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Expression of Kallikrein, Bradykinin B2 Receptor, and Endothelial Nitric Oxide Synthase in Placenta in Normal Gestation, Preeclampsia, and Placenta Accreta

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In an effort to define the varied expression of three vasoactive markers in the clinical models of normal placenta/normal invasion ($n = 11$), preeclampsia/restricted trophoblast invasion ($n = 15$), and placenta accreta/exaggerated invasion ($n = 6$), we performed semiquantitative immunohistochemistry for kallikrein, bradykinin B2 receptor, and endothelial nitric oxide synthase (eNOS). In the floating villi, the syncytiotrophoblast expressed more kallikrein in placenta accreta ($p < 0.05$), than in normal and preeclamptic placentas, while the bradykinin B2 receptor and eNOS were similarly expressed in all groups; in the fetal endothelium, the bradykinin B2 receptor was enhanced in placenta accreta ($p < 0.005$), but kallikrein and eNOS were similarly expressed in the other two groups. In the extravillous trophoblast, both kallikrein and eNOS expression were higher in placenta accreta ($p < 0.001$), while the bradykinin B2 receptor signal was only enhanced in preeclampsia ($p < 0.05$). The presence and localization of kallikrein, the bradykinin B2 receptor, and eNOS in the fetomaternal interface in the three study conditions supports a local role for interrelated vasodilatory/antiaggregating systems. This first report of the variations observed in kallikrein and eNOS in a condition of exaggerated trophoblast invasion supports the participation of vasodilatation in trophoblast migration.

Key Words: Kallikrein; bradykinin B2 receptor; endothelial nitric oxide synthase; normal placenta; preeclampsia; accreta.

Introduction

Adequate fetal development requires firm attachment of the placenta to the uterine wall, the replacement of the contractile spiral arteries by saccular dilated vessels and an extensive and intact surface of exchange of oxygen, nutrients, and waste products between the fetal and the maternal blood. In early pregnancy cytotrophoblasts have the capacity to switch their phenotype into syncytiotrophoblast, anchoring columns, and invasive trophoblasts that migrate from the base of the anchoring villi to finally replace the maternal endothelium *(1)*. In the second and third trimester, once the placenta is established, abundant patent fetal capillaries in the floating villi, a high uteroplacental blood flow, and an intact syncytium are required to permit an increasing contact between the blood of the fetus and his mother.

Vasodilator systems, with additional antiaggregating effects, are ideal candidates to prime the spiral arteries for trophoblast invasion, to prevent leukocyte and platelet adhesion and aggregation in the intricate intervillous space, and to vasodilate the fetal capillaries.

Tissue kallikrein is a serine protease that cleaves low-molecular-weight kininogen to generate kallidin and bradykinin. Kinins stimulate inflammation through the type 1 receptor (B1R), vasodilatation and increased vascular permeability through the type 2 receptor (B2R) *(2,3)*, and also enhance mitogenesis and angiogenesis *(3)*. In human placenta we have shown kallikrein in syncytiotrophoblast, intravascular trophoblast, fetal endothelium, and in the basal and chorionic plate *(4)*. The B2R and the mRNA for tissue kallikrein, detected by *in situ* hybridization, were observed in these cell types and additionally in the extravillous trophoblast *(5)*. Nitric oxide (NO) is a ubiquitous molecule, a potent vasodilator, and inhibitor of platelet aggregation, synthesized by NO synthase (NOS), from L-arginine *(6)*. NO diffuses into nearby cells and activates cytosolic guanylate cyclase, which converts GTP to cyclic GMP. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are predominantly constitutive, while iNOS is predominantly inducible *(6,7)*.

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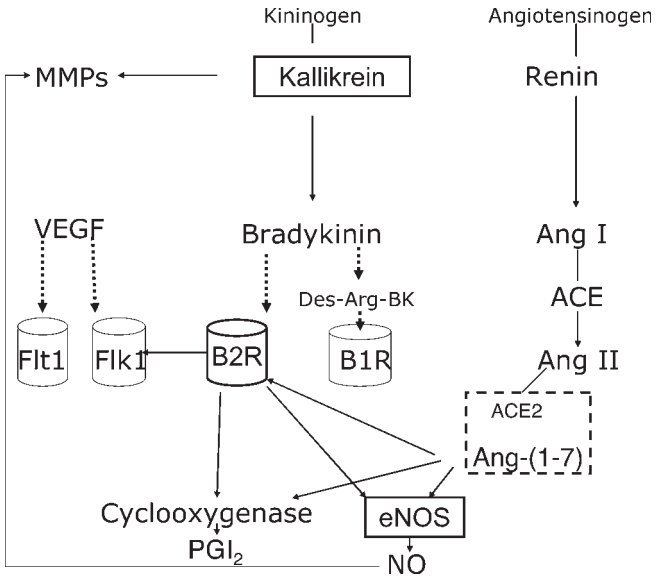

Fig. 1. Schematic of the kallikrein–kinin, renin–angiotensin, and nitridergic systems and their interactions with the VEGF receptor 2, cyclooxygenase, and metalloproteinases (MMPs). Continuous squares highlight the vasoactive markers included in this study, while dashed squares contain the vasodilator component within the renin–angiotensin system, Angiotensin-(1-7) [Ang-(1-7)] and converting enzyme 2 (ACE2) previously described by us in the same cell types expressing kallikrein, the bradykinin B2 receptor (B2R), and eNOS.

In the human placenta the three isoforms of NOS have been described, eNOS being the most extensively studied *(8–13)*. The kallikrein–kinin and the nitridergic systems are linked in their vasodilatory and antiaggregating effects through bradykinin that stimulates eNOS to increase NO synthesis *(14)* (Fig. 1).

Urinary kallikrein — reflection of renal synthesis — rises in normotensive gestation, attaining lower levels in preeclampsia, and has been used as a predictor index of the syndrome *(15–17)*. On the other hand, NO production, as determined by plasma nitrate/nitrite, rises in normal pregnancy; however, studies in preeclampsia have yielded normal or decreased levels *(18,19)*.

Given the need to advance in the understanding of the role of kallikrein, B2R and eNOS expression at the feto-maternal interface, and in order to test whether the previously described variations in the urinary or circulating levels of kallikrein and nitrates/nitrites are reflected locally, we have chosen to study their expression in the clinical conditions of normal pregnancy/normal invasion, preeclampsia/restricted invasion *(20)*, and placenta accreta/deep trophoblast invasion *(21)*.

Results

Chorionic villi expressed kallikrein in the syncytiotrophoblast and the endothelium of the fetal capillaries, with a punctuate and apical staining, respectively. The intensity of the syncytiotrophoblast staining was similar in normal and preeclamptic samples, while it was mildly increased in placenta accreta specimens ($p < 0.05$). The predominantly apical expression in normal and preeclamptic pregnancy differs from the more intense signal obtained in the whole syncytial cytoplasm in placenta accreta (Figs. 2A,B,C; Fig. 6). The bradykinin B2 receptor was similarly expressed in syncytiotrophoblast and fetal endothelium in normal and preeclamptic gestation, yielding a granular and thin apical staining, respectively. Placenta accreta presented similar syncytial signal, but greater fetal endothelial expression than normal gestation ($p < 0.005$) (Figs. 2D,E,F; Fig. 6). Endothelial nitric oxide synthase was similarly expressed in a granular pattern in syncytiotrophoblast and linearly in the apical border in fetal endothelium in the three groups (Figs. 2G,H,I; Fig. 6). Immunostaining controls in absence of first antibody showed no positive signals (Figs. 2J,K,L).

Extravillous trophoblasts in the placental bed of placenta accreta had a markedly higher expression of kallikrein in comparison to those in the basal plate of the other conditions studied ($p < 0.001$) (Figs. 3A,B,C; Fig. 7). In addition, endothelial nitric oxide synthase expression in the extravillous trophoblasts was markedly increased in placenta accreta ($p < 0.001$) (Figs. 3D,E,F; Fig. 7). A diffuse staining pattern for kallikrein and eNOS was seen in extravillous cytotrophoblasts. The expression of kallikrein and eNOS in cytokeratin positive extravillous cytotrophoblasts was demonstrated in sequential sections (Fig. 3) and by immunofluorescence double labeling (Fig. 5). The bradykinin B2 receptor was similarly expressed in extravillous trophoblasts in normal gestation and placenta accreta, but presented an increased signal in preeclampsia as compared to normal gestation ($p < 0.05$) (Figs. 4A,B,C; Fig. 7).

Discussion

In this study we demonstrate the colocalized expression of two interrelated local vasodilatory systems at the feto-maternal interface from normal gestations and pregnancies complicated by placentation defects.

The tissue kallikrein and the B2R immunoreactivity observed in syncytio and extravillous trophoblast in normal placentas agrees with our previous findings *(4,5)*. In addition, we now demonstrate that kallikrein and the B2R syncytial expression did not differ in preeclamptic gestations, while syncytial kallikrein and the fetal endothelial B2R were enhanced in accretas. In extravillous trophoblasts, kallikrein was increased in accretas and B2R in preeclampsia.

The eNOS expression in the syncytiotrophoblast agreed with that of other authors, and showed no difference between normal and preeclamptic pregnancies *(8,11–13)*. In extravillous trophoblast, the eNOS immunohistochemical and immunofluorescent signal is similar to that previously reported *(9,10)*. The extravillous expression of eNOS has also been confirmed by *in situ* hybridization and in a cell line

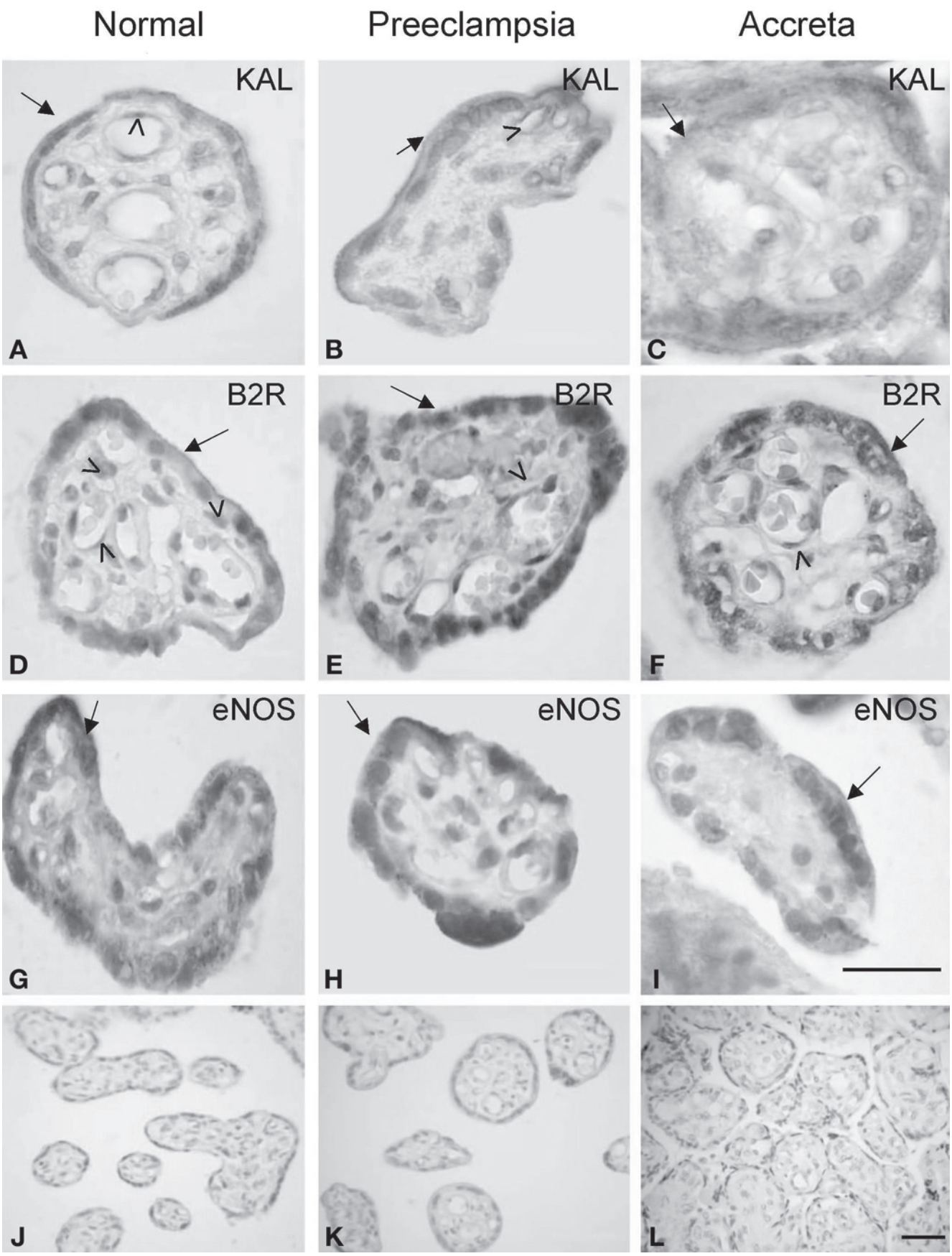

Fig. 2. Expression of kallikrein (KAL) (**A,B,C**), the bradykinin B2 receptor (B2R) (**D,E,F**), and eNOS (**G,H,I**) in villi from placentas obtained from normal (**A,D,G**), preeclamptic (**B,E,H**), and placenta accreta (**C,F,I**) complicated pregnancies. Arrows: syncytiotrophoblast, and arrowheads: fetal endothelium. Bar = 25 μm. Controls in absence of first antibody (**J,K,L**). Bar = 50 μm.

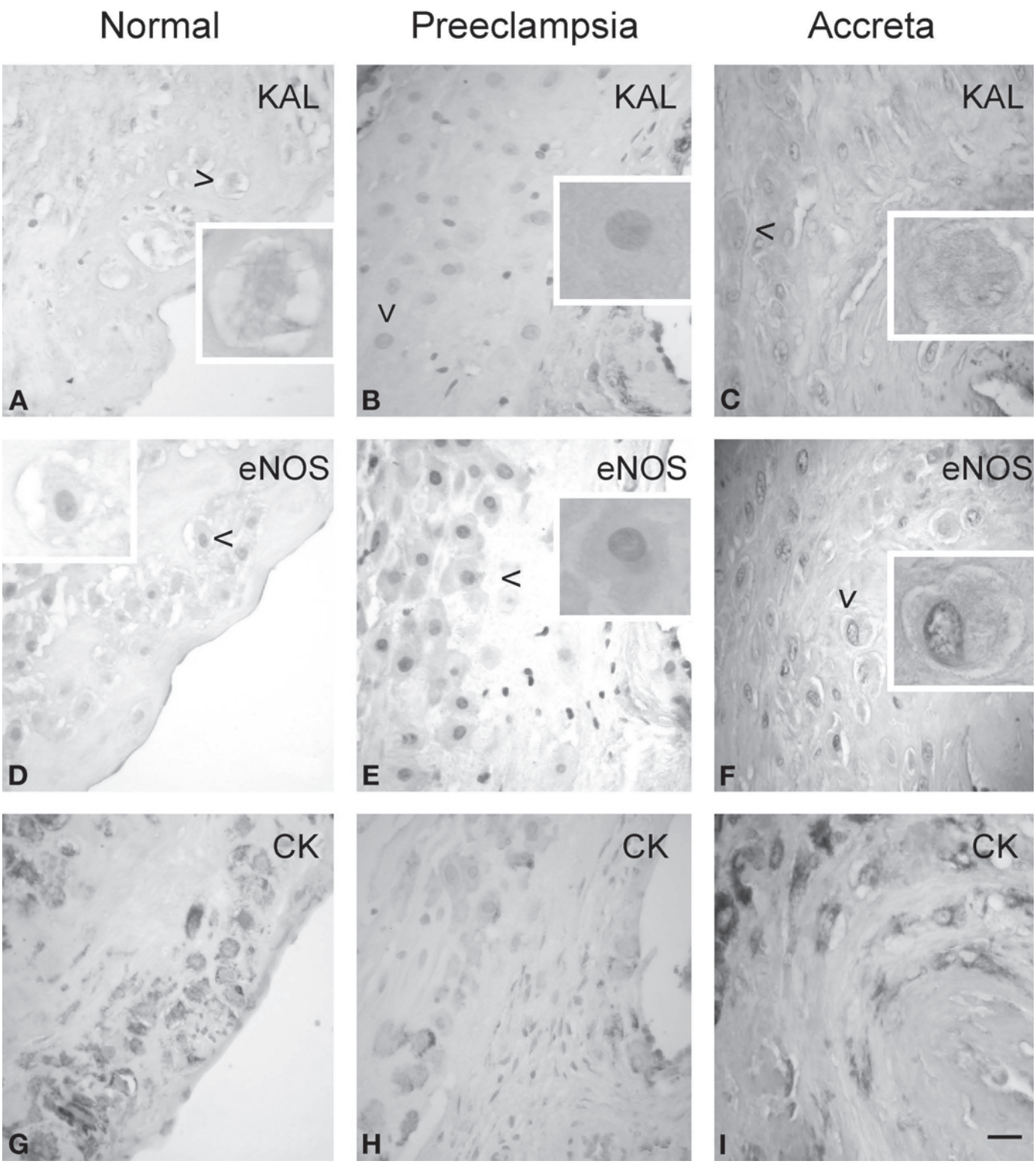

Fig. 3. Expression of kallikrein (KAL) (**A,B,C**), eNOS (**D,E,F**), and cytokeratin (CK) (**G,H,I**) in the extravillous trophoblast of the basal plate of normal (**A,D,G**) and preeclamptic (**B,E,H**) placentas, and of the placental bed from placenta accreta complicated pregnancies (**C,F,I**). Cytokeratin positive extravillous trophoblasts (**G,H,I**). Bar = 25 μm. Insets show high-power magnification of the extravillous trophoblast highlighted in the lower-power field (arrow heads).

that conserves the protein expression and invasiveness of extravillous trophoblasts *(10,22)*. Our finding of increased eNOS expression in the extravillous cytotrophoblast of placenta accreta adds to the qualitative description of Ariel et al., to our knowledge the only report on eNOS in this condition *(9)*.

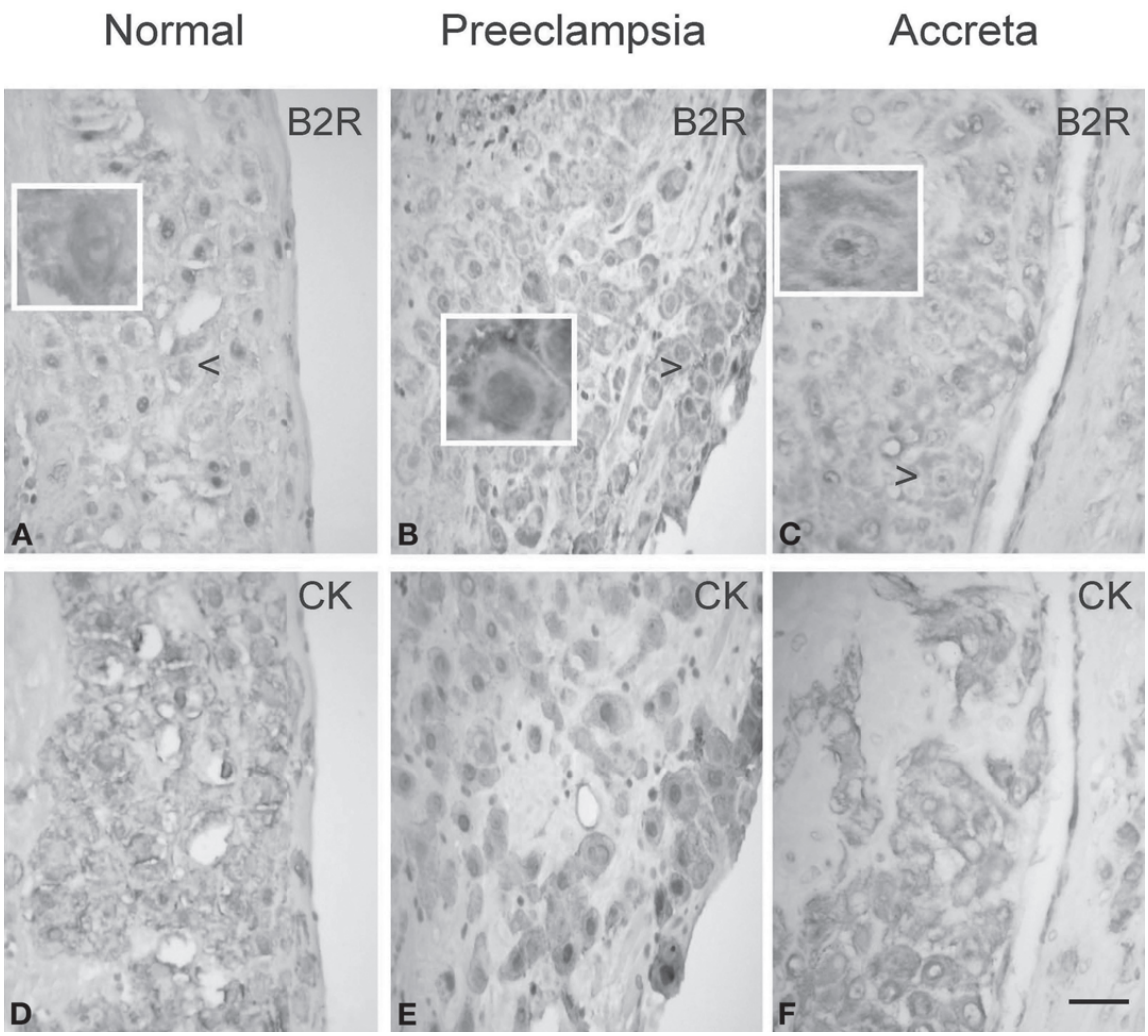

Fig. 4. Expression of the bradykinin B2 receptor (B2R) (**A,B,C**) and cytokeratin (CK) (**D,E,F**) in extravillous trophoblasts from placentas collected from normal (**A,D**), preeclamptic (**B,E**) pregnancies, and accreta placentas (**C,F**). Bar = 25 μm. Insets show high-power magnification of the extravillous trophoblast highlighted in the lower-power field (arrowheads).

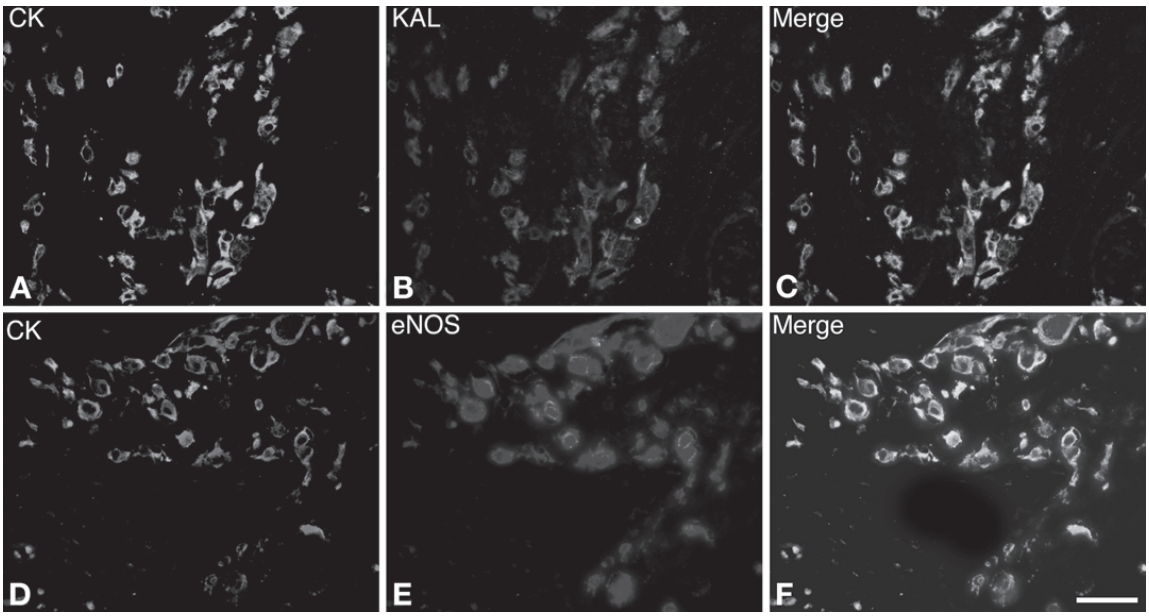

Fig. 5. Immunofluorescence of single and merged images showing the same cellular localization for kallikrein (KAL) (**A,B,C**) and eNOS (**D,E,F**) in cytokeratin-positive extravillous trophoblast cells. Bar = 40 μm.

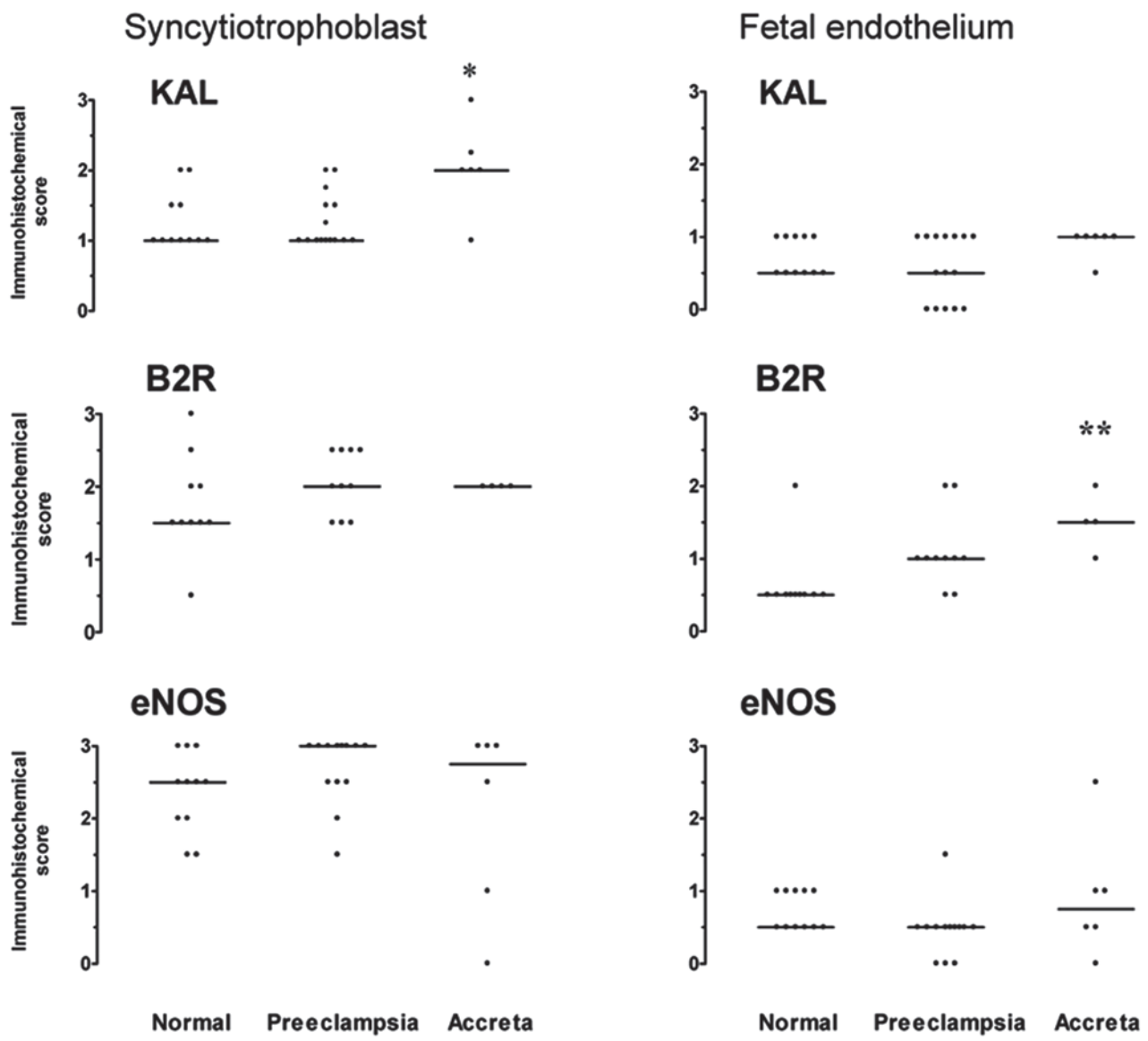

Fig. 6. Semiquantitative evaluation of the expression of kallikrein (KAL), bradykinin receptor (B2R), endothelial nitric oxide synthase (eNOS) in chorionic villous of normal, preeclamptic and placenta accreta. Bar represents median. Differences between groups were analyzed by Kruskall–Wallis and Dunn's post hoc test. $*p < 0.05$, $**p < 0.005$.

The link between the kallikrein–kinin and the nitridergic systems has been extensively demonstrated in other systems. In bovine aortic endothelial cells, bradykinin induces the translocation of eNOS from membrane caveolae to the cell cytosol through an intracellular Ca^{2+} mediated mechanism *(23)*, and stimulates NO synthesis by phosphorylation of eNOS *(24)*. Interestingly, in human uterine arteries, bradykinin, known to increase cGMP by releasing NO from the endothelium, produced a much greater increase in cGMP levels in the arteries of pregnant women than in those of nonpregnant women *(25)*. The presence of kallikrein and eNOS in syncytium favors the interaction between bradykinin and NO, and their predominant location at the apical border supports their release into the intervillous space. The syncytial surface, a morphologic and functional equivalent of the endothelium, is specially challenged to prevent cellular adhesion/aggregation as villous interdigitations originate variable intervillous blood flow patterns, thus requiring enhanced anticoagulant conditions.

The similar protein expression of kallikrein and eNOS in syncytium and extravillous trophoblast in preeclampsia and normal gestation at term does not preclude variations in this type of impaired placentation at the early stages. The differences previously observed in urinary (renal) kallikrein between normal and preeclamptic gestations *(15–17)*, in the face of similar trophoblastic expression in these same conditions, point to cell-specific regulation of the enzyme. On the other hand, the increase of kallikrein and eNOS in

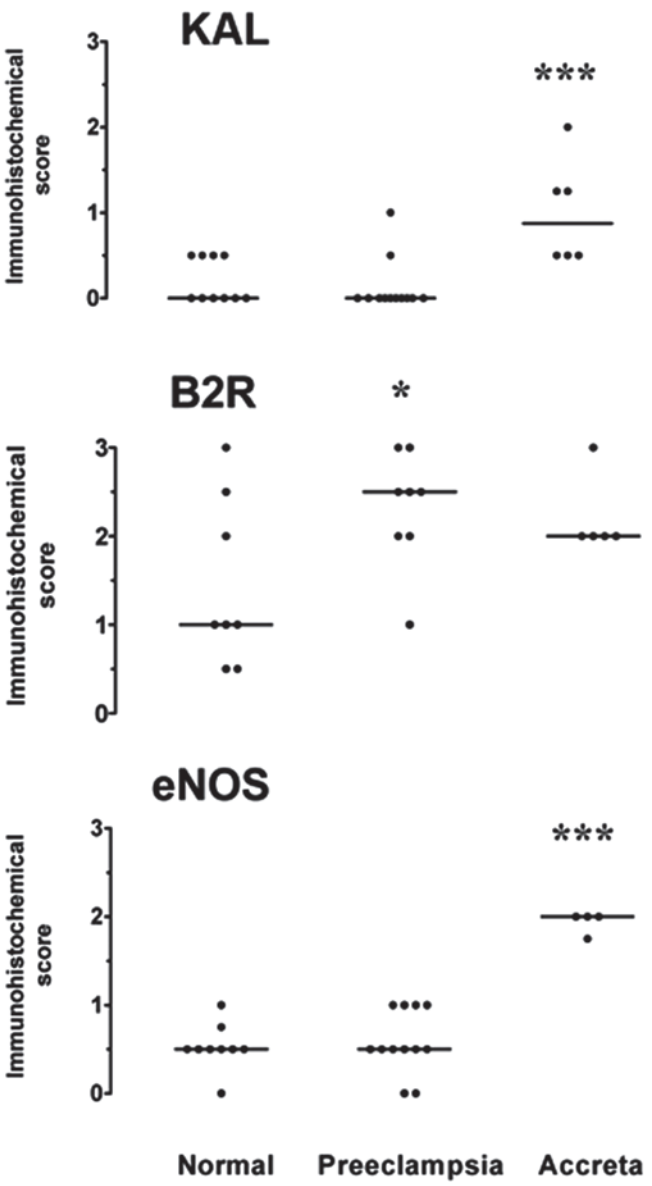

Fig. 7. Semiquantitative evaluation of the expression of kallikrein (KAL), bradykinin receptor (B2R), and endothelial nitric oxide synthase (eNOS) in extravillous cytotrophoblasts of normal, preeclamptic, and placenta accreta. Bar represents median. Differences between groups were analyzed by Kruskall–Wallis and Dunn's post hoc test. $*p < 0.05$, $***p < 0.001$.

placenta accreta, a condition of exaggerated invasion, suggests that the vasodilator enzymes favor the invasiveness of the extravillous cytotrophoblasts.

The increased expression of kallikrein in syncytiotrophoblast, and of kallikrein and eNOS in extravillous trophoblast in placenta accreta, could, on the other hand, contribute to the important and extended vasodilatation of noninvaded or partially invaded uteroplacental arteries described by Khong and Robertson *(21)* thus favoring vascular transformation. Endothelial NOS and the B2R in the fetal villous endothelium are strategically placed to control fetal blood flow. In placenta accreta, the bradykinin receptor up regulation in villous capillaries could enhance fetal blood flow, contributing to the good neonatal outcome in this group. It remains to be elucidated whether the upregulation of the bradykinin receptors in extravillous trophoblasts in preeclampsia reflects an attempt to improve fetal blood flow, in the face of an inability to increase the kinin-generating enzyme.

Further support for the functional importance of the expression of vasodilator-generating enzymes in different cell types of the fetomaternal interface is provided by the coincident cellular expression of angiotensin-(1-7) and of converting enzyme 2, its generating enzyme *(26)*. Angiotensin-(1-7) is a direct vasodilator *(27)* and also stimulates the release of bradykinin and NO, and potentiates their effects *(28)* (Fig. 1).

Having discussed the potential vasodilatory/antiaggregating effects of the kallikrein–kinin and the NOS–NO system, we would like to mention other actions that may pertain to placental development (Fig. 1). Kallikrein activates MMP-9 *(29)*, and NO stimulates the expression and activity of MMP-2 and MMP-9 in isolated trophoblasts *(30)*. Bradykinin, NO, and metalloproteinases promote angiogenesis *(3,31,32)*, an important mechanism of fetal and maternal vascular development and remodeling.

The concordance of eNOS, kallikrein, and bradykinin receptor signals in specific cell types that play primordial roles on the regulation of fetoplacental perfusion supports their autocrine and paracrine influences on uteroplacental hemodynamics. The increased expression of kallikrein and eNOS in extravillous trophoblasts in placenta accreta/exaggerated invasion, also suggest their participation in the invasion process. The demonstration of the vasoactive changes in the establishment and maintenance of the fetal blood flow, and of the interrelationships between the different components of this network, remain to be elucidated in animal models.

Methods

This study, approved by the Institutional Review Board, was conducted at the Clinical Hospital, Pontificia Universidad Católica de Chile and the Paula Jaraquemada Hospital, Universidad de Chile. Physiological pregnancies were defined by normotension, absence of proteinuria, and term birth of infants of adequate weight; the multiparas had previous normotensive gestations and no miscarriages. Preeclampsia was defined as *de novo* proteinuric hypertension (≥140 and/or ≥90 mmHg for systolic and diastolic blood pressures, respectively, and proteinuria greater than 300 mg/d). Placenta accreta samples were obtained from the tissue bank of the Department of Pathology, from postpartum hysterectomies due to bleeding. The characteristics of the studied groups are described in Table 1. The mean age was similar in the three groups; the preeclamptic group had lower gestational age at delivery and infant weight, higher

Table 1
Clinical Characteristics and Pregnancy Outcome of Study Groups (mean ± SD)

	Age (yr)	Gestational age at term (wk)	Infant birthweight (g)	Blood pressure (mmHg)	Primiparas vs multiparas
Normal gestation *(11)*	32.0 ± 5	38.8 ± 1	3385 ± 379	119/71 ± 7/6	2 vs 9
Preeclampsia *(15)*	29.1 ± 7	34.6 ± 3*	2242 ± 844*	177/109 ± 20/10**	8 vs 7
Placenta accreta *(6)*	32.5 ± 5	37.4 ± 2	3360 ± 616	115/72 ± 7/4 0 vs 6	

*$p < 0.05$, **$p < 0.001$ patients with preeclampsia vs normal gestation by one-way ANOVA and post hoc Dunn's test.

blood pressures, and an increased ratio of primiparas versus multiparas.

Placental specimens, including both the basal and the chorionic plate in normal and preeclamptic gestations, were obtained within 30 min of parturition and fixed with 4% formalin. Placenta accretas were obtained from the tissue bank of the Department of Pathology. The tissue blocks were dehydrated in a graded series of ethanol and embedded in Paraplast-Plus (Sigma, St. Louis, USA). Sections (5 μm) were mounted on polylysine-covered glass slides.

Immunohistochemistry

All immunostaining procedures were performed at room temperature as previously described *(33)*, with some modifications. Deparaffinized sections were rehydrated through ethanol. Samples were subjected to antigen retrieval by using 10 m*M* citrate, pH 6.0, and a microwave oven, except for kallikrein and vimentin immunostaining, and were then treated with 10% hydrogen peroxide for 10 min to block endogenous peroxidases. After rinsing three times in PBS–50 m*M* Tris-HCl, pH 7.8, the sections were incubated in a humid chamber for 30 min with protein block (DakoCytomation, CA, USA), then 18 h with either goat polyclonal antiserum against purified human urinary kallikrein (1:2000) (Protogen AG, Switzerland), monoclonal anti-eNOS/NOS Type III (2.5 μg/mL) (cat. no. 30020, BD Transduction Laboratories, USA), and polyclonal rabbit antiserum (1:2000) raised against the extracellular and intracellular domains of B2 kinin receptor *(34)*. Sections were immunostained using the biotin–streptavidin–peroxidase technique (Dako Cytomation). Finally, the samples were treated for 15 min with 0.1% (w/v) 3-3'-diaminobenzidine in buffer containing 0.05% H_2O_2. The slides were counterstained with Harris' hematoxylin.

Decidual cells and cytotrophoblasts were identified by staining sequential sections with anti-vimentin and anti-cytokeratin, respectively [goat anti-human vimentin (1:500) and monoclonal anti-pan cytokeratin (1:100) from Sigma]. The specificity of the staining was determined by incubation of sequential sections in the absence of the first antibody, or with antiserum preabsorbed with purified urinary kallikrein (50 μg/mL). All sections incubated with omission of the first antibody or preabsorbed showed no staining.

The semiquantitative evaluation of the intensity of the immunostaining in syncytiotrophoblasts, fetal endothelial cells, and extravillous trophoblasts was evaluated by a single observer, blinded to whether the placentas belonged to normal or preeclamptic pregnancies. The histology of placenta accretas precluded blinding. Grading ranged from 0 to 3, according to the following intensity score: 0 = absence of staining, 1 = faint or scant; 2 = moderate; 3 = intense.

Immunofluorescence

Deparaffinized and rehydrated sections were subjected to heat-antigen retrieval using 10 m*M* citrate, pH 6.0, treated with protein block and then incubated overnight with both anti-cytokeratin (DakoCytomation) (rabbit) and anti-eNOS (mouse) or anti-cytokeratin (rabbit) and anti-kallikrein (goat). Sections were then washed with PBS, 0.1% Tween-20, and incubated with FITC anti-rabbit (DakoCytomation) (1:20), rhodamine anti-mouse (1:50) (Pierce), and rhodamine anti-goat (Pierce) (1:20) for 1 h at room temperature in the dark, and then washed with PBS and mounted with Vectashield (Vector, California, USA).

Statistical Analysis

Gradings are expressed as medians. To analyze differences between groups we used the Kruskall–Wallis and Dunn's post-hoc tests in the semiquantitative evaluation of the immunoreactivity. Statistical significance was fixed at $p < 0.05$.

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