A new animal model for human preeclampsia: Ultra-low-dose endotoxin infusion in pregnant rats

Maria M. Faas, MSc,[a] Gerard A. Schuiling, PhD,[b] Julius F.W. Baller, AS,[a]
C. Annet Visscher, MSc,[b] and Winston W. Bakker, PhD[a]

Groningen, The Netherlands

OBJECTIVE: An animal model for preeclampsia was developed by means of an ultra-low-dose endotoxin infusion protocol in conscious pregnant rats.

STUDY DESIGN: Rats received a permanent jugular vein cannula on day 0 of pregnancy, through which endotoxin (1.0 μg/kg body weight) ($n = 10$) or saline solution ($n = 6$) was infused during 1 hour on day 14 of pregnancy. Blood pressure, albuminuria, and platelet counts were measured, and histopathologic studies was performed in these rats.

RESULTS: A significant increase of blood pressure ($p < 0.05$) and of urinary albumin excretion ($p < 0.05$) was observed in endotoxin-treated pregnant animals, in contrast to control pregnant rats receiving saline solution. Platelet coagulopathy was found and glomerular fibrinogen deposits could be detected only in the endotoxin-treated pregnant rats. In addition, the activity of the glomerular antithrombotic enzyme adenosine diphosphatase was decreased in endotoxin-treated pregnant rats compared with saline solution–treated pregnant rats.

CONCLUSION: Because histopathologic and clinical events in this model mimic predominant features of human preeclampsia, this model may enable further study into the pathophysiologic mechanisms of this complication of pregnancy. (Am J Obstet Gynecol 1994;171:158-64.)

Key words: Experimental preeclampsia, endotoxin, rat

Preeclamptic toxemia is a major complication of human pregnancy, with an incidence of 5% to 7% in the Western world.[1] The most important clinical symptoms (hypertension, proteinuria, and edema) are manifested in the third trimester of pregnancy.[1] The cause of the syndrome is unknown, and the pathogenesis is poorly understood.

Interestingly, preeclampsia shares a number of pathophysiologic phenomena with the so-called generalized Shwartzman reaction, which can be induced by endotoxin.[2] Diffuse intravascular coagulation, affecting the glomerular microvasculature of the kidneys in which cortical necrosis may eventually occur, is one such a common phenomenon.[3] It has also been recognized for several decades that in experimental animals the sensitivity for endotoxin is enhanced considerably during pregnancy.[4] Although in nonpregnant animals the generalized Shwartzman reaction is induced by two intravascular injections of endotoxin given in 24-hour interval, in the pregnant animal only one injection of endotoxin is sufficient to induce the generalized Shwartzman reaction.[5] However, as is the case for preeclampsia, the exact pathogenesis of the generalized Shwartzman reaction is also still obscure.

Until now experimental research with respect to preeclampsia has been complicated by the fact that suitable animal models for this disorder are lacking. In this study, however, we describe the animal model that we recently developed on the basis of the idea that possibly the pathogenesis of preeclampsia and the pathophysiologic process induced by endotoxin share a final common pathway. If so, endotoxin-treated pregnant rats might be expected to also exhibit other clinical features of preeclampsia (i.e., hypertension and proteinuria).

Material and methods

Animals. Female Wistar outbred rats, 200 to 220 gm, were kept in a temperature- and light-controlled room (lights on from 5:30 AM to 5:30 PM) with free access to food and water. Until selection for experiments vaginal smears were taken daily. Rats were rendered pregnant by housing them on proestrus with fertile males for 1

night. The day after proestrus was designated day 0 of pregnancy. In cyclic and pregnant rats, the latter ones on day 0 of pregnancy, a cannula was inserted into the right jugular vein with the rat under ether anesthesia according to the method of Steffens,[6] allowing stress-free infusion of either endotoxin or saline solution and stress-free blood sampling.

A total of 1.0 µg/kg body weight endotoxin (E-Coli, 0.55: B5, Whittaker MA Bioproducts, Walkerville, Md.) dissolved in 2 ml of pyrogen-free saline solution was infused through the jugular vein. The infused low dose of endotoxin (1.0 µg/kg body weight) was based on previous findings showing clear-cut histopathologic alterations at the end of pregnancy, whereas the pregnant state per se was not significantly affected.[7, 8] Control rats received either 6.5 µg/kg body weight endotoxin or pyrogen-free saline solution alone. Pregnant rats (on day 14 of pregnancy) or cyclic rats were infused for 1 hour by means of an infusion pump (infusion rate 2 ml/hr).

Experimental design. Pregnant rats were infused with 1.0 (group I, $n = 10$) or 6.5 µg/kg body weight endotoxin (group II, $n = 13$) or saline solution (group III, $n = 6$) on day 14 of pregnancy. Cyclic rats received infusion of either endotoxin, 1.0 µg/kg body weight (group IV, $n = 5$) or 6.5 µg/kg body weight (group V, $n = 5$), or saline solution (group VI, $n = 5$) on day 0.

Systolic blood pressure was measured in groups I to V between 9 AM and 12 noon on days 8 through 14 and days 15, 16, 19, 20, and 21 (pregnant rats) and 8 days before infusion until 7 days after infusion (cyclic rats).

Urinary albumin was determined in the urine of pregnant low-dose endotoxin-treated rats (group I) and in the urine of pregnant saline solution–treated rats (group III) on days 6, 12, 15, and 19 of pregnancy and in the urine of cyclic low-dose endotoxin-treated rats (group IV) and cyclic saline solution–treated rats (group VI) 1 and 5 days after infusion.

Blood samples for platelet counts were taken on day 14 (just before infusion) and on days 15 and 20 of pregnancy in groups I and III and from cyclic rats (groups IV and VI) on day 0 (just before infusion) and 1 and 6 days after infusion.

Seven days after infusion pregnant (i.e., day 21 of pregnancy, groups I and III) and cyclic rats (groups IV and VI) were bled while they were under ether anesthesia and the kidney fragments were snap-frozen and prepared for immunohistologic and enzyme histochemical studies, as described below.

Measurement of systolic blood pressure. Systolic blood pressure was measured by means of the tail-cuff method, according to the method of Pfefffer et al.,[9] with minor modifications. In brief, after the rats were trained for 8 to 9 days to accustom them to the manipulations necessary for measuring blood pressure, blood pressure

was measured with a 15 mm occlusion cuff and a pulse transducer. Within 5 minutes three measurements were made, and systolic pressure was expressed as the mean of these measurements.

Determination of urinary albumin excretion. Rats were placed in metabolic cages (between 4:30 and 8:30 PM), and urine was assayed for albumin content by rocket electrophoresis with rabbit antirat albumin antiserum (Nordic Immunology, Tilburg, The Netherlands), as described elsewhere.[10] Because blood pressure measurement was made in the same animals, it was necessary to avoid stress. Therefore the period of urine collection was limited to this relatively short time (4 hours).

Platelet numbers in peripheral blood. Blood samples (100 µl) were taken from the permanent vena jugularis cannula between 9 AM and 12 noon. Samples were mixed with 3 µl of ethylenediaminetetraacetic acid (0.17 mmol) and counted with a microcellcounter (model Sysmex F800, Toa Medical Electronics, Kobe, Japan). Both platelet numbers and mean platelet volume were measured.

Immunohistologic and enzyme histochemical studies. Seven days after infusion of endotoxin or saline solution pregnant (day 21 of pregnancy) and cyclic rats were killed, and specimens of kidneys were snap-frozen according to standard procedures in methylbutane and stores at $-80°$ C until processed for immunohistologic and histochemical studies. For each pregnant rat killed on day 21 the number of implantation sites was counted, reflecting the number of fetuses in the uterine horns. Living fetuses were collected and weighed. Also, the number of resorptions, characterized by intrauterine hemorrhage, was assessed.

Glomerular fibrinogen depositions. The presence of intraglomerular fibrinogen depositions was examined in 4 µm cryostat sections by fluorescein isothiocyanate–conjugated labeled goat antirat fibrinogen immunoglobulin G (Nordic) by means of standard fluorescence microscopy. Kidney sections were scored semiquantitatively for intraglomerular fibrinogen deposits (50 glomeruli per section per animal) in a double-blind manner by two independent observers. A kidney section was scored positively when $>10\%$ of the glomeruli contained fibrinogen deposits. Sections showing no staining or $<10\%$ staining of the glomeruli were scored negative.

Glomerular adenosine diphosphatase activity. Cryostat sections were stained for adenosine diphosphatase activity by means of the cerium method, as previously described by Poelstra et al.[11] In 20 glomeruli of one section of each rat the amount of reaction product after staining for adenosine diphosphatase activity was quantitatively evaluated at the light microscopic level by computerized image analysis and expressed as units.[11]
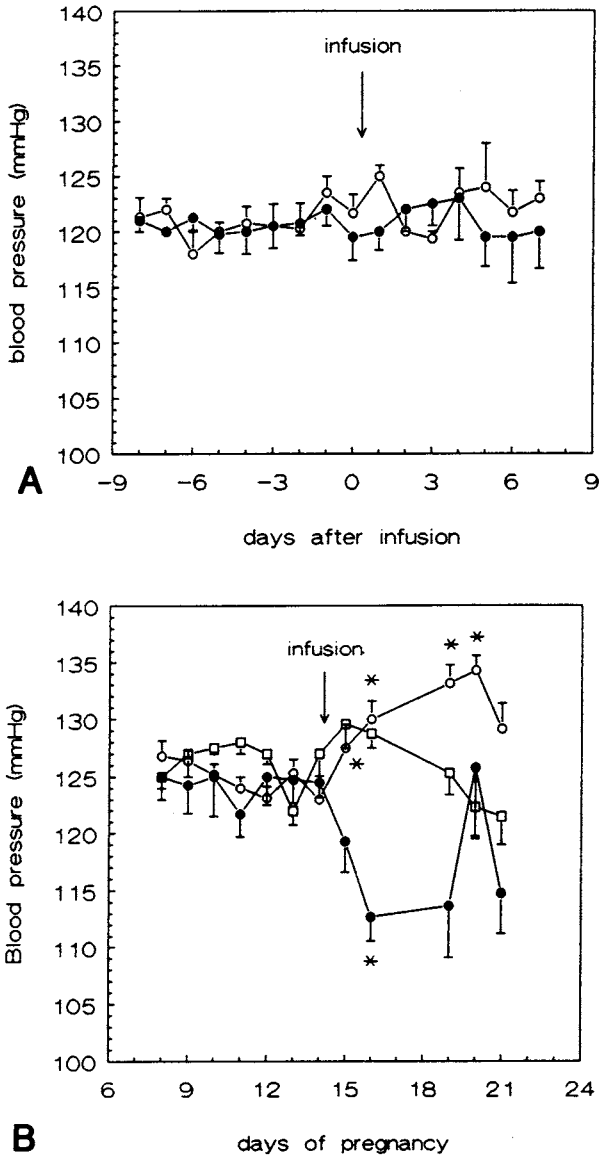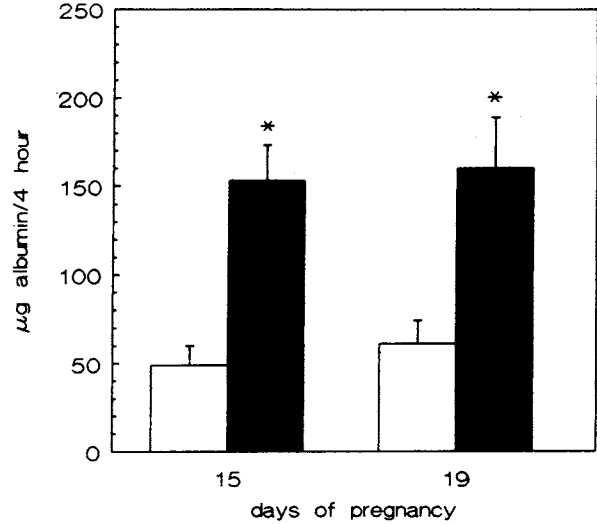
A

days after infusion

Fig. 2. Mean urinary albumin excretion (as measured between 4:30 and 8:30 PM) on days 15 and 19 of pregnancy after infusion on day 14 of 1.0 µg/kg body weight endotoxin (*solid columns*, group I) or saline solution (*open columns*, group III). *Columns* represent mean ± SEM. *Asterisk*, Significantly increased compared with saline solution–treated rats ($p < 0.05$, Mann-Whitney U test).

B

days of pregnancy

Fig. 1. A, Mean systolic blood pressures of cyclic rats measured 8 days before infusion until 7 days after infusion of 1.0 µg/kg body weight endotoxin (○, group IV, $n = 5$) or 6.5 µg/kg body weight endotoxin (●, group V, $n = 5$). Each *symbol* represents mean ± SEM. In none of the days studied were significant alterations of blood pressure compared with preinfusion value at day 0 observed ($p < 0.05$, Wilcoxon signed-rank test). **B,** Mean systolic blood pressures of pregnant rats before and after infusion of either 1.0 (○, group I, $n = 10$) or 6.5 (●, group II, $n = 13$) µg/kg body weight endotoxin or saline solution (□, group III, $n = 6$) on day 14 of pregnancy. Each *symbol* represents mean ± SEM. *Asterisk*, Significant alteration of blood pressure compared with preinfusion value at day 14 ($p < 0.05$, Wilcoxon signed-rank test).

Statistical analysis. Results are expressed as arithmetic means ± SEM. Statistical significance of differences between two means were analyzed by the Mann-Whitney U test or the Wilcoxon signed-rank test, as

indicated in the legends of the figures or in the text. Data of multiple groups were also compared by analysis of variance, as indicated in the text. Data on systolic blood pressure were analyzed by one-way analysis of variance for repeated measures (analysis of variance for repeated measures). Differences were considered significant when a value of $p < 0.05$ was found.

Results

Number of fetuses. No significant differences in mean fetal number or mean fetal weight was observed among the three groups of rats (analysis of variance, $p < 0.05$) (Table I). However, with respect to the number of resorbed fetuses (not shown in Table I) some variation was seen between the three groups; in one mother in group III (saline solution infused, $n = 6$) two fetuses had been resorbed, whereas in one low-dose endotoxin-treated animal (group 1, $n = 10$) one fetus had been resorbed. After infusion of 6.5 µg/kg endotoxin (group II, $n = 13$), however, resorption of one to 13 fetuses (median 8) occurred in six rats.

Systolic blood pressure. In cyclic rats blood pressure was measured during 8 days before and 7 days after infusion of endotoxin. Fig. 1, *A,* shows that the mean blood pressure did not vary significantly during the period before infusion (mean 121.2 ± 0.5 mm Hg) nor after infusion of 1.0 or 6.5 µg/kg body weight of endotoxin (Fig. 1, *A*) (analysis of variance for repeated measures). As can be seen in Fig. 1, *B,* no significant differences in mean blood pressure were observed be-
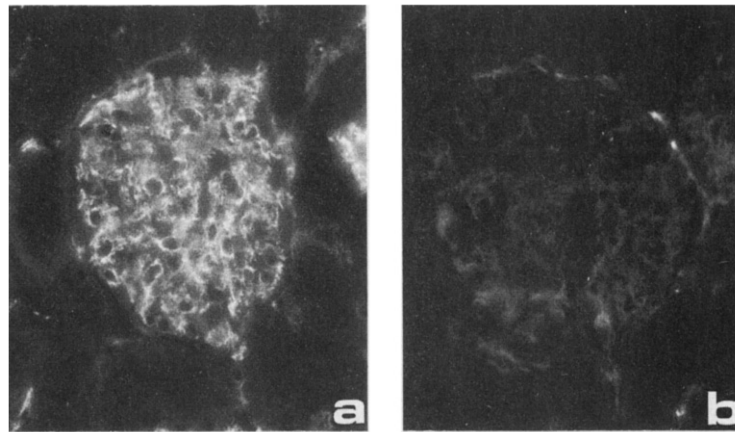
Fig. 3. Immunofluorescent photomicrographs showing renal glomeruli of pregnant rats stained with fluorescein isothiocyanate–labeled goat antirat fibrinogen immunoglobulin G. **a,** Glomerulus of low-dose endotoxin-treated pregnant rat 7 days after infusion (group I) showing fibrinogen deposits predominantly along capillary walls and to lesser extent in mesangial areas; **b,** glomerulus of saline solution–treated pregnant rat in which no significant fibrinogen deposits can be detected. (Original magnification ×250.)

tween the three groups of pregnant rats before infusion (analysis of variance for repeated measures, $p < 0.05$), whereas after infusion with saline solution, 1.0 μg/kg body weight endotoxin, or 6.5 μg/kg body weight endotoxin significant differences in mean blood pressure between the three groups could be observed (analysis of variance for repeated measures, $p < 0.05$). In the saline solution–treated pregnant rats mean blood pressure did not differ significantly from the preinfusion day 14 value throughout the experiment (analysis of variance for repeated measures). After infusion of 1.0 μg/kg body weight endotoxin, mean blood pressure gradually increased and was significantly elevated on days 15, 16, 19, and 20 compared with the preinfusion day 14 value. After infusion of 6.5 μg/kg body weight of endotoxin, on the other hand, mean blood pressure dropped to 112 mm Hg on day 16, followed by a gradual recovery of blood pressure.

Urinary albumin excretion. Before infusion of endotoxin or saline solution mean albumin excretion of pregnant rats was 39 ± 9 μg on day 6 and 73 ± 15 μg on day 12, which was not significantly different from days 15 (49 ± 11 μg) and 19 (61 ± 13 μg) after saline solution infusion (analysis of variance). After infusion of 1.0 μg/kg body weight endotoxin urinary albumin increased significantly on day 15 and day 19 compared with the level in saline solution–treated pregnant rats (Fig. 2).

Cyclic rats did not show increased albumin excretion after infusion of 1.0 μg/kg body weight endotoxin (58.4 ± 4.7 μg, 1 day after infusion and 63.8 ± 32 μg, 5 days after infusion) compared with saline solution–treated cyclic rats (69.07 ± 10 μg, 1 day after infusion and 70.93 ± 10 μg, 5 days after infusion) (Mann-

Table I. Mean fetal number and mean fetal weight on day 21 of pregnancy after infusion of 1.0 μg/kg body weight of endotoxin (group I), 6.5 μg/kg body weight of endotoxin (group II), or saline solution (group III) on day 14 of pregnancy

Group	No. of fetuses	Fetal weight (gm)
I ($n = 10$)	9.9 ± 1.3	5.1 ± 0.18
II ($n = 13$)	11.3 ± 0.48	4.7 ± 0.24
III ($n = 6$)	10.1 ± 0.49	5.09 ± 0.8

Values are mean ± SEM.

Whitney U test, endotoxin vs saline solution infused).

Platelet numbers in peripheral blood. As can be seen from Table II, in pregnant animals receiving low-dose endotoxin (group I) a significant decrease of platelet numbers occurred 1 and 6 days after infusion, in contrast to saline solution-infused pregnant rats (group III). In cyclic rats (group IV) 1 day after infusion of low-dose endotoxin a drop in platelet number could be observed, whereas 6 days after infusion platelet numbers reached normal levels compared with day 0 (before infusion) and in saline solution–treated cyclic rats no significant changes could be found. Mean platelet volume, expressed in femtoliters, showed a significant increase 6 days after infusion of endotoxin in pregnant rats (group I).

Immunohistologic and enzyme histochemical studies

Glomerular fibrinogen deposits. In 10 of 10 pregnant endotoxin-treated rats studied, fibrinogen deposition could be detected on day 21 of pregnancy (Fig. 3, *a*), in
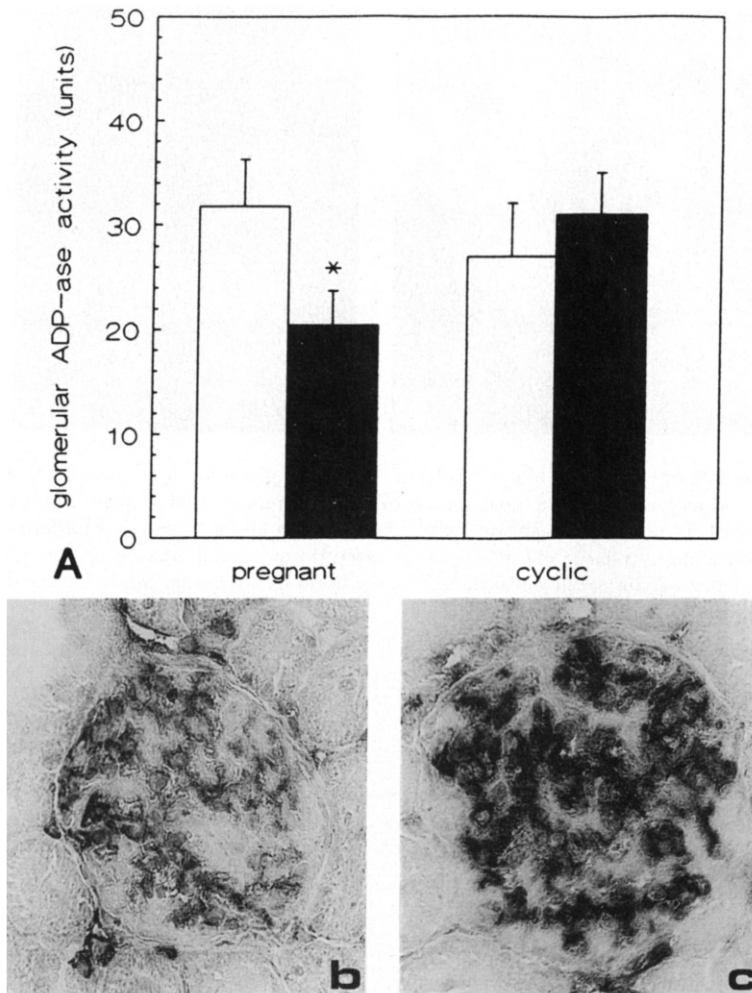
Fig. 4. A, Mean values of quantified intensity of reaction product of glomerular antithrombotic enzyme adenosine diphosphatase of pregnant rats *(left columns)* and cyclic rats *(right colums)* 7 days after infusion of saline solution *(open columns)* or 1.0 μg/kg body weight endotoxin *(solid columns)*. *Columns* represent mean ± SEM. *Asterisk,* Significantly decreased compared with saline solution–infused rats ($p < 0.05$, Mann-Whitney U test). **b** and **c,** Light microscopic photomicrographs of renal glomeruli of pregnant rats 7 days after infusion of 1.0 μg/kg body weight endotoxin **(b)** or saline solution **(c),** stained for adenosine diphosphatase activity. Decreased amount of reaction product can be seen after endotoxin treatment **(b),** whereas abundant reaction product is present in glomerulus of saline solution–treated pregnant rat **(c).** (Original magnification ×350.)

contrast to saline solution–treated pregnant rats in which one of 10 animals showed minor fibrinogen staining; in cyclic rats (endotoxin treated and saline solution treated), no significant fibrinogen depositions could be detected (Fig. 3, *b*).

Glomerular adenosine diphosphatase activity. In pregnant rats decreased glomerular adenosine diphosphatase activity on day 21 of pregnancy was observed after infusion of 1.0 μg/kg body weight endotoxin, compared with saline solution–treated pregnant rats ($p < 0.05$). In contrast, kidneys of cyclic rats did not show decreased adenosine diphosphatase activity after low-dose endotoxin infusion (Fig. 4) compared with saline solution–treated cyclic rats.

Comment

In this study we explored an experimental model for human preeclampsia in which both pathophysiologic and immunohistologic features are expressed. For several reasons the endotoxin-treated pregnant rat was investigated. First, the pregnant rat (like the human) carries a hemochorial placenta[12]; second, endotoxin may induce disseminated intravascular coagulation, which also occurs in preeclampsia[3]; and third, the rat is highly sensitive for endotoxin in the pregnant condition.[13] The (low) dose of endotoxin and the time of infusion used in the current study were explored in previous studies showing reproducible histopathologic alterations in kidney and placental microvasculature.[7, 8]

Table II. Mean platelet number and volume on days 0, 1, and 6 after infusion of saline solution (group III and group VI) or 1.0 μg/kg body weight endotoxin (group I and group IV)

	Mean platelet number ($\times 10^9$/L)			Mean platelet volume (fl)		
	0 Days*	1 Day*	6 Days*	0 Days*	1 Day*	6 Days*
Group I	726 ± 36	457 ± 29†	531 ± 42†	8.63 ± 0.12	8.86 ± 0.18	9.58 ± 0.2†
Group III	1004 ± 65	978 ± 44	893 ± 32	8.20 ± 0.26	8.23 ± 0.18	8.50 ± 0.18
Group IV	887 ± 68	559 ± 18†	900 ± 31	8.40 ± 0.30	8.06 ± 0.60	8.43 ± 0.16
Group VI	757 ± 72	649 ± 32	708 ± 116	8.07 ± 0.38	8.52 ± 0.57	8.15 ± 0.48

Values are mean ± SEM.

*Days after infusion refer for pregnant rats (groups I and III) to days 14, 15, and 20, respectively. Blood samples of day 0 were taken just before start of infusion.

†Significantly different from day 0 ($p < 0.05$, Wilcoxon signed-rank test).

As can be deduced from Table I, the low endotoxin dose (1.0 μg/kg body weight) does not interfere with the continuation of pregnancy, whereas the higher endotoxin dose (6.5 μg/kg body weight), which was tested for comparison in some experiments, showed considerably more fetal resorptions. In spite of histopathologic alterations in the placenta in this model,[8] fetal weight was the same in the groups infused with saline solution and low-dose endotoxin. Apparently in the current experiment the endotoxin-induced pathologic alteration in the placenta did not prevent normal fetal growth during the last week of pregnancy.

It is shown in Fig. 1, B, that 1.0 μg/kg body weight endotoxin, in contrast to 6.5 μg/kg body weight endotoxin, is able to induce a gradually increasing blood pressure during pregnancy. Although the drop in blood pressure after the higher dose of endotoxin in pregnant animals may readily be explained by the "classic" endotoxin effect of hypotension,[14] the increase in blood pressure after low-dose endotoxin infusion remains to be explained. This study, however, unequivocally shows that, depending on the dose, endotoxin is able to induce either hypotension or hypertension in the pregnant rat. Although this dual outcome after endotoxin treatment seems paradoxical, various data in the literature support this notion. For instance, endotoxin has been demonstrated to act predominantly by tumor necrosis factor-α production by macrophages and monocytes,[15] which may lead to release of vasorelaxing factors (i.e., nitric oxide).[16] However, induction by tumor necrosis factor-α of the release of vasoconstricting factors such as endothelin has also been described.[17]

It may be speculated that the increased vascular resistance induced by the current lower dose of endotoxin, which is an ultralow dose indeed, is related to predominant release of endothelin, whereas increased amounts of endotoxin may predominantly induce vasorelactants, leading to hypotension. This hypothesis has to be experimentally tested, notably in view of the conflicting data concerning the role of endothelin in human preeclampsia.[18, 19]

As can be seen from Fig. 2, low-dose endotoxin infusion induces significantly increased albumin excretion in pregnant animals. This finding matches clinical observations in which increased glomerular permeability is a prominent sign of human preeclampsia.[20] Neither in preeclampsia nor in the current model is the exact mechanism underlying increased glomerular permeability fully understood, and it needs further investigation. It is likely that depositions of fibrinoid material in the glomeruli of endotoxin-treated pregnant animals may promote increased glomerular permeability.[21] In addition, endotoxin-induced inflammatory injury of the glomerular filtration barrier may also play a role; exclusively in endotoxin-treated pregnant rats are significant intraglomerular fibrinogen deposits seen (Fig. 3) together with an inflammatory cell influx in the glomeruli (results not shown). Whether increased systemic blood pressure may also contribute to the increment in albumin remains to be tested.

The decreased glomerular adenosine diphosphatase activity observed in this study is also in line with the presence of the inflammatory microenvironment within the glomeruli. As described elsewhere,[22] the antithrombotic ectoenzyme adenosine diphosphatase is highly sensitive for oxygen-free radicals, as shown in other forms of inflammatory disease.[11] The exact mechanism, however, by which exclusively in the pregnant rat minor amounts of endotoxin may create this intraglomerular microenvironment remains to be established. Apparently in this condition even very small amounts of endotoxin are able to induce a cascade of cytotoxic activity (i.e., release of tumor necrosis factor-α from mononuclear cells, which are able to activate neutrophils to produce oxygen-free radicals). This oxygen stress provided by neutrophils in situ may inactivate vascular nucleotidase, leading to a prothrombotic and proinflammatory microenvironment. Additional toxic oxygen molecules may be supplied by increased prostaglandin I_2 synthesis related to pregnancy.[23]

As can be seen from Table II, platelet consumption occurs in this experimental disorder, as is often the case

in human preeclampsia as well.[24] Platelet numbers are decreased after endotoxin infusion, whereas younger thrombocytes also appear in the circulation, as reflected by increased mean platelet volume.[25] In human pre-eclampsia macrothrombocytosis is also seen.[26]

The current results raise the question as to the mechanism of induction of relatively long lasting pre-eclampsia-like features by extreme low doses of endo-toxin in pregnant rats. Neither in human preeclamp-sia[27] nor in this animal model can detectable plasma concentrations of endotoxin be found from 1 day after infusion onward (unpublished results). These data may suggest that endotoxin acts by initiating a self-support-ing process producing symptoms that bear a striking similarity to the clinical and histopathologic signs of preeclampsia. This may point to the existence of a final common pathway of the current low dose endotoxin-induced disorder and human preeclampsia. Although there is no evidence that human preeclampsia is caused by endotoxin, we feel that further investigation of the pathophysiologic mechanisms of this animal model may help elucidate the pathogenesis and cause of this hu-man disorder. Because in this model and in human preeclampsia oxygen-free radical formation has been implicated in the pathogenesis,[28] further experiments to evaluate a putative role of these molecules in the pathogenesis of both spontaneous and experimental preeclampsia are necessary. These data may show that the ultralow dose endotoxin–treated pregnant rat may serve as a suitable model for the study of preeclampsia.

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