CALL FOR PAPERS | Integrative and Translational Physiology: Inflammation and Immunity in Organ System Physiology

IL-17-mediated oxidative stress is an important stimulator of AT1-AA and hypertension during pregnancy

Pushpinder Dhillion,1 Kidra Wallace,1 Florian Herse,2 Jeremy Scott,1 Gerd Wallukat,2 Judith Heath,1 Janae Mosely,1 James N. Martin Jr.,1 Ralf Dechend,2 and Babbette LaMarca1

1Department of Obstetrics and Gynecology, Division of Maternal Fetal Medicine, Center for Excellence in Cardiovascular-Renal Research, University of Mississippi Medical Center, Jackson, Mississippi; and 2HELIOS Clinic, Experimental and Clinical Research Center, Max-Delbrueck Center, Berlin Germany

Submitted 15 February 2012; accepted in final form 17 June 2012

Dhillion P, Wallace K, Herse F, Scott J, Wallukat G, Heath J, Mosely J, Martin JN, Jr., Dechend R, LaMarca B. IL-17-mediated oxidative stress is an important stimulator of AT1-AA and hypertension during pregnancy. Am J Physiol Regul Integr Comp Physiol 303: R353–R358, 2012.—Preeclampsia is a condition associated with autoantibodies activating the angiotensin II type I receptor AT1-AA, and placental oxidative stress ROS). The objective of this study was to determine whether chronic IL-17 increases blood pressure by stimulating ROS and AT1-AAs during pregnancy. To answer this question four groups of rats were examined: normal pregnant (NP, n = 20), NP+IL-17 (n = 12), NP+tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) (n = 7) (a superoxide dismutase mimetic that scavenges ROS), and NP+IL-17+tempol (n = 11). IL-17 (150 pg/day) was infused into NP rats while tempol was administered via the drinking water ad libitum. On day 19 blood pressure (MAP) was recorded, and plasma, urine, and tissue were collected for isolation of ROS detected by chemiluminescent technique. Urinary isoprostane was measured by ELISA. AT1-AAs were determined via cardionomyocyte assay and expressed as beats per minute. MAP increased from 98 ± 3 mmHg in NP to 123 ± 3 mmHg in IL-17-infused NP rats. Urinary isoprostane increased from 1,029 ± 5 pg·mg−1·day−1 in IL-17-infused rats (P < 0.05). Placental ROS was 436 ± 4 RLU·ml−1·min−1 in NP and 702 ± 5 (n = 5) RLU·ml−1·min−1 in IL-17-treated rats. Importantly, AT1-AA increased from 0.41 ± 0.05 beats/min in NP rats (n = 8) to 18.4 ± 1 beats/min in IL-17 rats (n = 12). Administration of tempol attenuated the hypertension (101 ± 0.05 mmHg) ROS (459 ± 5 RLU·ml−1·min−1) and blunted AT1-AAs (7.3 ± 0.6 beats/min) in NP+IL-17+tempol-treated rats. Additionally, AT1 receptor blockade inhibited IL-17-induced hypertension and placental oxidative stress. MAP was 105 ± 5 mmHg and ROS was 418 ± 5 RLU·ml−1·min−1 in NP+IL-17 treated with losartan. These data indicate that IL-17 causes placental oxidative stress, which serves as stimulus modulating AT1-AAs that may play an important role in mediating IL-17-induced hypertension during pregnancy.

autoimmunity; preeclampsia; reduced uterine perfusion pressure

PREECLAMPSIA is one of the major causes of maternal and perinatal mortality and morbidity, and yet, the pathophysiology of this disease remains incompletely understood (5, 14). The initiating event of the disease is associated with abnormal cytotrophoblast invasion resulting in inadequate remodeling of the uterine spiral arteries leading to significantly reduced uterine perfusion pressure (RUPP) of the uteroplacental unit (5, 6). This cytotrophoblast invasion is tightly regulated between various immune cell types, such as monococytes, macrophages, uterine natural killer cells, T lymphocytes, and endothelial cells (5, 6, 8, 14). As preeclampsia develops, a dysregulation among these immune cell types begins early in implantation, thus lending to the overtactivation of a chronic inflammatory immune response (8). As a result the placenta becomes ischemic, releasing factors thought to contribute to the development of hypertension and other agents of disease expression such as proinflammatory cytokines, tumor necrosis factor (TNF)-α, and interleukin 6 (IL-6) being released into the maternal peripheral circulation (5, 6, 8, 14). Additionally, we have learned recently of autoimmune activities during preeclampsia such as the production of autoantibodies to the angiotensin II type I receptor (6, 8) and elevated circulating IL-17 and T_H17 cells (16).

One important physiological function of IL-17 producing T_H17 cells is to recruit leukocytes such as neutrophils to the site of infection as a major host defense mechanism against extracellular bacteria. At the site of recruitment, IL-17 stimulates a myriad of other cytokines such as TNF-α and IL-6, which are important for intercellular communication. Importantly, IL-17 also induces neutrophil release of antimicrobial substances and/or phagocytosis of microbes and dead tissues. Macrophages and neutrophils convert molecular oxygen into reactive oxygen species (ROS) by the phagocyte oxidase system catalyzed by the enzyme NADPH oxidase. Once neutrophils are activated, they can cause injury to normal host tissues, such as the placental unit, by the release of lysosomal enzymes, ROS, or nitric oxide. Many studies have indicated that preeclamptic women have elevated oxidative stress within the placental unit measured by increased NADPH subunits as well as elevated urinary 8-isoprostanes as a measure of whole body oxidative stress.

In accord with exacerbated immune function during preeclampsia, recent evidence implicates an imbalance between regulatory (Treg) and effector T cells, subclasses of CD4+ T lymphocytes, in preeclamptic women (15, 16). There is con-
sclerosis. In contrast, Tregs (foxp3) have anti-inflammatory properties and can cause quiescence of immune activation (4, 16). In agreement with recent clinical studies performed in preeclamptic versus normal pregnant patients, we have demonstrated abnormal ratios of circulating Tregs (foxp3+CD4+Tcells) have anti-inflammatory properties and can cause quiescence of immune activation (4, 16). In agreement with recent clinical studies performed in preeclamptic versus normal pregnant patients, we have demonstrated abnormal ratios of circulating Tregs (foxp3+CD4+Tcells)/T17 (ROR γ+CD4+Tcells) occurring in response to RUPP in the pregnant rat. In addition, we have shown that adoptive transfer of total CD4+ T cells from RUPP control rats into normal pregnant (NP) recipient rats causes hypertension associated with elevated IL-17 and AT1-AA. Therefore, the objective of this study was to determine whether chronic IL-17 during pregnancy leads to increased blood pressure, placental oxidative stress, and autoantibody production (AT1-AA) as mediators of the pathogenicity of preclampsia.

MATERIALS AND METHODS

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

Protocol 1a: Effect of IL-17 Infusion on MAP in Pregnant Rats

Recombinant mouse IL-17 (150 pg/day) (RnD Systems, Minneapolis, MN) was infused intraperitoneally from day 14 to day 19 of gestation via mini-osmotic pumps (model 2002, Alzet Scientific) into NP rats. IL-17 (150 pg/day) was also infused into virgin rats via mini-osmotic pumps for 5 days.

Measurement of mean arterial pressure in chronically instrumented conscious rats. Under isoflurane anesthesia on day 18 of gestation or the fifth day of IL-17 infusion for virgin rats, carotid arterial catheters were inserted for blood pressure measurements. The catheters inserted are V3 tubing (SCI), which is tunneled to the back of the neck and exteriorized. On day 19 of gestation mean arterial blood pressure (MAP) was analyzed after placing the rats in individual restraining cages. MAP was monitored with a pressure transducer (Cobe III Transducer CDX Sema) and recorded continuously after a 1-h stabilization period. Subsequently, a blood and urine sample was collected, kidneys and placentas were harvested, and litter size and pup weights were recorded under anesthesia (9, 12).

Determination of circulating T lymphocytes. Circulating CD4+ T cell populations were measured from peripheral blood leukocytes (PBL) collected at day 19 of gestation from NP rats and from pregnant IL-17-infused rats. We utilized flow cytometry analysis to detect specific CD4+ T cell populations; CD4+ RORγ+ (retinoic acid receptor-related organ receptor gamma) isolated from chronic IL-17-treated and NP rats PBLs. At the time of tissue harvest, plasma was collected and PBLs were isolated from plasma by centrifugation on a cushion of Ficoll-Hypaque (Lymphoprep, Accurate Chemical) according to the manufacturer’s directions. For flow cytometric analysis equal numbers of leukocytes (1x10^6) were incubated for 30 min at 4°C with antibodies against mouse CD4 (BD Biosciences, San Jose, CA). After washing was completed, cells were labeled with the secondary fluoroscein isothiocyanate (FITC) antibody (Southern Biotech, Birmingham, AL) for 30 min at 4°C. Cells were washed and permeabilized and stained with anti-rat RORγ conjugated to PE (BD Pharmingen) for 30 min at 4°C. As a negative control, for each individual rat, cells were treated exactly as described above except they were incubated with anti-FITC and anti-PE secondary antibodies alone. Subsequently, cells were washed and resuspended in 500 μl of Roswell Park Memorial Institute medium (RPMI) and analyzed for single and double staining on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The percentage of positive staining cells above the negative control was collected for each individual rat and mean values for each experimental group (NP and NP+IL-17) was calculated.

Determination of IL-6. An important function of IL-17 is to induce cytokines such as IL-6, which would induce expansion of the T17 and B lymphocytes; therefore, we utilized the rat IL-6 Quantikine ELISA. The assay displayed a sensitivity of 21 pg/ml, intra-assay variability is 7.4%, and interassay variability is 8.4%.

Determination of urinary isoprostane. On day 19 of gestation, urine was collected and utilized for determination of excreted isoprostanes measured via ELISA from Oxford Biomedical Research (Oxford, MI). The assay displayed a sensitivity of 0.05 ng/ml, inter-assay variability of 4.2%, and intra-assay variability of 4.7%.

Determination of tissue ROS. Superoxide production in the placenta was measured by using the lucigenin technique as we have recently described (10, 13). Rat placentas were snap frozen in liquid nitrogen directly after collection and stored at −80°C until further processing. Placentas were removed and homogenized in RIPA buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail; Santa Cruz, Santa Cruz, CA) as described previously (10, 13). The samples were centrifuged at 12,000 for 20 min, the supernatant aspirated, and the remaining cellular debris was discarded. The supernatant was incubated with lucigenin at a final concentration of 5 µmoll. The samples were allowed to equilibrate for 15 min in the dark, and luminescence was measured every second for 10 s with a luminometer (Berthold, Oak Ridge, TN). Luminescence was recorded as relative light units (RLU) per minute. An assay blank with no homogenate but containing lucigenin was subtracted from the reading before transformation of the data. Each sample was repeated five times and the average used for data transformation. The protein concentration was measured using a protein assay with BSA standards (Pierce, Rockford, IL; expressed as RLU·min·1·mg protein−1).

Determination of circulating AT1-AA. On day 18 of gestation blood was collected and immunoglobulin was isolated from 1 ml of serum by specific anti-rat IgG column purification. AT1-AA was purified from rat IgG by epitope binding to the amino acid sequence corresponding to the second extracellular loop of the AT1 receptor covalently linked to Sepharose 4B CNBr-activated gel. Unbound IgG was washed away and bound IgG was eluted with 3 M potassium thiocyanate. AT1-AA activity was measured utilizing a bioassay that evaluates the beats per minute (bpm) of neonatal cardiomyocytes in culture (2, 3, 9–12). AT1-As were assessed in NP controls and NP+IL-17-infused rats. Since AT1-AA is not present in nonpregnant rats, it was not determined in this study.

Protocol 2: Effect of Tempol on IL-17-Induced Hypertension

This protocol was performed to determine whether the effect of placental and cortical oxidative stress was generated in response to chronic IL-17 to increase blood pressure by administering a superoxide dismutase mimetic (tempol) to NP and IL-17-infused rats. NP rats treated with tempol added to the drinking water were used as controls, whereas IL-17-infused rats treated with tempol served as the experimental group. On day 14 of gestation, tempol (30 mg·kg−1·day−1) was administered in the drinking water. Mini-osmotic pumps (Alzet 2002) infusing 150 pg/day of IL-17 were surgically implanted intraperitoneally on day 14 into 12 pregnant rats. Arterial pressure was...
determined in all groups of pregnant rats at day 19 of gestation as previously described in protocol 1a.

Protocol 3: Effect of AT1 Receptor Blockade on IL-17-Induced Hypertension

This protocol was performed to determine whether activation of the AT1 receptor response to chronic IL-17 caused an increase blood pressure and placental ROS. In this protocol losartan (5 mg·kg⁻¹·day⁻¹) was administered to NP and IL-17-infused rats via the drinking water beginning on day 14 of gestation. Mini-osmotic pumps (Alzet 2002) infusing 150 pg/day of IL-17 were surgically implanted intraperitoneally on day 14 into five pregnant rats; losartan-treated NP served as controls (n = 4). Arterial pressure was determined in all groups of pregnant rats at day 19 of gestation as previously described in protocol 1a.

Protocol 4: B Cell Depletion Attenuates IL-17-Induced Increases in Blood Pressure

NP rats were treated with Rituximab (250 mg/kg) from day 14 to day 19 of gestation (n = 7) via osmotic minipump insertion. Rituximab is a chimeric monoclonal anti-CD20 antibody that is used to induce B cell depletion in vivo (10, 17). The dose used was based on the efficacy demonstrated in previous studies from our lab illustrating that administration of Rituximab depleted circulating B cells and suppressed AT1-AA in RUPP rats and RUPPCD4+ T cell-induced hypertensive pregnant rats. Arterial pressure and circulating TH17 cells were determined in pregnant rats at day 19 of gestation as described in protocol 1.

Statistical Analysis

All data are expressed as means ± SE. ANOVA was used to determine differences among blood pressure and TH17 cells between the groups of the multiple experimental groups. Difference between control and experimental tissues were analyzed using the t-test and was considered statistically significant at P values < 0.05. NP was compared with IL-17 infused, whereas tempol-treated NP was compared with tempol-treated IL-17-infused rats. Losartan-treated NP were compared with losartan-treated IL-17-infused pregnant rats.
IL-6 increased significantly in response to IL-17 infusion and was $323 \pm 83$ pg/ml in IL-17-infused rats ($n = 5$) and was $89 \pm 13$ pg/ml in NP rats ($P < 0.005$) ($n = 8$).

Protocol 2: Tempol Attenuates Placental Oxidative Stress, AT1-AA, and Hypertension During Pregnancy

Effect of tempol on placental oxidative stress. Administration of tempol decreased NADPH-stimulated placental production of ROS from IL-17-infused pregnant rats ($459 \pm 5$ RLU·min$^{-1}$·mg$^{-1}$) to levels no longer statistically different compared with placental ROS from tempol-treated NP controls ($474 \pm 5$ RLU·min$^{-1}$·mg$^{-1}$; $n = 3$) (Fig. 4B; $P = 0.096$). In addition, tempol administration significantly decreased circulating AT1-AAs produced in response to IL-17 to 7.3 ± 0.8 pmol/l ($n = 3$) (Fig. 4B; $P = 0.006$). Importantly, the blood pressure increase to chronic IL-17 was completely attenuated with tempol administration and was no different in tempol-treated IL-17-infused rats compared with tempol-treated control rats. MAP was $102 \pm 5$ mmHg ($n = 7$) in tempol control NP rats versus $101 \pm 2$ mmHg ($n = 7$) in IL-17-tempol-treated pregnant rats (Fig. 1).

Protocol 3: AT1 Receptor Blockade Attenuates IL-17-Induced Increases in Blood Pressure and Placental Oxidative Stress

Importantly, losartan administration attenuated the blood pressure increase to chronic IL-17; MAP was no different in losartan-treated IL-17-infused rats compared with losartan-treated control rats. MAP was $98 \pm 5$ mmHg ($n = 4$) in losartan control NP rats versus $105 \pm 2$ mmHg ($n = 5$) in IL-17 losartan-treated pregnant rats (Fig. 1). Furthermore, administration of losartan decreased both basal and NADPH-stimulated placental production of ROS from IL-17-infused pregnant rats ($185 \pm 38$ RLU·min$^{-1}$·mg$^{-1}$; $n = 3$, basal) to levels no longer statistically different compared with basal placental ROS from losartan-treated NP controls ($243 \pm 36$ RLU·min$^{-1}$·mg$^{-1}$; $n = 4$). NADPH-stimulated placental production of ROS from IL-17-infused pregnant rats ($418 \pm 5$ RLU·min$^{-1}$·mg$^{-1}$; $n = 5$) to levels no longer statistically different compared with placental ROS from losartan-treated NP controls ($619 \pm 5$ RLU·min$^{-1}$·mg$^{-1}$; $n = 4$).

DISCUSSION

IL-17 producing Th17 cells is a newly discovered subset of T helper cell, which is believed to be a mediator for many autoimmune diseases such as Crohn’s disease, psoriasis, rheumatoid arthritis, and multiple sclerosis. We are now learning of a potential role for IL17 and Th17 cells as mediators for the pathophysiology associated with preeclampsia (1, 7, 16). Although IL-17 and Th17 cells are elevated in preeclampsia, it remains unknown if either IL-17 or Th17 cells cause hypertension or any state of disease expression that is associated with preeclampsia. Therefore, in this study we demonstrate, for the first time, that chronic IL-17 increases blood pressure and Th17 cells in pregnant rats but not in virgin rats. It is important to note that infusion of this dose of IL-17 had no hypertensive effects on the nonpregnant rats, just as we have seen with both...
TNF-α and IL-6, indicating that moderate doses of cytokines cause hypertension during pregnancy most likely by stimulating some placental factor or factors to cause much of the disease characteristics.

IL-17 stimulates neutrophilic reactions and the production of antimicrobial substances such as defensins and ROS. We demonstrate that IL-17 stimulates oxidative stress as illustrated by the increased in urinary isoprostanes and NADPH-stimulated placental oxidative stress. With preeclampsia being associated with autoantibodies, and IL-17 being primarily an autoimmune cytokine, we wanted to determine whether IL-17-induced hypertension was mediated through production of AT1-AA. Indeed, as with TNF-α and IL-6-induced hypertension during pregnancy, IL-17-induced hypertension in pregnant rats is associated with production of the AT1-AA. Other investigators have demonstrated a clear link with IL-17- and ANG II-induced hypertension (17), therefore, we measured plasma ANG II and found only a small increase that was not significantly different between NP controls and IL-17-infused pregnant rats. Although, elevations in ANG II are not associated with preeclampsia, a link with increased ANG II sensitivity is speculated to play a role in this disease, we believe this may be due to production of the AT1-AA acting in concert with ANG II to activate the AT1 receptor. To determine a role for activation of AT1 receptors, we treated a group of rats with losartan in the presence or absence of IL-17 infusion. We found that losartan attenuated the blood pressure response to IL-17 during pregnancy but also surprisingly inhibited placental oxidative stress in IL-17-treated pregnant rats. This could be due to blockade of both AT1-AA and ANG II activation of the AT1 receptor.

To determine whether the increase in oxidative stress caused the hypertension observed with IL-17 infusion, we repeated the infusion study in pregnant rats treated with the SOD mimic in their drinking water (tempol). We found that administration of tempol not only attenuated the placental oxidative stress but also the increase in blood pressure in response to IL-17 infusion. Surprisingly, we found that administration of tempol and decreased ROS in the placenta was associated with significantly lowered AT1-AA in response to IL-17. These data indicate the importance of ROS as signaling molecules between immune cells and tissues to cause much of the pathology associated with preeclampsia and IL-17-induced hypertension during pregnancy.

In a previous study, we infused rat AT1-AA into NP rats and found that placental oxidative stress was significantly elevated when compared with the nonhypertensive control NP rats (13). In that study we administered tempol and found that we could attenuate the placental oxidative stress and the hypertension in that model. Therefore, in the current study, we examined the role of the AT1-AA to mediate the hypertension, stimulated in response to IL-17 as mediators of pathophysiology of preeclampsia. We have recently demonstrated that administration of Rituximab decreased circulating B cells and AT1-AA in two previous experiments. First, we administrated the B cell-depleting agent to RUPP rats and found that AT1-AA, local ET-1, and blood pressure were decreased in response to placental ischemia in this rat model of preeclampsia (10). Most recently, we administrated Rituximab to NP recipient rats of RUPP CD4 T cells. In this T cell-induced preeclampsia rat model, blood pressure and circulating AT1-AA were significantly elevated compared with NP recipients of NP CD4 T cells. Administration of losartan decreased the hypertension. Furthermore, administration of Rituximab was efficient at B cell depletion and AT1-AA suppression and attenuation of hypertension in response to CD4 T cells from RUPP rats (17). We demonstrate, in this study, that administration of Rituximab significantly decreased the blood pressure in response to IL-17 infusion, suggesting an important role for the AT1-AA to cause hypertension in this model. However, it is important to note that as seen in RUPP rats, B cell depletion by administration of Rituximab did not attenuate but significantly blunted the blood pressure response to IL-17; MAP in IL-17+Rituximab-treated rats was still significantly elevated compared with NP control rats. Furthermore, we demonstrate that administration of Rituximab significantly decreased circulating T<sub>H</sub>17 cells stimulated in response to IL-17 infusion. However, this response was not completely attenuated, and circulating T<sub>H</sub>17 cells stimulated in response to IL-17 infusion were still significantly higher than that of NP control rats. Therefore, we conclude that the remaining increased in blood pressure in this group could be due to the presence of excess circulating T<sub>H</sub>17 cells or ANG II or other inflammatory cytokines stimulated in response to IL-17 infusion. However, since the blood pressure response nor the T<sub>H</sub>17 stimulation was completely abolished, we did not examine these additional factors. Nevertheless, these data highlight the importance of many factors caused by IL-17 excess during pregnancy, such as IL-17-stimulated oxidative stress, other inflammatory cytokines, and T<sub>H</sub>17 cells to mediate the elevation in blood pressure and pathophysiology associated with preeclampsia.

Although we recognize that ROS is a product of neutrophils and monocytes, we did not determine nor isolate and culture these cell types from our IL-17-infused pregnant rats. These phagocytic cells contain NADPH subunits and produce ROS, therefore, they could be the producers of elevated whole body oxidative stress indicated by the sharp increase in urinary 8-isoprostanes. We did, however, demonstrate that in response to chronic IL-17 infusion, T<sub>H</sub>17 cells are elevated in the circulation of pregnant rats. The stimulus for such could be a typical cellular response resulting from IL-6 and other cytokines such as IL-8, TNF-α, or IL-1, all of which synthesis is induced by elevated IL-17. Importantly, we found that indeed in response to chronic IL-17 administration, IL-6 is significantly elevated above that observed in NP rats. IL-17 is critical...
for function of TH17 cells, which are associated with immune and autoimmune phenomena. It is accepted that elevated cytokines stimulate immune cells and intercellular/intracellular communication between leukocytes. Therefore, as demonstrated here, one result of chronic IL-17 infusion is elevated TH17 cells, oxidative stress, and AT1-AA as mechanisms of hypertension during pregnancy. A secondary mediator could be elevated IL-6, which facilitates proliferation of both TH17 cells and B cells, and as we have previously shown, is an important player in hypertension and AT1-AA production in response to placental ischemia (19). Future studies of interest would be to determine what immune cell type is the source of oxidative stress in the placenta and what importance do TH17 cells play in causing hypertension and AT1-AA production from B cells when IL-17 is elevated or in response to placental ischemia. Importantly, this study highlights the importance of IL-17-stimulated ROS as signaling molecules linking immune activation with the development of hypertension during pregnancy. Future studies blocking IL-17 and possibly TH17 cells in response to placental ischemia will be important to determine a causal role for IL-17 in placental oxidative stress and hypertension during preeclampsia.

GRANTS

This work was supported by American heart Association Grant SDG0835472N and by National Institutes of Health Grants HD-67541 and HL-51971.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES