure in preeclamptic women throughout gestation is more important in the development of neurologic complications than the absolute number and thereby should be assessed individually.

One important contributor of the development of vasogenic brain edema and eclampsia is breakthrough of cerebral blood flow autoregulation [3, 13, 15]. Above the autoregulatory pressure threshold, forced dilation of the cerebral vessels occurs, resulting in hyperperfusion, which may lead to significant brain tissue damage and vasogenic brain edema and life-threatening sequels [13, 21]. Several studies have found a considerable percentage of eclamptic women without significant hypertension [10, 22]. This has led to the concept that pregnancy may shift the upper limit of autoregulation. Although pregnancy was shown to extend the upper limit of the autoregulatory curve during acute hypertension in rats,[14] pregnant rats showed brain edema formation at significantly lower blood pressures compared to non-pregnant rats [23]. In addition, pregnancy both prevents and reverses medial hypertrophy of cerebral arteries resulting from chronic exposure to hypertension. This effect eliminates effectively the possible protective effect against forced dilatation of the preexistent vascular remodeling [24, 25]. Here, we speculate that women with a relatively low pre-pregnant blood pressure who develop preeclampsia during pregnancy have a higher risk for eclampsia by autoregulatory breakthrough at a lower blood pressure than their counterparts with a higher nonpregnant blood pressure. However, this hypothesis assumes that blood pressures measured remote from pregnancy reflect antepartum blood pressures and needs to be addressed in future studies.

Additionally, the incidence of postpartum eclampsia has increased at the cost of antepartum eclampsia, probably as a result of intensified surveillance and more active clinical management during pregnancy [26]. Here, we found about 40% of all cases of eclampsia to occur postpartum, which is in line with the 30-50% incidence

of postpartum eclampsia reported by others [26]. Importantly, previous reports indicated postpartum eclampsia often was not preceded by earlier diagnosed preeclampsia or hypertension [19, 27, 28]. It follows that women with mild hypertension during pregnancy may have blood pressures above their autoregulatory threshold putting them at particular risk during the puerperium, where blood pressure monitoring is decreased, yet with no clinical profile to differentiate them from low-risk women. This changing pattern of eclampsia confronts health professionals with the challenge of recognizing women in the puerperium with neurologic symptoms that need prompt referral.

Impaired cerebral autoregulation may not be the only factor associated with an increased risk for preeclampsia/eclampsia. Circulating factors have shown to increase BBB permeability, neuronal hyper-excitability and seizure activity [16, 17]. For example, physiological hyperlipidemia is a normal feature of pregnancy. However, this characteristic is much more pronounced during preeclampsia [29, 30, 31]. Recent studies demonstrated that elevated circulated levels of oxLDL increase BBB permeability and may thereby be an important contributor to vasogenic brain edema in severe early-onset preeclampsia [16, 32]. Our results suggest that antepartum eclampsia occurs more often in early-onset preeclampsia, however, did not indicate that eclamptic women already had an underlying metabolic disorder. We showed a trend, but not significant, towards higher nonpregnant circulating lipids in antepartum eclampsia compared to postpartum eclampsia. Nevertheless, previous studies support that women that suffered from severe early-onset preeclampsia have a higher risk of metabolic syndrome in later life [33, 34]. Further research is needed to investigate hyperlipidemia in severe early-onset preeclamptic patients, combined with elevated growth factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) that could interact with each other and induce significant oxidative stress. In fact, previous animal studies found that inhibition of VEGF receptor tyrosine kinase activity prevented BBB disruption induced by plasma from preeclamptic women [17]. The mechanism underlying VEGF-induced BBB permeability in preeclamptic plasma was shown to be due to VEGF receptor phosphorylation and release of nitric oxide that binds oxidative radicals generated by activated oxLDL to cause endothelial dysfunction through the formation of peroxynitrite [16, 35]. Thus, circulating factors that cause oxidative stress and endothelial dysfunction likely have a role in cerebral edema formation during eclampsia.

Additionally, our results showed that CRP was slightly but consistently higher in formerly eclamptics compared to formerly preeclamptic women. Previous studies found that CRP may have a predictive value in predicting severe preeclampsia [36, 37]. CRP binds to the same receptor as oxLDL, the so-called LOX-1 receptor, which subsequently induces endothelial dysfunction by releasing toxic oxidative radicals [16, 18, 32]. Also, CRP is a risk factor for cardiovascular events [38]. Thus, CRP may be involved in the oxLDL-LOX1 pathway that induces BBB disruption in women resulting in eclampsia or other neurologic sequels. Further research is necessary to examine the involvement and clinical significance of CRP in the pathogenesis of (pre)eclampsia.

Limitations

In this study, we were unable to access data from these women specifying their pre-pregnancy health status and medical check-ups during pregnancy before the onset of preeclampsia/eclampsia. Also, although we do not show any underlying metabolic disorder in these women, it may be possible that pregnancy itself unmasks metabolic syndrome that will re-emerge in later life, beyond the time frame for which this study was conducted. Another limitation is that we did not measure angiogenic factors, which made it impossible to assess the possible involvement of other growth factors such as VEGF, sFlt-1 and PLGF in the development of superimposed eclampsia. Finally, the fact that women with preeclampsia are increasingly managed more aggressively in the course of the last 2 decades may have led to a bias in the eclamptic group.

Conclusion

Formerly eclamptic women have significant lower non-pregnant blood pressure compared to formerly preeclamptic women with a negative correlation between blood pressure and the occurrence of eclampsia. This observation supports the concept that the rate and/or magnitude of the rise of blood pressure rather than the maximum blood pressure at the onset of eclamptic fits may be more important in triggering eclampsia. As about 40% of all eclamptic cases occur postpartum, surveillance of preeclamptic women during childbirth should include routine blood pressure monitoring postpartum.

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Chapter 7:

General Discussion

Introduction

Healthy human and rodent pregnancy is characterized by a maternal circulatory transition towards a high volume- low resistance state, a condition thought to be necessary for a normal development of the maternal-fetal unit, and is paralleled by changed vessel wall integrity, hemodynamics and coagulation [1-3]. Concomitantly, the maternal brain is challenged to maintain its constant blood supply and strict water homeostasis to prevent cerebral edema, and with it, seizures or other neurologic complications [4]. Currently, there is only limited understanding of the adaptation of the cerebral vasculature in pregnancy in humans with more data on rodents. Understanding how pregnancy and pregnancy-related conditions such as preeclampsia affect the cerebral vasculature is of great importance, considering neurologic complications to be a major cause of maternal death worldwide [5, 6]. In this manuscript, we investigated how the rat cerebral vasculature is affected by pregnancy and examined mechanisms of cerebral vascular dysfunction in pregnancy related to potentially life-threatening neurologic complications. In addition, we searched for potential cardiovascular differences in a cohort of women that were diagnosed with preeclampsia only or preeclampsia that was complicated by eclampsia.

Normal pregnancy: the adaptation of the blood-brain barrier.

The cerebral vasculature has several unique features compared to the other peripheral vascular beds [7]. First, the cerebral vasculature is the site of the blood-brain barrier (BBB). The BBB comprises an unique endothelial layer that lacks fenestrations, contains high electrical resistance tight junctions and has restricted transcellular and paracellular flux of ions and proteins, resulting in a strong protective mechanism against vasogenic brain edema [8-10]. During normal pregnancy, the placenta releases large amounts of permeability promoting factors that may affect the BBB permeability, however, vasogenic brain edema does not normally occur [11]. Apparently, the cerebral vasculature or its hemodynamics appears to adapt during normal pregnancy to maintain its protective state against vasogenic edema formation.

In women diagnosed with preeclampsia, development of neurologic complications due to BBB disruption and vasogenic brain edema may occur. As these women may develop epileptic seizures in absence of high blood pressure, these observations suggest pregnancy itself may affect the BBB. In **chapter 2**, we confirmed previous studies in that the permeability factor vascular endothelial growth factor (VEGF) is significantly increased in pregnancy. VEGF has been shown to be necessary for the development and maintenance of the maternal-fetal unit and uteroplacental circulation, however, other studies have shown that increased levels of VEGF can cause vasogenic brain edema during brain injury [12, 13]. We found that VEGF could induce increased BBB permeability in the nonpregnant state, but that VEGF was unable to increase BBB permeability during pregnancy. We also examined possible adaptive mechanisms that prevent the BBB during pregnancy. First, the BBB itself could adapt to increased VEGF by alteration of VEGF-receptor (VEGFR) expression. A previous study observed increased permeability in uterine vessels in pregnancy accompanied by increased mRNA-expression of VEGF-receptors [13]. In our study, no increased mRNA expression of VEGF-receptors was observed. This could reflect a possible adaptative mechanism where the uterine veins require increased vascular permeability for controlled extravasation of intravenous molecules into the maternal-fetal unit [14], while the BBB must maintain its protective state. A second explanation may be the presence of circulating factors in plasma during pregnancy preventing VEGF-induced BBB permeability. We found that sFlt1, a known regulator of the bioavailability of VEGF that is also increased in pregnancy [12], critically regulates VEGF action at the BBB by preventing VEGF-induced BBB permeability, even in excessive levels. This finding is interesting because it has been thought that excess levels of sFlt1 are involved in the pathogenesis of preeclampsia [15]. Although these data are limited to an acute exposure time, other studies also did not find BBB disruption in response to sFlt1 alone, but only in interaction with pro-inflammatory cytokines such as TNFα it appeared to cause damage [16, 17]. This could suggest that increased sFlt1 in pregnancy does not increase BBB permeability, but may be a protective mechanism for the threatened BBB in that release of sFlt1 is triggered by high levels of permeability factors such as VEGF.

Dyslipidemia in pregnancy, good or bad?

It has been thought that pregnancy might act as a 'stress test' that may unmask women's cardiovascular risk mediated through metabolic syndrome or other circulatory conditions related to high blood pressure [18, 19]. Metabolic syndrome

that includes dyslipidemia, jeopardizes endothelial cell function and may even be involved in endothelial dysfunction in preeclampsia [20, 21]. On one hand, during pregnancy, there is a physiological hyperlipidemia that is thought to encourage lipogenesis and fat storage in preparation for rapid fetal growth without affecting endothelial cell function [22]. On the other hand, in preeclampsia, previous studies have showed an excessive hyperlipidemia compared to normal pregnancy that may promote cerebral vascular dysfunction [21, 23]. Several reports investigating stroke have reported that dyslipidemia can cause cerebral vascular dysfunction [24, 25]. In **chapter 3**, we show that excessive hyperlipidemia caused greater inflammatory responses en oxidative stress in cerebral arteries during pregnancy compared to the nonpregnant state. The fact that dyslipidemia enhanced oxidative stress is confirmed in studies investigating other cardiovascular diseases and thereby suggesting that preeclampsia show similar etiologies compared to these conditions. In addition, we found in **chapter 4** that women diagnosed with early-onset preeclampsia showed excessive dyslipidemia in that levels of oxidized low density lipoprotein (oxLDL) were approximately 260% higher compared to women diagnosed with late-onset preeclampsia or women that experienced a normal pregnancy, suggesting that lipids are of great importance in the pathogenesis of pregnancy related disorders such as preeclampsia.

The pathogenesis of the development of neurologic complications in preeclampsia.

Although extensive research has been performed on the pathogenesis of preeclampsia, understanding of the development of the life-threatening complications of preeclampsia such as eclampsia has received less attention. In this manuscript we did not only focus on the adaptation of the cerebral vasculature in normal pregnancy, but also on the consequences of preeclampsia on the cerebral vasculature. Preeclampsia is a complex heterogenous disorder and its pathogenesis still remains to be elucidated [5, 26]. It has been thought that preeclampsia can progress to a severe state and eventually into eclampsia, thus affecting the brain. Nowadays is has become generally accepted that eclampsia can be considered as a form of posterior reversible encephalopathy syndrome (PRES) [27]. The hallmark event in the occurrence of neurologic complications may be the development of vasogenic brain edema, as is shown as PRES on MRI imaging in eclamptic patients [4, 28, 29]. However, the underlying mechanisms are still largely unknown. Before the onset of eclamptic seizures, one theory is that

elevated blood pressures causes cerebral autoregulation breakthrough resulting in increased cerebral blood flow leading to endothelial dysfunction and vasogenic edema [4, 30]. However, a large number of number of women that suffered from eclamptic seizures or other neurologic complications, such as uncontrolled vomiting, severe headache, cortical blindness, or cerebral hemorrhage, did not show elevated blood pressures [31, 32]. This may suggest other limits in the cerebral autoregulation curve or other factors that may be involved. **Chapter 6** describes that women that suffered from eclampsia had lower nonpregnant blood pressure compared to women that suffered from preeclampsia only. This observation suggest that those eclamptic women with lower baseline blood pressure have autoregulatory curve disruption at lower pressures. This was confirmed by a previous animal study investigating autoregulation in late pregnant rats showed brain edema at significantly lower blood pressures compared to nonpregnant rats [33]. In addition, previous studies demonstrated that pregnancy prevents and reverses medial hyperthrophy of cerebral arteries that occurs during chronic hypertension, effectively eliminating any protective effect of vascular remodeling on forced dilatation [34, 35]. Importantly, **chapter 6** also shows that the blood pressure is negatively correlated with the occurence of eclampsia. Because more agressive screening and clinical management have shifted the prevalence of eclampsia from antepartum to postpartum, health professionals are challenged with recognising women in the puerperium without hypertension but already have neurologic symptoms that need prompt referral.

Nevertheless, previous studies have shown that the BBB plays a central role in the etiology of vasogenic brain edema and neurologic complications. BBB disruption promotes the passage of damaging proteins and plasma constituents into the brain parenchyma that may activate microglia that promote seizure activity [36, 37]. In fact, a previous study has showed that circulating factors present in plasma from severe preeclamptic women increased BBB permeability, demonstrating the central role of the BBB in promoting neurologic complications in preeclampsia [38]. We found in **chapter 4** that oxLDL is substantially increased in plasma from women with severe, early-onset preeclampsia. We also found that exogenous oxLDL to the levels of preeclamptic plasma, but not native LDL (nLDL), causes BBB disruption, demonstrating the involvement of oxLDL in the onset of vasogenic brain edema and neurologic symptoms. The fact that oxLDL was mainly increased in early-onset preeclampsia was confirmed in that BBB permeability was significantly increased in

response to plasma from early-onset preeclamptic women compared to late-onset preeclamptic women, suggesting different etiologies. These results are supported by epidemiologic studies that demonstrated that neurologic complications in preeclampsia occurred most often in preeclampsia diagnosed before 34 weeks compared to preeclampsia that developed after 34 weeks [32, 39-41]. Also, previous studies have found that this difference may arise from a greater placental underperfusion and subsequent increased release of circulating vasoactive factors [40]. Thus, in this manuscript, we demonstrate oxLDL as an underlying mechanism of BBB disruption in early-onset preeclampsia and a possible new important therapeutic target. **Chapter 6** showed that women that suffer from severe early preeclampsia and eclampsia did not show underlying features of metabolic syndrome outside of pregnancy. Thus, women that develop severe preeclampsia and/or eclampsia may develop a more pronounced hyperlipidemia during pregnancy instead of suffering from dyslipidemia outside of pregnancy.

oxLDL is a known key factor in the pathogenesis of many cardiovascular diseases [42, 43]. Oxidative modification of physiological nLDL into oxLDL occurs in numerous disease states as a result of oxidative stress and the presence of reactive oxygen species. The formation of oxLDL initiates multiple pathways in both endothelial and vascular smooth muscle cells, mostly through binding to its receptor lectin-like oxLDL receptor (LOX-1). oxLDL binding to LOX-1 generates complex signaling cascades leading to induction of the inflammatory pathway and increased production of superoxide that can further promote vascular dysfunction [42, 43]. Previous studies already showed that LOX-1 could be involved in preeclampsia in that plasma from preeclamptic women caused upregulation of LOX-1, however, we support that also its ligand oxLDL increases and causing BBB disruption without upregulation of LOX-1 due to its short exposure time [44-46]. In **chapter 4** we show that oxLDL-induced BBB permeability could be prevented with a LOX-1 antibody, confirming the involvement of LOX-1 activation in BBB disruption. LOX-1 activation is known to increase production of superoxide and other reactive oxygen species. Several reports have shown in cell-culture that oxLDL-LOX-1 activation decreased NO by activation of NADPH oxidase, an important source of superoxide production [47, 48]. In fact, in **chapter 4 and 5**, we describe that oxLDL increases BBB permeability through NADPH oxidase activation and subsequent generation of peroxynitrite. In line with our findings, several other studies have showed in different cell-culture models that oxLDL is able to increase

NADPH oxidase activity and increase peroxynitrite [47, 48]. Because NADPH oxidase expression and activity are greater in the cerebral vasculature compared to the peripheral circulation [49], the brain may be especially vulnerable for activation of NADPH oxidase and subsequent peroxynitrite generation. Thus, we present a novel mechanism for the development of neurologic complications in early-onset preeclampisa in that increased oxLDL binds to LOX-1, to increase NADPH oxidase activity, that can produce excessive superoxide. Superoxide can bind rapidly nitric oxide (NO) to form the toxic radical peroxynitrite, resulting in BBB disruption and vasogenic brain edema.

In addition to the direct effects of oxLDL on the BBB, we also studied how oxLDL may impact the cerebral vascular reactivity that could contribute to the development of neurologic symptoms in pregnancy. In **chapter 5**, we found that oxLDL, but not native LDL, significantly decreased myogenic tone in posterior cerebral arteries, an effect that could lead to diminished cerebral vascular resistance, hyperperfusion, and BBB disruption due to high hydrostatic pressure. Together with the finding that oxLDL caused increased constriction to NOS inhibition, it may be possible that oxLDL-induced superoxide production combines with increased NO to generate peroxynitrite that is the vasoactive component. This hypothesis is supported by previous reports that found that peroxynitrite is a powerful vasodilator at high concentrations and decreases myogenic tone in the cerebral vasculature [50-52]. Thus, in addition to the direct detrimental effects of oxLDL-induced generation of peroxynitrite disrupting the BBB, it may also decrease myogenic tone in PCAs affecting cerebral circulatory autoregulation. Importantly, in **chapter 3**, we also found that in pregnancy, excessive hyperlipidemia also decreased myogenic tone due to presence of increased peroxynitrite. Because excessive levels of lipids also increased BBB permeability in pregnancy that could be inhibited by a LOX-1 antibody, it is tempting to speculate that excessive hyperlipidemia in pregnancy is accompanied by conversion of nLDL to the detrimental oxLDL leading to oxidative stress and peroxynitrite generation causing cerebrovascular dysfunction. Thus, in addition to direct impact on the endothelial layer of the BBB, oxLDL may also predispose the cerebral vascular reactivity to cerebral vascular dysfunction.

Magnesiumsulfate ($MgSO_4$) therapy as prevention of neurologic complications in preeclampsia

$MgSO_4$ is widely used and effective in the management of severe preeclampsia for prevention of neurologic complications such as seizures [53, 54]. Several studies examining brain injury and acute hypertension have shown $MgSO_4$ to be protective of the BBB, however, the exact mechanism remains unclear [53, 54]. $MgSO_4$ is a calcium antagonist and a potent vasodilator by inhibiting vascular smooth muscle contraction, however, these effects are considerably less effective in the cerebral vasculature compared to the systemic arteries, suggesting $MgSO_4$ is not likely acting as a vasodilator in the cerebral vasculature [55, 56]. In **chapter 5**, we found that $MgSO_4$ abolished oxLDL-induced BBB permeability without affecting oxLDL-induced changes in myogenic tone. These results confirm the earlier reports that $MgSO_4$ is protective of the BBB without causing significant dilation in the cerebral vasculature . However, it is not clear from this study if $MgSO_4$ directly antagonizes the mechanism of oxLDL that leads to BBB disruption or if $MgSO_4$ had an overall protective effect on the BBB. It may be possible that the mechanism by which $MgSO_4$ acts is through its ability to scavenge free radicals such as superoxide and decrease lipid-peroxidation. Although this may explain the protective effect of $MgSO_4$ on BBB disruption, oxLDL-induced decrease in myogenic tone was not prevented, suggesting $MgSO_4$ may act primarily to protect the cerebral endothelium.

Conclusions

1. Pregnancy causes a unique challenge to the maternal brain. While other organs undergo substantial changes to increase the maternal blood flow, the adaptation of the cerebral circulation appears to maintain a relative constant blood flow and maintain the protective state of the blood-brain barrier (BBB).
2. The BBB adapts in normal pregnancy and this may be the most important adaptation of the maternal brain in pregnancy to prevent increased BBB permeability, BBB disruption, vasogenic edema and neurologic complications. Although several permeability factors are released into the maternal circulation such as VEGF, BBB permeability remains unchanged compared to the nonpregnant state, where VEGF does cause significantly increased BBB permeability. It appears that the anti-angiogenic factor sFlt1

is critical in regulating and preventing VEGF-induced BBB permeability in pregnancy.

3. BBB disruption in pregnancy can lead to life-threatening neurologic symptoms such as seizure, edema, hemorrhage and coma. It appears to be that mainly severe early-onset preeclamptic women are at risk to develop neurologic symptoms. It may be that the physiologic hyperlipidemia in pregnancy, combined with oxidative stress during poor placentation, converts native LDL into oxidized LDL (oxLDL) and is significantly increased in plasma from early-onset preeclamptic women. oxLDL has shown to decrease cerebrovascular resistance that may impair cerebral autoregulation. Importantly, oxLDL causes significant BBB disruption through production of superoxide and subsequent generation of peroxynitrite, showing that oxLDL is not only an underlying mechanism of neurologic complications in preeclampsia, but also a possible important therapeutic target.
4. Magnesium sulfate ($MgSO_4$) directly prevents oxLDL-induced BBB permeability without preventing oxLDL-induced decrease in myogenic tone, suggesting $MgSO_4$ may act primarily to protect the cerebral endothelium without affecting active cerebral myogenic responses.
5. Women that suffered from eclampsia have lower nonpregnant blood pressure compared to women that suffered from preeclampsia only. Importantly, there is a negative correlation between blood pressure and the occurrence of eclampsia. Because eclampsia is not often preceded by hypertension and the prevalence of eclampsia is increasing in the puerperium, postpartum care should be intensified to identify women with neurologic symptoms that need acute referral to prevent eclampsia.

Future perspectives

The development of (pre)eclampsia during pregnancy remains a life-threatening complication, however its exact pathogenesis still remains to be elucidated. This manuscript has revealed new insight on the physiologic adaptations of the maternal brain during normal pregnancy. Importantly, this manuscript also unraveled a new mechanism that is involved in the pathogenesis of eclampsia. However, future animal and human studies should be performed to gain further insight in the phyiologic and pathologic changes in the maternal brain during pregnancy.

Adaptations of the maternal brain during normal pregnancy.

This manuscript demonstrates for the first time that the anti-angiogenic circulating factor sFlt1 is a critical regulator of the bioavailability of VEGF. It also demonstrates that PLGF is able to increase the BBB permeability. Several animal studies have demonstrated significant reversible structural changes in the maternal brain during normal pregnancy. Further animal studies are needed to gain further insight in the effect of circulating factors in the maternal brain during normal pregnancy combined with the structural cerebral changes that occur. It will be important to investigate the effects of longterm expression of sFlt1 on the BBB to translate its involvement in physiologic changes in the brain during pregnancy. Further, the exact role of PLGF on the BBB should be further investigated to increase our understanding if and how PLGF may be involved in the pathogenesis of neurologic complications in preeclampsia. It will be important to distinguish whether the release of specific circulating factors during normal pregnancy either have a protective or detoriating effect on the maternal brain. Understanding the physiologic changes in the maternal brain during normal pregnancy will help identify the pathologic circulating risk factors during pregnancy.

Cerebrovascular changes in the development of preeclampsia and eclampsia.

This manuscript demonstrates a novel mechanism in the pathogenesis of neurologic complications in severe early-onset preeclampsia. It demonstrates that especially early-onset preeclamptic women are at risk for neurologic complications through an increased level of oxidized LDL and subsequent generation of peroxynitrite that will lead tot BBB disruption. Further animal studies are needed to determine if this mechanism is initiated by a predisposition of underlying metabolic syndrome in women that have higher levels of LDL that become oxidized, or that the levels of LDL are similar with a higher level of oxidation. Also, further animal studies are needed to investigate the interaction of VEGF with oxLDL and the possible relevance of these interactions in the pathogenesis of preeclampsia. An important next step is to examine the levels of oxLDL in all pregnant women and compare these levels between women that experienced a normal pregnancy compared to a hypertensive pregnancy. It would be interesting to measure the levels of LDL before pregnancy as well. Also, women that have suffered from preeclampsia during pregnancy should be examined by MRI to investigate the occurence of PRES and compare early-onset preeclampsia to late-onset preeclampsia. Further, it would be interesting to measure the BBB in women during their pregnancy to

observe the changes in women that experience a pregnancy that is complicated by a hypertensive disorder. If we are able to identify the specific changes and riskfactors in these women, new therapeutic targets could be developed for treatment or even preventing neurologic complications in preeclampsia. Further, since we have demonstrated the protective effect of MgSO4, women could receive improved and individualised treatment with antihypertensives and MgSO4 in preventing neurologic complications during pregnancy.

Long term consequenses of (pre)eclampsia: follow up.

It has been known for over 4 decades that women that suffered from preeclampsia or eclampsia have an increased risk of cardiovacular disease in later life. More recently it has been described that only women that suffered from early-onset disease are at increased risk for cardiovascular disease in later life. Future large cohort studies could identify the exact risk factors in early-onset preeclampsia versus late-onset preeclampsia versus eclampsia. It would be important to identify possible differences in the risk profile of formerly preeclamptic versus formerly eclamptic patients. Since this manuscript already found differences in blood pressures between these two subgroups, it is very likely they have a different risk profile for developing cardiovascular disease in later life. Further, it would be interesting to know if changes in the maternal brain during eclampsia have left irreversible structural changes in the cerebral microcirculation that will lead to an increased risk for developing cerebral vascular disease in later life. A follow-up study of formerly preeclamptic and eclamptic women would be necessary to identify these possible differences. If these differences are identified, treatment or preventive lifestyle intervention programs could be developed to minimize the risk for cerebrocardiovascular disease in later life.

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Chapter 8:

Summary

The brain is one of the most highly perfused organs of the human body due to its high metabolic needs critical for normal functioning and its pivotal role in maintaining homeostasis throughout the whole body. Thus, its supply requires strict regulated transport of nutrients with exclusion of potentially harmful constituents. To accomplish this, the cerebral vasculature has unique features compared to the peripheral vascular beds. First, the cerebral vessels comprise an unique endothelial lining that forms the blood-brain barrier (BBB). The BBB is a tight regulator of transport of nutrients and forms a strong protective system against formation of vasogenic brain edema, a state involved in numerous pathologic neurologic conditions and thought to precede epileptic seizures. Second, the cerebral vasculature has the intrinsic ability to maintain a relatively constant cerebral blood flow (CBF) despite substantial changes in perfusion pressure.

Early pregnancy is accompanied by a sudden drop in total peripheral vascular resistance leading to decreased blood pressure and secondary increase in plasma volume and cardiac output. Also, there is an increased production of vasoactive permeability-increasing factors. However, increased BBB permeability does not normally occur during normal pregnancy and normal pregnant women do not suffer from neurologic complications. If and how the BBB and cerebral vasculature adapts to pregnancy-related changes is not clear.

Preeclamptic pregnancy is thought to be superimposed upon cardiovascular risk factors both jeopardizing placenta and endothelial function. These risk factors, often consistent with the metabolic syndrome, are commonly present during preeclamptic pregnancy, and put substantial oxidative burden upon the vessel wall. This oxidative stress has potential deleterious effects on the cerebral vasculature in that it may cause BBB disruption, vasogenic brain edema and life-threatening neurologic complications .To reduce the possibility of secondary neurologic complications in preeclampsia, $MgSo_4$ is often used as primary choice of treatment. However, its exact working mechanism remains to be elucidated. The current thesis concentrates on the adaptation the BBB in pregnancy and the mechanism of BBB disruption and cerebrovascular dysfunction in preeclampsia.
Chapter 1 presents a general introduction describing the cerebral vasculature and the impact of changes in the cerebral vasculature in normal pregnancy and preeclampsia. **Chapter 1** concludes with describing the following aims for this thesis:

1. To investigate the impact of increased angiogenic and anti-angiogenic factors on BBB permeability in pregnancy.

2. To investigate if and how high levels of lipids impact the cerebral vasculature in pregnancy compared the nonpregnant state.

3. To investigate the effects and underlying mechanisms of oxLDL on BBB permeability in preeclampsia.

4. To investigate the mechanism of oxLDL-induced BBB permeability and the effects of oxLDL on cerebral vascular reactivity.

5. To investigate the effect of $MgSO_4$ on oxLDL-induced cerebral dysfunction.

6. To investigate blood pressure and metabolic factors in a cohort of women that suffered from eclampsia compared to women that were diagnosed with preeclampsia only.

In **Chapter 2** we focused on the first aim of this thesis and investigated how the maternal brain and specifically the BBB may adapt during pregnancy to high levels of the permeability factor vascular endothelial growth factor (VEGF). Also, we determined the influence of soluble-Flt1 (sFlt1) on VEGF signaling at the BBB, because BBB disruption and vasogenic brain edema are important factors in the involvement of neurologic symptoms in preeclampsia. Lastly, we investigated the permeability-inducing potential of placental growth factor (PLGF). We found that both VEGF and PLGF are able to significantly increase BBB permeability, however through activation of different VEGF receptors in that PLGF increased BBB permeability through VEGF-receptor1 (Flt) and VEGF required both VEGF-receptor 1(Flt) and 2 (Flk). We found that circulating factors present in plasma from pregnant rats prevented VEGF-induced BBB permeability and preserved barrier function during pregnancy in the face of high circulating angiogenic factors such as VEGF. BBB permeability was similar in both the nonpregnant and late pregnant rats without the addition of exogenous VEGF, despite the finding that veins from late pregnant rats showed increased VEGF mRNA expression. Exogenous addition of VEGF initiated a significant increase in BBB permeability when exposed to a nonpregnant model, while it did not affect BBB permeability in a pregnant model.

Importantly, BBB permeability was also not affected to exogenous VEGF when mixed in plasma from pregnant rats but exposed to cerebral veins of nonpregnant animals, suggesting that VEGF-induced BBB permeability was specifically prevented due to the circulating factors present in the plasma from pregnant rats. Further, sFlt1 regulated VEGF-induced permeability similar to the plasma from pregnant rats in that it abolished permeability in response to VEGF. The finding that increased sFlt1 protects the BBB against VEGF-induced permeability raises some new questions about the role of high sFlt1 in the onset of neurologic complications in preeclampsia. As high sFlt1 appears to be protective of the BBB, there may be other pro-inflammatory factors released during preeclampsia, but not during normal pregnancy, that are responsible for increased BBB permeability and edema formation.

As we have found that sFlt1 was protective of the maternal BBB rather than causing BBB disruption we changed our focus from angiogenic imbalance to the effect of dyslipidemia on the maternal brain in preeclampsia. In preeclampsia, hyperlipidemia is enhanced compared to normal pregnancy that could adversely affect vascular function. In the cerebral vasculature, this could lead to dysregulation of cerebral blood flow resulting into neurological complications in preeclamptic women. In **Chapter 3,** we examined the second aim of this thesis and determined the effect of excessive hyperlipidemia on cerebral artery function in pregnant rats compared to the nonpregnant state by creating an in vivo high cholesterol model in pregnant and nonpregnant rats. As it is known that peroxynitrite is generated due to high levels of lipids, we also examined the effect of peroxynitrite generation on cerebral function during hyperlipidemia. Peroxynitrite is generated by superoxide and nitric oxide and has been known to have deliterious effects on endothelial function. We showed that an excessive hyperlipidemia caused cerebral arteries to have less myogenic tone in both pregnancy and nonpregnancy, that was more pronounced in the pregnant state due to a significant amount of peroxynitrite generation. These findings are confirmed by previous studies that showed that peroxynitrite can act as a powerfull vasodilator in the cerebral vasculature. We also found that excessive hyperlipidemia caused a greater inflammatory response in cerebral arteries in pregnancy compared to the nonpregnant state in that iNOS expression was increased a 12-fold.

One of many circulating factors that is increased in preeclampsia and is associated with hyperlipidemia is oxLDL. oxLDL is known to cause endothelial dysfunction in numerous cardiovascular disease such as hypertension, diabetes and cerebral vascular disease, however, its involvement in preeclampsia is not known. In **Chapter 4,** we investigated oxLDL and LOX-1 activation as an underlying mechanism to induced BBB disruption in preeclampsia. Also, we determined possible differences in BBB disruption between early-onset preeclampsia (preeclampsia diagnosed before 34 weeks of gestation) and late-onset preeclampsia (preeclampsia diagnosed later than 34 weeks of gestation). We provided new direct evidence for a different etiology between early-onset preeclampsia and late-onset preeclampsia and revealed a novel mechanism that is responsible for BBB disruption in women diagnosed with early-onset preeclampsia. Circulating factors in plasma from early-onset preeclamptic women significantly increased BBB permeability compared to late-onset preeclamptic women. The increased BBB permeability was prevented by inhibiting LOX-1 and was confirmed in vivo in pregnant rats with pathologically high levels of LDL that also showed LOX-1 dependent increased BBB permeability. Circulating oxLDL, a major ligand of LOX-1, was 260% increased in early-onset preeclampsia compared to late-onset preeclampsia. The fact that this increased level of oxLDL was able to cause BBB disruption was confirmed by experiments that showed that similar levels of isolated exogenous oxLDL increased BBB permeability comparable to plasma from early-onset preeclamptic women. Also, the selective peroxynitrite decomposition catalyst FeTMPyP inhibited the increased BBB permeability induced by plasma from EPE women or by exogenous oxLDL, confirming the involvement of peroxynitrite formation in oxLDL-induced BBB disruption. Thus, our results here showed for the first time that increased circulating oxLDL present in plasma from EPE women significantly increase BBB permeability through LOX-1 activation and subsequent increased peroxynitrite generation.

Because we wanted to further examine the mechanism of oxLDL-induced BBB permeability and the impact of oxLDL to cerebral vascular function, we examined the effect and mechanism of isolated oxLDL on posterior cerebral artery (PCA) permeability and vascular reactivity in **Chapter 5**. In **Chapter 4,** we found that oxLDL increased generation of peroxynitite, however, how this peroxynitrite generation occured was not investigated. In addition, in **Chapter 5** we also examined the possible beneficial effects of $MgSO_4$ on oxLDL-induced changes in BBB permeability

and cerebral reactivity, as it has been shown to primary treatment for preventing neurologic complications in severe preeclampsia. We showed that oxLDL, but not native LDL, significantly increased BBB permeability and decreased myogenic tone in posterior cerebral arteries that was prevented by apocynin, suggesting a role for NADPH oxidase activity and superoxide in these responses. Treatment with $MgSO_4$ protected the BBB and prevented oxLDL-induced BBB disruption without affecting the oxLDL-induced changes in myogenic tone. Thus, these results suggest that oxLDL causes BBB disruption and cerebrovascular tone dysregulation through activation of NADPH oxidase. We suggest that oxLDL increases NADPH oxidase to produce superoxide that will form peroxynitrite when combined to nitric oxide that will result in endothelial damage and cerebral vascular dysfunction. Also, $MgSO_4$ shows to be protective of the BBB whereas the effect of $MgSO_4$ treatment on oxLDL-induced vascular dysfunction appears more limited.

In **Chapter 6** we compared a large cohort of women that suffered either from preeclampsia only of preeclampsia complicated by eclampsia. We measured blood pressure in the nonpregnant state and we compared circulating factors that are features of the metabolic syndrome. This large cohort demonstrated that women that suffered from eclampsia had significant lower blood pressure outside of pregnancy compared to women that were diagnosed with preeclampsia only. Also, analysis showed a negative correlation between blood pressure and the occurence of eclampsia. We also found that the prevalence of eclampsia was 60% in the antepartum group and 40% in the postpartum group. These findings are of clinical significance because it is known that a significant percentage of eclampsia is not preceded by the definition of hypertension. Thus, it may be that women with lower base line pressure have a more pronounced risk to develop eclampsia. The 40% prevalence postpartum indicates that postpartum care should we intensified and changed to detect women with neurologic symptoms that could be warning signs for eclampsia, while blood pressure is still relatively low. Further, metabolic factors were compared to determine if women that have developed eclampsia already suffered from underlying metabolic syndrome. Our data did not show a significant difference between the formerly preeclamptic and eclamptic women, suggesting women do not already have underlying metabolic syndrome.

Chapter 7 describes a general discussion of the most important findings listed in this thesis and the following conclusions are presented:

1. Pregnancy causes a unique challenge to the maternal brain. While other organs undergo substantial changes to increased the blood flow, the adaptation of the cerebral circulation appears to maintain a relative constant blood flow and maintain the protective state of the BBB.

2. The BBB adapts in normal pregnancy and this may be the most important adaptation of the maternal brain in pregnancy to prevent increased BBB permeability, BBB disruption, vasogenic edema and neurologic complications. Although several permeability factors are released into the maternal circulation such as the permeability VEGF, BBB permeability remains unchanged compared to the nonpregnant state, where VEGF does cause significantly increased BBB permeability. It appears that the anti-angiogenic factor sFlt1 is critical in regulating VEGF-induced BBB permeability in pregnancy.

3. BBB disruption in pregnancy can lead to life-threatening neurologic symptoms such as seizure, oedema, hemorrhage and coma. It appears to be that mainly severe early-onset preeclamptic women are at risk to develop neurologic symptoms. It may be that the physiologic hyperlipidemia in pregnancy, combined with oxidative stress during poor placentation, converts native LDL into oxLDL and is significantly increased in plasma from early-onset preeclamptic women. oxLDL has shown to decrease cerebrovascular resistance that may impair cerebral autoregulation. More importantly, oxLDL causes significant BBB disruption through production of superoxide and subsequent generation of peroxynitrite, showing that oxLDL is not only a underlying mechanism of neurologic complications in preeclampsia, but also a possible important therapeutic target in severe preeclamptic women.

4. $MgSO_4$ directly prevents oxLDL-induced BBB permeability without, preventing oxLDL-induced decrease in myogenic tone, suggesting $MgSO_4$ may act primarily to protect the cerebral endothelium without affecting active cerebral myogenic responses.

5. Women that suffered from preeclampsia complicated by eclampsia have lower nonpregnant blood pressure compared to women that suffered from preeclampsia only. There is a negative correlation between blood pressure and the occurrence of eclampsia. Since eclampsia is often not preceded by hypertension and the prevalence of eclampsia is increasing in the puerperium, postpartum care should be intensified to identify women with neurologic symptoms that need prompt referral to prevent eclampsia.

Nederlandse Samenvatting

Het brein is een van de meest doorbloede organen van het menselijk lichaam. De hersenen hebben, om optimaal te functioneren, een hoge metabole vraag. De brandstofvoorziening van het brein heeft daarom een strikt gereguleerd transportsysteem van voedingsstoffen en zuurstof die daarnaast ook potentiele toxines snel af voert of buiten de hersenen houdt. Om dit regulatiesysteem goed te laten werken hebben de hersenen enkele unieke kenmerken in tegenstelling tot de perifere vasculaire vaatbedden. Ten eerste hebben de cerebrale vaten een unieke endotheliale cellaag die de bloed-hersen barriere (BBB) vormt. De BBB is een nauwkeurige regulator van het transport van voedingstoffen en vormt een sterke beschermend systeem om formatie van vasogeen cerebraal oedeem te voorkomen. Vasogeen oedeem is betrokken bij veel pathologische neurologische condities waaronder epileptische aanvallen. Daarnaast heeft het cerebrale vaatbed de intrinsieke capaciteit om een relatief constante bloedstroom te behouden ondanks schommelingen in de perfusiedruk.

De vroege zwangerschap gaat gepaard met een plotselinge daling in perifere vaatweerstand. Dit resulteert in eerste instantie in een daling van de bloeddruk en vervolgens een compensatoire stijging van het plasma volume en het hart-minuut volume. Daarnaast is er sprake van een stijging van vaso-actieve permeabiliteitsfactoren. Echter, tijdens een normale zwangerschap is er, ondanks de aanwezigheid van meer permeabiliteitsfactoren, geen sprake van een verhoogde BBB permeabiliteit waardoor zwangere vrouwen normaliter geen neurologische symptomen zullen vertonen. Of en hoe de BBB en de cerebrale vaten zich aanpassen aan zwangerschaps-gerelateerde veranderingen is niet duidelijk.

Er wordt gedacht dat hypertensieve complicaties in de zwangerschap zoals preeclampsie worden veroorzaakt door cardiovasculaire risicofactoren die de placentaire en endotheliale celfunctie negatief beïnvloeden. Deze cardiovasculaire risicofactoren, waarvan velen ook tot het metabool syndroom behoren, zijn vaak aanwezig tijdens preeclampsie en veroorzaken substantiële oxidatieve stress in de vaatwanden wat kan leiden tot endotheliale celdysfunctie. In het cerebrale vaatstelsel kan oxidatieve stress potentiële beschadigingen veroorzaken zoals BBB disruptie, vasogeen oedeem en levensbedreigende neurologische complicaties. Om vrouwen tegen neurologische complicaties van preeclampsie te beschermen worden vrouwen primair of secundair preventief behandeld met

Magnesiumsulfaat ($MgSO_4$). Het exacte werkingsmechanisme van $MgSO_4$ is echter niet volledig bekend. Dit manuscript concentreert zich op de adaptatie van de BBB in de zwangerschap en mogelijke mechanismes van BBB disruptie en cerebrale cardiovasculaire dysfunctie in preeclampsie.

Hoofdstuk 1 geeft een algemene introductie van het manuscript weer. Het beschrijft het cerebraal vaatsysteem en de impact van de veranderingen van het cerebraal vaatsysteem tijdens de normale zwangerschap en zwangerschappen gecompliceerd door preeclampsie. Daarnaast beschrijft **Hoofdstuk 1** de doelstellingen/hypotheses van het manuscript:

1. Vaststellen wat de impact is van gestegen angiogene en anti-angiogene factoren op de BBB permeabiliteit tijdens de normale zwangerschap.

2. Onderzoeken of en hoe hogere lipiden spiegels het cerebral vaatsysteem kan beïnvloeden tijdens de zwangerschap in vergelijking met de situatie buiten de zwangerschap.

3. Bepalen wat de mogelijke effecten zijn van geoxideerd low density lipoproteïne (oxLDL) op de BBB tijdens preeclampsie.

4. Onderzoeken wat het mechanisme is van oxLDL-geïnduceerde BBB permeabiliteit en het effect van oxLDL op de cerebrale vaatreactiviteit.

5. Het vaststellen van mogelijke verschillen van de bloeddruk en metabole circulerende factoren in een groot cohort tussen vrouwen die een preeclapsie gecompliceerd door eclampsie of louter een preeclampsie hebben doorgemaakt.

In **Hoofdstuk 2** werd er dieper ingegaan op de eerste doelstelling van dit manuscript. We hebben onderzocht hoe het maternale brein en specifiek de BBB zich tijdens de zwangerschap aanpast aan hoge concentraties van de permeabiliteitsfactor vascular endothelial growth factor (VEGF). Daarnaast hebben we gekeken naar de invloed van sFlt1 op de VEGF-geïnduceerde effecten op de BBB, wetende dat BBB doorbraak en het daaropvolgende vasogeen cerebraal oedeem belangrijke factoren zijn in het ontstaan van neurologische symptomen tijdens preeclampsie.

Als laatste hebben we het potentiële BBB-inducerende effect van placental growth factor (PLGF) onderzocht. We vonden dat zowel VEGF als PLGF de mogelijkheid bezitten om de BBB permeabiliteit significant te laten stijgen, maar wel door verschillende VEGF-receptor activatie. PLGF verhoogde de BBB permeabiliteit door activatie van VEGF-receptor 1 (Flt) terwijl VEGF zowel VEGF-receptor 1 (Flt) als VEGF-receptor 2 (Flk) moest binden om effect te hebben op de BBB. Daarnaast vonden we dat circulerende factoren die aanwezig zijn in het bloedplasma van zwangere ratten VEGF-geïndcueerde BBB permeabiliteit voorkwamen en de beschermende functie van de BBB behielden. BBB permeabiliteit was gelijk in zowel de zwangere als niet-zwangere ratten, ondanks het feit dat cerebrale venen van zwangere ratten verhoogd VEGF mRNA expressie vertoonden. Exogeen toegevoegde VEGF initieerde een significante stijging van de BBB permeabiliteit in een niet-zwanger rattenmodel, terwijl het in een zwanger rattenmodel geen veranderingen veroorzaakte. Daarnaast bleef de BBB ook onaangetast als VEGF werd toegevoegd in plasma van zwangere ratten blootgesteld aan een cerebrale vene van een niet-zwangere rat, wat suggereert dat de VEGF-geïnduceerde BBB permeabiliteit werd voorkomen door circulerende factoren die vrijkomen in het bloed tijdens de zwangerschap. Verder lieten we zien dat sFlt1 de VEGF-geïnduceerde BBB permeabiliteit op dezelfde wijze beïnvloedde als het plasma van zwangere ratten. De observatie dat verhoogd sFlt1 de BBB beschermt tegen VEGF-geïnduceerde BBB permeabiliteit creëert nieuwe vragen over de rol van sFlt1 in het ontstaan van neurologische complicaties bij preeclampsie. Omdat hoge concentraties sFlt1 de BBB lijken te beschermen, zijn er misschien andere pro-inflammatoire circulerende factoren betrokken bij het ontstaan van BBB disruptie en cerebraal vasogeen oedeem tijdens preeclampsie.

Omdat onze experimenten niet direct lieten zien dat sFlt1 de BBB schaadde maar zelfs beschermde, hebben we ons focus verlegd van angiogene/anti-angiogene imbalans naar de effecten van hyperlipidemie op het maternale brein tijdens preeclampsie. Tijdens de normale zwangerschap is er sprake van een fysiologische hyperlipidemie die in preeclampsie verder doorstijgt en schade kan aanrichten aan het cerebrale en perifere vasculaire stelsel. Dit kan in het cerebrale vaatbed leiden tot dysregulatie van de cerebrale bloedstroom wat kan resulteren in neurologische complicaties in vrouwen met preeclampsie. In **Hoofdstuk 3** onderzochten we de tweede doelstelling van dit manuscript en bekeken of hyperlipidemie tijdens de zwangerschap andere effecten heeft op het cerebrale vaatbed in

vergelijking tot buiten de zwangerschap. Voor deze experimenten gebruikten we zwangere en niet-zwangere ratten en creëerden een "in vivo" hoog-cholesterol rattenmodel. Daarnaast onderzochten we ook het effect van peroxynitriet op de cerebrale vaatfunctie, omdat peroxynitriet geproduceerd wordt bij hoge lipiden concentratie. Peroxynitriet wordt gevormd door superoxide en stikstof monoxide en is een bekende beschadiger van de endotheliale functie. We lieten zien dat een excessieve hyperlipidemie de spiertonus in zowel de cerebrale vaten van zwangere en niet-zwangere ratten verlaagde maar dat dit effect groter was in de cerebrale arteriën van zwangere ratten en dat dit toegeschreven was aan peroxynitriet. Deze bevindingen werden bevestigd door vorige studies die demonstreerde dat peroxynitriet een sterke vasodilator kan zijn in het cerebrale vaatbed. Daarnaast zagen we dat een excessieve hyperlipidemie een grotere inflammatoire respons veroorzaakte in cerebrale arteriën van zwangere ratten in vergelijking tot de arteriën van niet-zwangere ratten door middel van een 12 maal grotere expressie van iNOS.

Een van de vele circulerende factoren die zijn verhoogd tijdens preeclampsie en is geassocieerd met hyperlipidemie is geoxideerd LDL (oxLDL). Het is bekend dat oxLDL in staat is endotheelschade te veroorzaken in vele ziektebeelden zoals hypertensie, diabetes en cerebrale vasculaire ziekten. Echter, of oxLDL ook betrokken is bij de pathogenese van preeclampsie is onbekend. In **Hoofdstuk 4** hebben we onderzocht of oxLDL en LOX-1 (de oxLDL-receptor) activatie een mogelijk onderliggend mechanisme kan zijn in het ontstaan van BBB disruptie in preeclampsie. Daarnaast hebben we gekeken of er verschillen zijn in het ontstaan van BBB disruptie bij vroege preeclampsie (gediagnosticeerd < 34 weken) of late preeclampsie (gediagnosticeerd > 34 weken). We hebben nieuwe aanwijzingen gevonden dat er mogelijk sprake is van een verschillende etiologie tussen vroege en late preeclampsie en we onthullen een potentieel nieuw mechanisme in het ontstaan van BBB disruptie bij vrouwen gediagnosticeerd met vroege preeclampsie. Circulerende factoren aanwezig in het bloedplasma van vrouwen met vroege preeclampsie veroorzaakten een stijging van de BBB permeabiliteit in tegenstelling tot vrouwen met een late preeclampsie. Wanneer LOX-1 werd geblokkeerd werd de stijging in BBB permeabiliteit ongedaan gemaakt. Dit fenomeen van LOX-1 afhankelijke stijging van de BBB permeabiliteit werd bevestigd "in vivo" in zwangere ratten met pathologische LDL concentraties. Circulerend oxLDL, een van de belangrijkste bindingsfactoren van de LOX-1 receptor, was 260% hoger in

plasma van vrouwen met een vroege preeclampsie in vergelijking met plasma van vrouwen met late preeclampsie. We bevestigde de potentie van oxLDL om de BBB permeabiliteit te verhogen door middel van experimenten met geïsoleerd oxLDL(in vergelijkbare levels van plasma van vrouwen met vroege preeclampsie) welke een vergelijkbare significante stijging van de BBB permeabiliteit lieten zien. Daarnaast toonde we aan dat de selectieve peroxynitriet decompositie catalyst FeTMPyP de oxLDL-geïnduceerde BBB permeabiliteit afremt. Hiermee bevestigen we de betrokkenheid van de formatie van peroxynitriet in oxLDL-afhankelijke BBB disruptie. Onze resultaten laten voor de eerste maal zien dat verhoogd circulerend oxLDL in het bloedplasma van vrouwen met vroege preeclampsie de BBB permeabiliteit verhoogt via LOX-1 activatie en vervolgens formatie van peroxynitriet.

Na deze bevindingen wilden we het mechanisme van oxLDL-geïnduceerde BBB permeabiliteit verder onderzoeken en tevens bekijken welke effecten oxLDL heeft op de cerebrale vaatreactiviteit. In **Hoofdstuk 5** onderzochten we de effecten en onderliggende mechanismen van geïsoleerd oxLDL op de BBB permeabiliteit en vaatreactiviteit van cerebrale arteriën. In **Hoofdstuk 4** vonden we dat oxLDL een stijging veroorzaakte in de formatie van peroxynitriet, maar het onderliggende mechanisme was nog onduidelijk. Omdat magnesiumsulfaat de primaire behandeling is in het voorkomen van neurologische complicaties tijdens ernstige preeclampsie, bekeken we in **Hoofdstuk 5** ook the potentiële beschermende effecten van magnesiumsulfaat ($MgSO_4$) op oxLDL-geïnduceerde verandering van de BBB permeabiliteit en cerebrale vaatreactiviteit. We lieten zien dat oxLDL een daling van de spiertonus in cerebrale arteriën veroorzaakte dat kon worden voorkomen door apocynin (een antagonist voor NADPH oxidase). Niet-geoxideerd LDL toonde deze effecten niet. Dit suggereert een rol voor NADPH oxidase activiteit en superoxide. Behandeling met $MgSO_4$ beschermde de BBB en voorkwam oxLDL-geïnduceerde BBB disruptie. MgSO4 had geen invloed op de oxLDL-geïnduceerde veranderingen in de cerebrale vaattonus. Deze resultaten suggereren dat oxLDL BBB disruptie en cerebrovasculaire vaattonus dysregulatie veroorzaakt door middel van activatie van NADPH oxidase. We speculeren dat oxLDL de NADPH oxidase activiteit verhoogt met als gevolg een toegenomen productie van superoxide dat bindt met stikstof monoxide en peroxynitriet vormt, resulterend in endotheelschade en vasculaire dysfunctie. Daarnaast bewijst $MgSO_4$ dat het de BBB beschermt, maar niet veel invloed heeft op de cerebrale vaatreactiviteit.

In **Hoofdstuk 6** hebben we een groot cohort van vrouwen vergeleken die preeclampsie (n=698) of eclampsie (n=88) hebben doorgemaakt. We vergeleken de bloeddrukken en circulerende factoren die onderdeel zijn van het metabool syndroom van deze vrouwen buiten de zwangerschap. Dit grote cohort liet zien dat vrouwen die een eclampsie hadden doorgemaakt buiten de zwangerschap een lagere bloeddruk hadden dan vrouwen die alleen een preeclampsie hadden doorgemaakt. Tevens lieten deze data zien dat er sprake was van een negatieve correlatie tussen de bloeddruk en het ontstaan van eclampsie. Verder vonden we dat 60% van alles eclamptische insulten voor de bevalling ontstonden en 40% na de bevalling. Deze observaties zijn van klinisch belang gezien het algemeen bekend is dat een groot deel van de vrouwen die een eclamptisch insult doormaken voorafgaand geen hypertensie volgens strikte criteria hebben (<140/90 mm Hg). We speculeren dat vrouwen met een lagere uitgangsbloeddruk meer kans hebben om een eclamptisch insult te ontwikkelen. Het feit dat 40% van de eclampsie postpartum ontstaat stipt het belang aan om ook in het kraambed goede controles van de bloeddruk te verrichten en goede te letten op de prodromale symptomen van eclampsie. Als laatste keken we naar de metabole factoren om te bepalen of vrouwen met een voorgaande zwangerschap gecompliceerd met eclampsie misschien al aan een vorm van een onderliggend metabool syndroom zouden kunnen lijden. Uit onze data komen echter geen verschillen tussen de 2 subgroepen voor.

Hoofdstuk 7 geeft een algemene discussie van de meest belangrijke bevindingen in dit manuscript. De discussie wordt afgesloten met de volgende conclusies:

1. De zwangerschap stelt het brein voor een unieke uitdaging. Terwijl andere maternale organen veranderingen ondergaan om de bloedstroom te vergroten, past het brein zich zodanig aan dat de bloedstroom gelijkt blijft om de BBB te beschermen.

2. De BBB adapteert in de normale zwangerschap om een toename van de BBB permeabiliteit, BBB disruptie, cerebraal vasogeen oedeem en neurologische complicaties te voorkomen. Ondanks dat veel circulerende permeabiliteitsfactoren zoals VEGF zijn verhoogd tijdens de zwangerschap, verandert de BBB permeabiliteit niet. Buiten de zwangerschap veroorzaakt VEGF interessant genoeg wel een significante toename van de BBB

permeabiliteit. Het lijkt dat sFLT1 belangrijk is in het reguleren van de VEGF-geïnduceerde BBB permeabiliteit tijdens de zwangerschap.

3. BBB disruptie in de zwangerschap kan leiden tot levensbedreigende neurologische complicaties zoals stuipen, cerebraal oedeem, cerebrale bloedingen en coma. Vooral vroege preeclampsie lijkt een risico factor te zijn voor het ontwikkelen van neurologische complicaties. Fysiologische hyperlipidemie in de normale zwangerchap kan bij een toename van oxidatieve stress omgezet worden in oxLDL wat ook significant verhoogd is tijdens vroege preeclampsie. oxLDL verlaagt de spiertonus van cerebrale arterien dat mogelijk meewerkt aan cerebrovasculaire dysfunctie en verstoring van de cerebrale autoregulatie. Daarnaast verhoogt oxLDL significant de BBB permeabiliteit middels productie van superoxide en daaropvolgende formatie van peroxynitriet. Dit laat zien dat oxLDL niet alleen een onderliggend mechanisme is in het ontstaan van neurologische complicaties, maar mogelijk ook een belangrijke therapeutische target is in de behandeling van ernstige vroege preeclampsie.

4. Magnesium sulfaat ($MgSO_4$) beschermt de oxLDL- geïnduceerde BBB permeabiliteit rechtstreeks zonder de oxLDL-gerelateerde verlaging van de vaattonus te beïnvloeden. Dit suggereert dat $MgSO_4$ voornamelijk direct de BBB beschermt zonder veel invloed te hebben op de myogene veranderingen in de cerebrale vaten.

5. Vrouwen die een eclampsie hebben doorgemaakt, hebben buiten de zwangerschap een significant lagere bloeddruk in vergelijking met vrouwen die een preeclampsia hebben doorgemaakt. Er bestaat een negatieve correlatie tussen de bloeddruk en het voorkomen van eclampsie. Omdat eclampsie niet vaak wordt voorafgegaan door hypertensie en de prevalentie van eclampsie toeneemt in het kraambed, is routine bloeddrukcontrole in het kraambed geïndiceerd.

Chapter 9:

Valorisatie

Introductie

Het brein is een uniek orgaan in het menselijk lichaam dat een strikte regulatie vereist om continu van de juiste hoeveelheid zuurstof en voedingstoffen te worden voorzien. Om deze strikte regulatie ook onder omstandigheden zoals zwangerschap en de daarbij horende veranderingen in circulerende factoren en bloedsomloop te waarborgen, moeten de hersenen zich wel kunnen aanpassen. Bij vrouwen met zwangerschapvergiftiging (preeclampsie) raakt het autoregelulatiesysteem in het brein verstoord en raakt de bloed-hersenbarrière (BBB) beschadigd zodat er schadelijke stoffen de hersenen kunnen binnentreden. Het huidige proefschrift geeft relevante nieuwe informatie over het functioneren van het brein bij de vrouw tijdens de zwangerschap, welke aanpassingen het brein doet en wat er fout kan gaan in de regelsystemen van de hersenen met name door circulerend LDL cholesterol en vrije zuurstof radicalen. Daarnaast laat dit proefschrift zien dat vrouwen die preeclampsie hebben doorgemaakt met levensbedreigende complicaties in het brein (eclampsie) verschillen laten zien in hartslag en bloeddruk buiten de zwangerschap in vergelijking met vrouwen die een preeclampsie hebben doorgemaakt zonder deze complicaties. Dit maakt duidelijk dat er verschillende risicofactoren zijn voor het ontwikkelen van eclampsie bij vrouwen met preeclampsie en dat dit een individueel zorgplan vereist tijdens de zwangerschap en het kraambed. Maar wat betekenen deze resultaten die tot nu toe zijn behaald nu eigenlijk concreet voor onze maatschappij en onze huidige gezondheidzorg? In dit hoofdstuk zullen de resultaten van het proefschrift voor zover als mogelijk worden vertaald en worden geïmplementeerd in de huidige Nederlandse samenleving en gezondheidszorg.

Relevantie

Preeclampsie en eclampsie zijn levensbedreigende ziektebeelden die tijdens de zwangerschap en het kraambed kunnen ontstaan. Het lastige van deze ziektebeelden is dat ze een multifactoriële en nog niet volledig bekende oorzaak hebben waardoor gerichte behandeling of preventie nog altijd incompleet is. De ziektebeelden lijken vrij acuut te kunnen ontstaan en symptoomloos te verlopen totdat er al ernstige complicaties aanwezig zijn. Tot op heden zijn deze ziektebeelden ook de hoofdoorzaak van de maternale sterfte (sterfte van

vrouwen tijdens de zwangerschap of gedurende het kraambed) in Nederland en alle overige ontwikkelde landen in de wereld. Al geruime tijd geleden heeft men vast gesteld dat wereldwijd maar vooral in ontwikkelingsgebieden nog altijd te weinig aandacht besteed aan de gezondheid van vrouw en kind hoewel hun gezondheid aan de basis van het leven staat. Dit heeft zich vertaalt in terugdringing van de moedersterfte als concreet doel binnen de Verenigde Naties Millenium Doelstellingen. Ieder land, of zelfs regio, zal hiervoor eigen en passende oplossingen bedenken. Ondanks de toenemende aandacht van de gezondheid van vrouw, moeder en kind in ons land, blijft preeclampsie vooralsnog een groot maatschappelijk probleem die nog altijd veel onderzoek en aandacht behoeft. Dit proefschrift geeft nieuwe inzichten over het ontstaan van hersenoedeem dat vervolgens eclampsie kan veroorzaken. Er worden nieuwe circulerende factoren aangewezen die betrokken zijn in het verstoren van de BBB wat leidt tot een toename van hersenoedeem. Dit zijn met name vrije zuurstofradicalen die ontstaan door een verhoogd geoxideerd LDL cholesterol. Dit proefschrift toont dus aan dat de mate van dyslipidemie erg belangrijk is in het ontstaan van eclampsie. Dit legt tevens een belangrijke link tussen de pathogenese van preeclampsie en andere cardiovasculaire ziekten zoals atherosclerose, hypertensie en diabetes. Omdat deze resultaten gebaseerd zijn op dierstudies is het wel van belang dat deze resultaten worden vertaald naar humane studies. Desalniettemin zijn dierstudies de basis naar de ontwikkeling van nieuwe behandelmethodes en ontwikkeling van preventieprogramma's. Daarnaast demonstreert dit proefschrift ook nog resultaten van een humane studie die suggereert dat zowel de hoogte en ook de stijging van de bloeddruk gedurende de zwangerschap van belang is in de ontwikkeling van eclampsie. Dit is met name relevant gezien het feit dat de definities van preeclampsie hypertensie (of te wel een te hoge bloeddruk) beschrijven maar niet ingaan op de verandering van de bloeddrukstijging. De bloeddruk waarbij wij spreken van hypertensie is 140/90 mm Hg waarboven men antihypertensieve en preventieve behandeling start. Echter ons onderzoek toont aan dat jonge vrouwen die een preeclampsie gecompliceerd door een eclampsie hebben gehad buiten de zwangerschap een bloeddruk hebben die aanzienlijk lager is dan deze waarde en dat hoe dichter de niet zwangere bloeddruk bij de 140/90 mm Hg ligt hoe geringer de kans is dat preeclampsie wordt gecompliceerd door eclampsia. Deze gegevens suggereren dat ofwel de grens van eclampsie preventieve behandeling of lager moet worden gesteld of dat de stijging in bloeddruk, relatief of absoluut, dient te worden meegewogen in de diagnose hypertensie.

Doelgroep

De uitkomsten van dit huidige proefschrift zijn voor het overgrote deel het resultaat van dierstudies om meer inzicht te krijgen in de veranderingen van de BBB tijdens de zwangerschap en om de mechanismen die disruptie van de BBB veroorzaken te onderzoeken. De resultaten geven nieuwe belangrijke inzichten in de pathogenese van BBB disruptie en het ontwikkelen van eclampsie tijdens de zwangerschap en relateert de ontwikkeling van preeclampsie aan het voorkomen van toegenomen oxidatieve stress en dyslipidemie. De resultaten dienen wel nog verder te worden onderzocht in onze Nederlandse zwangerenpopulatie. Dus de huidige resultaten van de dierstudies zijn nog met name van belang voor de onderzoeksteams van de afdelingen obstetrie en gynaecologie en neurologie. Anderzijds zijn de overige resultaten gebaseerd op een groot cohort van zwangere vrouwen die zijn gediagnosticeerd met een preeclampsie of eclampsie wel van direct klinisch belang voor verscheidene groepen in de samenleving. De resultaten laten zien dat niet zozeer de hoogte van de bloeddruk maar ook de stijging van de bloeddruk gedurende de zwangerschap belangrijk is in het ontwikkelen van preeclampsie en eclampsie. Daarnaast laten onze resultaten van het Nederlandse cohort ook zien dat eclampsie ongeveer in 40% van de gevallen in het kraambed ontstond. Deze bevindingen zijn belangrijk voor ziekenhuizen en geboortecentra om in hun zorgprotocol op te nemen. Daarnaast zijn deze bevindingen ook belangrijk voor 1^{e} lijn verloskundigen en huisartsen. Gezien er een significant percentage van de vrouwen die eclampsie ontwikkelen al bevallen zijn, dient de zorg in het kraambed, ook voor ogenschijnlijk gezonde vrouwen zeker niet te verslappen. Artsen, verloskundigen en huisartsen dienen alert te zijn op de bloeddruk van vrouwen aan het begin van de zwangerschap in vergelijking met de bloeddruk na hun bevalling. Ook al is bloeddruk in het kraambed volgens de internationale hypertensie richtlijnen normaal, voor bepaalde vrouwen kan de bloeddruk al verhoogd zijn en lijken ze meer risico te lopen op het ontwikkelen van eclampsie in het kraambed. Gezien eclampsie in het kraambed zelfs nog weken later kan manifesteren, dienen met name huisartsen (als gynaecologen en verloskundigen hun zorgpad hebben afgesloten) goed inzicht te hebben in het verloop van de bloeddruk van hun patiënte, zeker indien zij met klachten komen die zouden kunnen passen bij preeclampsie en eventueel complicaties in het brein. . Tenslotte is deze informatie vanzelfsprekend met name belangrijk voor de patiënte zelf. Zij kan met deze informatie zelf haar bloeddruk in de gaten houden

(bij risicopatiënten eventueel met geleende gevalideerde apparatuur) en hulp inschakelen indien gewenst.

Activiteiten en producten

De resultaten uit de dierstudies van het huidige proefschrift tonen dat het brein zich aanpast tijdens de zwangerschap aan allerlei circulerende factoren die vrijkomen. Daarnaast tonen ze een nieuw mechanisme dat disruptie van de BBB kan optreden bij verhoogde levels van geoxideerd LDL cholesterol. De resultaten worden vertaald in verder onderzoek in cohortstudies van vrouwen die zijn gediagnosticeerd met preeclampsie of eclampsie. De resultaten zijn een volledig vernieuwend concept in de ontrafeling van de pathogenese van preeclampsie en eclampsie maar dienen verder worden uitgewerkt door onderzoeksteams van de obstetrie en gynaecologie en tevens eventueel de neurologie om hier uiteindelijk therapeutische of preventieve targets te ontwikkelen. De resultaten uit het cohort kunnen direct worden vertaald in protocollen van ziekenhuizen, 1e lijn verloskundigen en huisartsen. Daarnaast kunnen deze resultaten ook worden vertaald in voorlichtingsfolders over hypertensieve aandoeningen tijdens de zwangerschap en waar vrouwen op dienen te letten. Uiteindelijk om zorg op maat te creëren zou het mooi zijn om vrouwen thuis hun bloeddruk te kunnen laten monitoren die via email met een ziekenhuis of verloskundige praktijk in verbinding staan. Hier is dan wel gevalideerde apparatuur voor nodig en instructies aan alle zwangeren om dit te kunnen realiseren. Daarnaast zullen de bevindingen van dit proefschrift worden vertaald en vormgegeven in de follow-up studies van het cohort vrouwen die een preeclampsie of eclampsie hebben doorgemaakt.

Innovatie

De resultaten van dit proefschrift maken een belangrijke stap in het ontrafelen van de pathogenese van preeclampsie en eclampsie. Er wordt ontzettend veel onderzoek verricht naar de pathogenese van preeclampsie. Echter blijft eclampsie hierin vaak nog onderbelicht, terwijl eclampie juist de een van de levensbedreigende complicaties vormt. Wat onderzoek naar de complicaties in de hersenen nog lastiger maakt, is dat het vaatbed in de hersenen compleet

anders is gereguleerd dan het perifere vaatbed in de rest van het lichaam. Vandaar is dit proefschrift van waarde om meer begrip te krijgen over de fysiologische en pathologische mechanisme die ervoor zorgen dat de BBB zich aanpast of beschadigd raakt. Daarnaast wordt de tot nu toe onderschatte waarde van dyslipidemie en oxidatie tijdens de zwangerschap belicht en naar voren geschoven als sleutelfactor in het ontstaan van complicaties in de hersenen. Dit is ook belangrijk voor andere cerebrovasculaire aandoeningen en kan preeclampsie hier weer aan gerelateerd worden. Daarnaast lijkt naast chemische factoren het voorkomen van eclampsie tijdens preeclampsie ook door de hoogte van de niet-zwangere bloeddruk te worden beïnvloed, waarmee mogelijk de definities van preeclampsie en eclampsie en de daarmee samenhangende start van eventuele eclampsie preventieve maatregelen herzien moeten worden. Mogelijk speelt ook de stijging van de bloeddruk een rol en behoeft wellicht herijking. Samengevat bevat dit proefschrift dus een hoop nieuwe ontwikkelingen die uiteindelijk leiden naar een betere herkenning en behandeling van complicaties in de hersenen bij zwangere vrouwen en vrouwen in het kraambed.

Planning en Realisatie

De resultaten uit het cohort zullen direct kunnen worden geïmplementeerd in de protocollen van ziekenhuizen en verloskundigenpraktijken zonder extra kosten. Daarnaast lijkt awareness bij hulpverleners op zijn plaats bij vrouwen in het kraambed in combinatie met hun gemeten bloeddruk, ook als die nog geen 140/90 mm Hg heeft bereikt. Richtlijnen van verloskundigen, huisartsen en gynaecologen zouden hierin een waarschuwing kunnen opnemen. De verdere resultaten uit de dierstudies wordt beschouwd als pionierswerk. Voordat deze resultaten vertaald worden in onze zwangerenpopulatie zullen er zeker enkele jaren overheen gegaan zijn. Er blijft natuurlijk altijd financiering nodig om deze resultaten verder uit te werken in humane studies. Wij zijn er in ieder geval van overtuigd dat dit proefschrift ons wederom een stapje dichterbij de pathogenese van preeclampsie en eclampsie heeft gebracht, maar zoals vele studies weer meer vragen heeft gegenereerd dan antwoorden. Het legt nieuwe correlaties tussen cardiovasculaire ziekten waarin dyslipidemie ook een belangrijke rol speelt. Juist omdat een normale zwangerschap de basis vormt voor gezonde moeders en hun nakomelingen, verdient inzicht en daarmee onderzoek naar

de gezonde fysiologische aanpassingen aan de zwangerschap en de verschillen die waarneembaar zijn met de pathologische zwangerschap leidend tot bovengeschetste levensbedreigende beelden preeclampsie en eclampsie de hoogste prioriteit!

Dankwoord

Dankwoord

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Malou

Curriculum Vitae

Malou Schreurs werd geboren op 23 januari 1985 te Tegelen. Zij behaalde haar VWO diploma in 2003 aan het Valuascollege in Venlo. Direct na het behalen van dit diploma startte zij met de opleiding Geneeskunde aan de Universiteit van Maastricht. Door 2 keuze-coschappen te lopen in het onderzoek bij de Chirurgie in Maastricht en in het Maternity hospital in Malawi stond haar belangstelling in de Obstetrie en Gynaecologie en de wetenschap vast. In december 2009 behaalde zij haar artsexamen. Zij startte in Januari 2010 als ANIOS Obstetrie en Gynaecologie in het Flevoziekenhuis te Almere. In oktober 2010 verruilde zij deze baan om voor een jaar naar Burlington, Vermont in de Verenigde Staten te verhuizen om onderzoek te doen bij Professor Marilyn Cipolla naar de neurologische complicaties bij preeclampsie. Een jaar Verenigde Staten werd algauw 2 jaar gedreven onderzoek wat als resultaat in dit proefschrift staat beschreven. In Oktober 2012 keerde zij terug naar Nederland waar zij in Januari 2013 startte als ANIOS obstetrie en Gynaecologie in het MUMC+ te Maastricht. Daar werd haar onderzoek geadopteerd door Professor Marc Spaanderman en voortgezet zoals in dit proefschrift staat beschreven. In Januari 2014 begon ze aan de opleiding tot Gynaecoloog in het Viecuri te Venlo.

Malou Schreurs was born on the 23rd of January 1985 in Tegelen, the Netherlands. After finishing secondary school at the Valuascollege, Venlo, in 2003, Malou started her study Medicine at the University of Maastricht. When Malou decided to expand her medical curriculum by performing research at the department of Surgery and gaining experience in Obstetrics at the Maternity hospital in Malawi, Africa, she was convinced of her interest for science and obstetrics and gynecology. She graduated from the college of Medicine in December 2009. After graduating she started working as an intern in obstetrics and gynecology at the Flevoziekenhuis in Almere, the Netherlands. In October 2010 she moved to Burlington, Vermont , USA, to perform research under supervision of Professor Marilyn Cipolla about the development of neurologic complications during preeclampsia. She decided to stay in Vermont for 2 years and published 5 research papers which resulted in this thesis. She returned to the Netherlands in October 2012 and started working as a intern in Obstetrics and Gynecology in the academic hospital of Maastricht. She continued her research under supervion of professor Marc Spaanderman which is described in this thesis. Malou started the residency program Obstetrics and Gynecology to become a gynecologist at the VieCuri hospital, Venlo, the Netherlands, at January 2014.