Beneficial Effects of Metolazone in a Rat Model of Preeclampsia

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Received March 29, 2006; accepted May 18, 2006

ABSTRACT

Preeclampsia is a disorder that continues to exact a significant toll with respect to maternal morbidity and mortality as well as fetal wastage. Furthermore, the treatment of this disorder has not changed significantly in 50 years and is unsatisfactory. The use of diuretics in this syndrome is controversial because there is a concern related to potential baleful effects of volume contraction leading to a possible further decrement in the perfusion of the maternal-fetal unit. Metolazone is a diuretic/antihypertensive agent, which has a therapeutic effect on blood pressure (BP) in human essential hypertension without causing a natriuresis. We administered the drug in nondiuretic doses in a rat model of preeclampsia previously developed in this laboratory. The drug reduced BP without an accompanying natriuresis. Although there was a trend toward an improvement in intrauterine growth restriction, as determined by litter size and the number of pups demonstrating malformations, the values did not reach statistical significance. We conclude that metolazone, in low dosage, is an effective antihypertensive in this rat model. These studies have implications for the treatment of the human disorder.

Preeclampsia affects from 3 to 10% of all pregnant women in the United States and worldwide (Lenfant and Zuspan, 1990; Pridjian and Puschett, 2002a). Yet, it is still a disease treated by symptom-directed therapy, usually magnesium sulfate and hydralazine or labetolol. Specific therapy directed to the underlying cause has not been developed.

The emerging concept regarding the etiology of preeclampsia is that it is probably multifactorial (Page, 1972; Ness and Roberts, 1996; Pridjian, 1999; Pridjian and Puschett, 2002b). Currently, there is no one satisfactory treatment regimen, which is directed at the underlying cause (Pridjian and Puschett, 2002b), although certain treatments have been proposed in certain preeclamptic subtypes (Saisto et al., 2004). We believe that successful definitive treatment of preeclampsia will be developed aimed at different subtypes.

Women with preeclampsia are volume-expanded, as are all pregnant women (Rovinsky and Jaffin, 1965; Scott, 1972). However, the added fluid resides in the interstitial, rather than intravascular, extracellular compartment (Gallery, 1999). In the past, diuretics were avoided in preeclampsia so as not to cause further volume contraction of the intravascular compartment and potential compromise of the maternal-fetal circulation. However, volume contraction and decreased perfusion of the placenta with diuretics have never been proven.

We have postulated that at least some forms of preeclampsia are related to excessive expansion of the extracellular fluid (ECF) volume (Ianosi-Irimie et al., 2005; Vu et al., 2005). We hypothesize that women who develop this type of preeclampsia have an acquired or congenital defect in sodium transport, which prevents them from excreting the excess sodium. In most women, this defect would not become manifest until the patient is challenged with the 40 to 50% expansion of ECF volume that accompanies normal pregnancy (Gallery, 1999). The expansion process may actually result in the elaboration of one or more circulating factors (VanWijk et al., 2000; Hayman et al., 2001; Vu et al., 2005), which have both natriuretic and vasoconstrictive properties (Graves and Williams, 1984; Morris et al., 1988; Hilton et al., 1996; Lopatin et al., 1999). The authors have developed an animal model of preeclampsia (Ianosi-Irimie et al., 2005), which has many of the phenotypic characteristics of the hu-
man disease (Outland et al., 2005). It consists of two manipulations: 1) replacement of the drinking water of the pregnant rat with saline and 2) administration of the mineralocorticoid deoxycorticosterone acetate (DOCA) to ensure that the excess sodium is retained. Under these circumstances, the animals develop hypertension, proteinuria, and intrauterine growth restriction (IUGR) (Vu et al., 2005).

Metolazone is a diuretic/antihypertensive agent that has been on the market for a number of years. It is a quinethazone derivative, a congener of the thiazide drugs (Puschett, 1972). It is considered safe in human pregnancy; specifically rated class B by the Food and Drug Administration use in pregnancy rating scale (Physician’s Desk Reference, 2000). In low doses, the drug has antihypertensive activity with minimal diuresis and natriuresis (J. Puschett, unpublished observations). We reasoned that, if this were the case, it might be effective in treating our “preeclamptic” rats without compromising ECF, particularly intravascular volume.

**Materials and Methods**

**Experimental Protocols.** Female Sprague-Dawley rats (200–250 g) (Harlan, Indianapolis, IN) were housed in metabolic cages for a nonpregnant, 24-h baseline urine collection. A control (nonpregnant) group was established for comparison (n = 8). The remaining animals were then mated with male rats (275–300 g), and pregnancy was confirmed by the presence of vaginal plugs. Pregnant females were isolated from the males and randomly divided into the following groups: normal pregnant (NP) (n = 10) and pregnant + DOCA + saline (PDS) animals (n = 10). PDS rats were injected i.p., with 12.5 mg of a depot form of DOCA at the time of mating followed by a 6.5-mg injection on a weekly basis. In this group, drinking water was replaced with 0.9% saline. In addition, a separate group of PDS animals were given daily metolazone diluted in saline by gavage at 50 μg/kg body weight (PDSM) (n = 15). The dose of metolazone was chosen because it corresponds to a low normal dose for humans. All animals were allowed free access to standard rat chow (Purina Lab Diet 5001 Laboratory Rodent Diet; St. Louis, MO), maintained on a 12-h light/12-h dark cycle and acclimatized for 1 week before being studied. Animal care was conducted in accordance with institutional guidelines.

**Blood Pressure and Sodium Excretion Measurements.** Systolic blood pressure (BP) was measured using a tail-cuff method as described previously (Lanosi-Irimie et al., 2005), and 24-h urine collections were collected/diary. Sodium excretion was measured by flame photometry (IL 943; Instrumentation Laboratory Co., Lexington, KY).

Once animals in the PDS and PDSM groups became hypertensive (at approximately days 6–8), metolazone treatment commenced. The metolazone dose was adjusted daily according to the BP and to the Na+ excretion measurement to ensure that metolazone was administered in non-natriuretic doses. For example, if the Na+ concentration in the 24-h urine obtained just before dosing was either greater than the Na+ concentration obtained the day before or greater than the sodium concentration of the untreated PDS group, the dose of metolazone administered would be decreased by 5 to 8%. If the BP was elevated but no evidence of natriuresis, the metolazone dose would be increased by 5 to 8%. The dose was thus individualized but ranged from 35 to 80 μg/kg body weight during the course of the experiment.

**Protein, Creatinine, and Nitrite/Nitrate Assays.** At days 19 to 20, a 24-h urine was collected for protein, creatinine, and NO determinations. On the 20th day of pregnancy, animals were humanely euthanized, and blood samples were taken. Pups and placentas were separated, and any pup malformations were noted. Urinary protein was measured using the pyrogallol red method with a total protein kit (Sigma-Aldrich, St. Louis, MO). Blood and urine creatinine levels were measured using the picric acid method with a Beckman creatinine analyzer (Beckman Coulter, Fullerton, CA). Nitrite/nitrate (NOx) measurements in sera and in 24-h urine collections were performed using sulfanilamide and N-(naphthyl)-ethylenediamine with a NO colorimetric assay (Roche Diagnostics, Indianapolis, IN). Hematocrit was measured using an Autocrit Ultra 3 centrifuge (BD Diagnostics, Sparks, MD).

**Immunoblotting Analyses.** Kidneys were excised, weighed, dissected, and washed in ice-cold saline buffered with 10 mM Tris-HEPES before removal of the cortex and medulla. Kidney slices (cortex and medulla) were homogenized in 50 mM mannitol buffered with 20 mM Tris-HEPES in washed sea sand (Fisher, Pittsburgh, PA). Cell extracts were obtained after centrifugation (1000g; 10 min), and protein was measured with a BCA assay kit (Pierce Chemical, Rockford, IL) with bovine albumin as the standard. Protein samples for immunoblotting analysis (10–15 μg of protein/sample) were prepared in Novex LDS sample buffer (Invitrogen, Carlsbad, CA), separated on 7% Tris-acetate NuPAGE gels (Invitrogen) (Laemmli, 1970), and transferred to a 0.2-μm nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked (phosphate-buffered saline, 0.5% Tween 20, and 5% milk) for 1 h at room temperature; briefly rinsed (phosphate-buffered saline and 0.5% Tween 20); and stained with monoclonal anti-nitric-oxide synthase, endothelial (eNOS) or neuronal (nNOS), antibody (BD Transduction Labortatories, Lexington, KY). Membranes were then washed and incubated with a horseradish peroxidase-conjugated goat anti-mouse antibody (GE Healthcare, Piscataway, NJ) for 1 h. The chemiluminescent detection was performed by using ECL Western Blotting detection reagents (GE Healthcare), and autoradiographs were digitized by QuantiScan (Biosoft, Ferguson, MO). The results were normalized for β-actin (monoclonal anti-β-actin antibody, clone AC-15; Sigma-Aldrich).

**Statistical Analysis.** Values are presented as mean ± S.E.M. Statistical comparison analyses were performed using covariant analysis for multiple determinations. A p value of less than 0.05 was considered significant.

**Results**

Mean tail-cuff BP in the control, nonpregnant group (C) varied between 97 and 109 mm Hg (Fig. 1). None of the mean values for this group significantly differed from each other at any time during pregnancy. The NP mean BP fell from an initial mean value of 109 ± 6 to 88 ± 1 mm Hg after 19 days of pregnancy (p < 0.01). This decline is reminiscent of the fall in BP seen in human pregnancy as gestation proceeds. The PDS group (i.e., animals rendered “preeclamptic”) displayed a BP increase from 103 ± 6 to 126 ± 1 mm Hg (p < 0.001). In the metolazone-treated preeclamptic animals (PDSM group),
a statistically significant decrease in BP was noted (pretreatment mean BP of 110 ± 4 mm Hg and posttreatment BP of 94 ± 4 mm Hg; p < 0.05). The latter value was similar to that noted in the normal pregnant group (p > 0.05). The decrement in tail-cuff BP of the PDSM animals was observed by 24 h after initiation of metolazone. There were no statistically significant differences in BP between any of the four groups of animals at time t₀. However, at 4 to 7 days of gestation (t₁), mean BP values in PDS and PDSM groups were significantly increased (113 ± 4 and 110 ± 4 mm Hg, respectively), and these changes are in contrast to a decrease in the NP group (94 ± 4 mm Hg) (NP versus PDS, p < 0.01; NP versus PDSM, p < 0.05). At 10 to 13 days of gestation (t₂), BP of the PDSM animals was decreased (94 ± 5 mm Hg) after metolazone administration, but PDS BP continued to increase (118 ± 2 mm Hg) (PDS versus PDSM; p < 0.001). Animal weight did not demonstrate any significant difference among the pregnant groups of animals (NP, PDS, and PDSM) throughout the course of the experiments (Fig. 2).

Twenty-four-hour Na⁺ excretion values (millimoles of Na⁺/24 h) in the control and normal pregnant animals were similar throughout the experiment (C, 3.0 ± 0.1; NP, 4.2 ± 0.3; day 19; p > 0.05) (Fig. 3). The animals in the PDS and PDSM groups had significantly higher but similar 24-h urinary Na⁺ excretion values (15.9 ± 2.2 and 16.1 ± 3.1, respectively; day 19; p > 0.05). The day 19, urinary Na⁺ mean values were consistent with sodium excretion rates obtained throughout the experiment once the animals were rendered hypertensive.

The normal pregnant animals excreted more protein (milligrams/24 h) than nonpregnant controls (NP, 4.9 ± 0.1; PDSM or PDS versus NP; ††, p < 0.001; PDSM or PDS versus NP; †††, p < 0.001). There were no statistically significant differences between the metolazone and DOCA groups and between the normal pregnant and control nonpregnant groups (p > 0.05).

Serum creatinine values (milligrams per deciliter) in NP (0.6 ± 0.03), PDS (0.5 ± 0.1), and PDSM (0.6 ± 0.1) groups were not different from each other, but they were significantly lower than the nonpregnant C group (0.8 ± 0.1, p < 0.05 versus NP and PDSM, and p < 0.01 versus PDS). Creatinine clearance (milliliters per minute) was found to be increased (versus nonpregnant control animals) in the NP and PDS groups (p < 0.05 in each case), but it just missed statistical significance, although demonstrating a numerical increase in the PDSM rats (C, 0.8 ± 0.1; NP, 1.6 ± 0.2; PDS, 1.6 ± 0.4; PDSM, 1.4 ± 0.1).

Blood NO as estimated by NOₓ measurements (micromoles per liter) was significantly higher in the NP (42.4 ± 2.1; p < 0.001), PDS (34.3 ± 2.9; p < 0.01), and PDSM (31.7 ± 1.3; p < 0.05) groups compared with the nonpregnant control group (25.6 ± 2.4) (Fig. 5A). PDS animals had a significantly lower blood NO compared with NP (p < 0.01). PDSM animals had a similar lowering compared with NP (p < 0.001). There was no difference noted between the PDS and PDSM groups (p > 0.05).

There was no difference in urinary NO excretion (expressed in nanomolar NOₓ/milligram creatinine) between the C and NP groups (71.9 ± 11.8 versus 48.3 ± 10.5), but there was an increase in both PDS (151.1 ± 25.9) and PDSM (102.7 ± 19.1) groups versus NP (p < 0.001 and p < 0.05, respectively) (Fig. 5B). An increase in the urinary NOₓ versus controls of PDS animals was also present (p < 0.01). There was no difference in levels between PDS and PDSM groups (p > 0.05).

Pup number in the PDS group was significantly decreased compared with the NP rats (13.9 ± 0.4 versus 11.2 ± 0.9; p < 0.05). However, despite a trend toward an improvement in the PDSM rats (13.4 ± 0.9), this value did not reach statistical significance (p > 0.05). With regard to malformations, none was noted in the NP group. The number of developmental malformations in the PDS animals was increased compared with NP (Fig. 6, A and B). These consisted of grossly immature and growth-retarded forms, occasional limb hypoplasia, and evidence of intrauterine death. The metolazone
treatment resulted in a trend toward fewer growth malformations, but this value did not reach statistical significance.

Hematocrit values were as follows: C, 0.52/H11006 ± 0.01; NP, 0.45/H11006 ± 0.02; PDS, 0.41/H11006 ± 0.02; and PDSM, 0.41/H11006 ± 0.08. The mean value for the C, nonpregnant animals was not statistically significantly different from that for NP (p > 0.05), but it was different from PDS (p < 0.05) and PDSM (p < 0.05) groups. However, the latter three treatments did not differ from each other.

Discussion

Definitive therapy of preeclampsia awaits determination of its multiple etiologies and development of measures to directly counteract the pathophysiology of the syndrome. The animal model of preeclampsia used has many of the phenotypic characteristics of human preeclampsia (Outland et al., 2005). Given the effectiveness of metolazone in the therapy of essential hypertension, even when given in non-natriuretic and nondiuretic doses, we elected to examine its use in our model of preeclampsia. We found that the drug was effective in lowering BP to normal pregnant levels without either a natriuresis or the disruption of fluid balance as determined by similar weights in the treated versus untreated animals (Fig. 2). We did not see resolution of proteinuria, and despite a trend toward improvement in IUGR, the values for pup number and average number of malformations did not reach statistical significance (Fig. 5A).

However, it may be that the drug was given too late in the pathogenetic process to alter the abnormal glomerular permeability to protein and the vascular abnormalities leading to IUGR. We speculate that if we had instituted metolazone therapy before hypertension developed, we might have noted beneficial effects on these parameters.

The current therapy of uncomplicated mild-to-moderate preeclampsia does not include the use of antihypertensive agents. Once BP rises to levels seen in severe preeclampsia, hydralazine or labetalol is administered to control BP while delivery is planned. Avoidance of treatment of mild-to-moderate preeclamptic hypertension was practiced in the past, because despite improvement in BP, the disease process was thought to continue. Definitive proof that antihypertensive agents used in mild-to-moderate preeclampsia as a temporizing measure are harmful has never been well documented.

A general consensus (Lindheimer and Katz, 1973) that diuretics should not be used for treatment or prevention of preeclamptic hypertension and edema exists (Wesley and Douglas, 1962; Krause et al., 1966; Gray, 1968; Pitkin et al., 1972; Christianson and Page, 1976) despite some early reports of successful outcomes with these agents (Cuadros and Tatum, 1964; Finnerty and Bepko, 1966). This concept has resulted from the following observations. 1) Preeclamptic women already have a contracted intravascular volume despite expansion of the interstitial ECF space (Brown et al., 1989). 2) A rat model of pregnancy-induced hypertension, developed in the mid-1970s (Abitbol et al., 1976) and 1980s (Losonczy and Mucha, 1989) by reducing uterine perfusion pressure, has been used more recently as a paradigm for the late events of preeclampsia by Granger and coworkers. In this model, hypoperfusion of the maternal-fetal unit led to increased renal vascular resistance and hypertension (Alex-
ander et al., 2001). Therefore, any additional decrement in ECF volume due to the use of diuretics might be expected to further reduce blood flow to the uterus and exacerbate the pathophysiology of the preeclampsia.

Excessive volume expansion as an important etiologic factor in the pathogenesis of preeclampsia may not be a universal phenomenon in the human syndrome. In our rat model of preeclampsia, we have identified a circulating inhibitor of sodium-potassium ATPase, the excretion of which is elevated before hypertension develops (Vu et al., 2005). This substance, marinobufagin, could serve as a predictor of the later development of preeclampsia if the data obtained in rats is applicable to the human subject. Thus, therapy with metolazone might be attempted only in those patients in whom marinobufagin excretion is elevated.

Past studies of the treatment of preeclamptic hypertension with diuretics have been problematic. The literature is replete with anecdotal reports. There have been no double-blinded, randomized, controlled investigations with adequate numbers to achieve valid conclusions. Confounding the results of many of these reports is the fact that patients with preeclampsia, superimposed upon preexisting hypertension from various etiologies, were included. Finally, the doses of the diuretics used to treat the hypertension and edema of preeclampsia were natriuretic, and by today's standards rather large. For example, chlorothiazide was given in doses of 500 to 1000 mg/day, equivalent to a dose of hydrochlorothiazide of 50 to 100 mg/day. These dosages are no longer used even in the therapy of essential hypertension. Thus, any potentially beneficial effect of this class of drugs on the disease process was most probably vitiated by the introduction of ECF volume contraction. Only in recent years has it been recognized that small doses of these drugs are effective in the therapy of essential hypertension (Puschett, 1999).

IUGR is a common concomitant of preeclampsia (Eskenazi et al., 1993; Obegard et al., 2000; Xiao et al., 2003). In the animal model described herein (Ianosi-Irimie et al., 2005), IUGR occurred. Furthermore, although the values did not reach statistical significance, the data suggest that IUGR might be either prevented or mitigated in this rat model of preeclampsia by the administration of metolazone (Fig. 5).

It is not possible, given our results, to implicate NO as an important pathogenetic factor in these studies. Furthermore, because there were no differences, either in the blood or urine NO levels between the PDS and PDSM rats, it is clear that the mechanism by which metolazone lowered BP did not involve alterations in NO. These conclusions are verified by the fact that there were no changes either in eNOS or nNOS. We suspect that the increased excretion of NO in the expanded animals was the result of an increment in Na + excretion (Shultz and Tolins, 1993) and that the augmented clearance of this substance by the kidney may have led to a decline in the blood levels that were observed.

The mechanism by which metolazone reduced BP is currently unknown. However, because volume contraction was not involved, it must have represented a vasodilatory effect. Because a NO mechanism is not supported by our data, further studies will be required to determine the nature of this effect.

In conclusion, metolazone has proven effective in ameliorating the hypertension in this rat model of preeclampsia. There was a trend toward a reduction in pup malformations and an improvement in pup number with metolazone that did not reach statistical significance. Perhaps provision of the drug earlier in pregnancy would result in an improvement in these parameters. The latter studies are currently planned.

References


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