Bradykinin Exerts Independent Effects on Trophoblast Invasion and Blood Pressure in Pregnant Guinea Pigs

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Abstract

Introduction: The pleiotropic kininogen–kallikrein–kinin system is upregulated in pregnancy and localizes in the uteroplacental unit. To identify the systemic and local participation of the bradykinin type 2 receptor (B2R), this was antagonized by Bradyzide (BDZ) during 2 periods: from days 20 to 34 and from days 20 to 60 in pregnant guinea pigs. **Methods:** Pregnant guinea pigs received subcutaneous infusions of saline or BDZ from gestational day 20 until sacrifice on day 34 (Short B2R Antagonism [SH-B2RA]) or on day 60 (Prolonged B2R Antagonism [PR-B2RA]). In SH-BDZA, systolic blood pressure was determined on day 34, while in PR-BDZA it was measured preconceptionally, at days 40 and 60. On gestational day 60, plasma creatinine, uricemia, proteinuria, fetal, placental and maternal kidney weight, and the extent of trophoblast invasion were evaluated. **Results:** The SH-B2RA increased systolic blood pressure on day 34 and reduced trophoblast myometrial invasion, spiral artery remodeling, and placental sufficiency. The PR-B2RA suppressed the normal blood pressure fall observed on days 40 and 60; vascular transformation, placental efficiency, urinary protein, serum creatinine, and uric acid did not differ between the groups. The proportion of all studied mothers with lost fetuses was greater under BDZ infusion than in controls. **Conclusion:** The increased systolic blood pressure and transient reduction in trophoblast invasion and fetal/placental weight in the SH-B2R blockade and the isolated impact on blood pressure in the PR-B2R blockade indicate that bradykinin independently modulates systemic hemodynamics and the uteroplacental unit through cognate vascular and local B2R receptors.

Keywords

kallikrein–kinin system, pregnant guinea pig, vascular remodeling, blood pressure regulation, animal models

Introduction

Bradykinin, the main effector of the kallikrein–kinin system,¹ is a nonapeptide cleaved by kallikrein from kininogen. It is one of the first pharmacological tools that permitted the identification of endothelial synthesis of nitric oxide in vitro.² Later, its contribution to blood pressure regulation was demonstrated in vivo by converting enzyme inhibitors, by the blockade of the bradykinin type 2 receptor (B2R) using Icatibant (HOE-140), and by the very recent communication of the association between higher systolic and diastolic blood pressures in the DD genotype of the BDKRB2 rs5810/61 in individuals without overt heart disease.^{3,4,5} Moreover, bradykinin has been identified as the cause of hereditary or angiotensin-converting enzyme inhibitors angioedema,⁶ and Icatibant has been approved to manage acute episodes of the disease.⁷

We have postulated that during gestation bradykinin regulates maternal blood pressure and exerts autocrine/paracrine effects in the fetomaternal interface.⁸ This hypothesis is based on the fact that its main signaling receptor, the constitutive G protein-coupled B2R, has a spatial distribution and actions that

tally with the physiological adaptations of pregnancy (eg, vasodilation, angiogenesis, reduction in oxidative stress, and cell proliferation⁸⁻¹¹). The following findings strengthen this postulate. The B2R-mediated stimulation in vitro enhances trophoblasts migration and invasion.¹² The in situ and in vitro expression of the kinin B2R protein and messenger RNA (mRNA),¹³⁻¹⁵ the upregulated endometrial BDKR2 gene during decidualization, and its downregulation in early pregnancy in chorionic villi of women who later developed severe

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preeclampsia.¹⁶ Urinary kallikrein excretion, reflecting renal synthesis, attains a peak between weeks 8 and 12 of normal pregnancy,¹⁷ while its reduction precedes and accompanies the clinical expression of preeclampsia.^{18–20}

Since the human uterine–placental interface of an ongoing pregnancy is inaccessible, surrogate models need to be extended to further understand its complexity and dynamics. Women share with guinea pigs the hemochorial placenta,²¹ the priming of spiral arteries,^{22,23} the extensive trophoblast invasion and vascular transformation,^{8,24} the luteo–placental shift of progesterone synthesis, the temporal profile of circulating progesterone,^{25,26} a preeclamptic-like syndrome associated with reduced placental perfusion,^{27,28} the localization of tissue kallikrein and the B2R, and other vasoactive and angiogenic factors in functionally equivalent uteroplacental structures.^{8,29,30} These features, added to the high degree of common characteristics in fetoplacental development and metabolism, have prompted the extensive use of this species to study the developmental origin of health and disease.³¹

Pregnant guinea pigs receiving angiotensin II and Bradyzide (BDZ), a potent, rodent selective, nonpeptide B2R antagonist³² from days 20 to 34 exhibited a marked increase in maternal blood pressure on day 34 which did not persist to day 60. The absence of this pressor response in treated nonpregnant females supports a B2R-mediated counter-regulation of bradykinin when circulating angiotensin II exceeds the endogenous gestational levels.³³

This study was designed to continue evaluating the longitudinal pattern of the effects of the B2R on blood pressure, trophoblast invasion, and preeclampsia-like functional and morphological alterations. Therefore, BDZ was started on day 20, once a gestational sac was visualized, in 2 time frames: (1) till sacrifice in day 34, the period of maximal trophoblast invasion and placental development³⁰ or (2) by prolonging the infusion up to day 60 to include the maintenance of the placental flow and the maternal compliance to expanded blood volume.³⁴

Methods

Animals

All experiments were conducted according to the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington, District of Columbia) and were approved by the institutional review board for Ethics and Animal Welfare (Protocol 11-120 IRB) and by the ethics committee of FONDECYT (Fondo Nacional de Desarrollo Científico y Tecnológico, Chile).

Virgin Pirbright white guinea pigs of ~600 g were kept under controlled conditions of humidity and temperature (25°C) with a 12-hour light–dark cycle. Females were examined daily for perforation of the vaginal membrane; when this occurred, they were caged with fertile males, and the following day was defined as day 1. Pregnancy, which extends from 63 to 70 days, was confirmed between 18 and 20 days postmating by

echography (Aloka Flexus SSD-1100; Hitachi Aloka Medical, Tokyo, Japan).

Short and Prolonged B2R Antagonism

To evaluate the consequences of short B2R antagonism (SH-B2RA), Alzet 2ML2 osmotic pumps (Durect, Cupertino, California) were installed subcutaneously in the interscapular zone on gestational day 20—once implantation is complete—in animals anesthetized with intraperitoneal ketamine and xylazine (60 and 4 mg/kg, respectively). The pumps delivered saline solution ($n = 6$) or BDZ (Sigma, St Louis, Missouri) from day (D) 20 to 34 at a dilution that initially infused 62.5 $\mu\text{g/kg/day}$ ($n = 5$); the initial dose BDZ was defined after 40.2, 62.5, and 250.0 $\mu\text{g/kg/d}$ were tested and yielded no changes, intermediate effects, and an important reduction in nonviable fetuses, respectively. Systolic blood pressure was determined at D34 before the dams were killed (Figure 1A).

The prolonged B2R antagonism (PR-B2RA) was evaluated in a second stage, in which BDZ ($n = 6$) or saline infusions ($n = 7$) were maintained from D20 to D60. Systolic blood pressure was measured before mating and at gestational D40 and D60 (Figure 1B).

Noninvasive Systolic Blood Pressure Determination

Blood pressure was measured under anesthesia (intraperitoneal ketamine/xylazine 60/4 mg/Kg, respectively) in the pad of the right paw distal to a neonatal blood pressure cuff (Critikon; General Electric Healthcare, Connecticut) using a Power Lab 8 SP system. Blood pressure curves were analyzed with the Labchart graphic software 6.1 Pro (ADInstruments, Sydney, Australia).

Pre- and Post-Sacrifice Sampling

The dams were sacrificed on D34 or D60 after determining blood pressure and reinforcing the anesthesia with a half dose of ketamine/xylazine. On D60, urine was withdrawn from the bladder for determination of protein (Bradford method) and creatinine (Beckman Autoanalyser, Fullerton, California) and blood from the left ventricle for creatinine and uric acid determination (Beckman Autoanalyser). The animals were euthanized by exsanguination, and the uterus and kidneys were removed. Finally, the pump was extracted from the subcutaneous pocket, and the animals were included in the study if this presented no blood or signs of infection. The fetuses, placentas, and kidneys were weighed after trimming the umbilical cord, amniotic membranes, and perirenal fat. Because miscarriages in guinea pigs are not always accompanied by a persistent implantation site (Elger W, MD, personal communication, March 13, 2014), fetal losses were attributed to demises (atrophic or necrotic fetoplacental units) or to abortions (absence of fetuses observed by ultrasonography). Only units with a live fetus were included for measurements of fetal and placental weight and immunohistochemical studies. A sagittal

Figure 1. Schematic diagram depicting the protocols of the short (A) and prolonged (B) bradykinin type 2 receptor (B2R) antagonism. The asterisks represent the days in which systolic blood pressure was determined.

section through the placenta, subplacenta, implantation site, and underlying myometrium was fixed as a single block. In other fetoplacental units, the placenta was detached from decidua, and the fetal–placental weight ratio was calculated as a proxy of placental efficiency.³⁵ Reproductive tissues and one kidney were immediately fixed with phosphate-buffered 10% formalin for 24 hours, then dehydrated in a graded series of ethanol and xylene dilutions, and embedded in Paraplast-Plus (Sigma). Sections (6-µm thick) were mounted on silanized slides. All samples were stained with hematoxylin and eosin prior to immunohistochemistry.

Evaluation of Extravillous Trophoblast Invasion

Extravillous trophoblasts (EVTs) were identified by immunostaining with cytokeratin as in previous studies.^{14,29,30,33} Deparaffinized sections were rehydrated using ethanol, rinsed 3 times for 5 minutes each in phosphate-buffered saline with 50 mmol/L Tris-HCl, and submitted to heat-induced antigen retrieval using citrate buffer (pH 6.0). Endogenous peroxidases were blocked by exposure of tissue sections to 10% H₂O₂ for 10 minutes. Sections were then incubated in a humid chamber for 30 minutes with protein block (Cas-Block; Zymed, San Francisco, California), followed by incubation for 18 hours at 4°C with anti-pan-cytokeratin mouse monoclonal antibody (1:50, P2871; Sigma). Sections were immunostained using a biotin–streptavidin–peroxidase system (LSAB DakoCytomation, Santa Barbara, California). Finally, the samples were treated for 15 minutes with 0.1% (wt/vol) 3-3'-

diaminobenzidine in buffer containing 0.05% H₂O₂. The slides were counterstained with Harris hematoxylin (Sigma).

Quantitative analysis was performed on images of histological sections acquired with a Zeiss AxioImager AX.10 microscope (Carl Zeiss, California) coupled to a Nikon CoolPix 4500 camera (Nikon Inc, Tokyo, Japan) using Axiovision 4.8.2.0 LE (Carl Zeiss AG, Inc, Oberkochen, Germany). The observer who performed the quantitative determinations from the photographic images was blinded regarding the pharmacological intervention.

In the myometrium, the depth of EVT invasion was measured in the sagittal section of the uteroplacental unit from the inferior surface of the subplacenta to the most distal cytokeratin positive cell and expressed as a percentage of the longitude between the subplacenta and the outer border of the myometrium. The magnitude of myometrial invasion was graded from 0 to 4 (absent to total decidual invasion).

Spiral arteries of sagittal sections were characterized as lateral when localized in the periphery of the subplacenta or as myometrial when they were in the subplacental uterine smooth muscle. The EVT was defined as intramural when located in the media of the spiral artery and as endoluminal when replacing endothelial cells. The extension of EVT replacing endothelial cells was deducted by measuring the internal arterial perimeter and calculating the percentage occupied by cytokeratin-positive cells. The depth of the vascular smooth muscle was measured between the abluminal endothelium/luminal EVT and the outer limit of muscle cells in 3 sites of each artery. Mean values represent both lateral arteries and 2 to

4 myometrial arteries. The glomerular, tubular, and interstitial renal structures were also examined by light microscopy.

Statistics

Data derived from the short B2RA study were analyzed to determine the effects of interventions on systolic blood pressure, fetal, placenta, and kidney weights and indexes of trophoblast invasion using Student *t* test with aid of Graphpad Prism 6.01 software (GraphPad Inc., San Diego, California).

In the prolonged blockade of the B2R, the mixed effects model³⁶ was performed with IBM SPSS Modeler to test differences in the evolution of systolic blood pressure between the control and the treated group, with the basal blood pressure as fixed covariable with random effects for each guinea pig to account for individual specific effects.

The mixed-model analysis also tested the association of fetal weight and the fetal-placental weight ratio with blood pressure, urinary protein-creatinine ratio, and the indexes of arterial invasion in D60. Fetal losses during the short and the prolonged study were analyzed by χ^2 test.

Determination of the replacement of endothelial cells by trophoblasts in spiral arteries was performed by the same observer (S.A.) in the controls of this and our previous study³³ and was found to be similar by the Mann-Whitney *U* test.

Data in the text are expressed as mean $\pm$ standard error of the mean (SEM), with one exception as mean (95% confidence interval, CI). In Supplementary material, all data are expressed as mean $\pm$ SEM. Data were considered significantly different when the 2-tailed *P* was $<.05$.

Results

Effects of Short B2R Antagonism During the Period of Maximal Trophoblast Invasion

Maternal, fetal, and placental outcome. When comparing the systolic blood pressures in both groups, dams receiving BDZ for 14 days exhibited an increased systolic blood pressure compared to that of control animals (65.2 ± 3.5 vs 55.2 ± 2.3 mm Hg; $P < .05$; Figure 2).

In litters of mothers that received BDZ and saline (2.5 ± 0.7 and 3.1 ± 0.3 , $P < .08$), the fetal-placental weight ratio was reduced by B2R antagonism (2.8 ± 0.1 vs 3.5 ± 0.1 g; $P < .006$). In the same groups, fetal and placental weights did not differ (3.4 ± 0.3 vs 4.7 ± 0.9 g and 1.2 ± 0.1 vs 1.4 ± 0.1 g, respectively).

Myometrial invasion was reduced in the BDZ-treated dams (3.0 ± 0.7 vs 3.7 ± 0.5 grades; $P < .04$). The percentage of endoluminal EVT in the lateral spiral arteries in BDZ-treated dams compared to controls was 54.6 ± 6.3 versus $79.2 \pm 6.3\%$; $P = .0584$ (Figure 3). No differences were observed between groups in the depth of EVT distal to the subplacenta, placental, and maternal renal weights

Figure 2. Evolution of maternal systolic blood pressure in guinea pigs receiving saline (dots) or Bradyzide (BDZ; squares) from gestational days 20 to 34 (mean $\pm$ standard error of the mean [SEM]). In the short bradykinin type 2 receptor (B2R) antagonism (SH-B2RA), the systolic blood pressure on day 34 is depicted in red. In days 40 and 60, BDZ abolished the systolic blood pressure fall observed in control animals from pregestational levels ($<D0$). * $P < .050$; ** $P < .015$ represent the difference between groups for a given day (Student *t* test). § $P = .033$ represents the blood pressure difference between groups on days 40 and 60 in regard to pregestational values (mixed-model analysis for repeated measurements).

Effects of Prolonged B2R Blockade During the Last Two-Thirds of Pregnancy

Maternal, fetal, and placental outcome. The pregestational systolic blood pressures of both groups did not differ; however, the decrement observed in the control arm in D40 and D60 was completely abolished in the BDZ-treated group ($P = .033$). On gestational days 40 and 60, the difference between the means of the BDZ-treated and the control group, adjusted for pregestational values, was 16.3 mm Hg (95% CI: 8.94-39.45). The BDZ-treated dams had higher systolic blood pressures than controls in D40 (72.4 ± 5.4 vs 57.6 ± 1.6 mm Hg; $P < .015$) but not in D60 (84.2 ± 10.4 and 64.4 ± 0.9). Maternal kidney weight in BDZ-treated dams and controls was 3.1 ± 0.2 and 2.7 ± 0.2 g; $P = NS$.

Litters of BDZ- and saline-treated dams had similar fetal and placental weight and fetal-placental weight ratios (Supplementary Table 1). The fetal-placental weight ratio correlated with the percentage of luminal trophoblast in myometrial ($r = 0.51$; $P < .05$) but not in lateral spiral arteries ($r = 0.23$; $P = .25$).

No correlation was found between blood pressures and plasma creatinine. Controls and treated dams displayed no differences in proteinuria or uricemia. Neither systolic blood pressure nor lateral and myometrial spiral artery invasion was associated with proteinuria. Renal histology did not show differences in glomerular, tubular, and interstitial structures.

Remodeling of uterine arteries. The BDZ-treated group displayed similar percentage of intraluminal perimeter replaced by EVT in lateral and myometrial arteries compared to the control

Figure 3. Lateral spiral artery and myometrial trophoblast invasion in the short protocol. Representative sections on gestational day 34 in a control and a Bradyzide (BDZ)-treated dam. The percentage of endoluminal trophoblasts was marginally significant (54.6 ± 6.3 vs $79.2 \pm 6.3\%$; $P = .0584$), while the magnitude of myometrial invasion by extravillous trophoblast (EVT) is decreased by BDZ (3.0 ± 0.7 vs 3.7 ± 0.5 grades; $P < .04$); the control and the BDZ sections were graded as 4 and 3, respectively. Extravillous trophoblast and endoluminal cyokeratin positive trophoblasts (ET) in lateral spiral arteries and myometrium are in the left- and right-hand panels, respectively. In the left panel, the perimeter occupied by endoluminal trophoblast is depicted by an interrupted red line and the perimeter occupied by endothelial cells (EC) by an interrupted black line. The percentage of the total perimeter for each cell type (EC or ET) is shown in brackets. Inset represents a magnification of the area included in the square; arrow heads highlight EC (black) and ET (red). Arterial lumen (L), intramural trophoblast (IMT), vascular smooth muscle cells (VSM), myometrial cells (MC), erythrocytes (ery). Bars = 100 μ m.

group (Figure 4; Supplementary Table 1). In addition, the depth of vascular smooth muscle in myometrial spiral arteries did not vary among groups. The percentages of endoluminal EVT invasion in lateral and myometrial arteries showed a positive correlation ($R = 0.314$; $P = .019$). Supplementary Table 1 includes the mean $\pm$ SEM of all the parameters evaluated in this group.

Fetal losses. In dams belonging to both the short and the prolonged studies, the proportion of mothers with lost fetuses (abortions and fetal demises) versus those with complete viable litters was greater in the BDZ-treated dams (5 with complete viable litters vs 6 with fetal losses) than in the control groups (11 with complete viable litters vs 2 with fetal losses; $\chi^2 = 4.1119$; $P < .05$).

Discussion

Our main finding is that in guinea pigs B2R blockade abolished the blood pressure decrement observed in controls at gestational days 34, 40, and 60, with the antagonist that has contributed to elucidate the role of the B2R in the hypotensive

enhancement of converting enzyme inhibitors, inflammation, and pain.^{37,38} This observation proves that bradykinin modulates blood pressure in mid and late pregnancy, corresponding with a buffer role that is hard to expose under normal conditions but becomes evident when blood pressure control is stressed (eg, subpressor levels of angiotensin II or salt loading).^{39,40} In addition, in midpregnancy the short blockade of the B2R reduced the percentage of endoluminal EVT in the lateral spiral arteries, the magnitude of myometrial invasion, and placental efficiency. This transient reduction in the local adaptation of pregnancy in the stage in which EVT displays the maximal myometrial and vascular penetration³⁰ mimics the precursor disturbance of preeclampsia⁴¹ and coincides with the decreased migration and invasion observed by us in HTR-8/SVneo pretreated by HOE-140 or BDZ.¹²

The effects of bradykinin on trophoblast invasion can be attributed to the binding of bradykinin to the B2R localized in the EVTs that derive from the subplacenta, traverse the decidua, dilate the arteries, remodel their walls, and replace the endothelial cells; the limited time span of this effect could be attributed to the decrement of the B2R in syncytial streamers

Figure 4. Lateral and myometrial spiral artery trophoblast invasion in the prolonged protocol. Representative sections of spiral arteries on gestational day 60 in control and Bradyzide (BDZ)-treated dams. No significant differences were found between treated and control groups. Extravillous trophoblasts (EVT) and endoluminal (ET) cytokeratin positive trophoblasts in lateral and myometrial spiral arteries are in the left and right panel, respectively. The perimeter occupied by endoluminal trophoblast is highlighted by an interrupted red line and that occupied by endothelial cells (EC) is represented by an interrupted black line. The percentage of the total perimeter for each cell type (EC or ET) is shown in brackets. Inset represents a magnification of the area included in the square; arrow heads highlight EC (black) and ET (red). No differences in the percentage of ECs replaced by endoluminal trophoblasts (T) were observed between the treated and the control group (brackets). (Table 1, supplementary material). Arterial lumen (L), intramural trophoblast (IMT), vascular smooth muscle cells (VSM). Bars = 400 μ m for lateral spiral arteries; 100 μ m for myometrial arteries.

observed by us in D40 and D60.²⁹ It has to be taken into account that the cellular expression of the B2R in the uteroplacental unit is shared in guinea pigs by other factors that could undertake the remaining remodeling (eg, increased vascular endothelial growth factor [VEGF]) and the preservation of placental flow (eg, prostacyclin).⁸ The prolonged bradykinin-mediated effect on maternal blood pressure is explained by the B2R located in the endothelium of elastic vessels and in arteriolar smooth muscle across organs and species.⁴² In early pregnancy, the vasodilation of uterine spiral arteries increases uterine perfusion and in conjunction with neighboring EVTs facilitate invasion. Further studies are required to unravel the difference in the time frames of the systemic and local effects of the B2R (regulation of receptor expression, ligand-receptor binding, signal transduction pathways, among others).

The first limitation of this study is the insurmountable fixed delivery of B2R antagonist throughout pregnancy, which entails a progressive decline in circulating levels. But even if the drug could be adjusted to the daily weight of the dam, its circulating levels are impossible to regulate, given the increment in weight of the fetoplacental units and the expansion of

maternal volume. Second, the discontinuous blood pressure measurements do not reflect daily changes and missed identifying the nadir of the bradykinin effect. It is worthwhile that future studies include telemetry; this has been performed in nonpregnant guinea pigs and yielded a mean systolic pressure of 70 mm Hg,⁴³ value that with the 76.7 mm reported for anesthetized animals⁴⁴ fall within the present reported range of 68.4 to 79.2 and the previous value of 73.5 mm Hg.³³

Finally, the residual effect of BDZ, angiotensin II infusion, and the combined interventions from gestational D20 to D34 on endothelial cell replacement in spiral arteries on D60 was not reproduced.³³ However, the correlation between indexes of vascular remodeling of lateral and myometrial spiral arteries and the similarity with values observed in controls of our previous study endorses the consistency of the morphometric determination. A compensatory effect of other proinvasive/angiogenic factors under the persistence of BDZ could explain this change as postulated for increased placental VEGF mRNA expression in dams submitted to hypoxia.⁴⁵

The evaluation of the localized and systemic action of endogenous kinins in this study favors the use of the guinea pig that

provides a gradient between the trophoblast invasion of the numerous lateral and myometrial spiral arteries^{22,25} as opposed to other rodents that show invasion of a few spiral arteries.⁴¹ Although the B2R antagonist in the dose and time span used in this study did not reproduce a “preeclampsia-like syndrome,” its effects resemble a “nonproteinuric hypertension of pregnancy.” Future research needs to simultaneously address the kallikrein–kinin and the renin–angiotensin systems due to the circulating and local changes in components of renin–angiotensin cascade, the counterregulatory role of both systems,^{46–54} and the connection between differentially expressed genes of the substrates of the renin–angiotensin and the kallikrein–kinin systems in preeclampsia.⁵⁵

Authors' Note

GV designed the study, participated in blood pressure determinations, sample extractions and data analysis, drafted the manuscript, and wrote the final version. SA and DS implanted the osmotic pumps and conducted blood pressure measurements, animal dissections, immunohistochemistry, and digital processing of the images. OP performed the mixed-model analysis. RO carried out the ultrasonography and SA the acquisition of microphotographs. All authors read and approved the final manuscript.

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Supplemental Material

Supplemental material for this article is available online.

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