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 a ~12-fold increased messenger RNA (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.

Keywords

pregnancy, preeclampsia, hypercholesterolemia, posterior cerebral artery

Introduction

Preeclampsia is a complex, hypertensive disorder that affects 5% to 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 reemerge 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

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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 (nicotinamide adenine dinucleotide phosphate (NADPH)-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 messenger RNA (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 to 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; $n = 7$). Both NP-CTL and LP-CTL received Prolab 3000 (Labdiet, Scott Distributing Inc, Hudson, New Hampshire) 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 -80°C 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, Roche Diagnostics, Indianapolis, Indiana). Low-density lipoprotein cholesterol was calculated using the Friedewald equation according to the 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 $37.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. 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 mm Hg. Intravascular pressure was then increased in steps of 25 to 150 mm Hg, 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 mm Hg and the vessel diameter was compared to the vessel diameter at 75 mm Hg prior to the pressure steps to confirm that tone was restored. For the remainder of the experiment, the pressure was maintained at 75 mm Hg. A single high concentration (0.1 mmol/L) of the NOS inhibitor L-nitro-arginine (L-NNA) was added to the bath and the 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} - 10^{-5} mol/L) 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 mm Hg 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 50 μ mol/L FeTMPyP. This concentration was used based on one of our earlier studies where FeTMPyP was used in isolated arteries to scavenge ONOO⁻.²⁵ After equilibration, the intravascular pressure was increased in steps of 25 to 150 mm Hg. Active lumen diameter was recorded at each pressure once stabilized. Afterward, zero-Ca²⁺ PSS was added to the bath and passive diameters were recorded at pressures from 5 to 175 mm Hg.

Messenger RNA Expression of iNOS and NOX2

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 polymerase chain reaction (PCR) analysis of iNOS and NOX2. Total RNA was extracted from MCAs from NP-CTL (n = 6), NP-HC (n = 6), LP-CTL (n = 6), and LP-HC (n = 7) by using Trizol reagent (Life Technologies, Grand Island, New York) followed by purification using an Rneasy Micro Kit (Qiagen) per manufacturer's protocols. RNA concentrations and quality were determined using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, California). Real-time PCR was performed in a 2-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 complementary DNA (cDNA) was used to amplify the target genes *iNOS* and *NOX2* and 2 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): -GAGAAGTCCAGCCGACCAC- and (r): -GAACAA TCCACAACCTCGCCCAAG-; *NOX2* (f): -TTAGCATCCATATCCGCATTGTTG- and (r): CTATCTTAGGTAGTTTC-CAGGCATCTT; *Hprt1* (f): -CTC ATGGACTGTTATGGAC AGGAC- and (r): -GCAGGTCAGCAAAGAACTTATAGCC-; *Ywhaz* (f): -GATGAAGCCATT GCTGAAGCTTG- and (r): -GTCTCCTTGGGTATCCGATGTC-. One microliter of cDNA was used per reaction with 150 nmol/L of the forward and reverse primers and 12.5 μ L of Power Sybrgreen Master mix (Life Technologies) in a 25- μ L 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 (Applied Biosystems, Carlsbad, California). 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 1 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-Ca²⁺ PSS by the equation: $((\varnothing_{\text{passive}} - \varnothing_{\text{active}}) / \varnothing_{\text{passive}}) \times 100\%$, where $\varnothing_{\text{passive}}$ is the diameter in zero-Ca²⁺ PSS and $\varnothing_{\text{active}}$ is the diameter with tone. Percent constriction to L-NNA was calculated with the equation: $((\varnothing_{\text{baseline}} - \varnothing_{\text{drug}}) / \varnothing_{\text{baseline}}) \times 100\%$, where $\varnothing_{\text{baseline}}$ is the diameter before giving L-NNA and $\varnothing_{\text{drug}}$ is diameter with the presence of L-NNA. Percent sensitivity to the NO donor SNP using a concentration-response curve was calculated with the equation: $((\varnothing_{\text{drug}} - \varnothing_{\text{minimum}}) / (\varnothing_{\text{maximum}} - \varnothing_{\text{minimum}})) \times 100\%$ where $\varnothing_{\text{drug}}$ is the diameter at a specific concentration of SNP, $\varnothing_{\text{minimum}}$ is diameter at the lowest concentration of SNP, and $\varnothing_{\text{maximum}}$ is the diameter at the highest concentration of SNP. To calculate the effective concentration that produced half maximal dilation (EC₅₀) of SNP, the concentration-response curve was log transformed and plotted for each experiment separately. The EC₅₀ 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-based PSS buffer was used for all experiments. The composition consisted of (mmol/L) NaCl 119.0, NaHCO₃ 24.0, KCl 4.7, KH₂PO₄ 1.18, MgSO₄·7H₂O 1.17, CaCl₂ 1.6, EDTA 0.026, and glucose 5.5. The PSS was aerated with 5% CO₂, 10% O₂, and 85% N₂ to maintain pH at 7.40 $\pm$ 0.05 and stored without glucose at 4°C. Glucose was added before each experiment. Both L-NNA and SNP were purchased from Sigma (St. Louis, Missouri), and FeTMPyP was purchased from Calbiochem (Gibbstown, NJ).

Statistical Analysis

Data from the isolated artery experiments are presented as mean $\pm$ 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 1-way analysis of variance (ANOVA) and post hoc Student-Newman Keuls test for multiple comparisons where appropriate or with a Student's *t* test. Differences were considered statistically significant when *P* < .05.

Results

Plasma Cholesterol Levels in NP and LP Rats

Total cholesterol levels were similar in NP and LP control animals, being 77 $\pm$ 3 and 79 $\pm$ 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, the total cholesterol increased 550% to 500 ± 52 mg/dL and in LP-HC the 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 ($P = .12$). The LDL-c levels could not be calculated in the control animals because the levels of determinants needed for calculating LDL-c were too low. The LDL-c levels in NP-HC animals were 400 ± 40 mg/dL and in LP-HC animals 547 ± 78 mg/dL ($P = .13$). The 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 ($P < .05$). In NP-HC rats, the HDL-cholesterol remained stable compared to NP-CTL rats and was 50 ± 5 and 44 ± 5 mg/dL, respectively ($P = .27$). 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 toward decreased tone in PCAs from both groups of animals, although this decrease was not statistically different. In NP-CTL rats, the percent tone of PCAs at the physiological pressure of 75 mm Hg was $14\% \pm 6\%$ compared to $5\% \pm 2\%$ in NP-HC rats ($P = .18$). The percent tone in PCAs from LP-CTL rats was $11\% \pm 6\%$ compared to $4\% \pm 3\%$ in LP-HC rats ($P = .25$).

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. The 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 $20\% \pm 3\%$ compared to $18\% \pm 2\%$ in NP-HC animals ($P > .05$). In LP-CTL rats, the percent constriction to L-NNA was $22\% \pm 5\%$ compared to $18\% \pm 2\%$ in LP-HC rats ($P > .05$).

Effect of Hypercholesterolemia on NO-Mediated Vasodilation in the PCAs From NP and LP Animals

To determine whether 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 EC₅₀ of SNP showed no difference

between NP-CTL and NP-HC animals (Figure 1C). However, the EC₅₀ 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.

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 μ mol/L 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 and 150 mm Hg (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.

Effect of High-Cholesterol Diet on iNOS and NOX-2 mRNA Expression in MCAs 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 threshold 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 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

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 EC₅₀ of SNP (C, D) as a percent 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. SNP indicates sodium nitroprusside; 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; NOS, nitric oxide synthase; NO, nitric oxide. * $P < .05$ vs LP-CTL.

a ~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.

Discussion

In this 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 pregnant compared to the nonpregnant state.

Preeclampsia presents with an excessive hyperlipidemia compared to normal pregnancy that may adversely affect the overall vascular function as seen in atherosclerotic 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 this study, we sought to determine the effects of excessive hyperlipidemia in pregnant compared to the nonpregnant state by using a high-cholesterol rat model. Although we attempted to match the levels seen in women with preeclampsia, 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

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. ONOO⁻, indicates peroxynitrite; 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* < .05 vs NP-HC and LP-HC. #*P* < .05 vs all.

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

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 a ~6-fold increase in iNOS mRNA expression and a ~12-fold increase in iNOS mRNA expression when LP rats were treated with high cholesterol. High cholesterol caused a ~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. mRNA indicates messenger RNA; iNOS, inducible nitric oxide synthase; 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.

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 of 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 of the plasma cholesterol in HDL particles and a high rate of hepatic clearance.³⁵ Also, in this 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 the 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 the 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 this study, the effect of FeTMPyP was significantly greater in the LP-HC animals compared to the NP-HC animals, suggesting pathologically high levels of lipids in pregnancy result in higher generation of ONOO⁻ compared to NP-HC animals. The increased generation of ONOO⁻ 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.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 a ~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. Inducible NOS is known to produce excess 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 pregnant 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 women with preeclampsia 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 whether the 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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References

- Pennington KA, Schlitt JM, Jackson DL, Schulz LC, Schust DJ. Preeclampsia: multiple approaches for a multifactorial disease. *Dis Model Mech*. 2012;5(1):9-18.
- Roberts JM, Pearson G, Cutler J, Lindheimer M. Summary of the NHLBI working group on research on hypertension during pregnancy. *Hypertens*. 2003;41(3):437-445.
- Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughlin MK. Preeclampsia: an endothelial cell disorder. *Am J Obstet Gynecol*. 1989;161(5):1200-1204.
- Portelinha A, Belo L, Cerdeira AS, et al. Lipid levels including oxidized ldl in women with history of preeclampsia. *Hypertens Pregnancy*. 2010;29(1):93-100.
- Bellamy L, Casas JP, Hingorani AD, Williams DJ. Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. *BMJ*. 2007;335(7627):974.
- Roberts JM, Cooper DW. Pathogenesis and genetics of pre-eclampsia. *Lancet*. 2001;357(9249):53-56.
- Williams D. Pregnancy: a stress test for life. *Curr Opin Obstet Gynecol*. 2003;15(6):465-471.
- Enquobahrie DA, Williams MA, Butler CL, Frederick IO, Miller RS, Luthy DA. Maternal plasma lipid concentrations in early pregnancy and risk of preeclampsia. *Am J Hypertens*. 2004;17(7):574-581.
- Belo L, Caslake M, Gaffney D, et al. Changes in LDL size and HDL concentration in normal and preeclamptic pregnancies. *Atherosclerosis*. 2002;162(2):425-432.
- Basaran A. Pregnancy-induced hyperlipoproteinemia: review of the literature. *Reprod Sci*. 2009;16(5):431-437.
- Mazurkiewicz JC, Watts GF, Warburton FG, Slavin BM, Lowy C, Koukkou E. Serum lipids, lipoproteins and apolipoproteins in pregnant non-diabetic patients. *J Clin Pathol*. 1994;47(8):728-731.
- Staff AC, Dechend R, Pijnenborg R. Learning from the placenta: acute atherosclerosis and vascular remodeling in preeclampsia-novel aspects for atherosclerosis and future cardiovascular health. *Hypertension*. 2010;56(6):1026-1034.
- Var A, Kuscuk NK, Koyuncu F, et al. Atherogenic profile in pre-eclampsia. *Arch Gynecol Obstet*. 2003;268(1):45-47.
- Miller AA, De Silva TM, Judkins CP, Diep H, Drummond GR, Sobey CG. Augmented superoxide production by nox2-containing nadph oxidase causes cerebral artery dysfunction during hypercholesterolemia. *Stroke*. 2010;41(4):784-789.
- Kitayama J, Faraci FM, Lentz SR, Heistad DD. Cerebral vascular dysfunction during hypercholesterolemia. *Stroke*. 2007;38(7):2136-2141.
- Rubbo H, Batthyany C, Radi R. Nitric oxide-oxygen radicals interactions in atherosclerosis. *Biol Res*. 2000;33(2):167-175.
- Hayashi T, Yamada K, Esaki T, et al. Estrogen increases endothelial nitric oxide by a receptor-mediated system. *Biochem Biophys Res Commun*. 1995;214(3):847-855.
- Conrad KP, Joffe GM, Kruszyna H, et al. Identification of increased nitric oxide biosynthesis during pregnancy in rats. *FASEB J*. 1993;7(6):566-571.
- Buhimschi IA, Saade GR, Chwalisz K, Garfield RE. The nitric oxide pathway in pre-eclampsia: pathophysiological implications. *Hum Reprod Update*. 1998;4(1):25-42.
- Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol*. 1996;271(5 pt 1):C1424-1437.
- Roggensack AM, Zhang Y, Davidge ST. Evidence for peroxynitrite formation in the vasculature of women with preeclampsia. *Hypertension*. 1999;33(1):83-89.
- Miller AA, Drummond GR, Schmidt HH, Sobey CG. NADPH oxidase activity and function are profoundly greater in cerebral versus systemic arteries. *Circ Res*. 2005;97(10):1055-1062.
- Huang X, Tang J, Zhou Q, Lu H, Wu Y, Wu W. Polysaccharide from fuzi (fps) prevents hypercholesterolemia in rats. *Lipids Health Dis*. 2010;9:9.
- Xie Z, Wei M, Morgan TE, et al. Peroxynitrite mediates neurotoxicity of amyloid beta-peptide1-42- and lipopolysaccharide-activated microglia. *J Neurosci*. 2002;22(9):3484-3492.
- Palomares SM, Gardner-Morse I, Sweet JG, Cipolla MJ. Peroxynitrite decomposition with fetmppy improves plasma-induced vascular dysfunction and infarction during mild but not severe hyperglycemic stroke. *J Cereb Blood Flow Metab*. 2012;32(6):1035-1045.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods*. 2001;25(4):402-408.
- Cipolla MJ, Houston EM, Kraig RP, Bonney EA. Differential effects of low-dose endotoxin on the cerebral circulation during pregnancy. *Reprod Sci*. 2011;18(12):1211-1221.
- De Silva TM, Brait VH, Drummond GR, Sobey CG, Miller AA. Nox2 oxidase activity accounts for the oxidative stress and vasomotor dysfunction in mouse cerebral arteries following ischemic stroke. *PLoS one*. 2011;6(12):e28393.
- Jayakody L, Senaratne M, Thomson A, Kappagoda T. Endothelium-dependent relaxation in experimental atherosclerosis in the rabbit. *Circ Res*. 1987;60(2):251-264.
- Tyroler HA. Cholesterol and cardiovascular disease. An overview of lipid research clinics (LRC) epidemiologic studies as background for the LRC coronary primary prevention trial. *Am J Cardiol*. 1984;54(5):14C-19C.
- Chan YC, Leung FP, Tian XY, et al. Raloxifene improves vascular reactivity in pressurized septal coronary arteries of ovariectomized hamsters fed cholesterol diet. *Pharmacol Res*. 2012;65(2):182-188.
- Ramirez RJ, Novak J, Johnston TP, Gandle RE, McLaughlin MK, Hubel CA. Endothelial function and myogenic reactivity

- in small mesenteric arteries of hyperlipidemic pregnant rats. *Am J Physiol Regul Integr Comp Physiol*. 2001;281(4):R1330-R1337.
33. McCalden TA, Nath RG. Mechanisms of vascular supersensitivity in hypercholesterolemia. *Stroke*. 1989;20(2):238-241.
34. Didion SP, Heistad DD, Faraci FM. Mechanisms that produce nitric oxide-mediated relaxation of cerebral arteries during atherosclerosis. *Stroke*. 2001;32:761-766.
35. Fuster JJ, Castillo AI, Zaragoza C, Ibanez B, Andres V. Animal models of atherosclerosis. *Prog Mol Biol Transl Sci*. 2012;105:1-23.
36. Czako L, Szabolcs A, Vajda A, et al. Hyperlipidemia induced by a cholesterol-rich diet aggravates necrotizing pancreatitis in rats. *Eur J Pharmacol*. 2007;572(1):74-81.
37. Cuzzocrea S, Misko TP, Costantino G, et al. Beneficial effects of peroxynitrite decomposition catalyst in a rat model of splanchnic artery occlusion and reperfusion. *FASEB J*. 2000;14(9):1061-1072.
38. Miller AA, Drummond GR, Sobey CG. Reactive oxygen species in the cerebral circulation: Are they all bad? *Antioxid Redox Signal*. 2006;8(7-8):1113-1120.
39. Maneen MJ, Hannah R, Vitullo L, DeLance N, Cipolla MJ. Peroxynitrite diminishes myogenic activity and is associated with decreased vascular smooth muscle f-actin in rat posterior cerebral arteries. *Stroke*. 2006;37(3):894-899.
40. Li J, Li W, Altura BT, Altura BM. Peroxynitrite-induced relaxation in isolated canine cerebral arteries and mechanisms of action. *Toxicol Appl Pharmacol*. 2004;196(1):176-182.
41. Fitch R, Da Cunha V, Kauser K, et al. Increased nitric oxide accounts for decreased basal vascular tone and responsiveness in the resistance vessels of high-cholesterol-fed rabbits. *Pharmacol*. 2001;63(4):220-227.
42. Miller AA, Drummond GR, De Silva TM, et al. NADPH oxidase activity is higher in cerebral versus systemic arteries of four animal species: role of Nox2. *Am J Physiol*. 2009;296(1):H220-H225.
43. Matsubara K, Matsubara Y, Hyodo S, Katayama T, Ito M. Role of nitric oxide and reactive oxygen species in the pathogenesis of preeclampsia. *J Obstet Gynaecol Res*. 2010;36(2):239-247.
44. Aleman I, Alex R, Ramirez M, Hung A, Ramirez C. [Endothelial and inducible nitric oxide synthase expression in Venezuelan patients with pre-eclampsia]. *Invest Clin*. 2008;49(3):321-330.
45. Schiessl B, Mylonas I, Hantschmann P, et al. Expression of endothelial NO synthase, inducible NO synthase, and estrogen receptors alpha and beta in placental tissue of normal, preeclamptic, and intrauterine growth-restricted pregnancies. *J Histochem Cytochem*. 2005;53(12):1441-1449.
46. Gunnett CA, Lund DD, McDowell AK, Faraci FM, Heistad DD. Mechanisms of inducible nitric oxide synthase-mediated vascular dysfunction. *Arterioscler Thromb Vasc Biol*. 2005;25(8):1617-1622.