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ORIGINAL ARTICLE

Characterization of the L-arginine–NO–cGMP pathway in spontaneously hypertensive rat platelets: the effects of pregnancy

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Nitric oxide (NO) is a short-lived intercellular messenger that provides an efficient vascular regulatory mechanism to support homeostasis and prevent thrombosis. Endothelial dysfunction and reduced NO bioavailability have a central role in hypertension associated with pregnancy. The purpose of this study was to investigate the impact of pregnancy on the L-arginine–NO–cGMP pathway in platelets and its correlation to platelet function and blood pressure in normotensive rats and spontaneously hypertensive rats (SHRs). Platelets were obtained from blood on the 20th day of pregnancy from female SHRs (SHR-P) and normotensive controls (P) or age-matched nonpregnant rats (SHR-NP and NP). Intraplatelet NO synthase (NOS) activity was reduced in P compared to NP, despite unchanged L-arginine influx. The expression levels of endothelial NOS (eNOS) and inducible NOS (iNOS) were diminished during pregnancy in normotensive rats. Paradoxically, cyclic guanosine monophosphate (cGMP) levels were similar between NP and P, as were phosphodiesterase type 5 (PDE5) expression and platelet aggregation induced by adenosine diphosphate. In SHRs, L-arginine influx was reduced in SHR-P compared to SHR-NP. SHR-P exhibited impaired NOS activity and reduced iNOS expression compared with SHR-NP. Soluble guanylyl cyclase and PDE5 expression in platelets were lower in SHR-P than in SHR-NP, whereas no differences were noted between groups with respect to cGMP levels. However, increased levels of cGMP were observed in SHR-P compared to normotensive groups and platelet aggregability remained unaltered. In conclusion, these observations prompted the hypothesis that normal platelet aggregation in pregnant SHRs may be related to a reduction in PDE5 expression and consequently the maintenance of cGMP levels, independently of reduced platelet NO bioavailability.

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INTRODUCTION

Chronic hypertension imposes a high risk of developing adverse obstetric outcomes in humans and rats, such as abruptio placenta, superimposed preeclampsia, fetal loss, preterm labor, low birth weight and perinatal death.[1,2] It is also well known that hypertensive patients are at a high risk of developing thrombotic events,[3] and it has been suggested that platelet activation together with endothelial dysfunction may provide an important link between pregnancy and hypertension.[4]

In human platelets, nitric oxide (NO) is formed from the cationic amino-acid L-arginine by inducible NO synthase (iNOS) and endothelial NOS (eNOS).[5] Extracellular L-arginine transport into human platelets is mediated by systemic y^+L, which modulates NO production in these cells.[6] The majority of NO effects occur through a highly regulated interplay of cyclic guanosine monophosphate (cGMP) formation mediated by the activation of soluble guanylyl cyclase (sGC) and degradation by phosphodiesterase type 5 (PDE5).[7]

Previous studies by our group have shown that a reduction in intraplatelet NO bioavailability associated with platelet hyperaggregability might be involved in thrombotic events in patients with essential hypertension[8–10] because NO inhibits platelet adhesion,[11] aggregation[12] and recruitment,[13] as well as the formation of leukocyte–platelet aggregates.[14]

In spontaneously hypertensive rats (SHRs), systolic blood pressure (SBP) is reduced to normal values at the end of pregnancy,[15,16] even though cardiac output and total blood volume are increased. This phenomenon has been associated with decreases in responsiveness to vasoconstrictor agents and systemic blood pressure.[17–19] It has also been suggested that there is substantial production of NO and prostacyclin by the endothelium.[20] Conversely, the responsiveness to vasodilators such as bradykinin is decreased in resistance arteries of pregnant SHRs[21] as well as in small subcutaneous arteries and myometrial resistance arteries from women with preeclampsia.[22,23]

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Because bradykinin endothelium-dependent relaxation is mediated by NO and endothelium-derived hyperpolarizing factor, these findings suggest that endothelial dysfunction may contribute to the altered vasoreactivity seen in pregnant SHRs,[21] to preeclampsia and to decreased placental perfusion and its associated intrauterine growth restriction.[24]

The endothelium has a key role in maintaining blood fluidity and in preventing thrombus formation. NO contributes to such physiological features by inhibiting platelet aggregation and adhesion and inducing vasodilation.[25,26] Endothelial dysfunction is widely thought to have a role in the hypertensive disease associated with pregnancy. To date, there is no evidence of the role of the L-arginine–NO–cGMP pathway in platelets and their activation in pregnant SHRs. Using SHRs as a model of pregnancy associated with preexisting hypertension, we studied the intraplatelet L-arginine–NO–cGMP pathway and its correlation with platelet function and blood pressure at the end of gestation in normotensive rats and SHRs.

METHODS

Animals

All experiments were reviewed and approved by the ethics committee of Animal Experiments at Rio de Janeiro State University. The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animals were maintained under standard conditions (12 h light/dark cycles, 21 ± 2 °C, humidity 60 ± 10% and 15 min h^{-1} air exhaustion cycle). Twenty-week-old female Wistar normotensive rats (body weight 212 ± 7.2 g; n=7) and SHRs (body weight 200 ± 10 g; n=6) were matched with respective male strains, and day 1 of pregnancy was documented by the presence of spermatozoa after a vaginal smear. The animals were used at the end of pregnancy (20th day). Age-matched virgin Wistar normotensive rats (body weight 201 ± 4.7 g; n=9) and SHRs (body weight 173 ± 6 g; n=6) in the diestrus cycle from each group served as controls, which yielded four groups: nonpregnant normotensive rat (NP), SHR (SHR-NP), pregnant normotensive rat (P) and SHR (SHR-P). Body weight was also determined at the end of pregnancy.

Measurement of SBP

SBP was measured by the tail-cuff method using a Letica LE 5000 device (Panlab, Comella, Spain). Rats were trained for 2 weeks before starting the experimental protocol so that blood pressure could be recorded consistently with minimal restraint and stress to the animals. SBP was measured after mating and at 7, 14 and 20 days of pregnancy and in control nonpregnant rats; just before measurement of SBP, the rats were kept at 30–32 °C for 15 min to render the tail artery pulsations detectable. When three consecutive blood pressure values were obtained without disturbance of the signal, SBP was recorded.

Platelet suspension

Animals were anesthetized with thiopental (70 mg kg^{-1}), and blood was collected by puncture of the descending aorta, with citric acid-dextrose (mmol l^{-1}: citric acid (73.7), trisodium citrate (85.9), dextrose (111); pH 4.0) to prevent coagulation. Blood samples were centrifuged at 200 g for 15 min. The supernatant was collected and centrifuged again at 900 g for 10 min and then the pellet was resuspended in Krebs buffer (mmol l^{-1}: NaCl (119), KCl (4.6), $CaCl_2$ (1.5), NaH_2PO_4 (1.2), $MgCl_2$ (1.2), $NaHCO_3$ (15), glucose (11); pH 7.4).

Measurement of total L-[^{3}H]arginine influx in platelets

The platelet suspension was incubated with L-[^{3}H]arginine (100 μmol l^{-1}), followed by two washes in Krebs buffer, centrifugation (2000 g for 15 s) and lysis by Triton X-100 (0.1%) for β-scintillation counting (LS 6500 Liquid Scintillation Counter; Beckman Coulter, Fullerton, CA, USA).[27]

Measurement of platelet NOS activity

Total NOS activity was determined from the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline.[28,29] The platelet suspension was incubated at 37 °C in the presence of L-[^{3}H]arginine (1 μmol l^{-1}) for 15 min. All reactions were stopped by rapid centrifugation (2000 g, 15 s), followed by two washes with Krebs buffer. The platelet pellet was lysed with 0.1% Triton X-100 and applied to a Dowex cation exchange resin column. The L-[^{3}H]citrulline was eluted and radioactivity measured by liquid scintillation counting (LS 6500 Liquid Scintillation Counter; Beckman Coulter).

Assay of platelet cGMP levels

Basal cGMP content was determined in washed platelets using a commercial enzyme-linked immunosorbent assay kit (Cayman Chemical, Ann Arbor, MI, USA). The platelet suspension was incubated with 200 μmol l^{-1} of 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor) for 30 min. Ice-cold perchloric acid (150 mmol l^{-1}) was added to the samples, and platelets were lysed by sonication for 15 min followed by rapid freezing in liquid nitrogen. Cell debris was centrifuged at 2000 g for 10 min and supernatants containing cGMP were collected and stored at −80 °C until the enzyme-linked immunosorbent assay was performed.[9,30,31]

Platelet aggregation protocol

Platelet aggregation was evaluated in platelet-rich plasma by optical densitometry.[32] First, blood samples were anticoagulated with 3.8% trisodium citrate and centrifuged at 200 g for 15 min at room temperature. Platelet-poor plasma was obtained by centrifuging the remaining blood at 900 g for 10 min. The platelet concentration in platelet-rich plasma was adjusted with platelet-poor plasma to achieve a constant count of 250×10^9 per l. Aggregation was induced by adenosine diphosphate (12 μmol l^{-1}) and responses were monitored for 5 min in a four-channel optical aggregometer (Chrono-Log, Havertown, PA, USA). Tests were performed at 37 °C with a stirring speed of 1200 r.p.m. Maximal aggregation was expressed as a percentage.

Western blot

Platelets were isolated from the platelet-rich plasma by centrifugation, washed and lysed with lysis buffer. Protein was quantified using the BCA protein assay reagent (Thermo Fisher Scientific Pierce Protein Research, Rockford, IL, USA). Amounts of 10 μg protein were loaded on the gel, subjected to SDS–polyacrylamide gel electrophoresis 10% (Invitrogen, Carlsbad, CA, USA) and then transferred to polyvinylidene fluoride membranes. The membranes were incubated at room temperature overnight with mouse monoclonal antibodies (BD Bioscience, San Jose, CA, USA) against eNOS (1:1000) and iNOS (1:1000) or rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against PDE5 (1:500) and sGC β_1 subunit (1:1000). The WesternBreeze chromogenic system (Invitrogen) was used for detection of the proteins.

Statistical analysis

All data are expressed as mean ± s.e.m of measurements in 4–8 rats. Statistical analysis of all data was performed by one-way analysis of variance test, followed by Bonferroni's test, except for SBP, which was tested by a two-way analysis of variance with repeated measures. Statistical significance was set at $P < 0.05$.

RESULTS

Body weight and SBP

As expected, there was a significant increase in body weight at the end of pregnancy as compared to day 1 of pregnancy in normotensive rats (295.0 ± 12.3 *vs.* 212.1 ± 7.2 g) as well as in SHRs (265.0 ± 17.4 *vs.* 200.0 ± 10.2 g). SBP (mm Hg) measurements during the experimental period for both normotensive and hypertensive rats are shown in Table 1. The SBP was increased in SHR-NP compared to NP rats. The SBP in P rats as well as in SHR-P rats was significantly reduced at the end of pregnancy. The levels obtained from SHR-P were not significantly different from those of the normotensive groups.

Table 1 Measurements of systolic blood pressure (mm Hg)

Group	Day 1	Day 20
NP	137 ± 1.9	128 ± 3.0
P	131 ± 2.3	110 ± 2.7*
SHR-NP	182 ± 4.9†	179 ± 3.7†
SHR-P	183 ± 5.4†	128 ± 3.4*

Abbreviations: NP, nonpregnant normotensive (n=7); P, pregnant normotensive (n=8); SHR-NP, nonpregnant spontaneously hypertensive rat (n=6); SHR-P, pregnant SHR (n=6).
*$P<0.05$ compared to day 1.
†$P<0.05$ compared to NP and P on the same day of measurement.

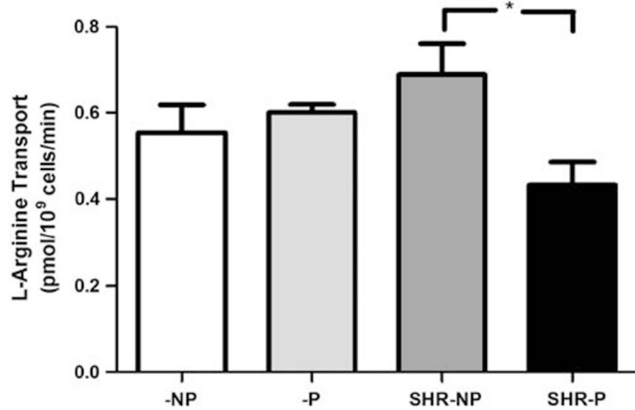

Figure 1 L-[^{3}H]arginine transport in platelets from normotensive nonpregnant (NP; n=7) and pregnant (P; n=8) or hypertensive nonpregnant (SHR-NP; n=8) and pregnant (SHR-P; n=5) rats. Values are reported as mean ± s.e.m. (*$P<0.05$).

Total L-[^{3}H]arginine transport in platelets

Total L-arginine transport in platelets is mainly mediated by system y^+L and diffusion. System y^+L transports cationic amino acids independent of Na^+ (for example, L-lysine and L-arginine) and neutral amino acids in the presence of Na^+ (for example, L-leucine) with high affinity, as described in classic experiments on human red blood cells and platelets.[27,33]

Total L-[^{3}H]arginine influx in platelets from SHR-P was significantly reduced when compared to SHR-NP rats, but no differences were observed between platelets from NP and P rats. L-[^{3}H]arginine transport in platelets from SHR-NP was not significantly different from that in NP rats (Figure 1).

Basal activity and expression of eNOS and iNOS in platelets

Platelets from P and SHR-P rats showed decreased NOS activity, as measured by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, when compared to NP and SHR-NP rats, respectively. NOS activity in platelets from SHR-NP was not significantly different from that observed in NP rats (Figure 2a). In normotensive animals, there was a significant decrease in platelet eNOS expression at day 20 of pregnancy. SHR-NP had a lower expression of eNOS in platelets than NP, but no significant change was observed for platelet eNOS expression in SHR-P as compared to SHR-NP (Figure 2b). The inducible isoform of NOS expression was decreased in platelets from P compared to NP rats. Similarly, in the SHR-P animals as compared to SHR-NP, a significant reduction in iNOS was observed. iNOS expression did not differ between the NP and SHR-NP groups (Figure 2c).

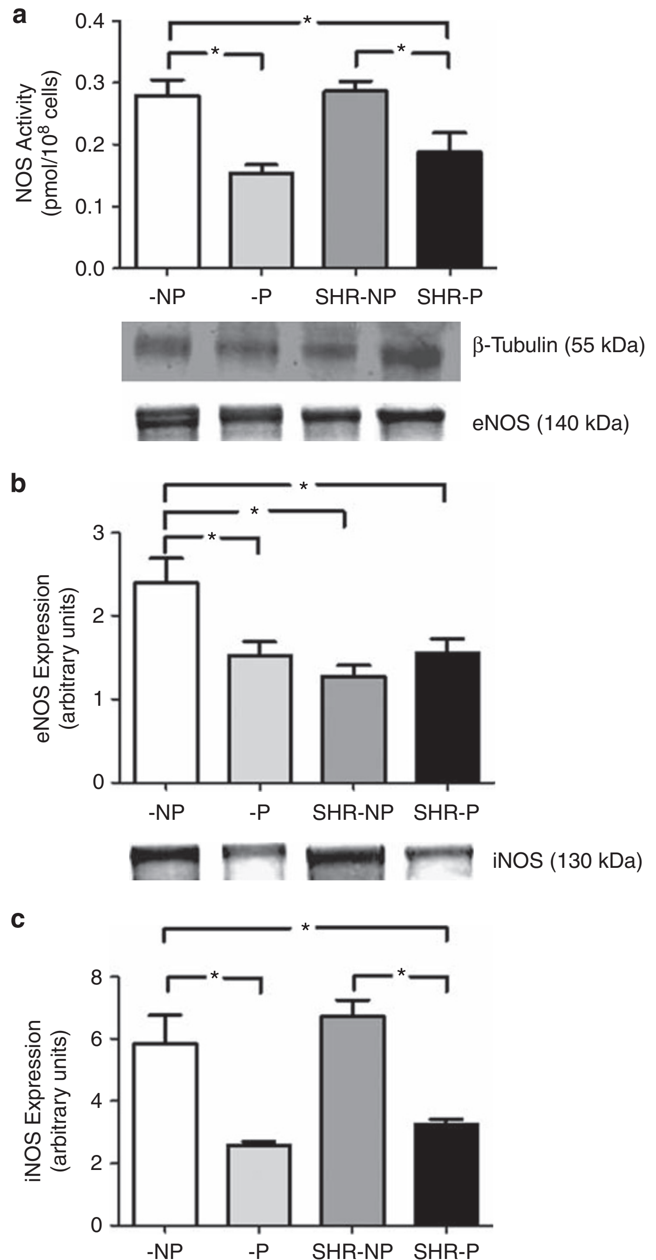

Figure 2 Total NOS activity (**a**), eNOS expression (**b**) and iNOS expression (**c**) in platelets from normotensive nonpregnant (NP; n=7) and pregnant (P; n=8) or hypertensive nonpregnant (SHR-NP; n=8) and pregnant (SHR-P; n=5) rats. Values are reported as mean ± s.e.m. (*$P<0.05$).

Intraplatelet cGMP levels and expression of sGC and PDE5

Intraplatelet cGMP levels did not differ among NP, P and SHR-NP groups. However, cGMP levels were increased in the SHR-P group compared to the normotensive groups but not to the SHR-NP group (Figure 3a). Expression levels of sGC (Figure 3b) and PDE5 (Figure 3c) were increased in the SHR-NP group as compared to NP and P rats. Pregnancy decreased the expression of both sGC and PDE5 in SHR rats.

Platelet aggregation

Platelet aggregation in platelet-rich plasma was induced by adenosine diphosphate and responses were monitored for 5 min. There was no significant difference in platelet aggregation among the groups (Figure 4).

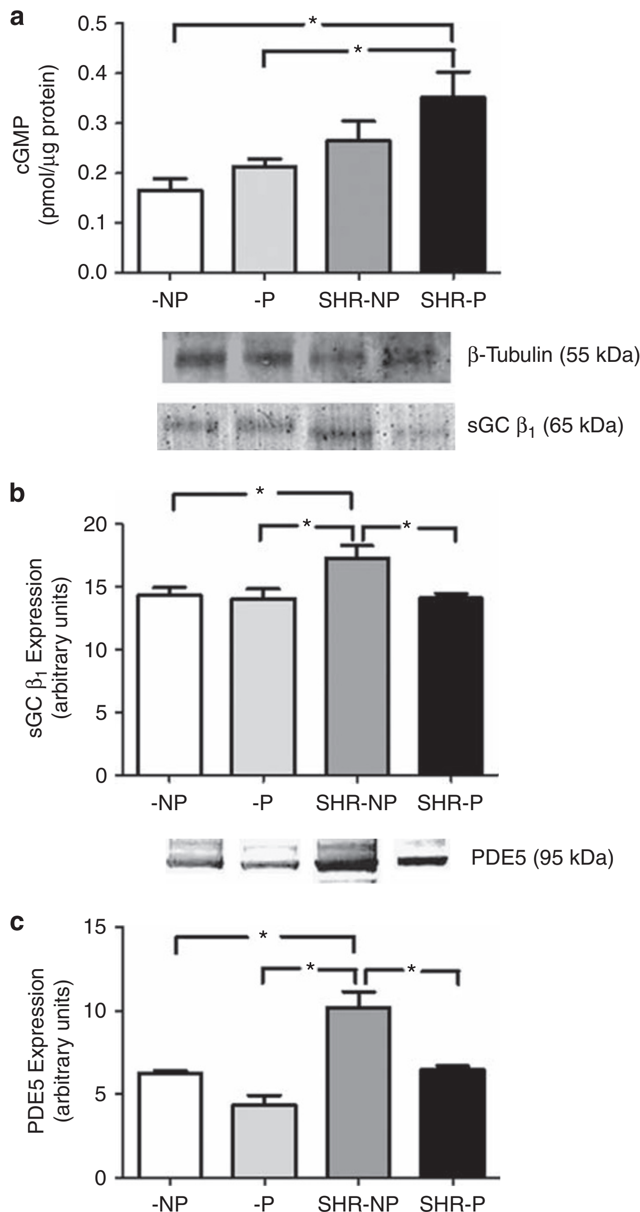

Figure 3 Basal cGMP levels (**a**), sGC expression (**b**) and PDE5 expression (**c**) in platelets from normotensive nonpregnant (NP; n=3–4) and pregnant (P; n=3–5) or hypertensive nonpregnant (SHR-NP; n=3–4) and pregnant (SHR-P; n=3–4) rats. Values are reported as mean ± s.e.m. (*$P<0.05$).

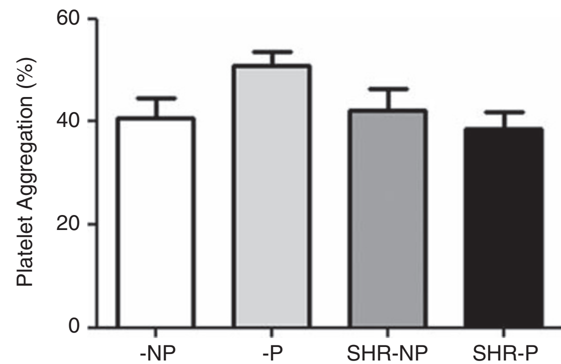

Figure 4 Platelet aggregation induced by adenosine diphosphate (ADP) (12 μmol l^{-1}) from normotensive nonpregnant (NP; n=7) and pregnant (P; n=7) or hypertensive nonpregnant (SHR-NP; n=8) and pregnant (SHR-P; n=8) rats. Values are reported as mean ± s.e.m.

DISCUSSION

This study investigated the impact of pregnancy on the L-arginine–NO–cGMP pathway in platelets from SHRs. The results show that, despite an inhibition of the intraplatelet L-arginine–NO pathway, platelet aggregation from pregnant hypertensive rats remained normal, which may be due to increased cGMP levels and decreased PDE5 expression.

Initially, we found that in normotensive and hypertensive rats, blood pressure was reduced at the end of pregnancy. The levels obtained from pregnant hypertensive rats were not significantly different from those obtained from the normotensive groups, which were in agreement with previous findings.[15,16,21] These results might be partially explained by an increased production of NO and prostacyclin from vascular endothelium[20] and a hyporeactivity to vasoconstrictors,[34] which may contribute to vasodilation and consequent reduction in peripheral vascular resistance. Although a specific role for NO in the induction of hemodynamic alterations in pregnancy is somewhat controversial, it is widely accepted that an excess of NO is generated by endothelial cells during normal pregnancy.[35,36]

Several studies have investigated L-arginine metabolism at gestation, but there was no evidence of NO synthesis in rat platelets. This is the first evidence that rat platelets, similar to human platelets, constitutively express both eNOS and iNOS, which actively generate NO from the semi-essential amino-acid L-arginine. Previous observations support a role for platelet-derived NO production in antiplatelet aggregation and adhesion properties.[37] The first step for NO production is the cellular uptake of L-arginine through amino-acid transporter systems.[38] Results from our study showed that total L-arginine transport and NOS activity were decreased in platelets from pregnant hypertensive rats as compared to nonpregnant hypertensive rats. Recent evidence has come to light that maternal aortic L-arginine uptake is profoundly attenuated in pregnancy, and the researchers of that study assume that rather than supplying its own endothelium, maternal L-arginine stores are depleted due to a preferential shift to the fetus.[39] These data could explain the reduction in L-arginine transport in platelets from pregnant hypertensive rats. Both influx and availability of intracellular L-arginine are limiting factors in NO production.[40] Therefore, it is possible that the reduced enzyme activity observed here is related to reduced levels of substrate together with decreased expression of iNOS. Nevertheless, we observed higher production of cGMP and no change in platelet aggregation during pregnancy in the hypertensive condition compared to the normotensive condition. Despite decreased activity of the L-arginine transporter and NOS, and the reduced expression of sGC, responsible for converting GTP in cGMP, one of the factors that could contribute to the increased cGMP levels is the reduction in PDE5 protein expression, an enzyme responsible for cGMP metabolism. There is evidence that oxidative stress markers are increased in maternal blood, urine, placenta and placental trophoblast cells, and that this state of oxidative stress is heightened in pregnancies complicated by preeclampsia and intrauterine growth restriction.[41] These findings suggest that pregnancy *per se* is a state of oxidative stress due to the high metabolic activity of the placenta and the maternal metabolism during pregnancy. However, there are few reports about oxidative damage in pregnant SHRs associated with the preexisting hypertension that persists mid-

way through pregnancy. There is recent evidence that the increased proinflammatory and oxidative markers (malondialdehyde content and protein nitrosylation) seen in SHRs are greatly ameliorated by pregnancy.[16] Together with changes in the renin–angiotensin system in the kidney, reduction of oxidative markers seems to contribute to the reduction of blood pressure near term in SHRs, as observed in our study. Future studies on oxidative stress in pregnant SHRs should be carried out in other tissues and platelets to support the hypothesis that a decrease in oxidative stress near term may contribute to the maintenance of NO and cGMP bioavailability.

Total L-arginine transport was maintained during pregnancy in the normotensive condition. However, similar to hypertensive rats, NOS activity, measured by the conversion of L-arginine to L-citrulline, was reduced. In addition, we observed that the expression of eNOS and iNOS isoforms was reduced in normal pregnancy. Aside from an apparent decrease in NO production, cGMP levels and platelet aggregation were not affected in this group. Therefore, reduced activity of NOS in platelets from normotensive pregnant rats may be related to lower expression of both NOS isoforms. Nevertheless, normal platelet aggregation is also sustained by unaltered cGMP levels. sGC is also regulated by a family of enzymatically formed guanylyl cyclase-activating factors that includes carbon monoxide and OH, which affect cGMP levels. Previous studies have shown that carbon monoxide and NO have similar effects on platelet aggregation,[42] vascular smooth muscle function and intracellular cGMP levels. OH^- was suggested to function as a physiological mediator of endothelium-dependent relaxation.[43] Thus, aside from NO, both carbon monoxide and OH^- may represent potential mediators of platelet function.

The interaction of NOS with a variety of proteins has an important role in regulating NO production. One of the proteins that interacts with NOS is heat shock protein 90, which appears to have an important role in the function and stability of the enzyme.[44] However, degradation of NOS has been proposed as a regulatory mechanism to prevent the toxic effects of this compound in conditions of high NO production[45,46] and appears to be associated with less interaction with heat shock protein 90.[47] As pregnancy is characterized by high NO production, mainly by endothelial cells, an excess of this compound could lead to degradation of NOS isoforms in platelets. Thus, reduced expression of NOS promotes reduced enzyme activity and consequently decreased intraplatelet NO production. Another possibility is that the increase of NO and prostacyclin production in pregnancy by endothelium[48] could participate in the maintenance of normal aggregation, even in the presence of reduced intraplatelet NO production. Therefore, even though the L-arginine pathway is inhibited in pregnancy with a consequent decrease in production of endogenous NO, there is probably compensation for the NO produced by endothelial cells.

Platelet antagonists inhibit platelet function by increasing intracellular levels of the cyclic nucleotides cAMP and cGMP through the activation of the respective cyclases. Cyclic nucleotide levels are downregulated by phosphodiesterase-mediated degradation. Platelets contain mainly PDE3, which preferentially hydrolyzes cAMP as a substrate, and PDE5, which preferentially catalyzes the breakdown of cGMP.[49] In addition, the effects of cGMP could involve the modulation of cAMP levels and PKA activity through inhibition of cAMP-hydrolyzing PDE3 activity.[50]

Finally, NO-independent mechanisms of cGMP formation in response to platelet agonists have been recently described,[51] which supports NO-mediated activation of cGMP in human platelets. These studies provide evidence that sGC can be activated independently of increased NO synthesis in response to the platelet activator adiponectin, leading to elevated cGMP levels and activation of cGMP-protein kinase G. The platelet cGMP signaling cascade seems to be activated by a novel tyrosine kinase-dependent mechanism in the absence of NO. Therefore, this novel mechanism may also explain the maintenance of cGMP levels in normal pregnancy or the increased production of cGMP associated with hypertension.

In conclusion, this study provides evidence for an active L-arginine–NO–cGMP pathway in rat platelets. Moreover, pregnancy decreases L-arginine transport, NOS activity and iNOS expression in platelets from hypertensive rats as compared to nonpregnant hypertensive rats. Despite reduced platelet NO bioavailability in pregnant hypertensive rats, platelet aggregability remains unaltered, which may be related to increased levels of cGMP and reduced expression of PDE5. This research provides new insights into the complex pathophysiology of gestational hypertension.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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