Nitric oxide and vascular reactivity in pregnant rats with adriamycin nephropathy

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1. In previous studies we have shown that, after the administration of adriamycin, hypertension developed in rats who became pregnant (adriamycin-pregnant rats), whereas virgin animals remained normotensive. Subsequently, we showed that this hypertension was prevented by administration of L-arginine, suggesting that deficient synthesis of nitric oxide may be pathogenetic in this model.

2. To further assess the role of nitric oxide in this model, we measured mean arterial blood pressure after administration of L-arginine to adriamycin-pregnant rats or of N\textsuperscript{G}-nitro-L-arginine-methyl ester (L-NAME) to normal pregnant rats. In other experiments, we assessed the response of isolated perfused arterial mesenteric vessels, precontracted with noradrenaline, to acetylcholine, L-arginine or L-NAME.

3. Blood pressure was decreased in normal pregnant rats, whereas it was elevated in adriamycin-pregnant rats. L-NAME treatment increased blood pressure in normal pregnant rats and L-arginine decreased it in adriamycin-pregnant rats.

4. Mesenteric vessels of adriamycin-pregnant rats exhibited an exaggerated vasoconstrictory response to noradrenaline, when compared with the blunted response observed in normal pregnancy. The addition of L-NAME \textit{in vitro} induced a further contraction, significantly greater in normal pregnant rats. The vasodilatory response to acetylcholine and L-arginine was greater in vessels from adriamycin-pregnant rats. In contrast, responses to either nitroprusside or diazoxide were similar in all groups.

5. The results suggest a state of reduced nitric oxide synthesis in rats with adriamycin nephropathy, leading to vascular maladaptation and hypertension in pregnancy.

INTRODUCTION

During pregnancy, profound changes occur in the maternal circulation. Blood volume at term exceeds the non-pregnant values by 45–55%. Stroke volume and cardiac output rise by about 40%, whereas blood pressure falls to a nadir in the second trimester, due to decreased peripheral vascular resistance. The responsiveness of the systemic vasculature to pressor agents, such as angiotensin II, vasopressin and catecholamines, is greatly reduced [1]. The vascular endothelium plays a central role in the regulation of peripheral resistance and blood pressure, by inactivating vasoactive substances such as serotonin and bradykinin and by producing pressor substances (endothelin-1 and angiotensin II) as well as the potent vasodilators prostacyclin and nitric oxide (NO). Convincing evidence supports the concept that the attenuated systemic vascular response associated with pregnancy results from changes of endothelial function [2–4]. This phenomenon has also been described in pregnant rats [5]. Recent studies have addressed the role of NO in the vascular adaptation of pregnancy. The biosynthesis of NO is increased during pregnancy [2], due to increased mRNA [6] and increased activity [7] of the endothelial nitric oxide synthase in the vasculature of pregnant rats and guinea pigs. Acetylcholine-mediated relaxations are potentiated in the mesenteric vessels of pregnant rats [8–10] and carotid artery rings of pregnant guinea pigs [11].

The haemodynamic changes occurring during normal pregnancy are reversed in pre-eclampsia, either primary or superimposed on pre-existing renal disease. Peripheral vascular resistance is increased and the response to angiotensin II is exaggerated [12], even if this seems to be quite a late phenomenon [13]. There is strong evidence for an imbalance in the endothelial synthesis of vaso-dilators and vasoconstrictors in pre-eclampsia [14]. In animals, inhibition of NO synthesis reproduced a clinical picture close to human pre-eclampsia [15, 16].

We have recently studied the influence of pregnancy in rats with adriamycin nephropathy. The intravenous injection of 3.5 mg/kg of body weight of

Key words: Mesenteric artery; noradrenaline; pregnancy; vascular reactivity.

Abbreviations: ADRP, rats mating 2 weeks after adriamycin administration; ADRV, virgin rats studied 5 weeks after adriamycin administration; MAP, mean arterial pressure; L-NAME, N\textsuperscript{G}-nitro-L-arginine-methyl ester; NP, normal pregnant.

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adriamycin produced minimal proteinuria, without changes in blood pressure. In animals who became pregnant 2 weeks after the administration of the drug, hypertension and heavy proteinuria ensued, as well as a lack of the expected increase in the glomerular filtration rate [17]. This picture was reversible 15 days after delivery [18], suggesting that, with adriamycin, a latent alteration of vascular function disturbed the adaptive processes required by pregnancy. In a previous study, we observed that the increase in blood pressure in pregnant rats with adriamycin nephropathy was prevented by L-arginine administration [19], suggesting that defective NO synthesis could play a role in the development of hypertension in these animals. In the present studies we have further investigated the possible role of NO in this model, by measuring the effects of stimulation or inhibition of NO synthesis on vascular reactivity of the isolated perfused arterial mesenteric bed. The results obtained were compared with the effects of these same manipulations in vivo.

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MATERIALS AND METHODS

Animals and protocols

Female Wistar rats, weighing about 200 g, received a normal diet (0.35 g of NaCl and 20 g of protein per 100 g) and tap water ad libitum. In some of the animals, adriamycin (3.5 mg/kg of body weight) was injected intravenously through one superficial femoral vein, under light ether anaesthesia. Rats were mated with a fertile male for 4 days and day one of gestation was documented by the presence of spermatozoa in the vaginal lavage. For all experiments, rats were divided into four groups: controls, normal pregnant rats (NP, 20–22 days of gestation), ADRV (virgin rats studied 5 weeks after adriamycin administration) and ADRP (rats mating 2 weeks after adriamycin, studies performed at 20–22 days of gestation). Virgin rats were age-matched with their respective pregnant groups.

Measurement of mean arterial pressure (MAP)

Rats were divided into six groups (six animals each): (i) controls; (ii) ADRV; (iii) NP; (iv) NP who received NO-nitro-L-arginine-methyl ester (L-NNAME) (100 mg/l) added to the drinking water from day 11 of pregnancy; (v) ADRP; and (vi) ADRP who received L-arginine (2 g/l) from day 11 of pregnancy. The animals were put into individual metabolic cages at days 7, 14 and 20–21 of pregnancy (or the corresponding day in non-pregnant animals) to monitor water and drug intake. The daily dosage of L-NNAME averaged 10 mg/kg of body weight and that of L-arginine 150 mg/kg of body weight. On the day after the last metabolic cage period, MAP was measured. One femoral vein and the tail artery were cannulated under light ether anaesthesia, the entire procedure lasting about 10 min. After surgery, the rats were allowed to recover in the plexiglass cage for 3–4 h, while receiving a continuous infusion of isotonic NaCl (3 ml/h). MAP was monitored continuously using a P 231 D Gould transducer and recorded with an M.G. Electronic Recorder (model B2599, Rehovoth, Israel). The average of ten consecutive measurements, made at 1 min intervals over a period of 10 min, was calculated. Rats were adapted to restraining plexiglass cages for a total of at least 10 h during the week before the measurements.

Perfusion studies in isolated perfused mesenteric vessels

For studies in vitro, rats were divided into the same four groups: controls, NP, ADRV and ADRP. The superior mesenteric vascular bed of the rat was prepared using the method of McGregor [20] with some modifications. Rats were anasthetized with pentothal (50 mg/kg of body weight intraperitoneally). After laparotomy, the superior mesenteric artery was rapidly cannulated and perfusion was immediately initiated at a rate of 1.4 ml/min. All the main branches, except two, were ligated. The vascular bed was then dissected out at the intestinal border. The final preparation comprised two main branches, with an internal diameter of approximately 200–250 μm, and third order arterioles were cut before their entry into the intestinal wall. The main mesenteric artery itself was completely occupied by the cannula. The vessels were perfused during dissection and throughout the successive experiments with a solution containing (mmol/l): NaCl 130, KCl 4.7, MgSO4 1.15, NaHCO3 15, Na2HPO4 1.15, KH2PO4 1.25, CaCl2 1.25 and glucose 5, at pH 7.4, po2 200 mmHg and temperature 37°C. The vessels were put in a tightly sealed chamber and perfused at a constant rate via two syringe pumps (GF-SP4, Foures S.A., France). The inflow line was connected via a lateral line to a mercury column, whose height was set at 80 mmHg, allowing the perfusate to overflow whenever vessel contraction increased resistance and intravascular pressure exceeded that of the mercury column. Outflow from the chamber occurred through a polyethylene tube (PE 50, i.d. 0.8 mm) and a 24 G needle. Perfusion was performed at constant pressure (80 mmHg) and inflow (1.4 ml/min). Under these conditions, perfusate outflow is inversely proportional to resistance. Experiments were started after allowing 60 min for equilibration. Concentrations of the different drugs employed were adjusted in the two syringes so that, by simultaneous change of infusion rates in opposite directions, concentrations were varied, whereas total inflow remained constant. Per fusate drops outflow-
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...ing from the chamber were counted with a drop counter connected to a PC computer, recorded continuously and transformed into a graphic representation of flow, so that the experiments could be followed on-line. Preliminary measurements showed that drop volume was remarkably constant (10 μl). In addition, outflow perfusate was collected for weighing as specified below. No experiment lasted more than 90 min. At the end of each experiment the presence of a viable endothelium was demonstrated by the presence of vasodilatation with acetylcholine (10⁻⁶ mol/l) (Fig. 1). Occasional preparations not responding to acetylcholine were not included in the study.

Fig. 1. Representative tracings for each of the four protocols in isolated perfused mesenteric beds, after allowing flow to stabilize for 1 h. B1 is the basal value of flow at the end of this period: note that outflow averages 1200 litres/min, being lower than the quantity furnished by the syringe pumps (1400 litres/min) due to the spontaneous tone of the vessels. Thus, under basal conditions, there is a small overflow through the mercury column and the vessels are pressurized at 80 mmHg. B2 represents the basal value of flow in the contracted state, used to assess the effect of the various substances tested. (a) Concentration–response curve with noradrenaline; (b) effect of L-arginine; (c) effect of L-NAME; (d) effect of nitroprusside and diazoxide. Abbreviations: NORAD, noradrenaline; ACChol, acetylcholine; NP, nitroprusside; DZ, diazoxide.
Protocols used

Concentration–response curves with noradrenaline (Fig. 1a). This protocol was performed in ten controls, ten NP, ten ADRP and six ADRV rats. One syringe contained noradrenaline (10 μmol/l), while the second contained buffer only. By changing the pumping rates of the two syringes in opposite directions, the final concentration of noradrenaline in the perfusate was increased every 7 min over a range from 1–10 μmol/l. The outflowing perfusate was collected during the last 2 min of each period and measured gravimetrically. The flow at each concentration was compared with that measured at the end of the equilibration period (baseline, Bl). At the end of each curve, after flow was stabilized with noradrenaline (10 μmol/l) (indicated as B2), acetylcholine (10^-6 mmol/l) was added and flow was measured after 5, 10 and 15 min.

Effects of l-arginine (Fig. 1b and Fig. 2). This protocol was performed in five controls, five NP, six ADRV and five ADRP rats. The mesenteric vessels were precontracted to flow values of 30–40% of baseline (corresponding to an increase in resistance 70–80% of the maximum response) with noradrenaline (2–3 μmol/l). Once perfusate outflow was stabilized (B2), l-arginine (10^-2 mol/l) was added and perfusate measured every 5 min for 25 min. Preliminary experiments showed that l-arginine at lower concentrations lacked any effect. However, its effect at a concentration of 10^-2 mol/l was sustained and specific, as D-arginine at similar concentration only produced a transient dilation followed by a rapid return to precontraction values (Fig. 2).

Responses to acetylcholine and l-NAME (Fig. 1c). Twenty-two rats were used for this protocol (five controls, seven NP, five ADRV and five ADRP). The vessels were precontracted with noradrenaline as described for l-arginine experiments. Once perfusate outflow was stabilized (B2), acetylcholine (10^-4 mol/l) was added and three collections were obtained as described above. Acetylcholine was then stopped and outflow was allowed to stabilize again with noradrenaline. l-NAME (10^-4 mol/l) was then added while the rate of noradrenaline remained the same. The perfusate was collected every 5 min for 20 min. After stopping l-NAME, outflow was allowed to stabilize again with noradrenaline, and then acetylcholine was added again as described above.

Effect of nitroprusside or diazoxide (Fig. 1d). For this protocol, 22 rats were used (five controls, seven NP, five ADRV and five ADRP). The vessels were maximally precontracted with noradrenaline (10 μmol/l). After flow was stabilized (B2), nitroprusside (10^-5 mol/l) was added for 10 min, and perfusate was collected every 2 min. After stopping nitroprusside, the vessels were allowed to stabilize again for 20 min (flow returning to the precontracted state after 3–4 min). Diazoxide (10^-5 mol/l) was then added for 10 min and perfusate collected as described above. After stopping diazoxide and stabilization for 20 min, the vessels were challenged with acetylcholine (10^-6 mol/l) as described above.

Drugs

Noradrenaline/HCl, l-NAME, l-arginine, D-arginine, nitroprusside and diazoxide were all from Sigma Co, St. Louis, MO, U.S.A. All drug solutions were prepared immediately before each experiment.

Statistical analysis

All values are expressed as means (SEM). For the studies in vitro, values of flow were calculated as percentages of the baseline (B1, i.e. no drug added for the noradrenaline curves; or B2, i.e. values during precontraction for the other protocols). Differences between groups were analysed with one-way analysis of variance and multiple comparisons using the method of least-protected differences. Differences between pre- and post-l-NAME responses to acetylcholine were analysed with a t-test for paired results.

RESULTS

MAP (Fig. 3)

Mean blood pressure was lower in NP rats than in controls (89.9 ± 1.0 compared with 106.0 ± 1.0 mmHg, P < 0.05). By contrast, ADRV rats had increased MAP (124.0 ± 2.5 mmHg, P < 0.05 compared with controls). ADRV rats had MAP values
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![Graph](image)

**Fig. 3.** Effect of L-NAME or l-arginine treatment on MAP. 
* = *P* < 0.05 comparing values of NP rats with those of controls; ** = *P* < 0.05 comparing values of L-NAME-treated animals with those of NP rats; # = *P* < 0.05 comparing values of untreated ADRP rats with those of controls and ADRV rats; ## = *P* < 0.05 comparing values of l-arginine-treated ADRP rats with those of untreated ADRP rats.

similar to controls (99.7 ± 1.6 mmHg, *P* = not significant compared with controls). L-NAME treatment increased MAP significantly in NP rats (154.5 ± 4.4 mmHg, *P* < 0.05 compared with untreated NP rats). In ADRP rats, l-arginine decreased blood pressure significantly (91.0 ± 2.1 mmHg, *P* < 0.05 compared with untreated ADRP).

**Perfusion studies in the isolated perfused mesenterial bed**

Concentration–response curves with noradrenaline and response to acetylcholine in maximally contracted vessels. Vessels from NP rats showed a blunted response to noradrenaline compared with non-pregnant controls, significantly so starting from 1.5 μmol/l. Vessels from ADRP rats responded to noradrenaline more intensely than those of NP rats even at low concentrations, the difference between them and NP rats being statistically significant at concentrations above 3.0 μmol/l. At concentrations above 3.0 μmol/l there was no significant difference between ADRP rats and non-pregnant rats. There was no difference between the curves of ADRV rats and those of normal virgin controls (Fig. 4). When acetylcholine was added to the vessels precontracted with 10 μmol/l noradrenaline, flow increased significantly in all preparations. There was no difference between the responses in non-pregnant controls, NP and ADRV rats, whereas the increase in flow was higher in ADRP rats, significantly so at 15 min (Fig. 5).

**Effect of l-arginine.** Per fusate outflow increased significantly in response to l-arginine in all the noradrenaline-contracted preparations. Again, the response was similar in the vessels from non-pregnant controls, NP and ADRV rats. By contrast, the increase in flow in vessels from ADRP rats was significantly greater than that of the other groups, significantly so at 5 and 10 min (Fig. 6).

![Graph](image)

**Fig. 4.** Percentage decrease of flow induced by increasing concentrations of noradrenaline. Basal values are those measured in buffer-perfused preparations in the absence of any vasoactive substance (BL). *P* < 0.05, comparing NP rats with the other groups; **P* < 0.05 comparing ADRP with NP rats.

![Graph](image)

**Fig. 5.** Percentage increase in flow induced by acetylcholine (10^–6 mol/l). For these experiments, basal values were measured after maximal precontraction with noradrenaline (10^–5 mol/l). *P* < 0.05, comparing ADRP rats with the other groups.
Effect of L-NAME. In preparations from all groups, the addition of L-NAME induced a further significant decrease in flow. This was similar in non-pregnant controls, ADRV and ADRP rats, whereas in NP animals the decrease was greater, significantly so at 10 and 20 min (Fig. 7). The degree of inhibition of NO synthesis, as stimulated by acetylcholine, was similar in all groups (Table 1).

Effect of nitroprusside or diazoxide. There were no differences in the responses to nitroprusside or diazoxide between the four groups. Fig. 8 summarizes the results, reporting peak values (percentage increase compared with the noradrenaline contracted status) obtained with L-arginine, acetylcholine, nitroprusside and diazoxide.

DISCUSSION

Hypertension in pregnancy occurs in about 4–8% of nulliparous women and remains a major cause of fetal and maternal morbidity and mortality. Pre-existing renal injury increases significantly the risk of this complication [21]. Its cause remains unknown. Recent studies in rats and primates have shown that inhibition of NO synthesis during normal pregnancy augments vascular reactivity [22, 23], increases arterial pressure in spontaneously hypertensive rats [24] and induces a clinical picture similar to pre-eclampsia [15, 16, 23, 25]. In human pregnancy, decreased NO production has been reported in pre-eclampsia [26], but this has been challenged by other studies [27, 28]. Taken together, these results suggest a new pathogenesis of hypertension in pregnancy. Interestingly, there are no data in the literature evaluating the involvement of the NO system in pre-eclampsia ‘superimposed’ on preexisting renal disease.

Recently, we have described the reversible effects of pregnancy in rats with adriamycin nephropathy, characterized by higher blood pressure, lack of hyperfiltration and enhanced proteinuria [17, 18]. This model is of particular significance, because, apart from L-NAME hypertension [15, 16, 23, 24], adriamycin nephropathy is the only form of hypertensive pregnancy in the rat. In contrast with other

![Fig. 6. Percentage increase in flow induced by carginine (10^-2 mol/l).](image)

For these experiments, basal values were measured after precontraction to 30–40% of initial flow with noradrenaline. * = P < 0.05, comparing ADRP rats with the other groups.

![Fig. 7. Percentage decrease in flow induced by L-NAME (10^-4 mol/l).](image)

For these experiments, basal values were measured after precontraction to about 30–40% of initial flow with noradrenaline. *P < 0.05, comparing NP rats with the other groups.

Table 1. Percentage increase in perfusate flow induced by acetylcholine (10^-4 mol/l) before and after exposure to L-NAME (10^-4 mol/l) in mesenteric vessels precontracted with noradrenaline (2-3 μmol/l). †Difference between pre- and post-L-NAME responses to acetylcholine. *P = post- compared with pre-L-NAME; there were no statistically significant differences between the groups.

<table>
<thead>
<tr>
<th>Increase in perfusate flow rate (%)</th>
<th>n</th>
<th>Pre-L-NAME</th>
<th>Post-L-NAME</th>
<th>Difference (%)†</th>
<th>P*</th>
</tr>
</thead>
<tbody>
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<td>Control 6</td>
<td></td>
<td>+321 ± 99</td>
<td>+170 ± 65</td>
<td>48 ± 12</td>
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<td>+144 ± 13</td>
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<td>+309 ± 83</td>
<td>+214 ± 59</td>
<td>45 ± 6</td>
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<tr>
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<td></td>
<td>+257 ± 46</td>
<td>+153 ± 27</td>
<td>40 ± 10</td>
<td>&lt;0.05</td>
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</table>
venous diseases in the rat, in the case of the adriamycin-induced lesion a form of clinical super-
renal diseases in the rat, in the case of the adria-
imposed pre-eclampsia is observed. In fact, several
rats, reminiscent of the decreased values of vasodila-
tory prostaglandins [17].
boxane B2 was diminished in glomeruli of ADRP
human condition have been found. For instance, the
hypertension [29], indicating that thromboxane also
plays a pathogenetic role.
synthesis of prostaglandin Ez relative to throm-
pressure in hypertensive ADRP animals. These
results may be interpreted to suggest a similarity
between ADRP rats and normal rats, in which an
antagonist, daltroban, prevented the development of
vessels 

In the present work, L-NAME induced hyper-
tension in NP rats and L-arginine reduced blood
pressure in hypertensive ADRP animals. These
results may be interpreted to suggest a similarity
between ADRP rats and normal rats, in which an
exogenous inhibitor depresses NO synthesis. In both
cases the rate of NO production would not be suffi-
cient to maintain an adequate vasodilatation during
pregnancy. This explanation is corroborated by our
recent findings of low levels of urinary nitrate (as
compared with normal pregnancy) in ADRP rats
[30].

We measured the response of resistance-sized
vessels in vitro. The conditions of the perfusion
system (fixed pressure in the physiological range for
these vessels [31] and uninterrupted presence of
intraluminal flow) mimic the conditions in vivo
reasonably well, which is especially important for
studying endothelium-related phenomena [32].
Vessels from NP rats behave clearly differently from
those of controls, whereas those of the ADRP
animals, while showing some degree of resistance to
noradrenaline at the lower concentrations, respond
like those of virgin animals at the higher concentra-
tions. This 'normal' behaviour is obviously inade-
quate during pregnancy and could well be the basis
of the hypertension observed in ADRP rats. In
order to elucidate whether this relatively increased
vasoconstrictory response is related to an alteration
of endothelial function or to a change in the con-
tactile properties of the arteries, we measured the
effect of vasodilators in vessels precontracted with
noradrenaline. Substances acting directly on the
smooth muscle, such as nitroprusside and diazoxide,
had the same effect in all groups. In contrast, a rela-
tively greater vasodilatation was observed with
acetylcholine or L-arginine in preparations from
ADRP rats compared with all the other groups.
The coexistence of increased vasoconstriction in preg-
nancy (possibly due to reduced NO) and enhanced
response to stimulation of the NO system by acetyl-
choline or excess L-arginine (concentrations much
higher than normally found in rat blood [33]) is
apparently paradoxical. These findings imply that, in
ADRP rats, the concentration of endothelial NO
synthase is intact or even increased (as is expected
during pregnancy [2, 7]). They also argue against a
state of arginine deficiency (which we also have
ruled out by measuring normal levels of arginine in
the blood of ADRP rats [30]). Possible alternative
explanations include: (i) a functional inhibition of
the endothelial NO synthase, overcome by
pharmacological stimulation, and (ii) an isolated
alteration of stress-dependent NO release, which is
known to follow a transduction pathway distinct
from that responsive to acetylcholine [32]. The
possibility that other endothelium-derived media-
tors, such as endothelin-derived hyperpolarizing
factor or prostaglandin I2, are involved in the
increased response to acetylcholine in ADRP
animals does not seem likely, in view of the fact that
the response was similarly reduced after L-NAME in
all groups (Table 1). Inhibition of NO synthase with
L-NAME augmented the effect of noradrenaline in
all groups, but the effect was significantly greater in
NP rats. This underscores the importance of
increased NO in the decreased vascular response to
vasoconstrictors in normal pregnancy. Thus, the fact
that ADRP rats responded essentially like virgin
animals, is logical, as NO production was already
inappropriately low to counteract the effects of nor-
adrenaline.

The data presented here demonstrate in our
model several points of agreement between the
results obtained in vivo and in vitro: (i) ADRP rats
develop hypertension and have increased reactivity
to noradrenaline (compared with NP rats); (ii)
L-arginine prevents the development of hypertension
in ADRP rats, i.e. L-arginine (or acetylcholine)
induces greater vasodilatation (in other words, cor-
rects the tendency to increased vasoconstriction) in these animals; (iii) L-NAME causes hypertension and has the maximal effect in vitro in NP rats. Our results thus confirm, in a different model, the data suggesting that abnormally depressed NO synthesis and/or actions may affect the normal course of gestation [15, 16, 23, 24] and strengthen the concept that endothelial cell dysfunction may lead to the development of hypertension in pregnancy. The interference of adriamycin with pregnancy-related endothelial adaptation is of particular interest because, in this model, the state of pregnancy triggers the clinical manifestations. The nature of the effect on endothelial function induced by adriamycin has not been studied until now. The only other report [34] concerns the analogue compound daunomycin, which induced hyperreactivity to noradrenaline in the thoracic aorta. In that report, cyclic GMP content was decreased, suggesting a decrease in either the stimulated or the spontaneous release of endothelium-derived relaxing factor.

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REFERENCES