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Role of Reactive Oxygen Species in Hypertension Produced by Reduced Uterine Perfusion in Pregnant Rats

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Abstract

BACKGROUND—Although recent studies indicate preeclampsia (PE) is associated with increased oxidative stress, the role of reactive oxygen species in the hypertension associated with PE remains unclear. We sought to test the hypothesis that placental ischemia increases oxidative stress which in turn, contributes to hypertension.

METHODS—Reduction in uterine perfusion pressure (RUPP) was induced by placing silver clips on the abdominal aorta and the ovarian arteries on day 14 of pregnancy. On day 20 of pregnancy, mean arterial pressure (MAP) was measured and oxidative stress was assessed in renal and placental tissues whereas systemic administration of tempol, a superoxide dismutase (SOD) mimetic, was used to evaluate the contribution of reactive oxygen species on RUPP-induced hypertension.

RESULTS—MAP (120 ± 2 mm Hg vs.106 ± 3 mm Hg), placental levels of 8-isoprostane (1.9 ± 0.4 ng/g tissue vs. 0.8 ± 0.1 ng/g tissue), and malondialdehyde (MDA) (6.9 ± 0.6 μmol/g tissue vs. 3.9 ± 0.4 μmol/g tissue) were increased, whereas renal cortical SOD activity was decreased in RUPP rats (1.2 ± 0.1 units/mg protein vs. 1.6 ± 0.1 units/ mg protein) at day 20 of gestation (20 dG) compared to controls. Chronic treatment with tempol attenuated the hypertension (RUPP + tempol 112 ± 2 mm Hg vs. RUPP, 120 ± 2 mm Hg) associated with RUPP, whereas tempol had no effect on MAP (NP, 106 ± 3 vs. NP + tempol, 108 ± 2) in control rats.

CONCLUSION—The results of this study indicate that placental ischemia decreases innate antioxidant activity resulting in elevated oxidative stress which appears to play a role in mediating hypertension associated with chronic RUPP in pregnant rats.

Preeclampsia is a major obstetric problem and a significant source of maternal and neonatal morbidity and mortality.1 Despite its position as a prominent disorder of pregnancy, the incidence of preeclampsia (PE) continues to increase and has risen 40% in the past decade.2 The preeclamptic syndrome has been described in detail with numerous studies indicating that poor placental perfusion along with endothelial cell dysfunction and a disturbed balance of angiogenic factors may all contribute to this disorder.3 Nevertheless, the pathophysiological mechanisms underlying this condition remain unresolved.

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Reactive oxygen species (ROS) are physiologically important signaling molecules and are typically generated at low amounts in a controlled manner.4 In hypertension, an accumulation of ROS may disturb the balance of cellular oxidative status and results in endothelial dysfunction. Recent studies have identified several markers of oxidative stress in preeclamptic women including 8-isoprostane, malondialdehyde (MDA), and increased myeloperoxidase activity. Although recent studies have postulated that a perturbation in the balance of oxidants and antioxidants may be involved in the pathogenesis of PE, the underlying mechanisms connecting placental ischemia and increased ROS to hypertension have not been clearly identified.5–8

Although there is evidence that oxidative stress occurs in PE, it remains unclear whether poor placental perfusion is a stimulus that increases ROS or whether oxidative stress is a contributing factor in the reductions of uterine perfusion observed in PE. Further, the importance of ROS in mediating the renal and cardiovascular abnormalities associated with placental ischemia is unknown. We have recently reported that chronic reductions in uterine perfusion pressure (RUPP) in pregnant rats results in cardiovascular and renal abnormalities similar to those found in preeclamptic women.3,9–11 Because of these robust similarities between RUPP in the rat and PE in the human, we used our well-characterized model of placental ischemia to test the following hypotheses: (1) chronic placental ischemia results in elevated oxidative stress and (2) treatment with a superoxide dismutase (SOD) mimetic would attenuate the hypertension associated with placental ischemia.

METHODS

All experimental procedures executed in the present study were in accordance with National Institutes of Health guidelines for care and use of animals and were approved by the Animal Care and Use Committee at the University of Mississippi Medical Center. Studies were performed in timed pregnant Sprague–Dawley rats (Harlan, Indianapolis, IN) in which day of gestation was determined by observation of a vaginal plug. Plug date was considered to be zero (0) dG. Animals were housed individually in a temperature-controlled room (23 °C) with a 12/12-h light/dark cycle and given *ad libitum* access to water and rat chow (Harlan Teklad, Madison, WI).

Experimental design

Two separate studies were performed using four experimental groups. The first study compared levels of oxidative stress between normal pregnant (NP; $n = 11$) and RUPP ($n = 15$) rats whereas the second study evaluated the effects of tempol (Sigma-Aldrich, St Louis, MO) on blood pressure in RUPP (RUPP+T; n = 13) and normal pregnant plus tempol, (NP + T; $n = 10$). Tempol, a SOD mimetic, was administered in drinking water at a dose of 30 mg/kg per day and treatment was initiated at day 12 of pregnancy. A pilot study determined daily water intake so that proper drug dosing could be achieved.

RUPP procedure

A chronic reduction in uteroplacental perfusion pressure in rats was achieved by a method previously reported by our laboratory.9–12 In brief, all rats undergoing RUPP or sham surgical procedures were anesthetized with 2% isoflurane. Pregnant rats entering the RUPP group underwent the following clipping procedure at 14 dG. After a midline incision, the lower abdominal aorta was isolated, and a silver clip (0.203 mm internal diameter) was placed around the aorta superior to the iliac bifurcation. Because compensation of blood flow to the placenta occurs in pregnant rats through an adaptive increase in ovarian blood flow, we also clipped branches of both the right and left ovarian arteries that supply the uterus with a silver clip (0.100 mm internal diameter). When the clipping procedure resulted

in total reabsorption of the conceptuses, rats were excluded from data analysis. To this end three RUPP rats were excluded from data analysis and one NP rat was excluded for having an unusually small litter size.

Measurement of arterial pressure in conscious rats

Mean arterial pressure (MAP) was measured using the method we have published previously in this model.9¯12 In brief, under isoflurane anesthesia (described earlier), 18 dG rats were surgically instrumented with catheters of V-3 tubing (SCI, Lake Havasu City, AZ) in the left carotid artery for blood pressure measurement. Catheters were tunneled to the back of the neck and exteriorized. On 20 dG, the rats were placed in modified restraining cages for arterial pressure monitoring in conscious rats with a pressure transducer connected to a pressure recorder for continuous recording.

Tissue collection

After blood pressure was measured at 20 dG, kidneys and placentas were collected from 10 NP rats and 12 RUPP rats. The largest and smallest placentas from each pregnancy were frozen in liquid nitrogen, whereas kidneys were dissected to obtain renal cortex and medulla before freezing.

Measurements of oxidative stress

To assess the effects of RUPP on oxidative stress in the placenta and the kidney, two organs complicit in the hypertension observed in this model, we measured several markers of oxidative stress. First, to determine SOD activity, an important marker of cellular antioxidant capacity, renal cortical and medullary tissues from 12 RUPP and 10 NP rats were minced and then homogenized in ice-cold 250 mmol/l sucrose buffer and centrifuged at 12,000*g* for 20 min at 4 °C and evaluated as described previously.13 SOD activity was then assessed using a commercially available kit (BIOXYTECH SOD-525; OXIS International, Portland, OR) according to the manufacturer's directions.

Plasma total antioxidant status was measured using a commercially available kit (Calbiochem Novobiochem, San Diego, CA)14 according to the manufacturer's instructions and the data are presented as mmol/l plasma. Although the total antioxidant status assay measures plasma antioxidant levels, the assay is not specific and may include (among others) selenium, flavonoids, β-carotene, carotenoids, vitamins C and E, and thiols.14

Lipid oxidation is recognized as a marker of oxidative stress; therefore we evaluated placental samples for two such markers. To determine local placental isoprostane production the following assay was performed as described previously.15 In brief, tissues were homogenized in phosphate-buffered saline (PBS, Sigma-Aldrich) and samples were extracted using ethanol containing 0.005% butylated hydroxytoluene (Sigma-Aldrich) to prevent oxidation during processing and with 5,000 cpm of ^{3}H-PGF_2 to determine recovery of 15-F_{2t}-IsoP. Isoprostane was measured using a commercially available EIA kit (Cayman Chemical; Ann Arbor, MI). The antibody was highly specific for 15-F_{2t}-IsoP (8-Iso PGF_2). 15 Third, we used an improved analysis of MDA for human body fluids previously published elsewhere.15 In brief, 0.005% butylated hydroxytoluene was added to prevent oxidation during the heating step with thiobarbituric acid and a sample size of 200 μl was used. Tetramethoxypropane was used to generate MDA for the standard curve.

Superoxide production in the placenta was also evaluated using the lucigenin technique. In brief, one placenta from each animal was homogenized (1:8 wt/vol) in RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail; Sigma, St Louis, MO). The samples were centrifuged at 12,000*g* for 20 min at 4 °C. The

supernatant was incubated with lucigenin at a final concentration of 5 μmol/l, the samples were allowed to equilibrate for 3 min in the dark, and luminescence was measured every minute for 5–15 min using a luminometer (Berthold, Oak Ridge, TN) and recorded as relative light units (RLUs) per min. An assay blank containing lucigenin without homogenate was subtracted from each RLU before transformation of the data. Protein concentration was measured using a Pierce (Rockford, IL) detergent compatible protein assay with bovine serum albumin standards. The data are expressed as RLU/min/μg protein.

Lastly, myeloperoxidase extraction was performed according to previously described methodology. Frozen placental tissues from 12 NP rats and 10 RUPP rats were thawed and placed in potassium phosphate buffer (pH 7.4). Homogenization with subsequent centrifugation at 20,000*g* relative centrifugal force was performed at 4 °C for 15 min. The supernatant was discarded and the pellet was resuspended in phosphate buffer (pH 6.0) containing 0.5% hexadecylmethylammonium bromide (HETAB; Sigma). Samples were recentrifuged and the supernatant was assayed for myeloperoxidase activity.16

Statistical analysis

A Grubb's test was used to identify statistical outliers and all data are expressed as mean ± s.e.m. Comparisons of control pregnant rats with RUPP rats both treated and untreated were analyzed using analysis of variance followed by Dunnett's multiple comparison test to compare all groups vs. the control (NP) group or unpaired *t*- test for data that were normally distributed or the Mann–Whitney *U*-test for data that were not normally distributed. A Welch's correction for unequal variances was applied to *t*-tests when appropriate. A value of $P < 0.05$ was considered statistically significant.

RESULTS

MAP in pregnant rats with chronic RUPP

MAP was increased in RUPP (120 ± 2 mm Hg vs. 106 ± 3 mm Hg; $P < 0.05$; Figure 1) compared to NP rats. Pretreatment of RUPP rats with tempol markedly reduced MAP (RUPP + T 112 ± 2 mm Hg; $P < 0.05$) compared to the RUPP rats. Pretreatment of NP dams with tempol did not alter MAP significantly.

Fetal and placental morphometry

Fetal weight was decreased in the RUPP compared to the NP rats (3.3 ± 0.2 g vs. 3.9 ± 0.2 g; $P < 0.05$) on 20 dG. Pretreatment with tempol attenuated the growth restriction RUPP + T compared to the NP dams (3.5 ± 0.1 g vs. 3.9 ± 0.2 g; $P > 0.05$). Pretreatment of NP dams with tempol had no effect on fetal growth (4.0 ± 0.1 g).

Placental weight was not different between the NP, NP + T, RUPP, and RUPP + T groups (0.59 ± 0.03 g vs. 0.58 ± 0.01 g vs. 0.56 ± 0.02 g vs. 0.56 ± 0.02 g), respectively.

Increased markers of oxidative stress in the serum and placentas of rats with chronic RUPP

Plasma total antioxidant status was decreased in the serum of the RUPP compared to the NP controls on 20 dG (0.92 ± 0.029 mmol/l vs. 1.06 ± 0.06 mmol/l; $P < 0.05$). Placental isoprostane was assayed to measure phospholipid oxidation. Figure 2a illustrates that placental isoprostane was increased in the RUPP (2 ± 0.35 ng/g tissue) compared to NP rats (0.84 ± 0.1 ng/g tissue; $P < 0.05$). MDA levels within the placenta were measured to assess lipid peroxidation. MDA level in the placenta was significantly increased in RUPP vs. NP rats (7.1 ± 0.6 μmol/g tissue vs. 4.0 ± 0.4 μmol/g tissue, $P < 0.01$; Figure 2b). Figure 2c illustrates the increased superoxide production in the placenta of the RUPP compared to the

NP dams (245 ± 66 RLU/min/μg vs. 67 ± 18 RLU/min/μg; $P < 0.05$). Myeloperoxidase activity was higher (253 ± 41 units/g tissue vs. 153 ±19 units/g tissue; $P < 0.05$) in placental tissue from pregnant RUPP rats compared to in NP rats.

Decreased SOD activity in the kidneys from rats with chronic RUPP

Cu/Zn SOD activity was lower (1.22 ± 0.09 units/mg protein vs. 1.56 ± 0.13 units/mg protein; $P < 0.05$) in the renal cortical tissue of the RUPP dams compared to those in the NP group. Tempol treatment did not stimulate Cu/Zn SOD activity in the RUPP + T renal cortex (1.26 ± 0.13 units/mg protein) compared to the RUPP rats. Cu/Zn SOD activity was unchanged in the renal medullary tissue from RUPP rats compared to NP rats.

DISCUSSION

Although a role has emerged for increased oxidative stress as a factor in the pathogenesis of PE, the mechanisms by which ROS are increased and the manner in which these effects are mediated remains unclear.6,8,17 The present study reports several new insights regarding this matter. First, we show that placental ischemia results in the generation of oxidative stress in the placenta and a decrease in the innate antioxidant capacity in the plasma and the renal cortex in the pregnant rat. Second, we show that chronic treatment with tempol, a SOD mimetic, attenuates the hypertension that is associated with placental ischemia induced by RUPP in the pregnant rat. Although the present study does not rule out the possibility that oxidative stress may be a contributing factor in the impaired placental perfusion of PE, the present study does demonstrate a clear role for placental ischemia as a cause of oxidative stress, which in turn contributes to the hypertension associated with RUPP.

In the present study, we show that placental ischemia–induced hypertension is associated with increases in many markers of oxidative stress in the plasma and the placenta and with decreased SOD activity in the renal cortex. The present observation that plasma total antioxidant status was decreased by chronic RUPP is similar to previous reports of women with PE18 and suggests that placental ischemia results in generalized oxidative stress. Placental isoprostane, a prostaglandin-like product with potent vasoconstrictor actions that is formed *in vivo* by free radical-catalyzed nonenzymatic peroxidation of arachidonic acid lipid oxidation, was increased 2.4-fold in the RUPP rats when compared to the NP controls. This is similar to the increases observed previously in preeclamptic human pregnancies.15 Although in the present study it remains unclear what the role of the increased isoprostanes are, previous work has shown that isoprostanes are potent vasoconstrictors in several vascular beds and that they also stimulate endothelin (ET-1) secretion from endothelial cells.19,20 We have previously shown that ET-1 stimulates oxidative stress and isoprostane production in a model of ET-1-induced hypertension.21 Thus, it is possible that a positive feedback loop may exist in which ET-1 both stimulates and is stimulated by oxidative stress. Further studies are needed to investigate this and other potential roles for isoprostanes in the cardiovascular dysfunction associated with placental ischemia.

We also report that three other markers of oxidative stress, placental MDA concentrations, MPO activity, and superoxide production, are increased in the RUPP compared to the NP controls. Again, these observations are in agreement with previous reports in preeclamptic women as previous studies have shown increases in ROS in both serum and the placenta.22,23 Interestingly, a previous study has shown that placental MDA production correlates with placental isoprostane production although no causal relationship was proposed by those authors.15 Myeloperoxidase is an oxidant enzyme that is reportedly increased in the circulation of patients with PE and eclampsia.22 Similar to the aforementioned studies, the present work found that myeloperoxidase activity was elevated in placental tissue from pregnant rats with RUPP compared to the NP controls. Further, placental superoxide

production as determined by a lucigenin assay was also increased 3.5-fold in the RUPP rats, yielding additional evidence that placental ischemia generates increased oxidative stress in this model. Taken together, placental ischemia appears to provide a strong stimulus for the generation of oxidative stress in the RUPP model.

Previous experimental data have revealed a close relationship between ROS, nitric oxide (NO), and blood pressure in rats.24 We have also previously reported that RUPP is associated with decreased neuronal nitric oxide synthase expression in the kidney as well as decreased glomerular filtration rate and renal plasma flow.10 Taken together with the observations that all forms of chronic hypertension observed to date demonstrate to some extent an altered capacity of the kidney to maintain normal Na^+ excretion,25 we explored whether the innate antioxidant capacity of the kidney was compromised in the RUPP model. Indeed, we found that Cu/Zn SOD activity was significantly lower in renal cortical tissue from the RUPP rats compared with the control NP rats. Thus it is possible that increased oxidative stress may decrease bioavailable NO in the kidney and have deleterious effects on renal function in the RUPP rat. In fact, preliminary studies in our laboratory suggest that RUPP rats do exhibit a hypertensive shift in the pressure-natriuresis curve (unpublished observations); however, further studies are needed to elucidate the mechanisms responsible for this observation.

In previous work, we have shown that fetuses of RUPP were smaller than those of NP control rats.11,12 In the present study, we show that treatment with tempol attenuates this decrease in fetal growth. The mechanism underlying this observation is unclear although one possibility is that decreased oxidative stress in the placental vasculature could allow for greater placental perfusion and increased delivery of nutrients to the growing fetus. Nevertheless, this is an interesting observation that will require further investigation.

In the present work we sought to determine the systemic effects of chronic daily treatment with the SOD mimetic tempol beginning before the onset of RUPP-induced hypertension and found that it results in decreased MAP in the affected rats. Moreover, tempol treatment during the same time period did not decrease MAP in the NP controls. Because similar observations have been reported in other models of experimental hypertension,26 it appears that increased oxidative stress is a significant factor in the hypertension associated with RUPP. In contrast, recent data from several clinical trials suggest that antioxidant treatment in the forms of vitamins A and C may not protect women from PE.27–29 Although it remains unclear why these reports differ, species differences may be a primary factor for this disparity. Although within the clinical studies there are several factors such as parity, ethnicity, genetic predisposition, and lifestyle factors that may contribute to these observations.

One such additional factor that might be involved is the soluble VEGF receptor (sFlt-1) that we have recently shown is increased in the plasma and the placental of the RUPP rat.11 Previous studies have shown that under hypoxic conditions ROS may stimulate sFlt-1, and that this may occur via stabilization of HIF-1α a transcription factor for sFlt-1.30,31 Similarly, a recent study has also shown that both heme oxygenase-1 (HO-1) and carbon monoxide exert protective effects against oxidative stress and inhibit the production of sFlt-1 *in vitro*.32 Further studies are underway to examine these possible connections between placental ischemia, ROS, sFlt-1, and hypertension.

In summary, we report that chronic RUPP in the pregnant rat was associated with significant increases in arterial pressure, placental isoprostane and MDA levels, increased placental MPO activity, and reduction in renal cortical SOD activity. Chronic administration of tempol markedly attenuated the increase in MAP observed in the pregnant rats with chronic

RUPP. However, tempol had no significant effect on blood pressure in the NP rats. These results suggest that oxidative stress due to placental ischemia may alter renal function and ultimately play a major role in mediating the hypertension produced by chronic RUPP. Nevertheless, it remains to be determined how other factors such as, HIF-1α, HO-1, and sFlt-1 may interact with increased ROS production and placental ischemia in hypertension during pregnancy.

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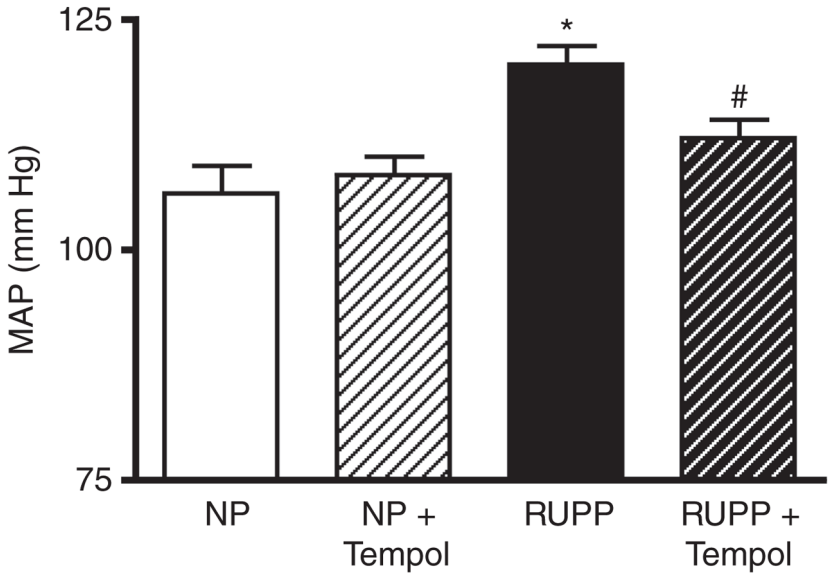

Figure 1.
Mean arterial pressure (MAP) in late gestation rats. Reduced uterine perfusion pressure (RUPP; $n = 15$) increased MAP compared to normal pregnant (NP; $n = 11$) control rats, whereas treatment with tempol in the RUPP group (RUPP + T; $n = 13$) attenuated the increased MAP but had no effect in the normal pregnant + tempol rats (NP + T; $n = 10$). All data are expressed as mean ± s.e.m. $^{*}P < 0.001$ RUPP vs. NP rats; $^{\#}P < 0.05$ RUPP + T vs. RUPP.

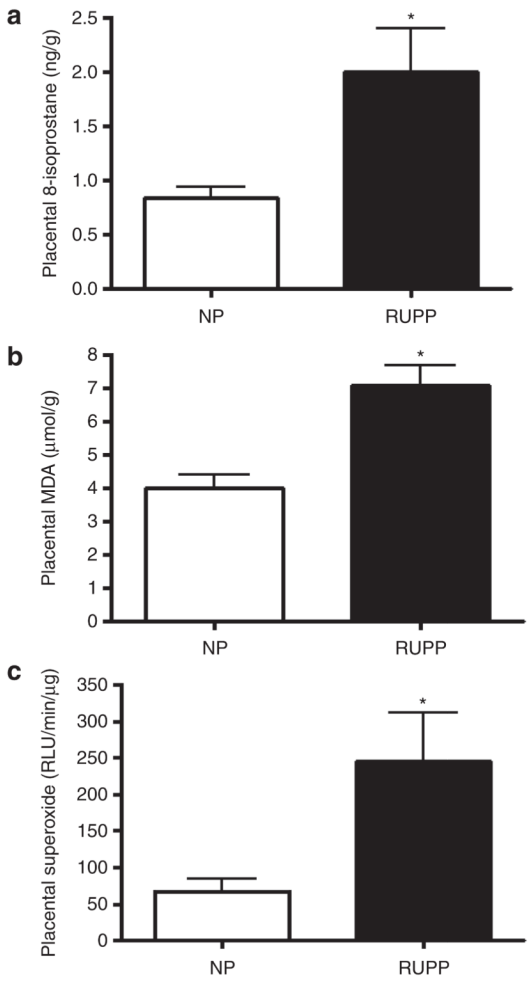

Figure 2.

Changes in placental isoprostane, malondialdehyde (MDA) and superoxide in response to chronic RUPP. (**a**) Placental isoprostane, (**b**) MDA, and (**c**) superoxide were increased ($P < 0.05$) in the RUPP ($n = 12$) compared to the NP ($n = 10$) dams. *$P < 0.05$ RUPP vs. NP. All data are expressed as mean ± s.e.m.