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Progesterone supplementation attenuates hypertension and the autoantibody to the angiotensin II type I receptor in response to elevated interleukin-6 during pregnancy

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Abstract

OBJECTIVE—Preeclampsia is a multisystem disorder recognized as hypertension with proteinuria developing >20 weeks' gestation. Preeclampsia is associated with chronic immune activation characterized by increased T and B lymphocytes, cytokines, and antibodies activating the angiotensin II type I receptor (AT1-AA). Hypertension in response to elevated interleukin (IL)-6 during pregnancy occurs with increased renin activity and AT1-AA, and reduced kidney function.

STUDY DESIGN—We aim to determine whether 17-alpha-hydroxyprogesterone caproate (17-OHPC), progesterone, improved inflammatory pathways during elevated IL-6 in pregnant rats. IL-6 (5 ng/d) was infused via miniosmotic pumps into normal pregnant (NP) rats beginning on day 14 of gestation and 17-OHPC (3.32 mg/kg) was diluted in normal saline and injected on day 18. Blood pressure (mean arterial pressure [MAP]) determination and serum collection were performed on day 19 of gestation.

RESULTS—MAP in NP was 100 ± 3 mm Hg, which increased with IL-6 to 112 ± 4 mm Hg ($P < .05$). Pregnant rats given 17-OHPC alone had a MAP of 99 ± 3 mm Hg and MAP increased to 103 ± 2 mm Hg in IL-6OHPC. AT1-AA was 1.2 ± 0.5 bpm in NP rats, increased to 17 ± 9 bpm with IL-6 infusion but administration of 17-OHPC significantly blunted AT1-AA to 4 ± 0.8 bpm in NP6OHPC. Total circulating nitrate/nitrite was significantly decreased and placental Ser¹¹⁷⁷-phosphorylated-eNOS/eNOS was lowered with IL-6 infusion. Supplementation of 17-OHPC

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significantly improved placental Ser¹¹⁷⁷-phosphorylated-eNOS/eNOS however, circulating nitrate/nitrite was unchanged with 17-OHPC supplementation.

CONCLUSION—This study illustrates that 17-OHPC attenuated hypertension, decreased AT1-AA activity, and improved placental nitric oxide in response to elevated IL-6 during pregnancy and could lend hope to a new potential therapeutic for preeclampsia.

Keywords

cytokines; hypertension; inflammation; nitric oxide; pregnancy; progesterone; renin angiotensin system

Preeclampsia is an important complication of pregnancy and is defined as new-onset hypertension usually with proteinuria or other evidence of maternal organ dysfunction that develops >20 weeks' gestation.¹ While preeclampsia and other hypertensive disorders of pregnancy are a leading cause of maternal and neonatal morbidity and mortality, a complete understanding of the mechanisms responsible for disease pathogenesis remains elusive.²⁻⁴ Furthermore, improvements in treatment strategies are dampened by the lack of knowledge of the underlying cause of this disease.

Many investigators have proposed preeclampsia as a disease of immunological origin. These women exhibit a chronic innate inflammatory response and have increased circulating and placental levels of tumor necrosis factor (TNF) alpha and interleukin (IL)-6, and endothelial activation and dysfunction exhibiting high levels of endothelin (ET)-1, intracellular and vascular adhesion molecules, IL-8, and monocyte chemotactic protein.⁴⁻⁷ Furthermore, women with preeclampsia display marked levels of autoantibodies that activate the angiotensin II type 1 receptor (AT1-A).⁸⁻¹⁰ We reported that chronic infusion of rat AT1-AA into pregnant rats from day 12–19 of gestation activates many systems, such as ET-1, reactive oxygen species, sFlt-1, and endoglin during pregnancy, all of which are suspected of playing an important role in the pathogenesis of preeclampsia.¹¹⁻¹³ Moreover, it is expected that vasodilators, such as nitric oxide (NO), are decreased and thus may have an important role in this process as well. Mechanisms involving altered formation of NO have been implicated in preeclampsia.¹⁴⁻¹⁷

Research efforts throughout the world have focused on effective low-cost alternative therapies for the treatment of preeclampsia that may help to prolong the pregnancy and avoid early delivery of the fetus and placenta. Seventeen-alpha-hydroxyprogesterone caproate (17-OHPC) is a synthetic metabolite of progesterone used effectively for the prevention of recurrent preterm birth in singleton pregnancies.¹⁸⁻²⁰ Furthermore, 17-OHPC is suspected to have vasodilatory and antiinflammatory effects, both of which could prove beneficial during preeclampsia. We have shown circulating progesterone to be decreased in preeclamptic patients.²¹ Interestingly, progesterone supplementation of cell culture media decreased ET-1 secretion from vascular endothelial cells activated by serum from preeclamptic patients.²¹ Previously, we identified a potential role for 17-OHPC in vivo. The 17-OHPC acted like an antiinflammatory substance that decreased hypertension, inflammatory cytokines (TNF alpha and IL-6), and ET-1 in response to placental ischemia in the reduced uterine perfusion pressure (RUPP) rat model of preeclampsia.^{21,22} Although

certainly not the only culprit, production of AT1-AA during pregnancy is a common pathway mediating hypertension in RUPP rats as well as in response to elevated cytokines, TNF alpha, IL-6, or IL-17.²³ Therefore, in the present study, we examined a role for 17-OHPC supplementation to decrease AT1-AA and blood pressure during IL-6-induced hypertension in pregnant rats. While the exact mechanism whereby 17-OHPC may decrease blood pressure is unclear, it has been suggested that progesterone can improve NO availability. Progesterone affects function of eNOS by both genomic and nongenomic mechanisms, involvement of PI3K/Akt leading to eNOS activation through phosphorylation of eNOS at serine 1177 (Ser¹¹⁷⁷-P-eNOS), resulting in increased enzyme activity.^{24–27} An up-regulation of eNOS, resulting in increased NO production has been shown to contribute to increases in uteroplacental blood flow via changes in vascular tone.²⁸ For these reasons circulating nitrate/nitrite and placental expression of eNOS and ser177 phosphorylated eNOS was evaluated during IL-6-induced hypertension during pregnancy with and without 17-OHPC supplementation.

Materials and Methods

All studies were performed in age-matched, timed pregnant Sprague Dawley rats purchased from Harlan Sprague Dawley Inc (Indianapolis, IN). Animals were housed in a temperature-controlled room (23°C) with a 12:12 hour light/dark cycle. All experimental procedures executed in this study were in accordance with National Institutes of Health guidelines for use and care of animals and the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center approved all protocols.

Experimental design

Effect of progesterone on mean arterial pressure in response to IL-6 in pregnant rats

This experimental protocol was performed to determine whether progesterone supplementation blunts hypertension in response to elevated IL-6 during pregnancy. Experiments were performed in 4 groups of pregnant rats: normal pregnant (NP) (n = 5); NPOHPC (n = 6); NP6 (n = 10); NP6OHPC (n = 10). IL-6 (5 ng/d) (Recombinant Rat IL-6; R&D Systems, Minneapolis, MN) was infused via miniosmotic pumps (model 2002; Alzet Scientific Corp, Palo Alto, CA) for 5 days into NP rats during days 14–19 of gestation. The 17-OHPC (Marty's Compounding Pharmacy, Jackson, MS) was diluted in normal saline and administered intraperitoneally as 0.5-cm³ solution of 3.32 mg/kg 17-OHPC to pregnant rats. We chose the 1-time 17-OHPC dose to be the weight equivalent of a typical human dose for the prevention of preterm labor and what was previously shown to be effective in RUPP rats. Carotid catheters were inserted on day 18 of gestation and mean arterial pressure (MAP) was determined on day 19 as described previously.²⁹

Effect of progesterone on AT1-AA in pregnant rats

This experimental protocol was performed to determine the role of progesterone to lower activity of AT1-AA in response to IL-6 in pregnant rats. Maternal serum was analyzed for AT1-AA by cardiomyocyte assay. Antibodies were detected by the chronotropic responses to AT1 receptor-mediated stimulation of cultured neonatal rat cardiomyocytes coupled with

receptor-specific antagonists as previously described.^{11,12} Chronotropic responses were measured and expressed in beats per minute (bpm).

Effect of progesterone on placental eNOS and Ser¹¹⁷⁷-P-eNOS

This experimental protocol was performed to determine whether the improvements seen due to progesterone administration are due to an increase in eNOS. Briefly, placental extracts were homogenized in cold radioimmunoprecipitation assay buffer. A total of 100 µg of protein extracts were separated by SDS-PAGE using a polyacrylamide gel (4–20%). The proteins were transferred onto nitrocellulose membranes (BioRad, Hercules, CA). Different membranes were blocked with Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature and incubated overnight at 4°C with primary antibody directed against eNOS (1:250; BD Transduction Laboratories, San Jose, CA) and Ser¹¹⁷⁷-P-eNOS (1:250; BD Transduction Laboratories), respectively. Then the membranes were incubated with secondary antibody (IRDye700-conjugated affinity-purified antimouse IgG, 1:5000; Rockland, Gilbertsville, PA) and scanned using Odyssey Infrared Imaging System (LI-COR Biosciences). The intensity of specific bands was quantified by densitometry using Image J (National Institutes of Health, Washington, DC) and placental eNOS and eNOS phosphorylated expression were normalized with respect to β-actin expression, respectively (1:5000, Cambridge).

Effect of progesterone on circulating nitrate/nitrite bioavailability

A total of 40 µL maternal plasma was used to measure circulating total nitrate/nitrite evaluated by Nitrate/Nitrite Colorimetric Assay Kit from Cayman Chemical (Ann Arbor, MI) following instructions outlined by the manufacturer. The interassay coefficient of variation is 3.4% while intraassay coefficient of variation is 2.7%.

Statistical analysis

All data are expressed as mean ± SEM. Comparisons between groups were assessed by 2-way analysis of variance. A Student *t* test was used for comparison of circulating nitrate/nitrite and AT1-AA among NP and IL-6-infused groups. A value of *P* < .05 was considered statistically significant.

Results

Administration of 17-OHPC blunted hypertension in response to elevated IL-6 during pregnancy

As in previous studies with RUPP or TNF alpha–infused pregnant rats,²⁸ blood pressure in response to elevated IL-6 during pregnancy was significantly decreased with 17-OHPC administration to IL-6-treated pregnant rats. MAP in NP was 100 ± 3 mm Hg, which increased with IL-6 to 112 ± 4 mm Hg (*P* < .05). Pregnant rats given 17-OHPC alone had a MAP of 99 ± 3 mm Hg and MAP increased to 103 ± 2 mm Hg in IL-6OHPC (*P* < .05) (Figure 1).

Administration of 17-OHPC decreased activity of AT1-AA in response to elevated IL-6 during pregnancy

We have recently shown that AT1-AA is a mediator of hypertension when IL-6 is elevated during pregnancy.^{11,13} To determine the role of progesterone in decreasing the activity of AT1-AA in response to IL-6 in pregnant rats we analyzed AT1-AA in the presence and absence of 17-OHPC. Surprisingly, 17-OHPC significantly decreased autoantibody production in IL-6-treated rats. Activity of AT1-AA was 1.2 ± 0.5 bpm in NP rats and increased to 17 ± 9 bpm with IL-6 infusion. Administration of 17-OHPC significantly blunted AT1-AA activity to 4 ± 0.8 bpm in NP6OHPC ($P < .05$) (Figure 2).

Administration of 17-OHPC increased placental ratio of eNOS but not circulating nitrate/nitrite in response to elevated IL-6 during pregnancy

To determine whether progesterone should be improving placental endothelial NO (eNOS) in response to IL-6 in pregnant rats, we analyzed endothelial NO synthase (eNOS) and endothelial NO synthase phosphorylated at serine 1177 (Ser¹¹⁷⁷-P-eNOS) expression in protein isolated from the placentas collected on day 19 of gestation. There were no significant differences in placental levels of eNOS protein between all groups ($P > .05$) (Figure 3). However, placental levels of Ser¹¹⁷⁷-P-eNOS protein in NP was 0.46 ± 0.06 arbitrary units (AU), which decreased with IL-6 to 0.20 ± 0.04 AU ($P < .05$) (Figure 3). Considering Ser¹¹⁷⁷-P-eNOS/eNOS as an index of eNOS activation status, we analyzed whether progesterone treatment changes this parameter. Ser¹¹⁷⁷-P-eNOS/eNOS ratio in NP6 was 0.47 ± 0.10 AU, and 17-OHPC treatment significantly increased Ser¹¹⁷⁷-P-eNOS/eNOS ratio to 1.68 ± 0.35 ($P < .05$) (Figure 3). Total nitrate/nitrite bioavailability was lowered significantly by IL-6-induced hypertension. In NP, nitrate/nitrite was 21 ± 1.4 $\mu\text{mol/L}$ and was 13 ± 3 with IL-6-induced hypertension ($P < .04$). Supplementation of 17-OHPC during IL-6-induced hypertension increased nitrate/nitrite to only 14 ± 2.5 $\mu\text{mol/L}$, which was not different from IL-6-induced hypertensive pregnant rats.

Comment

Preeclampsia is a complication of pregnancy and multiple hypotheses have been proposed to elucidate its pathogenesis. These include abnormal cytotrophoblast invasion resulting in inadequate remodeling and narrow uterine spiral arteries, which in turn could cause increased resistance or less blood volume and oxygen delivery to the growing uteroplacental unit compared to a normal placenta in which greater vascular remodeling has occurred.^{1,4,6} Alterations in the renin-angiotensin system play an important role in the development of hypertension and in recent years researchers have investigated the role AT1-AA plays in preeclampsia. Severity of preeclampsia has been strongly correlated with the levels of AT1-AA³⁰ and we and others have shown that infusion of AT1-AA into pregnant rats stimulates hypertension, ET-1, antiangiogenic factors, and inflammatory cytokine pathways during pregnancy.¹¹⁻¹³ We recently demonstrated that IL-6 promotes hypertension during pregnancy, decreases renal pressure natriuresis, stimulates plasma renin activity, and increases AT1-AA in pregnant rats while having no effect in virgin rats.^{28,29}

Our current findings support previous conclusions that the hypertension associated with IL-6 infusion in pregnant rats results in production of the AT1-AA.²⁸ In addition, we demonstrate that IL-6-induced hypertension reduced maternal circulating nitrate/nitrite (Figure 4) and placental eNOS availability (Figure 3). Administration of 17-OHPC improved placental bioavailability, however, 17-OHPC did not improve total circulating nitrate/nitrite. In addition, another important finding from this study is that 17-OHPC blunted hypertension and significantly decreased circulating AT1-AA in response to elevated IL-6 during pregnancy. These data correlated with improved eNOS bioavailability within the placental unit thereby indicating multiple mechanisms were improved during IL-6-induced hypertension when 17-OHPC is administered. The decrease in AT1-AA with 17-OHPC would be an important mechanism whereby blood pressure is improved in response to elevated cytokines or placental ischemia during pregnancy.

Although administration of 17-OHPC has routinely been used for prevention of preterm birth, the exact mechanism has yet to be completely understood. We illustrated that 17-OHPC significantly decreased circulating maternal AT1-AA bioactivity in response to elevated IL-6 during pregnancy. In addition, IL-6 is known to be a major regulator of B-cell proliferation and T-cell signaling processes. Therefore, future studies are designed to examine a role for progesterone to suppress T-cell–B-cell populations and/or communication as a mechanism to improve pregnancy outcomes in response to placental ischemia or elevated cytokines.

NO derived from eNOS is an important regulator of vasodilatation and dysfunction of endothelium-derived NO has been implicated as a potential factor in the development of preeclampsia.^{17,31} In addition to decreased AT1-AA, 17-OHPC seems to improve placental NO availability, however, had no impact to improve total circulating nitrate/nitrite in the mother. While previous studies have implicated endothelial dysfunction and generalized vasoconstriction to be involved in preeclampsia pathogenesis, this is the first study to show IL-6 to decrease nitrate/nitrite and placental levels of Ser¹¹⁷⁷-P-eNOS protein. Furthermore, this is the first study to show that 17-OHPC supplementation could increase Ser¹¹⁷⁷-P-eNOS/eNOS ratio and inhibit AT1-AA activity in NP6OHPC rats. These results suggest a significant contribution of progesterone supplementation to decrease MAP in this animal model through decreasing AT1-AA, which was also associated with modulation of the placental NO synthase (eNOS) pathway.

Previous studies have examined that progesterone has protective effects on the cardiovascular system that are independent of those in combination with estrogen.³² Indeed, circulating progesterone is lower in preeclamptic women when compared with NP.²¹ Other investigators demonstrate that in rat aorta, progesterone treatment causes an acute increase in the activity of eNOS.³³ Moreover, in porcine and ovine arteries progesterone treatment is associated with NO production and vasodilation.³⁴ In line with this previous study, we found increased placental Ser¹¹⁷⁷-P-eNOS/ eNOS ratio with progesterone supplementation in IL-6-induced hypertensive model of preeclampsia, however, found no improvement of total circulating nitrate/nitrite.

In conclusion, 17-OHPC supplementation decreased blood pressure and activity of AT1-AA, which was associated with improved placental endothelial Ser¹¹⁷⁷-P-eNOS/eNOS ratio in response to elevated IL-6 during pregnancy. Thus, our findings may have important clinical implications because they suggest that progesterone supplementation may be a relevant strategy to add to the management of this critical condition, especially preterm preeclampsia or superimposed preeclampsia associated with severe features of the syndrome.

In a previous study, we provided evidence that preeclampsia is a state of progesterone deficiency and we demonstrated the efficacy of 17-OHPC to blunt hypertensive actions in response to placental ischemia, without further reducing pup weight and litter size, or inducing fetal malformations.²⁷ We have previously demonstrated that 17-OHPC blunts inflammatory cytokine secretion, hypertension, and renal ET-1 in a pregnant rat model of preeclampsia.²¹ Furthermore 17-OHPC attenuated hypertension in chronic TNF alpha-treated pregnant rats and progesterone had a significant impact to reduce TNF alpha-stimulated ET-1 in human umbilical vein endothelial cells.²¹ Previous investigators show that progesterone treatment increases production of NO and eNOS activity in human endothelial cells.³⁵ In our current study we demonstrate that progesterone increased placental Ser¹¹⁷⁷-P-eNOS/eNOS ratio and decreased AT1-AA and improved hypertension in the IL-6-induced hypertensive pregnant rat. Collectively, these data suggest an important role for progesterone to blunt inflammatory responses that lead to many pathological changes observed in preeclampsia and suggest that 17-OHPC could be a potential treatment of hypertension associated with elevated cytokines during pregnancy. Importantly, although 17-OHPC is safely administered to prevent preterm labor in patients, human studies evaluating the safety of 17-OHPC administration on maternal and fetal outcome during preeclampsia are necessary to better ascertain the safety of progesterone to manage this disease. Data from this study emphasize the need to evaluate the use of progesterone and/or 17-OHPC in a larger trial to determine the efficacy of this treatment as a possible therapy for primary or superimposed preeclampsia.

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FIGURE 1. Supplementation of 17-OHPC blunts hypertension in response to elevated IL-6 during pregnancy

Data are shown as means $\pm$ SEM (n = 6–10/group).

IL, interleukin; *MAP*, mean arterial pressure; *17-OHPC*, 17-alpha-hydroxyprogesterone caproate.

* $P < .05$ vs normal pregnant (NP) group; # $P < .05$ vs NP6 group.

Amaral. Progesterone effects on IL-6-induced hypertension. *Am J Obstet Gynecol* 2014.

FIGURE 2. Supplementation of 17-OHPC decreased activity of AT1-AA in response to elevated IL-6 during pregnancy

Data are shown as means $\pm$ SEM (n = 4–6/group).

IL, interleukin; *17-OHPC*, 17-alpha-hydroxyprogesterone caproate.

* $P < .05$ vs normal pregnant (NP) group; # $P < .05$ vs NP6 group.

Amaral. Progesterone effects on IL-6-induced hypertension. *Am J Obstet Gynecol* 2014.

FIGURE 3. Placental protein expression of eNOS and Ser¹¹⁷⁷-P-eNOS in IL-6-induced hypertension rats

NOS protein expression was determined by Western blot analysis as described in “Materials and Methods” section. **A**, Representative Western blots showing eNOS and Ser¹¹⁷⁷-P-eNOS expression in the placentas from rats. **B**, Bar graph showing the densitometric data. β-Actin content was used for normalization to both gels. Data are shown as means ± SEM (n = 4–6/group). Arrow indicates the band of interest.

IL, interleukin; OHPC, alpha-hydroxyprogesterone caproate; Ser¹¹⁷⁷-P-eNOS, phosphorylated.

**P* < .05 vs normal pregnant (NP) group. #*P* < .05 vs NP6 group.

Amaral. Progesterone effects on IL-6-induced hypertension. Am J Obstet Gynecol 2014.

FIGURE 4. Total circulating nitrate/nitrite is reduced in IL-6-induced hypertension

Nitrate/nitrite was determined via enzyme-linked immunosorbent assay and data are shown as $\pm$ SEM ($n = 4-6$ /group). There was no difference when 17-alpha-hydroxyprogesterone caproate (OHPC) was administered to pregnant rats.

IL, interleukin.

* $P < .05$ vs normal pregnant (NP) group.

Amaral. Progesterone effects on IL-6-induced hypertension. *Am J Obstet Gynecol* 2014.