Nitric Oxide 57 (2016) 71–78

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Sodium nitrite attenuates hypertension-in-pregnancy and blunts increases in soluble fms-like tyrosine kinase-1 and in vascular endothelial growth factor

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ARTICLE INFO

Article history:
Received 6 March 2016
Received in revised form
5 May 2016
Accepted 11 May 2016
Available online 12 May 2016

Keywords:
Hypertension-in-pregnancy
Sodium nitrite
N(G)-nitro-L-arginine methyl ester
Rats

ABSTRACT

Preeclampsia is a pregnancy-associated disorder characterized by hypertension with uncertain pathogenesis. Increases in antiangiogenic soluble fms-like tyrosine kinase-1 (sFlt-1) and reductions in nitric oxide (NO) bioavailability have been observed in preeclamptic women. However, the specific mechanisms linking these detrimental changes to the hypertension-in-pregnancy are not clearly understood. In this regard, while recent findings have suggested that nitrite-derived NO formation exerts antihypertensive and antioxidant effects, no previous study has examined these responses to orally administered nitrite in hypertension-in-pregnancy. We then hypothesized restoring NO bioavailability with sodium nitrite in pregnant rats upon NO synthesis inhibition with N(omega)-nitro-L-arginine methyl ester (L-NAME) attenuates hypertension and high circulating levels of sFlt-1. Number and weight of pups and placentae were recorded to assess maternal-fetal interface. Plasma sFlt-1, vascular endothelial growth factor (VEGF) and biochemical determinants of NO formation and of antioxidant function were measured. We found that sodium nitrite blunts the hypertension-in-pregnancy and restores the NO bioavailability, and concomitantly prevents the L-NAME-induced high circulating sFlt-1 and VEGF levels. Also, our results suggest that nitrite-derived NO protected against reductions in litter size and placental weight caused by L-NAME, improving number of viable and resorbed fetuses and antioxidant function. Therefore, the present findings are consistent with the hypothesis that nitrite-derived NO may possibly be the driving force behind the maternal and fetal beneficial effects observed with sodium nitrite during hypertension-in-pregnancy. Certainly further investigations are required in preeclampsia, since counteracting the damages to the mother and fetal sides resulting from hypertension and elevated sFlt-1 levels may provide a great benefit in this gestational hypertensive disease.

1. Introduction

Preeclampsia and related hypertensive pregnancy disorders affect 5–8% of all births in the United States, resulting in 15–20% of maternal deaths worldwide [1]. These disorders present serious complications to the mother and the baby, and the mechanisms involved are not clearly understood [2]. Currently, despite intense investigation, definitive treatment is limited to preterm delivery of the baby and placenta, suggesting that the causative symptoms of preeclampsia may be dependent on the presence of the placenta [3]. In fact, placental ischemia is thought to be an initiating event, leading to the release of circulating biomarkers of inflammatory response, oxidative stress and antiangiogenic factors in the maternal circulation [4,5].

There may be an imbalance among the pro- and antiangiogenic factors, in which the circulating antiangiogenic protein sFlt-1 (soluble fms-like tyrosine kinase-1) binds and sequesters the vascular endothelial growth factor (VEGF), causing endothelial dysfunction and producing preeclampsia-like symptoms [6]. Accordingly, the extracorporeal removal of circulating sFlt-1 in preeclamptic patients may improve the symptoms [7], thus, confirming the key role of sFlt-1 in the maternal side of the disorder.

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http://dx.doi.org/10.1016/j.niox.2016.05.004
1089-8603/

However, it has been shown that the high levels of circulating sFlt-1 and uteroplacental circulation are not affected by the most commonly antihypertensive drugs used in clinic to treat pre-eclampsia [2,4,6]. In addition, reductions on the formation of nitric oxide (NO) may be inversely related to serum levels of sFlt-1, highlighting that hypertension-in-pregnancy during preeclampsia may be explained, at least in part, by reductions of NO bioavailability [8–10].

Importantly, accumulated experimental evidences have showed a potential role for the anion nitrite, being more than a simple biomarker of NO formation. Nitrite is recycled back to NO as a physiological alternative to NO formation independent of NO synthase (NOS)-related pathways, restoring the vasodilator actions of the NO [11–13]. In this context, recent studies have suggested that NO generation from nitrite may occur in conditions such as hypoxia and that sodium nitrite may selectively deliver NO to ischemic/hypoxic tissues [12,13]. Therefore, if uteroplacental ischemia is thought to play a major role in preeclampsia [4,5], we hypothesized that these conditions would create the ideal biochemical environment for the *in vivo* reduction of nitrite to NO, thus, attenuating hypertension (mother side) and concomitantly improving fetal detrimental changes caused by hypertension-in-pregnancy.

In order to confirm this hypothesis, pregnant rats were treated with N(omega)-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis, during mid-to late gestation, in which hypertension, reductions of litter size [14] and placental weight [15] are manifested. We have also examined the circulating levels of sFlt-1 and VEGF, and biochemical determinants of oxidative stress [16], and if the attenuation of hypertension with sodium nitrite would be associated with reduced levels of sFlt-1 and antioxidant effects [11].

2. Materials and methods

2.1. Animals and experimental protocol

Wistar rats (200–250 g) were housed in cages at 22 ± 2 °C on a 12-hr light/dark cycle and given free access to water and rat chow. Each female rat was separately mated overnight. Day 0 of pregnancy was defined as the day when spermatozoa were found in a vaginal smear.

On pregnancy day 14, each pregnant rat mother was first placed into a single cage and randomized to one of the four treatment groups (n = 10 per group, total of 40 rats): Norm-Preg, Preg + Sodium nitrite, HTN-Preg and HTN-Preg + Sodium nitrite groups. Pregnant rats received daily 0.9% saline solution by gavage and by via intraperitoneal (i.p.) in **Norm-Preg group**; or sodium nitrite by gavage (Sodium nitrite; Sigma, St. Louis, MO, #S2252; 15 mg/kg/day for 7 days) and saline injections by via i.p. in **Preg + Sodium nitrite group**; or i.p. injections of N(G)-nitro-L-arginine methyl ester (L-NAME; Sigma, St. Louis, MO, # 5751; 60 mg/kg/daily [17]) and saline solution by gavage in **HTN-Preg group**; or i.p. injections of L-NAME (60 mg/kg/daily) and sodium nitrite by gavage (15 mg/kg/day for 7 days) in **HTN-Preg + Sodium nitrite group**.

The dose of sodium nitrite (15 mg/kg or 0.217 mmol/kg; by gavage) was chosen with basis on previous studies showing that this dose exerts relevant antihypertensive and antioxidant effects in rats [11,18–21].

Rats were euthanized on gestation-day 21 under overdose of isoflurane followed by exsanguination. Blood samples were collected in lyophilised ethylenediaminetetraacetic acid (EDTA) (Vacuntainer Becton-Dickinson, BD, Oxford, UK) and immediately centrifuged and plasma was separated and stored at −80 °C until use for biochemical analysis.

All procedures for animal experimentation were approved by the Ethics Committee, Biosciences Institute of Botucatu, State University of Sao Paulo (Protocol #618/2014), which is complied with international guidelines of the European Community for the use of experimental animals.

2.2. Blood pressure measurements

Systolic blood pressure (mmHg) was measured on gestational day 13 (baseline with absence of gavage or i.p. injections) and days 14, 16, 18 and 20, 6 h after drugs administration, using tail-cuff plethysmography (Insight, Ribeirao Preto, Sao Paulo, Brazil, # EFF 306). Briefly, all pregnant rats were first acclimated in a quiet room, conditioned and restrained for 5–10 min in a warm box (Insight, Ribeirao Preto, Sao Paulo, Brazil, # EFF-307) to the measurements for 3 days before the pregnancy day 14 (these data were discarded) and then the baseline systolic blood pressure was determined as the average of the cuff inflation-deflation (3–6) cycles by a trained operator on pregnancy day 14 [22].

2.3. Effects on placenta and fetuses

On gestational-day 21, after euthanasia, animals were placed in supine position and cesarean section was performed. The averages of total number of viable fetuses, litter size, fetal weight and placental weight of each mother were recorded. Viable fetuses were determined as those which showed no macroscopical sign of malformation and could apparently have a normal outcome with the progression of the pregnancy, as previously reported [23].

2.4. Measurement of plasma NOx (nitrate + nitrite) concentrations

The plasma NOx concentrations were determined in duplicate by using the Griess reaction, as previously described [24]. Briefly, 40 μL of plasma were incubated with the same volume of nitrate reductase buffer (0.1 M potassium phosphate, pH 7.5, containing 1 mM β-nicotinamide adenine dinucleotide phosphate and 2U of nitrate reductase/mL) in individual wells of a 96-well plate. Samples were allowed to incubate overnight at 37 °C in the dark; 8 μL of freshly prepared Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid) were added to each well and the plate was incubated, for 15 additional minutes, at room temperature. A standard nitrate curve was obtained by incubating sodium nitrate (0.2–200 μM) with the same reductase buffer. The NOx levels in plasma were expressed in μmol/L.

2.5. Determination of sFlt-1 and VEGF

Commercial enzyme immunoassay (ELISA) kits for sFlt-1 (*R&D Systems Inc*, Minneapolis, MN, USA #MVR100) and VEGF (*R&D Systems Inc*, Minneapolis, MN, USA #RRV00) were used to determine plasma levels. Assays were performed according to manufacturer's instructions. Plasmatic levels of sFlt-1 and VEGF were expressed in pg/mL.

2.6. Determination of myeloperoxidase (MPO) activity

Circulating plasma levels of MPO reflect the inflammatory response, as according to the method previously proposed by Suzuki [25]. Briefly, 30 μL of centrifuged plasma samples received 100 μL of TMB (tetramethyl benzidine) and 0.04% of H_2O_2. Posteriorly, microplate was incubated for 10 min at 37 °C, protected from light. The reaction was stopped with 100 μL of H_2SO_4 (2N) and the absorbance at 450 nm with correction to 630 nm was read with the spectrophotometer (Synergy 4, BIOTEK, Winooski, VT, USA). The