Endothelial Cell Vasodilator Dysfunction Mediates Progressive Pregnancy-induced Hypertension in Endothelial Cell Tetrahydrobiopterin Deficient Mice

Surawee Chuaiphichai[1], Yasmin Dickinson[1], Christopher Whiteman[1], Desson Au-Yeung[1], Keith Channon[1], and Gillian Douglas[1]

[1]University of Oxford

October 19, 2022

Abstract

Background and purpose: Pregnancy-associated vascular remodeling is essential for both maternal and fetal health. We have previously shown that maternal endothelial cell tetrahydrobiopterin (BH4) deficiency causes poor pregnancy outcomes. Here, we investigated the role and mechanisms of endothelial cell-mediated vasorelaxation function in these outcomes. Experimental approach: The vascular reactivity of mouse aortas and uterine arteries from non-pregnant and pregnant endothelial cell-specific BH4 deficient mice (Gch1fl/flTie2cre mice) was assessed by wire myography. Systolic blood pressure was assessed by tail cuff plethysmography. Key results: Key results: In late pregnancy, systolic blood pressure was significantly higher (˜24 mmHg) in Gch1fl/flTie2cre mice compared with wild-type littermates. This was accompanied by enhanced vasoconstriction and reduced endothelial-dependent vasodilation in both aorta and uterine arteries from pregnant Gch1fl/flTie2cre mice. In uterine arteries loss of eNOS-derived vasodilators was partially compensated by upregulation of intermediate and large-conductance Ca2+-activated K+ channels. In rescue experiments, oral BH4 supplementation alone did not rescue vascular dysfunction and pregnancy-induced hypertension in pregnant Gch1fl/flTie2cre mice. However, combination with the fully reduced folate, 5-methyltetrahydrofolate (5-MTHF), restored endothelial cell vasodilator function and blood pressure. Conclusions and implications: We identify a critical requirement for maternal endothelial cell BH4 biosynthesis in endothelial cell vasodilator function in pregnancy. Targeting vascular Gch1 and BH4 biosynthesis with reduced folates may provide a novel therapeutic target for the prevention and treatment of pregnancy-related hypertension.

Introduction

Hypertensive disorders of pregnancy such as preeclampsia and gestational hypertension are associated with a higher risk of cardiovascular disease for both mother and offspring in later life. Women with these complications during pregnancy have a 4-fold increased risk of developing hypertension (Bellamy, Casas, Hingorani & Williams, 2007; McDonald, Malinowski, Zhou, Yusuf & Devereaux, 2008) and a 2-fold increased risk of developing stroke after pregnancy (Bellamy et al., 2007). Offspring from mothers with hypertensive disorders of pregnancy are also likely to have a higher risk of hypertension and other cardiovascular disease in later in life (Ferreira, Peeters & Stehouwer, 2009; Kajantie, Eriksson, Osmond, Thornburg & Barker, 2009).

The maternal cardiovascular system must undergo significant remodelling to support the growing fetus. By term, the uteroplacental bed receives more than 12% of cardiac output. In order to accommodate this increase in flow, vascular resistance is reduced by outward remodeling and vasodilatation of more proximal uterine arteries, together with trophoblast invasion and remodelling of spiral arteries (Kelly, Stone & Poston, 2000). Failure of this vascular adaptation is associated with adverse outcomes including pregnancy induced hypertension and pre-eclampsia. Therefore, understanding the mechanisms underlying vascular adaptations and how they impact blood pressure regulations during pregnancy are of great importance.

Nitric oxide (NO), generated by endothelial nitric oxide synthase (eNOS), is a key mediator of vascular adaptation in pregnancy. During pregnancy, enhanced uteroplacental vasodilation is driven by increased NOS-derived NO in humans, mice, and rats (Anumba, Robson, Boys & Ford, 1999; Miller, Jenkin & Walker, 1999; Sladek, Magness & Conrad, 1997; Williams, Vallance, Neild, Spencer & Imms, 1997). Increased uterine artery calibre is associated with both enhanced eNOS expression, activity, and NO bioavailability (Cooke & Davidge, 2003; Nelson, Steinsland, Wang, Yallampalli, Dong & Sanchez, 2000; Sullivan, Grummer, Yi & Bird, 2006) (Dimmeler, Fleming, Fisslthaler, Hermann, Busse & Zeiher, 1999; Kublickiene, Cockell, Nisell & Poston, 1997). Loss of eNOS leads to impaired uterine artery remodelling during pregnancy mediated in part by a reduced activation of matrix metalloproteinase in eNOS- knockout mice (van der Heijden, Essers, Fazzi, Peeters, De Mey & van Eys, 2005). NO bioavailability is also central to physiological adaptation in the more distal uteroplacental circulation with increased expression of eNOS observed in remodelling spiral arteries and in cytotrophoblasts and syncytiotrophoblasts (Lyall, Bulmer, Kelly, Duffie & Robson, 1999). However, simple strategies attempting to restore or augment NO with NO donors in pre-eclampsia have been disappointing (Meher & Duley, 2007). One possible explanation for these results is the failure to specifically target eNOS uncoupling and consequently altered NO/ROS signalling. To date, there is less certainty regarding the role of eNOS coupling and NO bioavailability in the uterine circulation in pregnancy induced hypertension and pre-eclampsia.

The generation of NO by eNOS requires the small molecule tetrahydrobiopterin (BH4). Loss of BH4 results in eNOS uncoupling, loss of NO generation and increased production of reactive oxygen species (ROS). Biosynthesis of BH4 is catalyzed by GTPCH (GTP cyclohydrolase 1, encoded by *Gch1* , the rate-limiting enzyme for de novo BH4 biosynthesis. We have previously shown that *Gch1* expression is a key determinant of BH4 bioavailability, NOS regulation and NO and superoxide generation (Crabtree et al., 2009; Vasquez-Vivar, Martasek, Whitsett, Joseph & Kalyanaraman, 2002). Recently, we have demonstrated that maternal endothelial cell BH4 deficiency, due to loss of *Gch1* , leads to progressive hypertension and fetal growth restriction during pregnancy (Chuaiphichai et al., 2021). However, the mechanism mediating these changes and how loss of endothelial cell-specific *Gch1* alters uterine artery function both before and during pregnancy and if these changes are reversable are yet to be answered. To address this question, we assessed vascular function and blood pressure in pregnant and non-pregnant endothelial cell specific *Gch1* knock out mice.

Methods

Generation of Endothelial Cell –Targeted Gch1 Knockout Mice

Endothelial cell–specific BH4 deficient mice and their littermates controls were generated by crossing $Gch1^{fl/fl}$ females with $Gch1^{fl/fl}$ Tie2cre male mice as described previously (Chuaiphichai et al., 2014). Mice were housed in ventilated cages with a 12-hour light/dark cycle and controlled temperature (20–22°C), and fed normal chow and water ad libitum. Female $Gch1^{fl/fl}$ Tie2cre mice and their $Gch1^{fl/fl}$ littermates (thereafter referred to as wild-type) were used for all experiments at 10 to 16 weeks. The generation and phenotyping of the knock-out model were carried out in accordance with the Animal (Scientific Procedures) Act 1986, with procedures reviewed by the clinical medicine animal care and ethical review body, and conducted under project licenses PPL 30/3080 and P0C27F69A. Mice were genotyped by polymerase chain reactions using DNA prepared from ear biopsies. For $Gch1^{fl/fl}$ genotyping, PCR was performed using the following primers:$Gch1^{fl/fl}$ -Fw 5'-GTC CTT GGT CTC AGT AAA CTT GCC AGG-3', $Gch1^{fl/fl}$ -Rv 5'-GCC CAG CCA AGG ATA GAT GCA G-3'. The *Gch1* floxed allele showed a 1030 bp. For Tie2cre genotyping, PCR was performed using the following primers: Tie2cre Fw 5'-GCA TAA CCA GTG AAA CAG CAT TGC TG-3'. Tie2cre Rv 5'-GGA CAT GTT CAG GGA TCG CCA GGC G-3'. The Tie2cre allele amplified as 280 bp fragment.

Timed mating

Pregnancy was achieved by mating either virgin female $Gch1^{fl/fl}$ Tie2cre or $Gch1^{fl/fl}$ (wild-type) females (aged between 10 to 16 weeks old) with a $Gch1^{fl/fl}$ male. Detection of a vaginal plugs indicated successful conception and was taken as 0.5 day of gestation (E0.5).

Vasomotor function studies

Vasomotor function in uterine arteries (main branch) and aortas from both non-pregnant and pregnant (E18.5) $Gch1^{fl/fl}$ Tie2cre and wild-type littermates was examined using isometric tension studies in a wire myograph (MultiMyogrph 610M, Danish Myo Technology, Denmark). Briefly, mice were culled by overdose of inhaled isoflurane and vascular rings were isolated from the uterine horns or thoracic aorta. The 2-mm rings were mounted in a wire myograph containing 5 ml of ice-cold Krebs-Henseleit buffer (KHB [in mmol/l]: NaCl 120, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, CaCl2 2.5, NaHCO3 25, glucose 5.5) at 37°C, gassed with 95% O_2/5% CO_2. After allowing vessels to equilibrate for 30 minutes, the optimal resting tension equivalent to 100 mmHg was set. Concentration-response contraction curves were established using cumulative half-log concentrations of U46619 (thromboxane A2 receptor agonist; uterine artery) and phenylephrine (aorta) respectively. Vessels were washed three times with fresh KHB, equilibrated for 20 minutes, and then precontracted to approximately 80-90% of maximal tension with U46619 for uterine arteries or with phenylephrine for aortas. Acetylcholine was used to stimulate endothelium-dependent vasodilatations in increasing cumulative concentrations. Responses were expressed as a percentage of the pre-contracted tension. Finally, the NO donor sodium nitroprusside (SNP) was used to test endothelium-independent smooth muscle relaxation in the presence of L-NAME. All pharmacological drugs were pre-incubated at least 20 min before the dose-response curves were determined. L-NAME was used at 100 µM, apamin at 50 nM, charybdotoxin at 100 nM, and indomethacin at 10 µM. All drugs used were purchased from Sigma Chemical Company.

Blood pressure measurement by tail-cuff plethysmography

Systolic blood pressure was determined using a computerized tail-cuff system (Visitech, USA) in conscious mice. Experiments were performed between the hours of 8 and 12 am. The animal tails were passed through a cylindrical latex tail-cuff and taped down to reduce movement. Twenty readings were taken per mouse of which the first 5 readings were discarded. The remaining 15 readings were used to calculate the mean systolic blood pressure and in each mouse.

Statistical analysis

Data are presented as mean ± SEM. Normality was tested using D'Agostino and Pearson omnibus normality test. Groups were compared using the Mann–Whitney U test for non-parametric data or an un-paired Student's t -test for parametric data. When comparing multiple groups data were analysed by analysis of variance (ANOVA) with Newman–Keuls post-test for parametric data or Kruskal–Wallis test with Dunns post-test for non-parametric data. When more than two independent variables were present a two-way ANOVA with Tukey's multiple comparisons test was used. When within subject repeated measurements were present a repeated measures (RM) ANOVA was used. A value of $P < 0.05$ was considered statistically significant.

Results

Loss of endothelial cell Gch1 and BH4 lead to progressive hypertension during pregnancy.

Consistent with our previous reports (Chuaiphichai et al., 2021), we observed a small but significant increase in basal systolic blood pressure (˜5-7 mmHg) in $Gch1^{fl/fl}$ Tie2cre mice compared with wild-type littermates (WT: 103 +- 2 mmHg, vs $Gch1^{fl/fl}$ Tie2cre mice: 110 +- 1 mmHg, P <0.05, n=6; Figure 1A). In wild type mice, systolic blood pressure was unchanged during pregnancy (baseline WT: 103 +- 2 mmHg, vs pregnant WT: 103 +- 3 mmHg, n=6; Figure 1A). In contrast, loss of endothelial cell *Gch1* resulted in a significantly marked increase (˜24 mmHg) in systolic blood pressure in later pregnancy in $Gch1^{fl/fl}$ Tie2cre (Figure 1A).

Deficiency in endothelial cell Gch1/BH4 during pregnancy leads to impaired vascular function in aortas from pregnant $Gch1^{fl/fl}$Tie2cre mice.

First, we determined the physiological requirement for endothelial cell-specific *Gch1* /BH4 biosynthesis in vasomotor function in conduit arteries from both nonpregnant and pregnant $Gch1^{fl/fl}$ Tie2cre and wild-type mice using wire myography. Isometric tension studies in isolated aortas demonstrated no difference in vasoconstriction responses, expressed as percentage contraction to KCl, prior to pregnancy between the genotypes (Figure 1B and C). However, at late stage of pregnancy loss of endothelial cell *Gch1* lead to a significant increase in vasoconstriction to phenylephrine (Figure 1B and C). This difference was unlikely due to structural difference between genotypes in pregnant mice as constrictive responses to KCl were similar across all groups (nonpregnant WT: 4.68 +- 0.41mN, vs. nonpregnant $Gch1^{fl/fl}$ Tie2cre: 5.13 +- 0.32mN, Pregnant WT: 4.35 +- 0.19mN, vs. Pregnant $Gch1^{fl/fl}$ Tie2cre: 4.74 +- 0.19,$P<0.05$; Figure 1D). The difference between genotypes was normalized in the presence of L-NAME (Figure 1E), indicating that the increased constrictor response in pregnant $Gch1^{fl/fl}$ Tie2cre aortas is likely mediated by tonic eNOS-mediated vasodilatation.

There was no difference in endothelium dependent vasodilatation to acetylcholine between the genotypes prior to pregnancy. However, in late stage of pregnancy, endothelium-dependent vasodilatation to acetylcholine was significantly impaired in pregnant $Gch1^{fl/fl}$ Tie2cre aortas when compared with aortas from pregnant wild-type mice and aortas from non-pregnant $Gch1^{fl/fl}$ Tie2cre mice (Figure 1F and G). In the presence of L-NAME, endothelium-dependent vasodilatation to acetylcholine in pregnant and nonpregnant mice of both genotypes was totally abolished (Figure 1H), indicating that eNOS is the major source of vasodilators in mouse aortas in both pregnant and non-pregnant mice. Interestingly, we found that pregnancy significantly increased the potency of endothelium-independent vasodilatation to the nitric oxide donor, sodium nitroprusside (SNP) in aortas from both wild-type and $Gch1^{fl/fl}$ Tie2cre mice when compared to aortas from nonpregnant wild-type mice (Figure 1I), suggesting that a normal pregnancy is associated with an increased sensitivity to nitric oxide downstream signalling pathway in conduit arteries.

Loss of endothelial cell BH4 during pregnancy caused vascular dysfunction uterine arteries from pregnant $Gch1^{fl/fl}$Tie2cre mice.

We next determine how loss of endothelial cell *Gch1* impacts on pregnancy induced vascular adaptations of the uterine artery. Firstly, the lumen diameter of uterine arteries, as determined by the length-tension relationship, was significantly increased in pregnancy in both genotypes with no difference between genotypes observed in either pregnancy or non-pregnancy mice (Pregnant WT: 258 +- 12.6 μm, Pregnant $Gch1^{fl/fl}$ Tie2cre: 276 ± 12.7 μm; Nonpregnant WT 155 ± 6.6 μm, nonpregnant $Gch1^{fl/fl}$ Tie2cre: 153 ± 3.1*; $P<0.05$*) (Figure 2A). This was accompanied by a greater KCl response (˜2-fold increased) in uterine arteries from pregnant wild-type and $Gch1^{fl/fl}$ Tie2cre mice (Pregnant WT: 5.8 ± 0.7 mN, Pregnant $Gch1^{fl/fl}$ Tie2cre: 5.1 ± 0.4 mN, nonpregnant WT: 2.6 ± 0.3 mN, nonpregnant $Gch1^{fl/fl}$ Tie2cre: 2.3 ± 0.4;$P<0.05$) (Figure 2A), indicating that a normal pregnancy is associated with increased lumen size and media thickness of the uterine artery. However, loss of endothelial cell *Gch1* in pregnancy lead to an increase in uterine artery stiffness, with increased wall tension in response to increasing stretch observed in uterine arteries from pregnancy $Gch1^{fl/fl}$ Tie2cre mice compared with wild type controls (Supplementary Figure 1).

In contrast to the aorta, uterine arteries from pregnant wild type mice had a significantly attenuated contractile response, with a corresponding increase in the EC_{50} and decrease in Emax (% maximum contraction) to the TxA2 receptor against, U46619, (Figure 2B, C, and D). Incubation with the NOS inhibitor L-NAME lead to a significant augmentation of the contractile response (Figure 3E), indicating that the pregnancy induced attenuation was due in part to increased NOS-derived NO.

However, uterine arteries from pregnant $Gch1^{fl/fl}$ Tie2cre mice showed incomplete adaptation to pregnancy with a significantly greater contractile response observed compared with arteries from pregnant wild type mice, as demonstrated by an increased maximum constriction and reduced EC_{50} (Figure 2B, C, and D). This difference appeared to be driven in part by a reduced NOS dependent production of vasodilators in

uterine arteries from pregnant $Gch1^{fl/fl}$ Tie2cre mice as in contrast to wild type mice. L-NAME did not alter the vasoconstrictor response in uterine arteries from pregnant $Gch1^{fl/fl}$ Tie2cre mice (Figure 2E).

In wild-type uterine arteries, endothelium-dependent vasodilatation to ACh was significantly enhanced in pregnancy compared to nonpregnant wild-type controls (Figure 2F; $P<0.05$). This was accompanied by a significantly enhanced endothelium-independent vasodilatation in response to nitric oxide donor, sodium nitroprusside (SNP), indicating increased vascular smooth muscle sensitivity to downstream NO signalling pathway during pregnancy (Figure 2I).

In contrast, pregnancy induced enhanced endothelium-dependent vasodilatations was blunted in pregnant $Gch1^{fl/fl}$ Tie2cre uterine arteries with no difference in endothelial cell mediated dilation between pregnancy and non-pregnant uterine arteries from knock out mice (Figure 2F, G and H). This blunted pregnancy induced vascular remodelling was observed despite the presence of pregnancy induced enhanced endothelium-independent vasodilatation in response to nitric oxide donor, SNP (Figure 2I).

Contribution of NO, Prostacyclin and Endothelium-derived Hyperpolarizing factors (EDHF) in uterine arteries from pregnant mice deficient in endothelial cell BH4.

We next determined the relative contributions of the endothelium-derived vasodilators NOS, prostacyclin and EDHF in uterine arteries of pregnant mice lacking endothelial cell BH4. Firstly, we found that in the presence of L-NAME, endothelium-dependent vasodilatation was significantly inhibited in uterine arteries from pregnant and non-pregnant wild-type and non-pregnant $Gch1^{fl/fl}$ Tie2cre mice, but was unaltered in uterine arteries from pregnant $Gch1^{fl/fl}$ Tie2cre mice (Figure 3A and B), adding further evidence to the loss of eNOS-mediated vasodilator function in uterine arteries of pregnant $Gch1^{fl/fl}$ Tie2cre mice.

In the presence of L-NAME, addition of indomethacin had minimal effect on endothelium-dependent vasodilation in uterine arteries from either pregnant or non-pregnant wild-type and $Gch1^{fl/fl}$ Tie2cre mice (Figure 3A and B) . In contrast, addition of EDHF blockers (combination of apamin and charybdotoxin) totally inhibited the non-NOS mediated component of endothelium-dependent vasodilation in uterine arteries from both non-pregnant and pregnant mice of both genotypes with a significantly greater augmentation observed in uterine arteries from $Gch1^{fl/fl}$ Tie2cre mice (Figure 3A, B, and C) . Systematic quantification contribution of the vasodilator responses revealed a marked reduction that in contrast to wild-type mice, uterine arteries from pregnant $Gch1^{fl/fl}$ Tie2cre mice revealed a striking loss of NOS-derived NO and a significant increase in the EDHF component (Figure 3C), suggesting loss of endothelial cell *Gch1* /BH4 leads to a compensatory upregulation of EDHF in $Gch1^{fl/fl}$ Tie2cre uterine arteries. However, this compensatory upregulation was insufficient to fully correct for the loss of NOS mediated dilators.

To further investigate which specific components of the EDHF response is affected in $Gch1^{fl/fl}$ Tie2cre mice, endothelium-dependent vasodilatation was assessed in the presence of L-NAME and indomethacin with either charybdotoxin or apamin. First, we found that in a normal pregnancy in wild-type mice, intermediate and large Ca^{2+}-activated K^+ channels (charybdotoxin-sensitive component) make up the majority of the EDHF component (Figure 4A and B) with the apamin-sensitive component accountable for approximately a third of the EDHF response (Figure 4A and B). In contrast to wild-type mice, loss of endothelial cell *Gch1* leads to a significant reduction in the apamin-sensitive component with the charybdotoxin-sensitive component dominating. This effect was seen even when the order of the inhibitors used was changed (Supplementary Figure 2). Taken together, these observations indicated that loss of endothelial cell BH4 impacts on the ability of endothelial cells to upregulate small Ca^{2+}-activated K^+ channel mediated EDHF responses.

Supplementation with BH4 fails to prevent vascular dysfunction and pregnancy-induced hypertension in $Gch1^{fl/fl}$Tie2cre mice, but is rescued by the addition of reduced folate, 5-Methyltetrahydrofolate.

In our previous study, we showed that treatment of pregnant $Gch1^{fl/fl}$ Tie2cre mice with BH4 and 5-MTHF was sufficient to restoration BH4 levels and prevented fetal growth restriction and increased blood pressure in late pregnancy (Chuaiphichai et al., 2021; Landmesser et al., 2003; Williams, Vallance, Neild, Spencer & Imms, 1997). Given the results of our current study we hypothesized that BH4 and 5-MTHF supplementa-

tion is acting to restored blood pressure and fetal growth via normalization of pregnancy induced vascular remodelling in *Gch1*$^{fl/fl}$ Tie2cre mice. To address this, we treated *Gch1*$^{fl/fl}$ Tie2cre and wild-type mice with either oral BH4 or BH4 with 5-MTHF for 3 days prior to mating, and throughout pregnancy.

We found that oral BH4 supplementation alone was not sufficient to prevent progressive hypertension throughout pregnancy or restore vascular function in uterine arteries from *Gch1*$^{fl/fl}$ Tie2cre mice. With an enhanced contractile response and reduced sensitivity to the endothelial cell dependent dilator acetylcholine still observed in pregnant *Gch1*$^{fl/fl}$ Tie2cre mice compared to their wild type littermates (Figure 5A, B, C and D).

However, the addition of reduced folate, 5-MTHF to BH4 supplementation resulted in a striking normalization of both the constriction responses to U46619 and relaxation responses to ACh, in *Gch1*$^{fl/fl}$ Tie2cre mice, restoring the responses to those observed in wild-type animals. The combination of 5-MTHF to BH4 oral supplementation just prior to conception was sufficient to prevent progressive pregnancy induced hypertension in *Gch1*$^{fl/fl}$ Tie2cre mice with no difference in blood pressure observed between wild type and *Gch1*$^{fl/fl}$ Tie2cre mice from 2.5 days post-conception until the end of the experiment at gestational day 18.5 (Figure 5C and F).

Discussion

To the best of our knowledge, this is the first study evaluating the effect of endothelial cell BH4 deficiency and the underlying mechanisms on maternal vascular adaptations in uterine arteries during pregnancy. The key findings are 1) Selective deficiency in maternal endothelial cell *Gch1* /BH4 biosynthesis during pregnancy leads to vascular dysfunction due to loss of NOS-derived vasodilators in both in aortas and uterine arteries from *Gch1*$^{fl/fl}$ Tie2cre mice; 2) In uterine arteries the vascular impairment is incompletely compensated for by an increase in EDHF-mediated vasodilation mediated by an increase in intermediate and large-conductance Ca^{2+}-activated K^+ channels. 3) Oral supplementation of BH4 and 5MTHF, but not BH4 alone, preserves vascular endothelial cell vasodilator function, and thus prevent progressive pregnancy-induced hypertension in mice with endothelial cell BH4 deficiency. Taken together, these findings identify a novel role for endothelial cell *Gch1* and BH4 biosynthesis in vascular adaptations to pregnancy.

Pregnancy is associated with a 10-fold increase in uterine artery blood flow. In order to accommodate this without increases in systemic blood pressure the uterine artery undergoes significant changes with enhanced vasodilation and reduced constriction, leading to reduced vascular resistance and increased blood flow (Cooke & Davidge, 2003; Nelson, Steinsland, Johnson, Suresh, Gifford & Ehardt, 1995). Nitric oxide is a key mediator of this adaptive response. Plasma NO levels are higher in viable than non-viable pregnancies and is inversely correlated uterine artery pulsatility index (Battaglia, Morotti, Montaguti, Mariacci, Facchinetti & Pilu, 2022) and loss of NOS3 (eNOS) associated with uterine artery dysfunction, reduced placenta nutrient transport and fetal growth restriction (Kusinski et al., 2012). In the current study, we have shown that loss of maternal endothelial specific BH4 is alone sufficient to causes maladaptive uterine artery remodeling with enhanced constrictor and reduced dilator response in both aorta and uterine artery. The enhanced constrictor response observed in endothelial cell *Gch1* knockout mice is likely mediated in part by reduced tonic production of NOS derived vasodilators as the difference between genotypes were abolished in the presence of the NOS inhibitor L-NAME. This is in keeping with clinical studies where acute administration of L-NAME greatly reduced forearm blood flow in pregnant women compared to non-pregnant controls (Anumba, Robson, Boys & Ford, 1999; Williams, Vallance, Neild, Spencer & Imms, 1997). In addition, uterine arteries from pregnant endothelial cell *Gch1* knockout mice had increase passive stiffness. Previous, studies in aorta have shown that increase vascular stiffness is associated with enhance agonist mediated contractile response (Zhang, Li, Gao, Wang, Cheng & Wang, 2022) and thus it is possible that this mechanism may also contribute to the increase contractile response observed in our current study.

Interestingly, prior to pregnancy compensatory changes in NOS mediated dilation were sufficient to maintain normal vasoconstrictor and dilator responses in endothelia cell *Gch1* knockout mice. We have previously shown in aorta that in the absence of endothelial cell BH4, eNOS become uncoupled and produces

H_2O_2instead of NO, which acts as an endothelium-dependent vasodilator partially compensating for the loss of eNOS derived NO (Chuaiphichai et al., 2017; Chuaiphichai et al., 2014). However, during pregnancy this compensatory mechanism is lost with NOS mediated vasodilation making minimal contribution to uterine artery vasodilation in endothelial cell *Gch1* knockout mice. Pregnancy is a state of mild oxidative stress (Morris et al., 1998; Palm, Axelsson, Wernroth & Basu, 2009; Toescu, Nuttall, Martin, Kendall & Dunne, 2002). Increase expression of antioxidant defenses have previously been observed in pregnancy (Jenkins, Wilson, Roberts, Miller, McKillop & Walker, 2000). Interestingly, catalase levels have been found to be increased in pre-eclampsia as compared to normal pregnancy (Gohil, Patel & Gupta, 2011). It is possible that in this altered redox environment, NOS derived H_2O_2 is no longer an effective vasodilator in endothelial cell *Gch1* knockout mice.

We show that pregnancy is associated with an increase in EDHF mediated vasodilation in uterine arteries. This is consistent with other studies which have shown enhanced EDHF mediated dilation in pregnancy (Luksha, Nisell & Kublickiene, 2004; Zhu et al., 2013). However, EDHF upregulation in the absence of endothelial cell BH4, was not sufficient to compensate fully for the loss of NOS mediated vasodilation in the uterine artery. In uterine arteries from both wild type and $Gch1^{fl/fl}Tie2cre$ mice both large and intermediate conductance Ca^{2+}-activated K^+ channels were responsible for the majority of the EDHF mediated dilation. This is in keeping with previous studies which have shown an increased expression and activity of BK_{Ca}channels (Hu et al., 2017) and SK_{Ca} channels (Zhu et al., 2013) in uterine arteries of pregnant sheep. Further interrogation of EDHF response showed in wild type mice SK_{Ca} channels made up approximately 40% of the EDHF response, however, in endothelial cell *Gch1* knockout mice this component was markedly reduced to only 20%. Recent studies have shown metabolic regulation of SK_{Ca} channel in coronary endothelial cells with reduced expression observed endothelial cells from diabetic arteries (Liu et al., 2020). Pregnancy represents a significantly altered metabolic state, further studies interrogating if similar mechanisms are driving reduced SK_{Ca} channel activity in our currently study will be key to address this.

Reduced vascular BH4 is a hallmark of multiple cardiovascular conditions (Hink et al., 2001; Landmesser et al., 2003; Li et al., 2006; Mollnau et al., 2003). Oxidative stress causes oxidation of BH4 to BH2 and B, which are incapable of acting as a cofactor for NOS leading to uncoupled NOS. Markers of oxidative stress are present in the placenta and maternal circulation of patients with pre-eclampsia (Raijmakers, Dechend & Poston, 2004) and we have previously shown reduced BH4 in placental extravascular vesicles from women with hypertensive pregnancies (Chuaiphichai et al., 2021). Yet in a landmark clinical trial the antioxidant vitamins C and E failed to prevent the development of pre-eclampsia in high risk pregnancies (Poston, Briley, Seed, Kelly & Shennan, 2006), and simple strategies attempting to restore or augment NO with NO donors in pre-eclampsia have been disappointing (Meher & Duley, 2007). One possible explanation for these results is the failure to specifically target eNOS uncoupling and consequent altered NO/ROS signalling. Thus, augmenting BH4 levels may be a rational therapeutic strategies to treat vascular complication in pregnancy. However, in this study we found that oral BH4 supplementation alone was not sufficient to restore vascular function in uterine arteries and thus prevent progressive hypertension in $Gch1^{fl/fl}$ Tie2cre mice. We have previously shown that oral supplementation of BH4 is not a consistent approach to increase vascular BH4 levels, either in mice or in patients (Chuaiphichai et al., 2021; Cunnington et al., 2012a; Cunnington et al., 2012b), due to oxidation of BH4 to BH2 and B. Interestingly, vascular supplementation of BH4 can be achieved by combining the BH4 with the 5-methyltetrahydrofoalte. The enzyme dihydrofolate reductase (DHFR) reduces dihydrofolate to the fully reduced folate, tetrahydrofolate, and can also reduce oxidized BH2 to regenerate BH4. We have previously demonstrated that supplementation of BH4 with 5-MTHF, restored BH4 levels in pregnancy (Chuaiphichai et al., 2021). In this study we show for the first time that that the combination of BH4 + 5-MTHF is sufficient to restore vascular function in $Gch1^{fl/fl}$ Tie2cre mice. 5-MTHF has been shown to be an effective treatment to augment vascular BH4 levels in patients (Antoniades et al., 2006), exemplifying the early translational potential of this approach – particularly since folates are already approved for use by pregnant women.

Taken together, this study demonstrated that deficiency in maternal endothelial cell BH4 biosynthesis leads to systemic vascular dysfunction and progressive pregnancy-induced hypertension, which could be reversed

by supplementation with BH4 and 5-MTHF. Thus, targeting endothelial cell *Gch1* and BH4 biosynthesis by supplementation with BH4 and 5-MTHF may provide a novel therapeutic target for the prevention and treatment of pregnancy-related hypertension such as pre-eclampsia.

Figure Legends

Figure 1: Blood pressure and vasomotor function in aortas from non-pregnant and pregnant wild-type and *Gch1*$^{fl/fl}$ Tie2cre mice at E18.5 day of gestation.

(**A**) Systolic blood pressure prior to conception and at E18.5, a significant increase in blood pressure was observed in *Gch1*$^{fl/fl}$ Tie2cre mice both before conception and at E18.5 compared with their wild type littermates (*$P<0.05$; n=6 to 8 animals per group). (**B**) Vascular function of in aorta from non-pregnant (NP) and pregnant (P) mice at E18.5 day of gestation. Vasoconstrictions in response to phenylephrine (PE) was significantly enhanced in aortas from pregnant *Gch1*$^{fl/fl}$ Tie2cre mice compared to pregnant wilt-type mice and non-pregnant *Gch1*$^{fl/fl}$ Tie2cre mice (*$P<0.05$; n=6 animals per group). (**C**) EC_{50} and maximum contraction in response to PE. (**D**) Absolute contraction (mN) in response to KCL response (mN) in non-pregnant and pregnant mice from both WT and *Gch1*$^{fl/fl}$ Tie2cre mice. (**E**) Vasoconstrictions in response to phenylephrine (PE) in the presence of 100 μM L-NAME in aortas from non-pregnant (NP) and pregnant (P) mice at E18.5 day of gestation of both genotypes. (**F**) Endothelium-dependent vasodilatation in response to ACh was markedly impaired in aortas from pregnant *Gch1*$^{fl/fl}$ Tie2cre mice compared to pregnant WT and non-pregnant *Gch1*$^{fl/fl}$ Tie2cre mice ($P<0.05$; n=6 animals per group). (**G**) EC_{50} and maximum relaxation in response to ACh. (**H**) Endothelium-dependent vasodilatations to ACh were totally inhibited in all four groups in the presence of L-NAME. (**I**) Endothelium-independent vasodilatations in response to the nitric oxide donor, sodium nitroprusside (SNP) were significantly enhanced in both pregnant wild-type and *Gch1*$^{fl/fl}$ Tie2cre mice compared to non-pregnant wild-type and *Gch1*$^{fl/fl}$ Tie2cre mice (*$P<0.05$, significant difference between non-pregnant WT and pregnant WT, #$P<0.05$, significant difference between non-pregnant *Gch1*$^{fl/fl}$ Tie2cre vs pregnant *Gch1*$^{fl/fl}$ Tie2cre mice; n=6 animals per group).

Figure 2. Effect of endothelial cell BH4 deficiency on vascular uterine artery function in pregnancy. Vascular function of isolated uterine arteries (UA) from non-pregnant (NP) and pregnant (P) mice at E18.5. (**A**) Uterine artery diameters as determined by the length-tension relationship at 100 mmHg. A significant increase in diameter was observed with pregnancy in both groups (*$P<0.05$; n=6 to 8 animals per group). Vasoconstrictions in response to KCL response (45 mM) were significantly increased in pregnant UA from both genotypes compared to non-pregnant controls (*$P<0.05$; n=6 to 8 animals per group). (**B**) Cumulative dose response curved to the TxA2 mimetic, U46619 in non-pregnant and pregnant arteries from wild-type and *Gch1*$^{fl/fl}$ Tie2cre mice. (**C and D)**EC_{50} and maximum contraction in response to U46619. (**E**) Vasoconstriction in response to U46619 in pregnant uterine arteries in the presence or absence of non-selective NOS inhibitor, 100 μM L-NAME (*$P<0.05$; n=6 to 8 per group). (**F**) Endothelium-dependent vasodilatation to acetylcholine (Ach) in uterine arteries from non-pregnant and pregnant mice from both genotypes, submaximally constricted with U46619. (**G** and **H**) EC_{50} and maximum vasodilatation in response to ACh. (**I**) Endothelium-independent vasodilatations in response to the nitric oxide donor, sodium nitroprusside (SNP) were significantly enhanced in both pregnant wild-type and *Gch1*$^{fl/fl}$ Tie2cre mice compared to non-pregnant wild-type and *Gch1*$^{fl/fl}$ Tie2cre mice (*$P<0.05$; n=6 to 8 animals per group).

Figure 3. Contribution of eNOS-derived vasodilators, prostacyclin, and EDHF in non-pregnant and pregnant wild-type and *Gch1*$^{fl/fl}$ Tie2cre uterine arteries at E18.5 day of gestation . (**A** and **B**) Endothelium-dependent vasodilatations to acetylcholine (ACh) were determined in the presence of the nitric oxide synthase inhibitor, L-NAME (100 μM) alone, or L-NAME and the cyclooxygenase inhibitor, indomethacin (10 μM), or L-NAME, indomethacin and EDHF blockers (apamin and charybdotoxin). (**C**) Percentage contribution of NOS-derived vasodilators, prostacyclin and EDHF-sensitive components (*$P<0.05$; n=6 to 8 animals per group).

Figure 4. Contribution of SK_{ca}, IK_{ca} and BK_{ca} channels in pregnant wild-type and *Gch1*$^{fl/fl}$

Tie2cre uterine arteries at E18.5 day of gestation. (**A**) Endothelium-dependent vasodilatations to acetylcholine (ACh) were determined in the presence of the nitric oxide synthase inhibitor, L-NAME (100 μM) and the cyclooxygenase inhibitor, indomethacin (10 μM), or L-NAME, indomethacin and small-conductance Ca^{2+}-activated K^+ channel (SK_{ca}) blocker, apamin (AP; 50 nM), or L-NAME, indomethacin, apamin and non-selectively intermediate and large-conductance Ca^{2+}-activated K^+ channels (IK_{ca} and BK_{ca} , respectively) blocker, charybdotoxin (ChTx; 100 nM). (**B**) Percentage contribution of apamin-sensitive component (SK_{ca}), charybdotoxin-sensitive component (IK_{ca} and BK_{ca}) in pregnant wild-type and *Gch1*$^{fl/fl}$ Tie2cre uterine arteries at E18.5 day of gestation (*$P<0.05$; n=6 to 8 animals per group).

Figure 5. Supplementation of BH4 and 5MTHF but not BH4 alone restores vascular function and prevents pregnancy-induced hypertension in pregnant mice with endothelial cell BH4 deficiency. Non-pregnant *Gch1*$^{fl/fl}$ Tie2cre and *Gch1*$^{fl/fl}$ (Wild-type; WT) mice were supplemented with BH4 (200 mg/kg/day) alone or BH4 with the fully reduced folate, 5-methyltetrahydrofolate (5-MTHF; 15 mg/kg/day) or control for 3 days before timed-matings, and throughout their subsequent pregnancies. Blood pressure was determined before and throughout pregnancy by non-invasive tail-cuff plethysmography. Vascular function of isolated uterine arteries (UA) from pregnant *Gch1*$^{fl/fl}$ Tie2cre and wild-type mice treated with either BH4 alone or BH4+5MTHF or control was assessed by wire myography at 18.5 day of gestation. (**A** and **C**) Oral BH4 supplementation alone was not sufficient to prevent progressive hypertension throughout pregnancy or (**B** and **D**) restore vascular dysfunction in uterine arteries from *Gch1*$^{fl/fl}$ Tie2cre mice (*$P<0.05$; n=6 animals per group). (**E** and**F**) The combination of 5-MTHF to BH4 oral supplementation was sufficient to prevent progressive pregnancy-induced hypertension and vascular dysfunction in UA from pregnant *Gch1*$^{fl/fl}$ Tie2cre mice (*$P<0.05$; n=6 animals per group).

REFERENCES

Antoniades C, Shirodaria C, Warrick N, Cai S, de Bono J, Lee J*, et al.* (2006). 5-methyltetrahydrofolate rapidly improves endothelial function and decreases superoxide production in human vessels: effects on vascular tetrahydrobiopterin availability and endothelial nitric oxide synthase coupling. Circulation 114**:** 1193-1201.

Anumba DO, Robson SC, Boys RJ, & Ford GA (1999). Nitric oxide activity in the peripheral vasculature during normotensive and preeclamptic pregnancy. Am J Physiol 277**:**H848-854.

Battaglia C, Morotti E, Montaguti E, Mariacci G, Facchinetti F, & Pilu G (2022). Plasma and amniotic fluid concentrations of nitric oxide: Effects on uterine artery and placental vasculature in women who underwent voluntary pregnancy termination and in women with missed and threatened abortion. A pilot study. Eur J Obstet Gyn R B 270**:** 105-110.

Bellamy L, Casas JP, Hingorani AD, & Williams DJ (2007). Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. BMJ 335**:** 974.

Chuaiphichai S, Crabtree MJ, Mcneill E, Hale AB, Trelfa L, Channon KM*, et al.* (2017). A key role for tetrahydrobiopterin- dependent endothelial NOS regulation in resistance arteries: studies in endothelial cell tetrahydrobiopterin-deficient mice. Br J Pharmacol 174**:** 657-671.

Chuaiphichai S, McNeill E, Douglas G, Crabtree MJ, Bendall JK, Hale AB*, et al.* (2014). Cell-autonomous role of endothelial GTP cyclohydrolase 1 and tetrahydrobiopterin in blood pressure regulation. Hypertension 64**:** 530-540.

Chuaiphichai S, Yu GZ, Tan CMJ, Whiteman C, Douglas G, Dickinson Y*, et al.* (2021). Endothelial GTPCH (GTP Cyclohydrolase 1) and Tetrahydrobiopterin Regulate Gestational Blood Pressure, Uteroplacental Remodeling, and Fetal Growth. Hypertension 78**:** 1871-1884.

Cooke CL, & Davidge ST (2003). Pregnancy-induced alterations of vascular function in mouse mesenteric and uterine arteries. Biol Reprod 68**:** 1072-1077.

Crabtree MJ, Tatham AL, Al-Wakeel Y, Warrick N, Hale AB, Cai S*, et al.* (2009). Quantitative regulation of intracellular endothelial nitric oxide synthase (eNOS) coupling by both tetrahydrobiopterin-eNOS stoichiometry and biopterin redox status: Insights from cells with tet-regulated GTP cyclohydrolase I expression. J Biol Chem 284**:** 1136-1144.

Cunnington C, Van Assche T, Shirodaria C, Kylintireas I, Lindsay AC, Lee JM*, et al.* (2012a). Systemic and vascular oxidation limits the efficacy of oral tetrahydrobiopterin treatment in patients with coronary artery disease. Circulation 125**:** 1356-1366.

Cunnington C, Van Assche T, Shirodaria C, Kylintireas I, Lindsay AC, Lee JM*, et al.* (2012b). Systemic and Vascular Oxidation Limits the Efficacy of Oral Tetrahydrobiopterin Treatment in Patients With Coronary Artery Disease. Circulation 125**:** 1356-1366.

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, & Zeiher AM (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 399**:** 601-605.

Ferreira I, Peeters LL, & Stehouwer CDA (2009). Preeclampsia and increased blood pressure in the offspring: meta-analysis and critical review of the evidence. Journal of Hypertension 27**:** 1955-1959.

Gohil JT, Patel PK, & Gupta P (2011). Evaluation of oxidative stress and antioxidant defence in subjects of preeclampsia. J Obstet Gynaecol India 61**:** 638-640.

Hink U, Li HG, Mollnau H, Oelze M, Matheis E, Hartmann M*, et al.* (2001). Mechanisms underlying endothelial dysfunction in diabetes mellitus. Circulation Research 88**:** E14-E22.

Hu XQ, Dasgupta C, Chen M, Xiao DL, Huang XH, Han LM*, et al.* (2017). Pregnancy Reprograms Large-Conductance Ca2+-Activated K+ Channel in Uterine Arteries Roles of Ten-Eleven Translocation Methylcytosine Dioxygenase 1-Mediated Active Demethylation. Hypertension 69**:** 1181-+.

Jenkins C, Wilson R, Roberts J, Miller H, McKillop JH, & Walker JJ (2000). Antioxidants: Their Role in Pregnancy and Miscarriage. Antioxidants & Redox Signaling 2**:**623-628.

Kajantie E, Eriksson JG, Osmond C, Thornburg K, & Barker DJP (2009). Pre-Eclampsia Is Associated With Increased Risk of Stroke in the Adult Offspring The Helsinki Birth Cohort Study. Stroke 40**:** 1176-1180.

Kelly BA, Stone S, & Poston L (2000). Cardiovascular adaptation to pregnancy: the role of altered vascular structure. Fetal and Maternal Medicine Review 11**:**105-116.

Kublickiene KR, Cockell AP, Nisell H, & Poston L (1997). Role of nitric oxide in the regulation of vascular tone in pressurized and perfused resistance myometrial arteries from term pregnant women. Am J Obstet Gynecol 177**:** 1263-1269.

Kusinski LC, Stanley JL, Dilworth MR, Hirt CJ, Andersson IJ, Renshall LJ*, et al.* (2012). eNOS knockout mouse as a model of fetal growth restriction with an impaired uterine artery function and placental transport phenotype. Am J Physiol-Reg I 303**:** R86-R93.

Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM*, et al.* (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. Journal of Clinical Investigation 111**:** 1201-1209.

Li HG, Witte K, August M, Brausch I, Godtel-Armbrust U, Habermeier A*, et al.* (2006). Reversal of endothelial nitric oxide synthase uncoupling and up-regulation of endothelial nitric oxide synthase expression lowers blood pressure in hypertensive rats. Journal of the American College of Cardiology 47**:** 2536-2544.

Liu YH, Kabakov AY, Xie A, Shi GB, Singh AK, Sodha NR*, et al.* (2020). Metabolic regulation of endothelial SK channels and human coronary microvascular function. International journal of cardiology 312**:** 1-9.

Luksha L, Nisell H, & Kublickiene K (2004). The mechanism of EDHF-mediated responses in subcutaneous small arteries from healthy pregnant women. Am J Physiol-Reg I 286**:**R1102-R1109.

Lyall F, Bulmer JN, Kelly H, Duffie E, & Robson SC (1999). Human trophoblast invasion and spiral artery transformation: the role of nitric oxide. Am J Pathol 154**:**1105-1114.

McDonald SD, Malinowski A, Zhou Q, Yusuf S, & Devereaux PJ (2008). Cardiovascular sequelae of preeclampsia/eclampsia: a systematic review and meta-analyses. Am Heart J 156**:** 918-930.

Meher S, & Duley L (2007). Nitric oxide for preventing pre-eclampsia and its complications. Cochrane Database Syst Rev**:** CD006490.

Miller SL, Jenkin G, & Walker DW (1999). Effect of nitric oxide synthase inhibition on the uterine vasculature of the late-pregnant ewe. Am J Obstet Gynecol 180**:**1138-1145.

Mollnau H, Schulz E, Daiber A, Baldus S, Oelze M, August M, *et al.* (2003). Nebivolol prevents vascular NOSIII uncoupling in experimental hyperlipidemia and inhibits NADPH oxidase activity in inflammatory cells. Arterioscl Throm Vas 23**:** 615-621.

Morris JM, Gopaul NK, Endresen MJR, Knight M, Linton EA, Dhir S*, et al.* (1998). Circulating markers of oxidative stress are raised in normal pregnancy and pre-eclampsia. Brit J Obstet Gynaec 105**:** 1195-1199.

Nelson SH, Steinsland OS, Johnson RL, Suresh MS, Gifford A, & Ehardt JS (1995). Pregnancy-Induced Alterations of Neurogenic Constriction and Dilation of Human Uterine Artery. Am J Physiol-Heart C 268**:** H1694-H1701.

Nelson SH, Steinsland OS, Wang Y, Yallampalli C, Dong YL, & Sanchez JM (2000). Increased nitric oxide synthase activity and expression in the human uterine artery during pregnancy. Circ Res 87**:** 406-411.

Palm M, Axelsson O, Wernroth L, & Basu S (2009). F-2-Isoprostanes, tocopherols and normal pregnancy. Free Radical Research 43**:** 546-552.

Poston L, Briley AL, Seed PT, Kelly FJ, & Shennan AH (2006). Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo-controlled trial. Lancet 367**:** 1145-1154.

Raijmakers MT, Dechend R, & Poston L (2004). Oxidative stress and preeclampsia: rationale for antioxidant clinical trials. Hypertension 44**:** 374-380.

Sladek SM, Magness RR, & Conrad KP (1997). Nitric oxide and pregnancy. Am J Physiol 272**:** R441-463.

Sullivan JA, Grummer MA, Yi FX, & Bird IM (2006). Pregnancy-enhanced endothelial nitric oxide synthase (eNOS) activation in uterine artery endothelial cells shows altered sensitivity to Ca2+, U0126, and wortmannin but not LY294002–evidence that pregnancy adaptation of eNOS activation occurs at multiple levels of cell signaling. Endocrinology 147**:** 2442-2457.

Toescu V, Nuttall SL, Martin U, Kendall MJ, & Dunne F (2002). Oxidative stress and normal pregnancy. Clinical endocrinology 57**:** 609-613.

van der Heijden OW, Essers YP, Fazzi G, Peeters LL, De Mey JG, & van Eys GJ (2005). Uterine artery remodeling and reproductive performance are impaired in endothelial nitric oxide synthase-deficient mice. Biol Reprod 72**:**1161-1168.

Vasquez-Vivar J, Martasek P, Whitsett J, Joseph J, & Kalyanaraman B (2002). The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. Biochemical Journal 362**:** 733-739.

Williams DJ, Vallance PJ, Neild GH, Spencer JA, & Imms FJ (1997). Nitric oxide-mediated vasodilation in human pregnancy. Am J Physiol 272**:** H748-752.

Zhang W, Li QQ, Gao HY, Wang YC, Cheng M, & Wang YX (2022). The regulation of yes-associated protein/transcriptional coactivator with PDZ-binding motif and their roles in vascular endothelium. Front Cardiovasc Med 9.

Zhu RH, Hu XQ, Xiao DL, Yang SM, Wilson SM, Longo LD, *et al.* (2013). Chronic Hypoxia Inhibits Pregnancy-Induced Upregulation of SKCa Channel Expression and Function in Uterine Arteries. Hypertension 62**:** 367-374.

Acknowledgements

This study was supported by a British Heart Foundation (BHF) Programme Grants (RG/12/5/29576 and RG/17/10/32859), BHF Project Grant (PG/19/48/34433), BHF Chair award (CH/16/1/32013), Oxford BHF Centre of Research Excellence (RE/13/1/30181), and the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre.

Author contributions

S.C. and K.M.C conceived the study and designed the experiments, with contributions from G.D. Mouse experiments and analyses were done by S.C. with help from G.D., C.W., and Y.D. Vascular function studies were undertaken by S.C., C.W., and D.A.Y. The manuscript was drafted by S.C., K.M.C., and G.D. All authors discussed the results and had the opportunity to contribute to the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Data availability statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

Figure 1

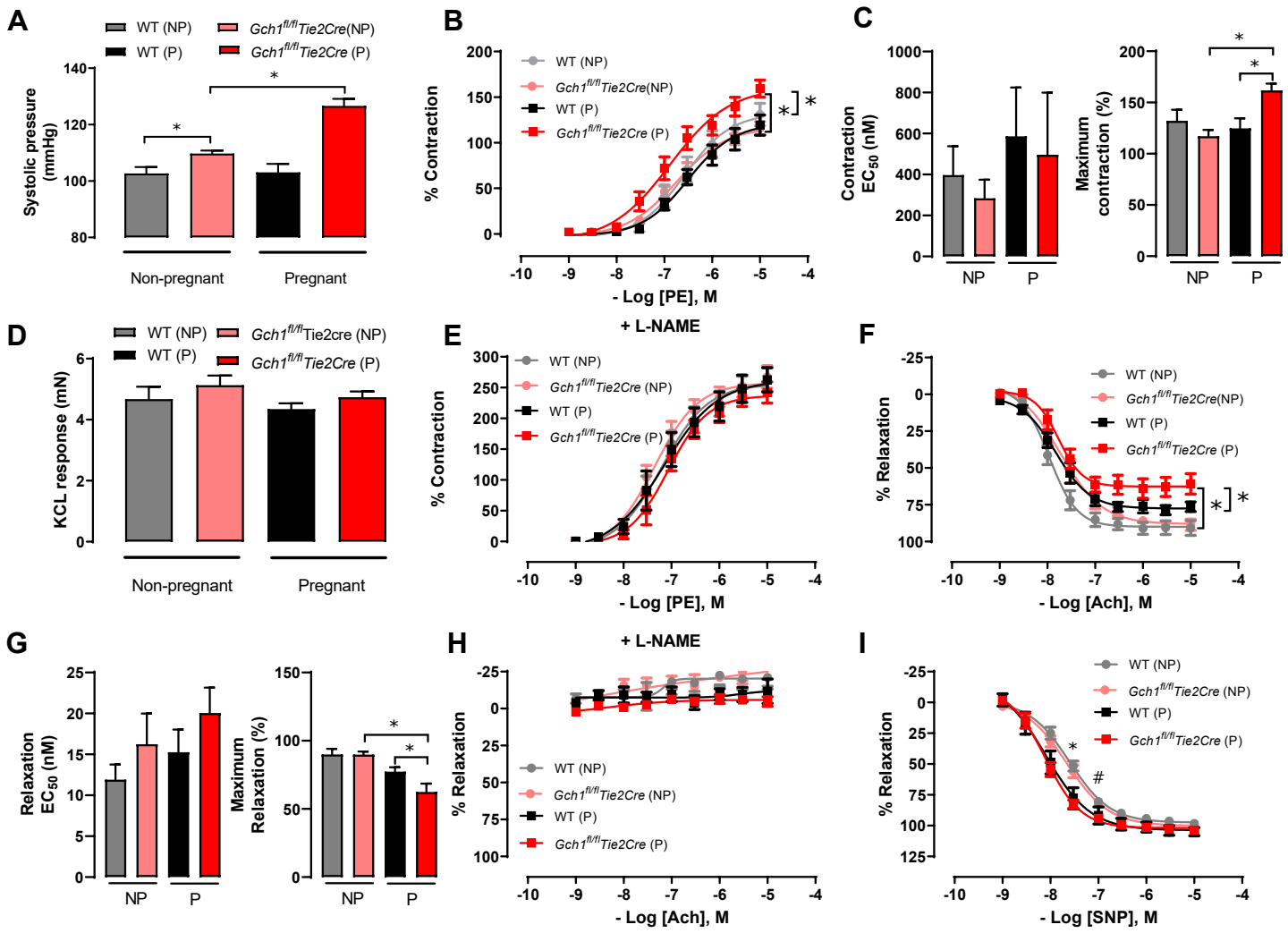

A
WT (NP)
WT (P)
Gch1fl/flTie2Cre(NP)
Gch1fl/flTie2Cre (P)
Systolic pressure (mmHg)
Non-pregnant
Pregnant
B
% Contraction
- Log [PE], M
C
Contraction EC50 (nM)
Maximum contraction (%)
NP
P
D
KCL response (mN)
E
+ L-NAME
F
% Relaxation
- Log [Ach], M
G
Relaxation EC50 (nM)
Maximum Relaxation (%)
H
I
- Log [SNP], M

Figure 2

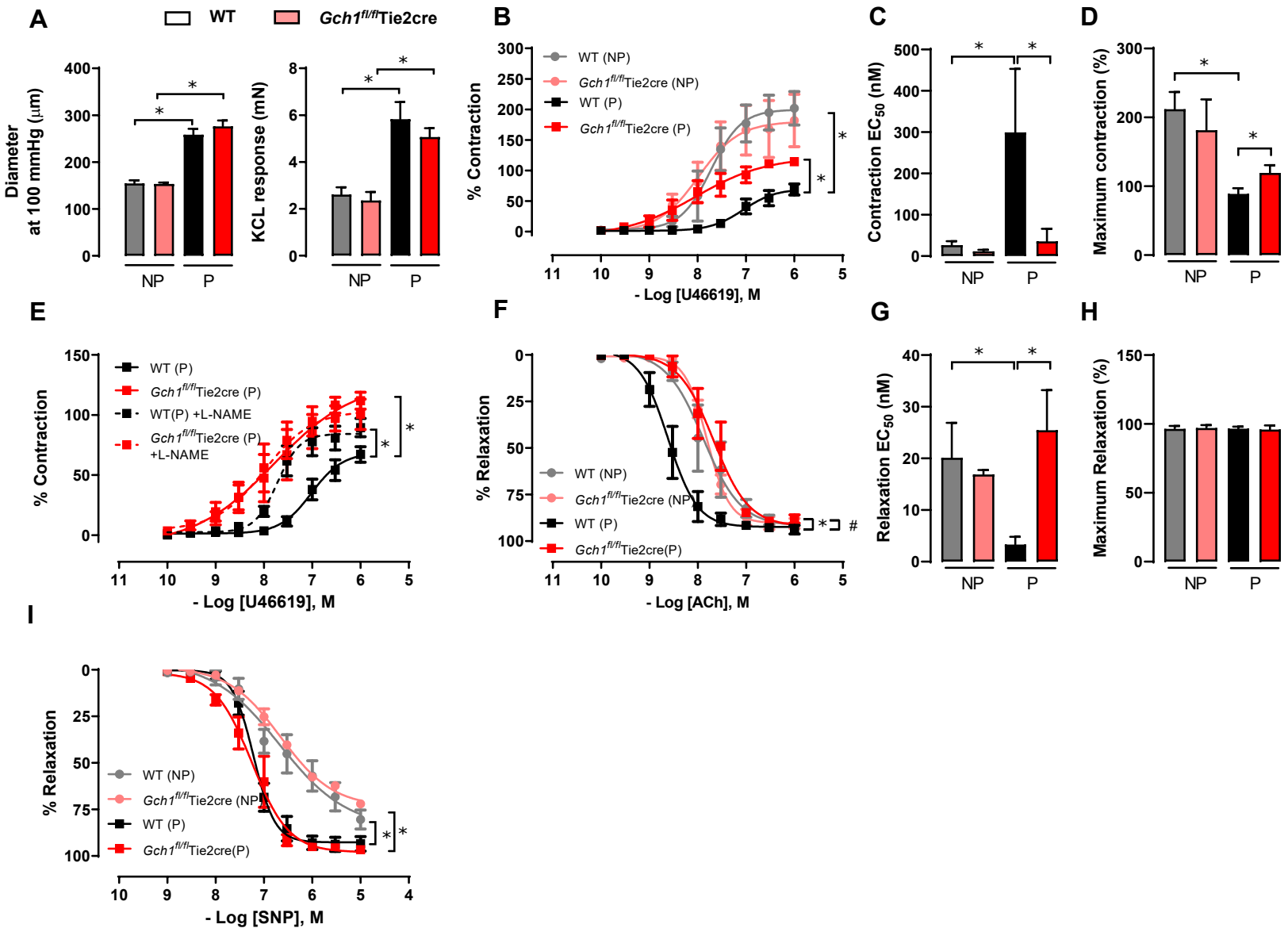

A
WT
Gch1fl/flTie2cre
Diameter at 100 mmHg (μm)
KCL response (mN)
NP
P
B
WT (NP)
Gch1fl/flTie2cre (NP)
WT (P)
Gch1fl/flTie2cre (P)
% Contraction
- Log [U46619], M
C
Contraction EC50 (nM)
D
Maximum contraction (%)
E
WT (P)
Gch1fl/flTie2cre (P)
WT(P) +L-NAME
Gch1fl/flTie2cre (P) +L-NAME
% Contraction
- Log [U46619], M
F
WT (NP)
Gch1fl/flTie2cre (NP)
WT (P)
Gch1fl/flTie2cre(P)
% Relaxation
- Log [ACh], M
G
Relaxation EC50 (nM)
H
Maximum Relaxation (%)
I
WT (NP)
Gch1fl/flTie2cre (NP)
WT (P)
Gch1fl/flTie2cre(P)
% Relaxation
- Log [SNP], M

Figure 3

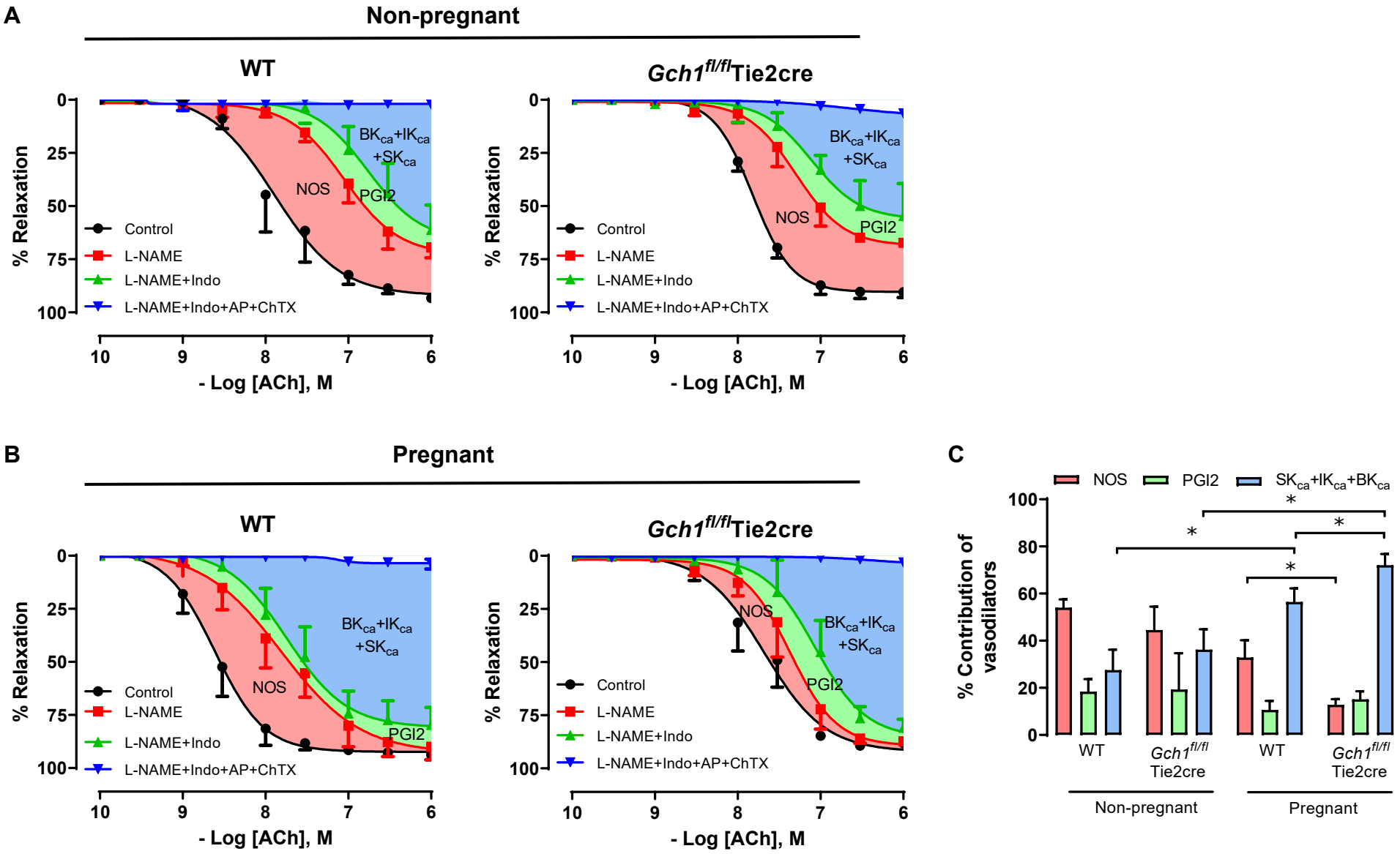

A
Non-pregnant
WT
Gch1fl/flTie2cre
% Relaxation
0
25
50
75
100
Control
L-NAME
L-NAME+Indo
L-NAME+Indo+AP+ChTX
BKca+IKca
+SKca
NOS
PGI2
10
9
8
7
6
- Log [ACh], M
B
Pregnant
WT
Gch1fl/flTie2cre
C
NOS
PGI2
SKca+IKca+BKca
% Contribution of vasodilators
100
80
60
40
20
0
*
WT
Gch1fl/fl Tie2cre
Non-pregnant
Pregnant

Figure 4

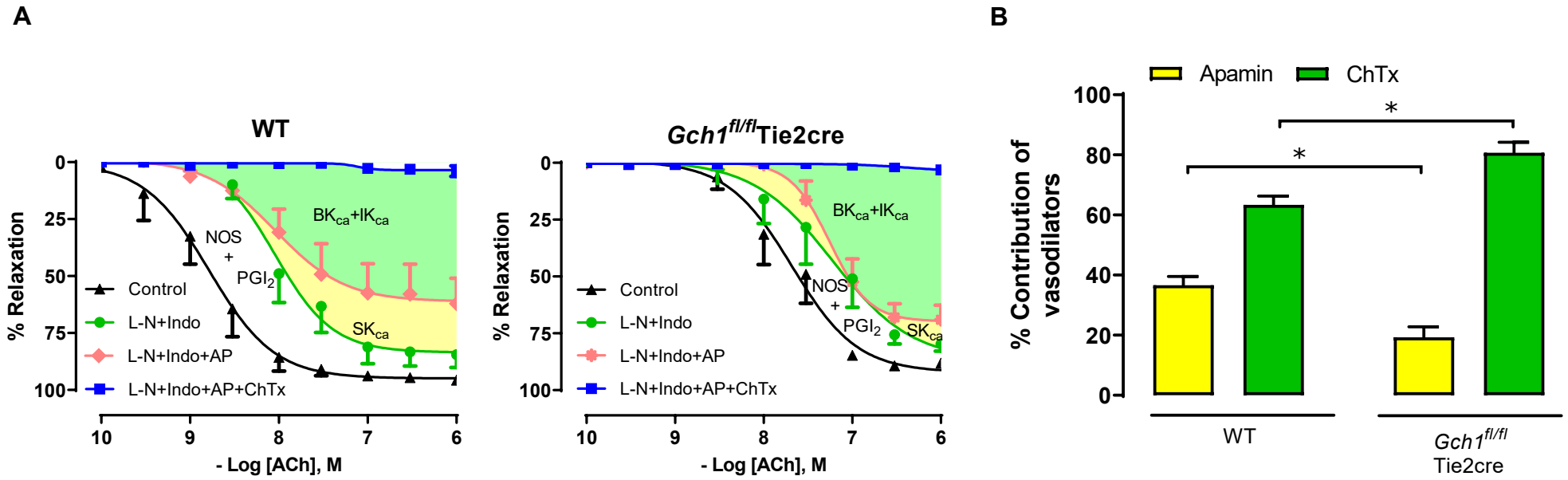
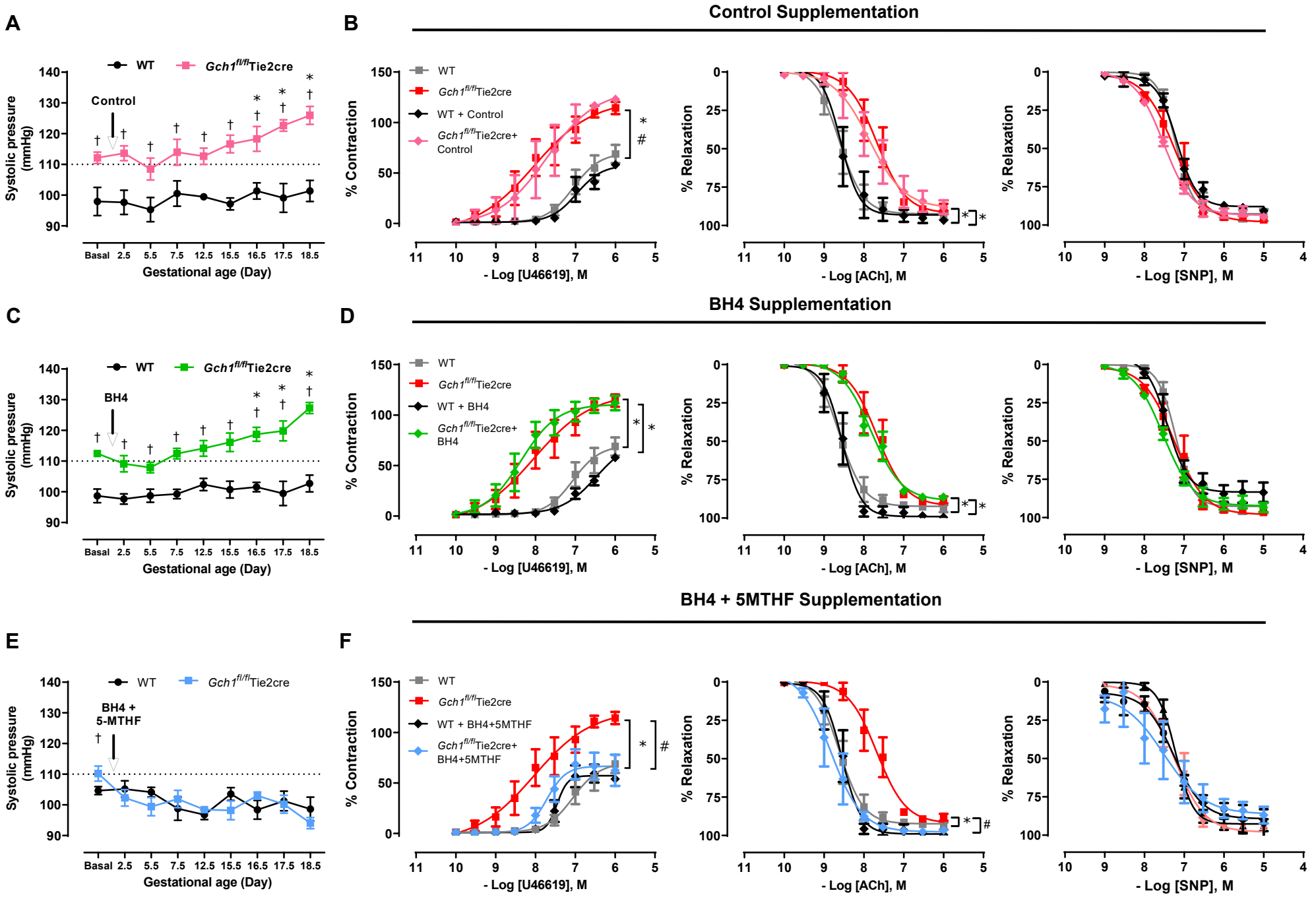

A
WT
Gch1fl/flTie2cre
% Relaxation
0
25
50
75
100
10
9
8
7
6
- Log [ACh], M
Control
L-N+Indo
L-N+Indo+AP
L-N+Indo+AP+ChTx
BKca+IKca
SKca
NOS
+
PGI2
B
Apamin
ChTx
% Contribution of vasodilators
100
80
60
40
20
0
*
*
WT
Gch1fl/fl
Tie2cre

Figure 5


A
B
Control Supplementation
WT
Gch1fl/flTie2cre
Control
Systolic pressure (mmHg)
Gestational age (Day)
Basal 2.5 5.5 7.5 12.5 15.5 16.5 17.5 18.5
WT + Control
Gch1fl/flTie2cre+ Control
% Contraction
- Log [U46619], M
% Relaxation
- Log [ACh], M
- Log [SNP], M
C
D
BH4 Supplementation
BH4
WT + BH4
Gch1fl/flTie2cre+ BH4
E
F
BH4 + 5-MTHF Supplementation
WT + BH4+5MTHF
Gch1fl/flTie2cre+ BH4+5MTHF