Extracellular ATP induces albuminuria in pregnant rats

Marijke M. Faas1, Gerda van der Schaaf2, Theo Borghuis2, Rianne M. Jongman2, Maria G. van Pampus3, Paul de Vos1, Harry van Goor2 and Winston W. Bakker2

1Division of Medical Biology, Department of Pathology and Medical Biology, 2Department of Pathology and Medical Biology and 3Department of Obstetrics and Gynaecology, University Medical Centre Groningen and University of Groningen, Groningen, The Netherlands

Correspondence and offprint requests to: Marijke M. Faas; E-mail: m.m.faas@med.umcg.nl

Abstract

Background. As circulating plasma ATP concentrations are increased in pre-eclampsia, we tested whether increased plasma ATP is able to induce albuminuria during pregnancy.

Methods. Pregnant (day 14) and non-pregnant rats were infused with ATP (3000 µg/kg bw) via a permanent jugular vein cannula. Albuminuria was determined, and blood samples were taken for leukocyte counts, plasma ATP and plasma haemopexin activity. At Day 20 of pregnancy, rats were sacrificed, fetuses and placentas weighed and kidney and placental tissue were snap frozen for immunohistology.

Results. ATP infusion induced albuminuria exclusively in pregnant rats, together with increased neutrophil counts, decreased staining for glomerular sialoglycoproteins and CD39 expression, significant intraglomerular monocyte infiltration and increased glomerular intracellular adhesion molecule-1 (ICAM-1) expression. Plasma haemopexin activity was increased in saline-infused pregnant rats as compared to non-pregnant rats but was inhibited in pregnant ATP-infused rats (to non-pregnant levels). At the end of pregnancy (Day 20), increased plasma ATP level was exclusively seen in ATP-infused pregnant rats. In pregnant rats as compared with non-pregnant rats, we found decreased expression of glomerular AT-1 receptors, which was increased after ATP infusion exclusively in pregnant animals.

Conclusion. The present study shows that ATP infusion induced albuminuria exclusively in pregnant rats, together with increased neutrophil counts, decreased staining for glomerular sialoglycoproteins and CD39 expression, significant intraglomerular monocyte infiltration and increased glomerular intracellular adhesion molecule-1 (ICAM-1) expression. Plasma haemopexin activity was increased in saline-infused pregnant rats as compared to non-pregnant rats but was inhibited in pregnant ATP-infused rats (to non-pregnant levels). At the end of pregnancy (Day 20), increased plasma ATP level was exclusively seen in ATP-infused pregnant rats. In pregnant rats as compared with non-pregnant rats, we found decreased expression of glomerular AT-1 receptors, which was increased after ATP infusion exclusively in pregnant animals.

Keywords: albuminuria; extracellular ATP; haemopexin activity; inflammation; pregnancy

Introduction

Haemopexin (Hx), which belongs to the family of acute phase proteins, shows significant protease activity [1]. Accordingly, active Hx is able to induce proteinuria in rats after intra-renal infusion of Hx in saline [2]. Since proteinuria during pregnancy is a hallmark of pre-eclampsia (PE), a common complication of pregnancy, we tested whether Hx activity was increased during pre-eclampsia. In contrast to our expectation, plasma Hx activity appeared to be significantly lower in patients with pre-eclampsia as compared to normal pregnant control individuals of the same gestational age [3]. Thus, in pre-eclampsia, proteinuria appeared not to be associated with increased plasma Hx activity.

However, in plasma samples from healthy pregnant women, we observed enhanced plasma Hx activity [3] as compared with non-pregnant women. We have shown that Hx activity can be inhibited with serine protease inhibitors or nucleotides like extracellular ATP [4,5]. Indeed, the decreased Hx activity during pre-eclampsia appeared to be due to an increased titre of plasma ATP observed in patients with pre-eclampsia [3]. In vitro treatment of plasma samples from pre-eclamptic patients with ATP-hydrolyzing phosphatases, like apyrase or alkaline phosphatase, leads to reactivation of the Hx activity in these plasma samples [3]. This is in line with previous data, showing that Hx activity can be inhibited by ATP in vitro and subsequently reactivated by apyrase or by endothelial cells expressing ecto-apyrase (CD39) [6]. The possibility of reactivating inactivated Hx by alkaline phosphatase could be the base of a future therapeutic approach, since alkaline phosphatase is a naturally occurring enzyme, the concentration of which is increased in pregnancy and decreased in pre-eclampsia [7]. Interestingly, alkaline phosphatase is presently used in phase II clinical trials for other disorders (http://clinicaltrials.gov/ct2/show/related/NCT00727324).

The observation that pre-eclampsia is associated with increased plasma ATP concentrations has led to the hypothesis that extracellular ATP per sé may be toxic, particularly...
during pregnancy. Therefore, we now conducted experiments in which pregnant and non-pregnant rats were infused with ATP (3000 µg/kg BW) during 1 h at Day 14 of pregnancy. Urinary protein excretion, glomerular inflammation and expression of glomerular angiotensin II receptor 1 (AT(1)-R) as well as various inflammatory parameters of the circulation were evaluated.

Materials and methods

Animals

All animal experiments were approved by the Animal Care Ethics Committee of our University. Female Wistar outbred rats (about 200 g) were kept in a temperature- and light-controlled room (lights on from 7:30 AM till 7:30 PM) with free access to food and water. Until selection for experiments, vaginal smears were taken daily, and rats were rendered pregnant by housing them on pro-oestrus with fertile males for one night.

When spermatozoa were detected in the smear the next day, this day was designated as Day 0 of pregnancy. In all rats, a cannula was inserted into the right jugular vein under fluothane/oxygen anaesthesia according to the method of Steffens [8]. This cannula allows stress-free blood sampling and infusions.

Experimental design

Pregnant rats were infused with 3000 µg/kg bw ATP in 2.0 ml saline (n = 10) or with 2.0 ml saline alone (n = 10) on Day 14 of pregnancy. Non-pregnant rats were infused with 3000 µg/kg bw ATP in 2.0 ml saline (n = 8) or saline alone (n = 8) on di-oestrus. For determination of albumin excretion, rats were placed in metabolic cages for 24 h 2 days before (Day 12), immediately after (Day 15), 3 days after (Day 17) and 6 days after the infusion (Day 20). At this time, none of the rats had delivered or started parturition. At the same days, blood samples (0.4 ml in EDTA) for white blood cell counts, plasma ATP concentrations and plasma Hx activity were taken from the jugular vein cannula. Six days after the infusion (i.e. Day 20 in pregnant rats, which is almost the end of pregnancy in the rat, delivery is at Day 21), rats were sacrificed by bleeding under fluothane/oxygen anaesthesia. Fetuses and placenta were collected and weighed, and kidney and placenta tissue samples were snap frozen and prepared for immunohistochemistry.

Measurement of urinary albumin

Rats were placed in metabolic cages for 24 h from 11:00 AM until 11:00 AM the next day. The volume of urine samples was measured, and urinary albumin levels were determined using rocket electrophoresis (Promega, Madison, WI, USA) as described before [9].

Leukocyte counts and differential leukocyte counts

Blood leukocytes were counted using a microcellcounter (model Sysmex F800, Toa Medical Electronics, Kobe, Japan). A smear was made from each blood sample and stained according to standard methods with the May–Grunwald–Giemsa method. After evaluation of 500 cells per smear, relative and absolute numbers of neutrophils, lymphocytes and monocytes were calculated according to standard methods.

Evaluation of plasma Hx activity

Protease activity of plasma Hx was evaluated by the ‘glomerular ECM stripping assay’ as described previously [2,5,10]. This in vitro assay is based on the potential impairment by Hx of glomerular extracellular matrix molecules (ECM), such as expression of sialo glycoproteins or CD39 expression. Acetone-fixed cryostat normal rat kidney tissue is incubated with either (1:8) diluted plasma samples (100 µl/section for 60 min; plasma from 3 and 6 days after the infusion) from pregnant rats infused with saline, pregnant rats infused with ATP non-pregnant rats infused with saline and non-pregnant rats infused with ATP. Following incubation, sections were washed and stained for glomerular ECMs (i.e. sialo glycoproteins, using standard histochemical methods [2,5,10]). To check for the specificity of the effect, incubations were done with or without supplementation of monoclonal anti-Hx IgG (150 µg/ml; kindly provided by Dr. E. Hansen, Southwestern Medical Center, University of Texas, Dallas). The inhibition by monoclonal anti-Hx IgG of the enzymatic impairment of glomerular ECM by plasma in vitro showed that the impairment is the result of Hx activity rather than activity of other plasma enzymes. Reaction product was semi-quantitatively evaluated in a double-blind fashion, with approximately 20 representative glomeruli per section (four sections per animal were scored, and the arithmetic mean of each group was expressed as arbitrary units). Evaluation was done using an arbitrary scale: 0–2 abundant staining; 2–4 clearly detectable staining; 4–6 faint staining; 6–8 very faint staining; 8–10 very faint to undetectable staining [2,5,10]. H-associated protease activity was calculated (a relatively low amount of reaction product reflects relatively high Hx protease activity).

Evaluation of plasma ATP

EDTA blood samples were diluted (1:3) with PBS supplemented with EDTA (1.0 mM). Extracellular ATP was assayed according to Gorman et al. [11], with minor modifications according to the manufacturer’s instructions (Promega, Madison, WI, USA). Diluted plasma (150 µl) was incubated with 150 µl substrate, i.e. 87.0 µg/ml luciferin (Beetle E 1602, Promega, USA) supplemented with MgCl2 (10.0 mM), and luciferase (500 000 RLU/ml in PBS; Quantum e-luciferase; Promega, Madison, WI, USA). The relative light units (RLU) were detected using a luminometer (Thermo Luminoskan Ascent, Thermo Fisher Scientific, Waltham, MA, USA) in 96-well ELISA trays.

Immunohistochemistry and enzyme histochemistry

Staining for CD39, 5’-nucleotidase activity (CD73), the presence of sialoglycoproteins (colloidal iron staining) and for monocytes, neutrophils, lymphocytes, ICAM-1 and the angiotensin II receptor were done according to standard methods (see supplemental material).

Intraglomerular inflammatory cell infiltration, glomerular ICAM-1, glomerular CD39 and glomerular staining for sialoglycoproteins

Kidney sections stained for monocytes, neutrophils and lymphocytes were quantitatively scored by light microscopical examination in a double-blind manner by two independent observers. Positive cells were counted in 100 glomeruli, and results are expressed as number of positive cells per glomerulus. Kidney sections stained for glomerular sialoglycoproteins, glomerular ICAM-1 expression or glomerular CD39 expression were semi-quantitatively graded by scoring a total of 100 glomeruli per section using an arbitrary scale from 1 to 5 as described before [12]: 1: no staining; 2: very little staining; 3: weak staining; 4: moderate staining; 5: bright staining. The mean of two sections per rat was taken, and results are expressed as mean arbitrary units per glomerulus.

Placental CD39 and CD73

Placental sections stained for CD39 expression and CD73 activity were semi-quantitatively graded by two independent observers by scoring the staining intensity per section using an arbitrary scale from 1 to 5 as described previously [12]: 1: no staining; 2: very faint staining; 3: weak staining; 4: moderate staining; 5: bright staining. Each observer scored two sections per rat. For each rat, the mean of these four scores was calculated.

Statistics

Statistical evaluation of the differences between experimental and control groups were done using the Mann–Whitney U-test. Differences between various days of the experiments (i.e. for albumin excretion and white blood cell counts) were evaluated using the Wilcoxon’s signed rank tests as indicated in the legends to the figures. Differences were considered to be significant if P < 0.05. To evaluate the relationship between albuminuria and glomerular monocyte number, we performed linear regression analysis (least sum of squares method). We calculated R² and the slope and tested whether the slope was statistically significantly different from zero.
Results

Urinary albumin excretion

Figure 1 shows that ATP infusion increased 24-h albumin excretion, peaking at Day 17, exclusively in pregnant rats. No significant alterations after ATP infusion in non-pregnant rats were observed; no effect of saline infusion was observed.

Colloidal iron staining

To demonstrate that loss of glomerular charge selectivity can play a role in the albuminuria observed, we stained kidney sections for glomerular sialoglycoproteins, reflecting glomerular anionic sites. Mean arbitrary scores for glomerular sialoglycoprotein staining are shown in Figure 2a. It can be observed that the amount of reaction product is significantly decreased in pregnant ATP-infused rats as compared with pregnant saline-infused rats. No difference of glomerular anionic sites between saline-infused pregnant and saline-infused non-pregnant rats could be observed; nor did we observe an effect on glomerular anionic sites after ATP infusion with respect to this staining in non-pregnant rats.

Representative glomeruli stained for sialoglycoproteins; glomerulus of a pregnant rat infused with saline (left photomicrograph) and a pregnant rat infused with ATP (right photomicrograph). Strong staining along the capillary walls and the mesangial area of the glomerulus of a pregnant rat infused with saline can be seen in the left micrograph, while a clear loss of stainability for sialoglycoproteins of the capillary walls and the mesangium in a glomerulus of an ATP-infused pregnant rat is shown in the right micrograph.

Fig. 1. Mean (± SEM) urinary 24-h albumin excretion in saline-infused pregnant rats (black bars), ATP-infused pregnant rats (open bars), saline-infused non-pregnant rats (dotted bars) and ATP-infused non-pregnant rats (striped bars). *P < 0.05, Wilcoxon, significantly different from pre-infusion value.

Fig. 2. (a) Mean (± SEM) arbitrary units of glomerular staining for sialoglycoproteins in saline-infused pregnant rats (black bars), ATP-infused pregnant rats (open bars), saline-infused non-pregnant rats (dotted bars) and ATP-infused non-pregnant rats (striped bars). *P < 0.05, Mann–Whitney U-test, significantly different from saline-infused pregnant rats. (b) Representative glomeruli stained for sialoglycoproteins; glomerulus of a pregnant rat infused with saline (left photomicrograph) and a pregnant rat infused with ATP (right photomicrograph).
crographs show strong staining along the capillary walls and the mesangial area of the glomerulus of a pregnant rat infused with saline (left photomicrograph) and a clear loss of stainability for sialoglycoproteins of the capillary walls and the mesangium in a glomerulus of an ATP-infused pregnant rat (right photomicrograph).

Glomerular inflammation

To evaluate whether the loss of glomerular sialoglycoproteins reflecting that affected charge selectively is induced by an inflammatory response and/or oxidative stress, we evaluated glomerular inflammation by staining kidney sections for intraglomerular influx of monocytes, neutrophils and lymphocytes as well as for the expression of ICAM-1, indicating endothelial cell activation. As a parameter for the presence of oxidative stress, we measured the expression of CD39, an ecto-enzyme which is extremely sensitive to oxidative stress [13].

It can be seen that the mean number of intraglomerular monocytes increased after ATP infusion in pregnant rats as compared to non-treated pregnant rats (Figure 3). We found no effect of ATP infusion on glomerular monocyte number in non-pregnant rats. At this interval after infusion, i.e. 6 days, we only found a single neutrophil or lymphocyte in the glomeruli of the kidneys in the various groups of rats; no differences between the four groups of rats were observed for neutrophil or lymphocyte numbers (results not shown).

Since we found increased numbers of monocytes but not of neutrophils or lymphocytes in the glomeruli of pregnant rats following ATP infusion, we evaluated whether there was a relationship between the number of infiltrated glomerular monocytes and the amount of albumin excreted in the urine (Figure 4). In Figure 4, the regression line represents a linear relationship between the number of ED-1 positive cells per glomerulus at Day 20 vs the 24-h urinary albumin excretion at the same day in pregnant ATP-treated rats ($R^2 = 0.68$; slope 492.4, significantly different from zero, $P < 0.05$). No relationship was found between intraglomerular ED-1 positive cells and 24-h albumin excretion in pregnant saline-treated or non-pregnant ATP- or saline-treated rats.

Upregulation of endothelial ICAM-1 indicates pro-inflammatory activation of these cells. Figure 5 shows increased glomerular ICAM-1 expression in pregnant ATP-infused rats as compared with saline-infused pregnant rats. No effect of ATP infusion was seen upon glomerular ICAM-1 expression in non-pregnant rats. Figure 5b shows representative glomeruli of pregnant rats, infused with saline (left panel) or ATP (right panel) stained for ICAM-1. ICAM-1 expression can be seen in the glomerular tuft in pregnant rats following ATP infusion, indicating activation of endothelial cells but not in pregnant rats following saline infusion.

Thus decreased expression of CD39 may reflect endothelial injury induced by oxygen free radicals released during a local inflammatory response. It can be seen that
glomerular CD39 expression is significantly decreased in pregnant ATP-treated rats as compared with saline-infused pregnant rats (Figure 6a), indicating oxidant injury within glomeruli of pregnant ATP-infused rats. No significant alterations of glomerular CD39 expression were observed after infusion of ATP into non-pregnant rats as compared with saline-infused non-pregnant rats. Figure 6b shows representative glomeruli from pregnant rats stained for CD39 expression. The pregnant saline-infused rat shows a linear staining pattern along the capillary walls (left panel). After infusion of ATP in pregnant rats, significant loss of CD39 throughout the glomerular tuft can be seen (right panel) as compared with glomeruli of pregnant saline-infused rats.

ATP-induced inflammatory cells in the circulation
To evaluate the potential systemic nature of the ATP-induced inflammatory response, we studied the number of circulating total leukocytes, neutrophils, monocytes and lymphocytes in ATP-infused pregnant rats vs control animals. The numbers of peripheral leukocytes counted at various intervals after ATP or saline infusion in pregnant and non-pregnant rats are shown in Figure 7. After infusion of ATP in pregnant rats, a significant increase of the mean leukocyte number was seen 1 and 3 days after ATP infusion, while the mean leukocyte number was decreased 6 days after ATP infusion as compared with the mean leukocyte number before infusion. In normal pregnant rats, the mean leukocyte number was also significantly decreased 6 days after the infusion of saline, i.e. at the end of pregnancy. No significant effect was observed regarding mean leukocyte number in non-pregnant rats infused with ATP or saline. As can be seen in the upper right panel of Figure 7, the mean neutrophil number was increased 1, 3 and 5 days after ATP infusion in pregnant rats as compared with the pre-infusion level. Mean monocyte number was decreased in pregnant rats after ATP infusion at Day 20 as compared with the pre-infusion level. Mean lymphocyte number increased 3 days after infusion and decreased 6 days after infusion of ATP as compared with pre-infusion levels (Day 12). No effect of ATP or saline infusion was seen on mean levels of leukocytes, neutrophils, lymphocytes or monocytes in non-pregnant rats.

Plasma Hx activity and plasma ATP titre
Next to its pro-inflammatory activity, ATP may act through inactivation of Hx, an effect which has been demonstrated in vitro [4,5]. We therefore measured plasma Hx activity and plasma ATP concentrations in the experimental and control groups. As can be seen from Figure 8, mean plasma Hx activity was increased in saline-infused pregnant rats as compared to saline-infused non-pregnant rats. On the other hand, mean plasma Hx activity is significantly
lower in ATP-treated pregnant rats as compared to saline-infused pregnant rats. Figure 9 shows that 6 days after ATP infusion, mean plasma ATP level was significantly increased in pregnant ATP-treated rats as compared to saline-infused pregnant rats and ATP-treated non-pregnant rats. In saline-infused non-pregnant rats, plasma ATP concentrations were not affected by the infusion.

Glomerular expression of the AT-1R

As activated Hx is able to decrease the expression of the AT-1R upon various cells in vitro [14], it was expected that the AT-1R was diminished in the tissue of control pregnant rats with increased Hx activity but not in ATP-infused rats in which circulating Hx was inhibited. Therefore, we stained kidney sections of the experimental and control rats for the presence of the AT-1R. Figure 10 shows representative micrographs of glomeruli immunohistologically stained for AT-1R expression. In non-pregnant control animals (Figure 10c), positive staining for AT-1R in a mesangial pattern can be observed. In line with our expectations, it can be observed that the glomerular expression of AT-1R is decreased in normal pregnant rats (with circulating active Hx) as compared with control non-pregnant rats, with inactivated Hx (Figure 10a and c). Pregnant rats, in contrast to non-pregnant animals, showed enhanced expression of AT-1R after infusion of ATP as compared to saline infusion (Figure 10b and d).

Bodyweight of fetuses

Finally, we studied the effect of ATP infusion on the weight of fetuses and placentas. Figure 11 shows that fetal weight was slightly but significantly decreased in pregnant rats infused with ATP as compared to saline-infused pregnant rats. No significant effect was seen on placental weight. There were no differences in mean numbers of fetuses between the ATP and saline-infused rats, and we found no fetal resorptions or fetal death in either group (results not shown).

Placental expression of CD39 and CD73

Although placental weight is not affected by ATP infusion, the fact that fetal weight is decreased suggests that placental function is impaired after ATP infusion. As affected placental function may be caused by ischaemia, we evaluated placental expression of CD39 and as well as CD73 activity (Figure 12). Decreased CD39 expression together with increased CD73 activity has been recognized as a hallmark of ischaemia [13,15]. Photomicrographs of representative placental tissue stained for CD39 or CD73 are shown in Figure 12a–f, while semi-quantitative evaluation of the staining intensity, using an arbitrary scale, is shown in Figure 12g. ATP-infused rats showed decreased placental CD39 expression as compared to control rats. It can be seen that CD39 expression is decreased in the maternal vasculature (V) of ATP-treated rats vs saline-infused rats.
(D vs C), while also in the microvasculature of the labyrinth (L), expression of CD39 is decreased in ATP-infused pregnant rats vs control pregnant rats (B vs A). Panels E and F show representative micrographs of enhanced placental activity of CD73 in a pregnant ATP-infused rat (panel F) vs a pregnant saline-infused rat (panel E). In contrast to saline-infused pregnant animals, ATP-treated pregnant rats showed increased CD73 activity in giant trophoblast cells (GT) and between the decidua (DB) and the giant trophoblast cell layer (GT). As can be seen in Figure 12g, the CD73 activity was significantly increased in placental sections of ATP-infused pregnant rats as compared with saline-infused pregnant rats (Figure 12g).

**Discussion**

As we previously observed enhanced plasma ATP levels in subjects with PE as compared with healthy pregnant women of the same gestational age [3], the notion emerged that extracellular ATP may be a potentially toxic molecule for the pregnant rat. The aim of the present study was to sub-

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**Fig. 7.** Mean (± SEM) peripheral WBC counts and mean granulocyte, lymphocyte and monocyte counts in saline-infused pregnant rats (black bars), pregnant ATP-infused rats (open bars), saline-infused non-pregnant rats (dotted bars) and non-pregnant ATP-infused rats (striped bars). *P < 0.05, Wilcoxon, significantly different from pre-infusion value.

**Fig. 8.** Mean (± SEM) plasma haemopexin activity in saline-infused pregnant rats (black bars), pregnant rats infused with ATP (open bars), saline-infused non-pregnant rats (dotted bars) and non-pregnant rats infused with ATP (striped bars). Plasma haemopexin activity is increased in saline-infused pregnant rats as compared with saline-infused non-pregnant rats, while in pregnant ATP-infused rats, plasma haemopexin activity is decreased vs saline-infused pregnant rats. *P < 0.05, Mann–Whitney U-test, significantly different from saline-infused pregnant rats. a, significantly different from saline-infused non-pregnant rats (P < 0.05, Mann–Whitney U-test).
tostantiate this hypothesis. Therefore, pregnant rats infused with ATP on Day 14 of pregnancy were compared with control pregnant rats infused with saline and with non-pregnant rats which also were given a single infusion of ATP or saline. Since at Day 14 of pregnancy the placenta of the rat has fully developed, we selected this gestational age for the ATP infusion. Slow infusion was carried out via a permanent jugular vein cannula in conscious conditions, which is preferable over a single injection in view of the short half-life of extracellular ATP in vivo [16]. It appeared that exclusively in pregnant rats, urinary albumin excretion associated with decreased loss of glomerular anionic sites occurred after ATP infusion. Next to systemic
as well as glomerular inflammation, also placental ischaemia was observed in ATP-infused pregnant rats vs saline-infused pregnant rats. Finally, the drop of mean fetal weight observed in ATP-treated pregnant rats may be considered a consequence of the pro-inflammatory and ischaemic events occurring in these animals.

Since systemic inflammation seems to be elicited by ATP infusion in pregnant rats as reflected by increased numbers of circulating inflammatory cells and lymphocytosis, we feel that albuminuria may be due to this inflammatory response. This notion is supported by the observations that glomerular ICAM-1 expression is upregulated exclusively in pregnant ATP-infused rats and that the mean intraglomerular influx of ED-1 positive monocytes correlates with the albumin excretion. The absence of significant enhancement of monocytes in the circulation in ATP-treated pregnant rats, in contrast to neutrophils and lymphocytes, is in line with the influx of these cells into the glomeruli of the...
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of a single ultra-low dose of the pro-inflammatory lipopolysaccharide (LPS) resulted in a relatively strong and persistent inflammatory response in pregnant rats in contrast to non-pregnant rats showing little response [12,35].

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

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Conflict of interest statement. None declared.

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