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ORIGINAL ARTICLE

# Impaired $A_{2A}$ adenosine receptor/nitric oxide/VEGF signaling pathway in fetal endothelium during late- and early-onset preeclampsia

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**Abstract** To investigate whether fetal endothelial cell proliferation and migration are modulated by the $A_{2A}$ adenosine receptor ($A_{2A}$AR), nitric oxide (NO) and the vascular endothelial growth factor (VEGF) signaling pathway, we isolated human umbilical vein endothelial cells from normal pregnancy (*n*=23), preterm delivery (*n*=4), and late-onset (LOPE, *n*=10) and early-onset preeclampsia (EOPE, *n*=8). We used the non-selective adenosine receptor agonist (NECA) and the selective agonist (CGS-21680) and/or selective antagonist (ZM-241385) for $A_{2A}$AR. Also, the nitric oxide synthase (NOS) inhibitor, L-NAME, was used in co-incubation with CGS-21680. Compared to normal pregnancy, EOPE exhibited low cell proliferation and migration associated with reduced expressions of $A_{2A}$AR and VEGF and NO synthesis (i.e., total and phosphorylated serine$^{1177}$ endothelial NOS and nitrite formation). In contrast, LOPE exhibited the opposite behavior in all these markers compared to normal pregnancy or EOPE. Cell proliferation and migration were increased by CGS-21680 (or NECA) in all analyzed groups (EOPE>LOPE>normal pregnancy) compared to their respective basal conditions, an effect that was associated with high NO and VEGF synthesis and blocked by ZM-241385 with significantly different $IC_{50}$ for each group (EOPE>LOPE>normal pregnancy). The differences seem independent of gestational age. L-NAME blocked the CGS-21680-mediated cell proliferation and migration in normal pregnancy and LOPE ($IC_{50}$=36.2±2.5 and 8.6±2.2 nM, respectively) as well as the VEGF expression in normal pregnancy. Therefore, the $A_{2A}$AR/NO/VEGF signaling pathway exhibits a pro-angiogenic effect in normal pregnancies and LOPE, whereas impairment in this pathway seems related to the reduced angiogenic capacity of the fetal endothelium in EOPE.

**Keywords** Adenosine · Adenosine receptor · Nitric oxide · Angiogenesis · Placental · Early- and late-onset preeclampsia

**Abbreviations**

| | |
|---|---|
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| VEGF | Vascular endothelial growth factor |
| $A_{2A}$AR | A2A adenosine receptor |
| LOPE | Late-onset preeclampsia |
| EOPE | Early-onset preeclampsia |

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## Introduction

Preeclampsia affects 5–10 % of pregnancies worldwide [1, 2], being the principal cause of maternal–neonatal morbidity and mortality [2]. There is significant evidence linking the genesis of preeclampsia and its associated perinatal outcomes with placental alterations, as we have recently reviewed [3]. One of the main features in the placenta from a preeclamptic pregnancy is failure of spiral artery remodeling, a phenomenon linked not only with reduced maternal blood flow toward the placenta [4] but also with high velocity and pressure of maternal inflow that may impair the placental villous structure [5]. Both a reduction in placental blood flow and impaired placental structure have been associated with neonatal complications following preeclampsia, such as growth retardation [4–7]. Specifically, Doppler studies reveal that impaired feto-placental blood flow is mainly evident in women with early-onset preeclampsia (EOPE) [8] rather than late-onset preeclampsia (LOPE) [9]. Moreover, EOPE exhibits placental histological changes related with reduced perfusion [10–13] as well as the anomalous structure of the peripheral villi and vasculature compared to respective controls identified by stereological analysis following EOPE, but not LOPE [13]. In addition, low placental levels of several pro-angiogenic factors have been reported in EOPE compared to LOPE or age-matched controls [14, 15]. In particular, microarray analysis has described the lower expression of at least two angiogenesis-associated transcripts (Egfl7 and Acvrl1) in EOPE compared to LOPE or age-matched controls [15]. Therefore, reduced placental angiogenesis could be responsible for the impaired placental blood flow observed in EOPE, while the observed normal blood flow in LOPE could result from either no alterations or increased placental angiogenesis [3, 6, 13].

Alternatively, impaired placental blood flow is associated with high levels of adenosine in the umbilical blood in preeclampsia [16, 17]. Adenosine is an endogenous purinergic nucleoside involved in angiogenesis via the activation of adenosine receptors (ARs) in both normal and pathological conditions (see [18]), which may include the human placenta in preeclampsia [6, 16, 19]. More specifically, using primary culture of microvascular placental endothelial cells (hPMEC), our group has described elevated levels of adenosine in culture medium and reduced expression of $A_{2A}AR$ ($A_{2A}AR$) without changes in $A_{2B}AR$ ($A_{2B}AR$) [20]. Moreover, George et al. [21] found an increase in the expression of vascular endothelial growth factor (VEGF) in rat placental explants exposed to hypoxia, a phenomenon that depended on the activation of AR. In addition, it is well described that stimulation of $A_{2A}AR$ triggers the activation of endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) synthesis in the endothelium [22–25]. Taking this evidence into account, we postulate that a dysfunctional $A_{2A}AR$/NO/VEGF signaling pathway may be involved in the alterations of placental angiogenesis observed in preeclampsia [6]. Therefore, our aim was to investigate whether fetal endothelium proliferation and migration are modulated by the $A_{2A}AR$/NO/VEGF signaling pathway during EOPE and LOPE.

## Patients and methods

### Patients

The Ethical Committee from the Universidad del Bío-Bío approved this cohort study, and informed consent was obtained from each participant. Pregnant women who attended the Obstetric and Gynecology Department of the Herminda Martin Clinical Hospital, Chillan, Chile, for their delivery were included in this study. The exclusion criteria included chronic hypertension, altered renal function, diabetes, chronic disease, twin pregnancies, recurrent miscarriages, and abruption placenta. Women were classified into: normal pregnancy (maternal blood pressure <140/90 mmHg, absence of proteinuria, and no medical complications) and preeclampsia (new-onset hypertension defined as blood pressure ≥140/90 mmHg with at least two measurements 6 h apart and proteinuria >300 mg/24 h after 20 weeks of gestation). Moreover, the preeclamptic group was divided into EOPE (≤34 weeks of gestation) or LOPE (>34 weeks of gestation). Women with preterm delivery ($n$=4) and without any clinical history of premature rupture of membranes or corioamnionitis were also included (see Electronic supplementary material, ESM). The gestational age was defined as the period of time from the first day of the mother's last menstrual period and the delivery date, confirming this age by a first trimester ultrasound. Moreover, according to the standard protocol, all patients with preeclampsia received anti-hypertensive treatment, magnesium sulfate to prevent convulsions in case of severe preeclampsia and/or corticoids for pulmonary maturation in the case of EOPE.

### Reagents

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO) $A_{2A}$ adenosine receptor-selective agonist, 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride hydrate (CGS-21680); non-selective agonist for adenosine receptor, 5′-(*N*-ethylcarboxamido) adenosine (NECA); non-selective inhibitor of nitric oxide synthase, $N_{\omega}$-nitro-L-arginine methyl ester hydrochloride (L-NAME); selective $A_{2A}AR$ antagonist, 4-(2-[7-Amino-2-(2-furyl)(1,2,4)tri-azolo(2,3-*a*)(1,3,5)triazin-5-ylamino)ethyl)phenol (ZM-241385) was from Tocris Biosciences, UK. The following antibodies were used: anti-$A_{2A}AR$ and anti-nitrotyrosin (Merck Millipore, USA), anti-eNOS and anti-serine$^{1177}$ phospho-eNOS (Transduction

Laboratories, USA), and anti-VEGF (Cell Signaling, USA) and anti-β-actin (Sigma Aldrich).

HUVEC culture

Endothelial cells were isolated from the human umbilical vein by digestion with collagenase (0.25 mg/mL) and then cultured (37 °C, 5 % $CO_2$) in medium 199 (M199) as previously described [26]. All experiments were performed in duplicate, after overnight serum deprivation. Cells were used in passage 2.

Cell proliferation

Cell proliferation was analyzed after treatment (24 h) with an AR agonist and/or antagonist by: (a) cell counter using a hemocytometer. Thus, cells were seeded at a density of $50\times 10^3$/mL and, after treatment, were counted for estimating cell proliferation. (b) MTS assay was performed using the available kit (Promega, USA) following the manufacturer's instruction. The correlation ($r^2$) of cell count and MTS analysis was 0.93.

Cell migration

Cell migration was analyzed after treatment (24 h) with an AR agonist and/or antagonist by: (a) wound assay. Briefly, cells were allowed to reach confluence in the growth medium and then switched to a serum-free medium prior to the addition of the test molecules. The monolayer was wounded with a single sterile cell scraper of constant diameter. Cells were observed in ×40 augmentation in a phase-contrast inverted microscope (Olympus, Japan) and six random images were taken using a digital camera (MShot MD90, Guangzhou Micro-shot Technology Co., Ltd, China) immediately after wound generation and 24 h after treatment. The cell count plugin from ImageJ software was used for counting cells crossing the wound. Analysis was blinded. (b) Transwell chambers assay: human umbilical vein endothelial cells (HUVECs) were trypsinized and seeded in the upper compartment of the transwell (Corning, USA) at a density of $150\times 10^3$ cells/well in M199. The lower compartments were loaded with the culture medium (control) or an AR agonist and/or antagonist. After 25 h, cells that had migrated to the bottom of the transwell membrane (8 μm) were stained using hematoxylin (Winkler, Chile). Membranes were observed at ×40 magnification using a light microscope (Olympus) and three photos were taken from each preparation.

Immunocytochemistry

Detection of $A_{2A}$AR in HUVECs was performed using a commercial kit (Vector Laboratories, USA) following the manufacturer's protocol. Briefly, HUVECs were fixed in 4 % paraformaldehyde prepared in phosphate buffer (in millimolars: NaCl, 13.7; KCl, 2.7; $Na_2HPO_4$, 0.9; $KH_2PO_4$, 1.8, pH 7.4, 4 °C) for 20 min. After blocking unspecific binding, cell preparations were incubated overnight with primary $A_{2A}$AR antibody. Antigen–antibody reaction was further revealed by diaminobenzidine reaction and densitometry was analyzed using ImageJ software as described previously [27]. In brief, analysis was blinded and performed using a bright-field microscope (Olympus). Three random pictures from each preparation were taken using a digital camera (MShot MD90, Guangzhou Micro-shot Technology Co., Ltd.). Estimation of the intensity of staining in the pictures was analyzed using ImageJ software (NIH, Bethesda, MD). Thus, the luminance of the incident light was calibrated for each section in order to assign pixel values from 0 (no light transmission) to 255 (full light transmission). After that, the immunostained signal was extracted from the images with a color deconvolution algorithm integrated in the ImageJ software. Values are expressed as the ratio between the area of positive brown stain divided by the total area of the reference field.

Western blot

Cell protein extracts (70 μg) were separated by SDS-PAGE (10 %) transferred to nitrocellulose membranes and probed with primary anti-$A_{2A}$AR, anti-nitrotyrosin, anti-eNOS, anti-serine$^{1177}$ phospho-eNOS, anti-VEGF, and anti-β-actin antibodies. Then, a horseradish peroxidase-conjugated secondary antibody was used for visualization.

Non-quantitative PCR

Non-quantitative RT-PCR was performed using commercially available kits (Fermentas, USA) and sequence-specific oligonucleotide primers for human VEGF: sense 5′-GGGCAGA ATCATCACGAAGTG-3′; antisense 5′-ATTGGATGGCAG TAGCTGCG-3′; or β-actin: sense 5′-CGCCCCAGGCAC CAGGGC-3′, antisense 5′-GGCTGGGGTGTTGAAGGT-3′ (0.5 μM). Cycles for PCR include: 4 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, then 30 s at 58 or 60 °C (for VEGF and β-actin, respectively) and later 30 s at 72 °C, and, finally, 7 min extension at 72 °C. β-Actin mRNA was used as the internal reference.

Nitrite measurement

Nitrite levels were measured by the Griess reaction using a commercially available kit (Promega). In brief, confluent cells were incubated (30 min) in the presence or absence of CGS-21680 (100 nM) and/or ZM-241385 (100 μM); then, according to the manufacturer's protocol, 100 μL of M199 was collected for nitrite quantification. A spectrophotometer

(Phomo, Autobio) was used to read the samples. It was set at 540 nm. The limit of detection of the Griess assay was 1.5 μM. Inter- and intra-assay variations were 17.2±2.3 and 4.5±0.4 %, respectively.

### Statistical analysis

Comparisons between the studied groups were performed using analysis of variance (ANOVA). If the ANOVA demonstrated a significant difference between variables, post hoc analyses were performed using the Bonferroni test. We used $\chi^2$ test to analyze proportions. Values are the mean ± SEM, where $n$ indicates the number of the different cell cultures (in duplicated). A value of $p<0.05$ is considered statistically significant. The statistical software GraphPad Instat 3.01 and GraphPad Prism 5.00 (GraphPad Software Inc., California, USA) were used for data analysis.

## Results

### Clinical characteristics of patients

Forty-five women were included in the study and divided into normal pregnancy ($n$=23), preterm delivery ($n$=4), LOPE ($n$=10), and EOPE ($n$=8) (see ESM Table). According to the diagnosis criteria, women with preeclampsia exhibited higher systolic and diastolic blood pressure than normal pregnancy or preterm delivery. As expected, newborns from pregnant women with EOPE and preterm delivery had reduced anthropometric parameters including lower birth weight and height and cephalic perimeter than newborns from normal pregnancy. The percentile of weight according to gestational age was reduced in newborn from EOPE compared to normal or LOPE. Diagnosis of small for gestational age (<10th percentile) was present in 10 % of women with LOPE and 25 % of women with EOPE. In addition, newborns exposed to EOPE showed perinatal stress according to the Apgar scale at 1 or 5 min. Moreover, placentas from EOPE and preterm pregnancies exhibited lower weight and had reduced area compared to those from normal pregnancies. Clinically, EOPE was a more severe condition and required a longer hospitalization period than LOPE or normal pregnancy.

### Expression of $A_{2A}AR$

In cells from EOPE, a lower protein abundance of $A_{2A}AR$ was observed by immunocytology (70±4 %, $p<0.05$) and Western blot (52±6 %, $p<0.05$) compared to cells derived from normal pregnancy (Fig. 1). This percentage of reduction was even higher when cells from EOPE were compared to those from LOPE (83±6 and 62±8 %, respectively, $p<0.05$). However, there were not significant differences between normal and LOPE.

### AR stimulation increases cell migration and proliferation

In basal condition media, cell migration (Fig. 2a, b) and proliferation (Fig. 2c, d) were significantly reduced in EOPE compared to normal or LOPE ($p<0.05$ in all cases). On the other hand, LOPE and preterm delivery exhibited the highest proliferation amongst the studied groups (see ESM), reaching as high as 1.6±0.04- and 2.8±0.06-fold in LOPE (Fig. 2d) and 1.6±0.03- and 2.9±0.07-fold in preterm delivery compared to normal pregnancy or EOPE, respectively (see ESM Fig. S1a, b).

Besides, both CGS-21680 (100 nM, $A_{2A}AR$ agonist) and NECA (10 μM, non-selective AR agonist) significantly increased cell migration (Fig. 2a, b) and proliferation (Fig. 2c, d) in normal pregnancy, LOPE, and EOPE (ANOVA, $p<0.001$). A similar trend in cell proliferation/migration was observed in the two different approaches that were used for each one of the cell analyses. However, a high migratory response induced by either CGS-21680 or NECA was observed in cells derived from EOPE in the transwell assay (Fig. 2b) compared to the wound assay ($p<0.05$, Fig. 2a). Moreover, considering that cells from EOPE exhibited the lowest migration basally, the magnitude of response in those cells tends to be higher than the others groups, in particular in the transwell assay.

Likewise, in proliferation (Fig. 2c), cells from EOPE exhibited the highest response to CGS-21680 (2.9±0.3-fold) and NECA (2.1±0.4-fold) compared to their own basal condition, while in LOPE, the proliferative responses in the presence of CGS-21680 and NECA were 1.6±0.2-fold for each; in normal pregnancy, the magnitudes of responses were 1.5±0.2- and 1.4±0.1-fold, respectively (ANOVA, $p<0.05$). These stimulatory effects of both agonists on cell migration (by wound assay) and cell proliferation (by MTS assay) were blocked by co-incubation with ZM-241385 (100 nM) in normal pregnancy only (Fig. 3a, b). On the other hand, in pathological cells, ZM-241385, at the initial concentration used, did not block the proliferation induced by CGS-21680. Therefore, we performed a dose–response curve of ZM-241385 for blocking the stimulatory effect of CGS-21680 on cell proliferation (Fig. 3c). In those experiments, the calculated inhibitory concentration 50 ($IC_{50}$) for ZM-241385 in EOPE was 11.1±0.2 μM, which was significantly higher than in both normal pregnancy (2.6±0.5 μM) and LOPE (4.2±1.3 μM; ANOVA, $p<0.001$). Then, all the experiments were further performed using 100 μM of ZM-241385. Complementary to these assays, CGS-21680 (100 nM) increased (2.1±0.4-fold) cell proliferation in preterm delivery compared to its own basal condition, an effect blocked by ZM-241385 (100 μM; see ESM Fig S1c).

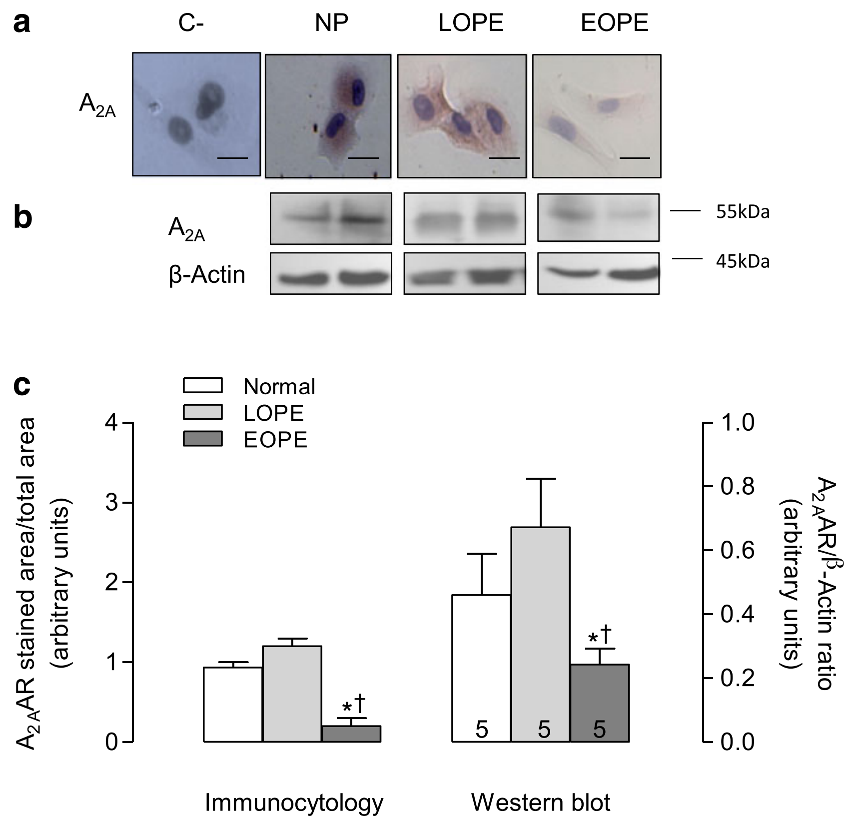


**Fig. 1** $A_{2A}$ adenosine receptor protein expression in early- and late-onset preeclampsia. HUVEC were isolated from normal (*NP*, *white bars*), late-onset preeclampsia (*LOPE*, *gray bars*), and early-onset preeclamptic pregnancies (*EOPE*, *darkest bars*) and used for immunocytochemistry (**a**) and Western blot analysis (**b**) looking for $A_{2A}$ adenosine receptor (*$A_{2A}AR$*) expression as described in "Patients and methods." **a** Representative images of immunocytochemistry. *Line* represent 10 μm. **b** Representative images of immunoblot for $A_{2A}$ (55 kDa) and β-actin (43 kDa) in the analyzed groups as described in (**a**). **c** Digital semiquantitative analysis using color deconvolution of images obtained from immunocytochemistry on the *left side* and densitometry of the $A_{2A}$AR/β-actin ratio form the images obtained by Western blot on the *right side*. In (**a**), C− is the negative control without primary antibody. *$p<0.05$ vs. normal pregnancy; †$p<0.05$ vs. LOPE. Values are the mean ± SEM. $n=5$ different umbilical cords in each group. All experiments were performed in duplicate

## $A_{2A}$AR stimulation and VEGF expression

Compared to the percentage of change with respect to normal pregnancy (41±7 %, $p<0.05$) or LOPE (78±9 %, $p<0.05$), it was found that VEGF expression in HUVEC was significantly lower in EOPE. Nevertheless, VEGF expression in HUVEC was higher in LOPE (2.6±0.7-fold, $p<0.05$) compared to normal pregnancy (Fig. 3d). Additionally, in total placental homogenate, a low expression of VEGF was observed in EOPE compared to normal pregnancy or preterm delivery, whereas VEGF protein in LOPE was not significantly different with respect to normal or preterm delivery (see ESM Fig. S1a, b).

CGS-21680 (12 h) increases the protein abundance of VEGF in normal and EOPE, which was later blocked by ZM-241385. Despite CGS-21680 not showing any effect on VEGF expression in the LOPE group, co-incubation with ZM-241385 exhibited a reduction (41±6 %, $p<0.05$) in the level of this protein compared to its respective basal condition. In fact, ZM-241385 by itself reduced the protein abundance of VEGF in normal and preeclamptic pregnancies (Fig. 3d).

## $A_{2A}$AR stimulation generates a NO-dependent signaling

Basally, the protein levels of eNOS were significantly lower in EOPE compared to normal pregnancy (51±16 %, $p<0.05$) or LOPE (47±8 %, $p<0.05$). Nevertheless, there are no changes in the amount of this protein when we compared the last two groups to each other (Fig. 4a, b). Moreover, incubation (12 h) with CGS-21680 or ZM-241385 did not significantly modify the amount of eNOS protein either in normal or preeclamptic pregnancies.

On the other hand, the ratio of phosphorylated eNOS (serine$^{1177}$, the active form) and total eNOS was increased in LOPE compared to normal pregnancy or EOPE (2.2±0.3- and 2.6±0.5-fold, respectively; Fig. 4c, d). Moreover, CGS-21680 (30 min) increased the

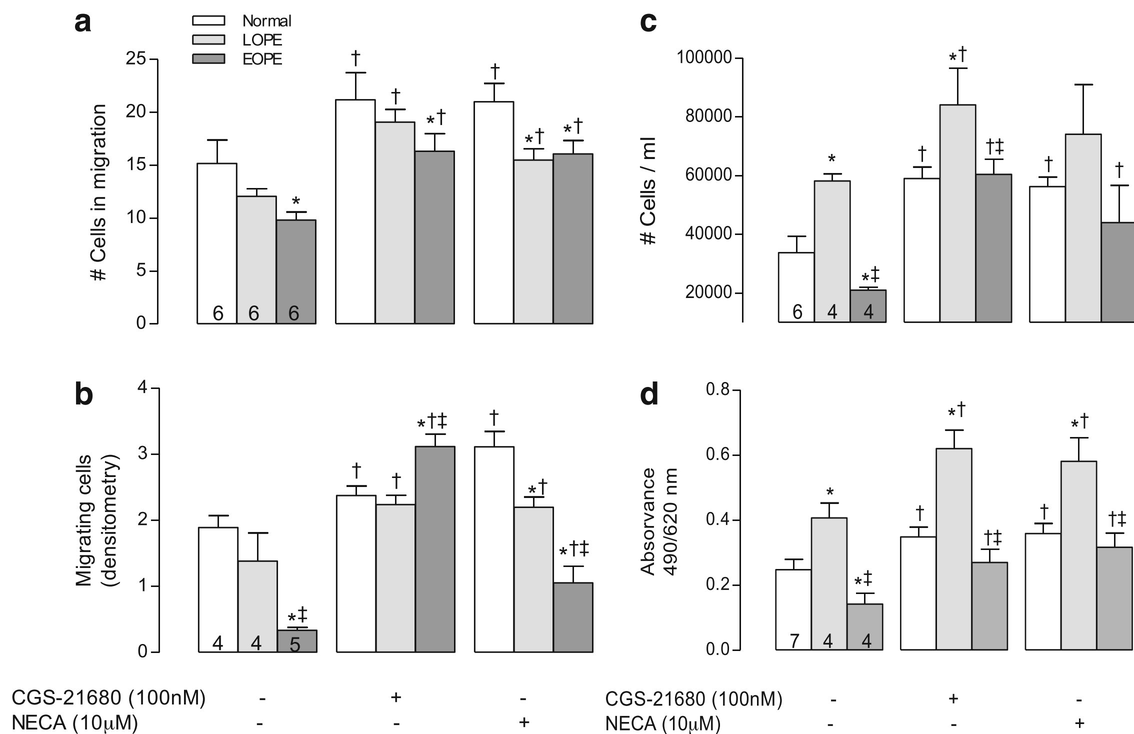


**Fig. 2** Cell migration and proliferation mediated by adenosine receptors in early- and late-onset preeclampsia. Cell migration was analyzed by two different techniques—wound technique (**a**) and transwell migration assays (**b**)—in the studied groups. In addition, cell proliferation was analyzed by counting cell (**c**) using hemocytometer and MTS analysis (**d**) as described in "Patients and methods." Experiments were performed after overnight serum deprivation and in the absence (basal, *negative sign*) or presence (*positive sign*) of CGS-21680 (selective $A_{2A}$AR agonist, 100 nM) and NECA (non-selective adenosine receptors agonist, 10 μM) for 24 h. *$p<0.05$ vs. respective value in normal pregnancy; $^{\dagger}p<0.05$ vs. respective value in their own basal conditions; $^{\ddagger}p<0.05$ vs. respective value in LOPE. Values are the mean ± SEM. $n$= 4–7 different umbilical cords in each group. Exact $n$ is included in the respective bar of basal conditions. All experiments were performed in duplicate

activation of eNOS (i.e., p-eNOS/eNOS ratio) in normal (1.5±0.1-fold) and EOPE (1.6±0.2-fold), but not in LOPE. This last stimulatory effect observed in normal and EOPE was blocked by ZM-241385 co-incubation. Otherwise, in LOPE, ZM-21680 by itself or co-incubated with CGS-21680 reduced the eNOS activation in 30±8 % compared to the respective basal condition. Moreover, cells from EOPE exhibited a significantly low nitrite level measured in the culture media (Fig. 4e) and nitrotyrosine formation in cell extractions (Fig. 4f) compared to LOPE and normal pregnancy (ANOVA, $p<0.05$), while both markers were higher in LOPE than normal pregnancy. Despite preterm delivery exhibiting a higher nitrite level compared to EOPE ($p<0.05$; see ESM Fig. S1d), there were no significant differences with respect to normal pregnancy or LOPE. Furthermore, CGS-21680 enhances the level of nitrite and nitrotyrosine formation in both normal pregnancy and EOPE, but not in LOPE. Nevertheless, GCS-21680 fails to enhance the nitrite level in preterm delivery; however, co-incubation with ZM-241385 or L-NAME reduced significantly the basal level of nitrite in those cells (see ESM Fig. S1d).

### Effect of blockage of the NO synthesis in $A_{2A}$AR-mediated proliferation and migration

Pre-incubation with L-NAME (100 μM) significantly reduced cell migration (Fig. 5a) and proliferation (Fig. 5b) mediated by CGS-21680 in normal pregnancy and LOPE, but not in EOPE. This blocking effect of L-NAME upon CGS-21680-mediated cell proliferation was also observed in preterm delivery (see ESM Fig. S1c). The $IC_{50}$ for L-NAME in CGS-21680-mediated cell proliferation was higher in LOPE than normal pregnancy (36.2±2.5 vs. 8.6 ±2.2 nM, $p<0.001$); however, in EOPE, the $IC_{50}$ for L-NAME was ambiguous (Fig. 5c).

### Effect of blockage of NO synthesis in $A_{2A}$AR-mediated VEGF expression

As detailed before, in HUVECs from normal pregnancy, CGS-21680 (12 h) by itself increased the expression of mRNA (1.4±0.2-fold) and protein (1.9±0.2-fold) of VEGF compared to their own basal conditions (Fig. 5d). The stimulatory effect of CGS-21680 was reverted by L-NAME,

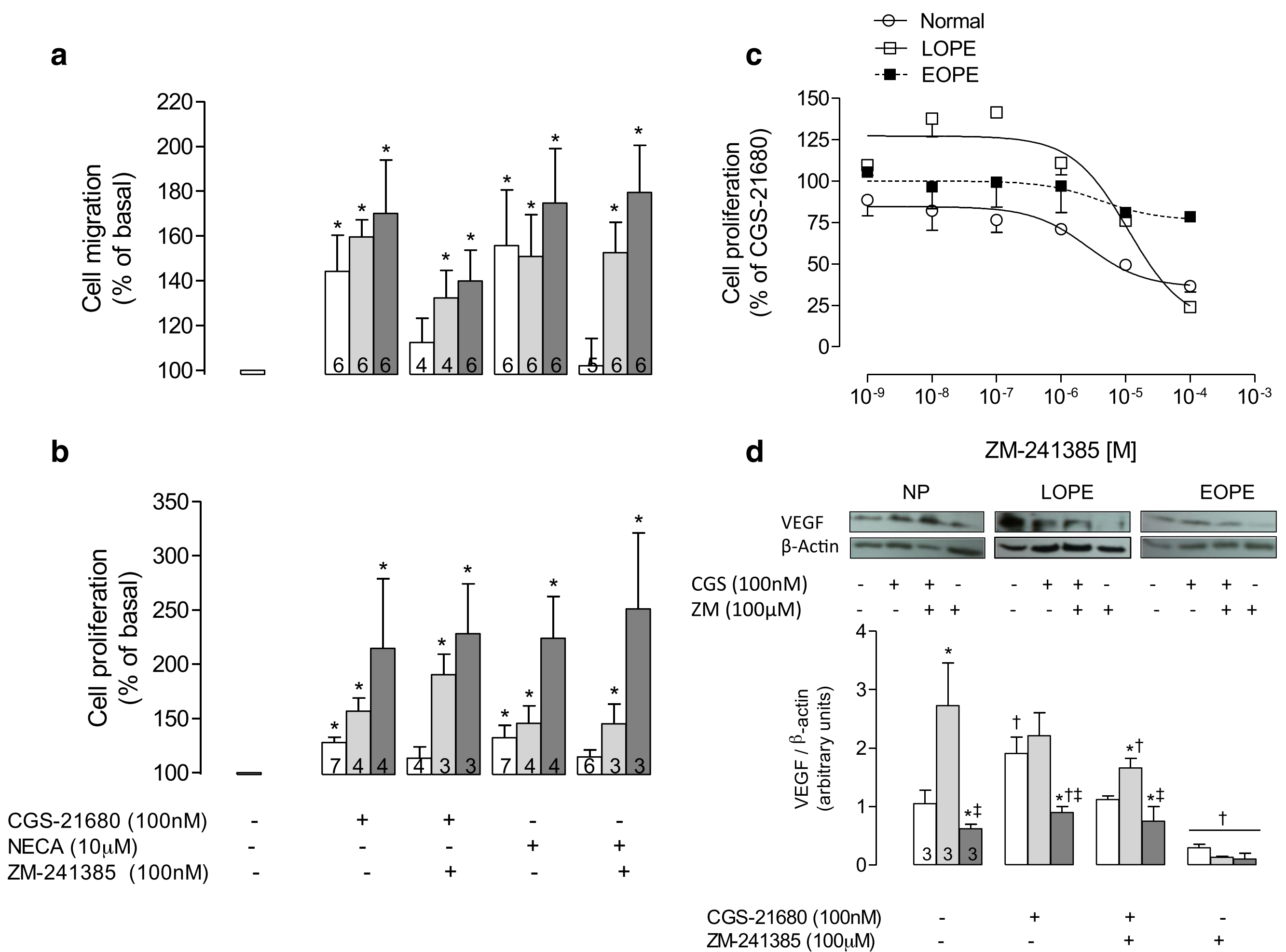


**Fig. 3** Cell migration and proliferation mediated by $A_{2A}$ adenosine receptor involves VEGF synthesis. **a** Percentage of cell migration considering respective basal condition (i.e., without agonist or antagonist, *negative sign*) or in the presence (*positive sign*) of CGS-21680 (100 nM), NECA (10 μM), and/or ZM-241385 (100 nM) for 24 h in the analyzed groups. **b** Cell proliferation as described in (**a**). **c** Inhibitory response of the CGS-21680-mediated proliferation using a dose–response curve of ZM-21680. **d** *Upper panel* Representative image of Western blot for VEGF (55 kDa) and β-actin (43 kDa) in the analyzed groups in absence (*negative sign*) or presence of CGS-21680 (*CGS*) and/or ZM-241385 (*ZM*). *Bottom panel* Densitometry of the VEGF/β-actin ratio as shown in the *upper panel*. In (**a**) and (**b**), $^{*}p<0.05$ vs. the respective value in their own basal conditions. In (**d**), $^{*}p<0.05$ vs. the respective value in normal pregnancy; $^{\dagger}p<0.05$ vs. the respective value in their own basal conditions; $^{\ddagger}p<0.05$ vs. the respective value in LOPE. Values are the mean ± SEM. $n$=3–7 different umbilical cords in each group. Exact $n$ is included in either the respective basal condition or respective bars. All experiments were performed in duplicate

especially at concentrations of 100 and 10 μM, where the percentages of reduction with respect to the basal condition were 25±3 and 62±4 % for both mRNA and protein, respectively.

## Discussion

Preeclampsia is characterized by placental insufficiency, mainly during early onset [8], which has been associated with low placental angiogenesis [3, 4, 6, 28]. We have studied the cell proliferation/migration of HUVECs as the initial steps linked with angiogenesis. In addition, we have focused on the study of the functional presence of the $A_{2A}$AR/NO/VEGF signaling pathway and its alterations as the underling mechanisms behind the differential angiogenic processes in normal and preeclamptic pregnancies. Thus, the results presented in this work describe the activation of $A_{2A}$AR as associated with the following cascade: eNOS activation (i.e., ser$^{1177}$ phosphorylation), NO synthesis, VEGF expression, and cell proliferation/migration in normal pregnancy. Considering this paradigm, cells derived from EOPE, LOPE, or preterm delivery exhibit a different behavior. Thus, whereas EOPE presents a low $A_{2A}$AR expression, reduction in the NO/VEGF synthesis, and cell proliferation/migration, LOPE leads to high cell proliferation/

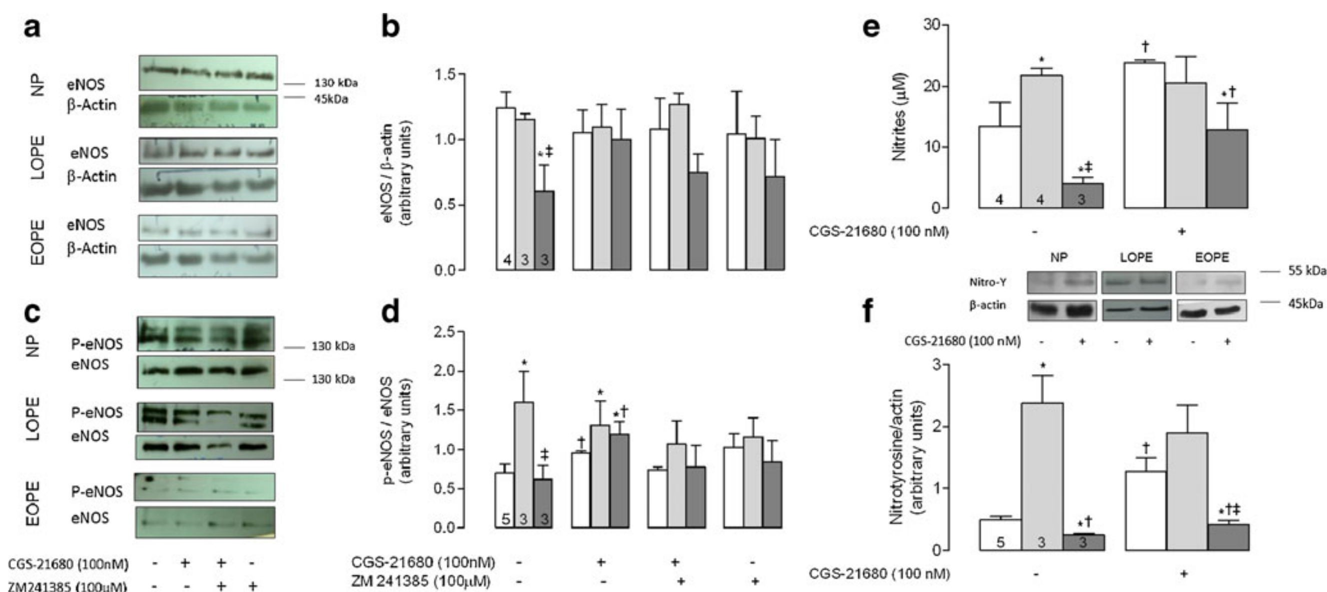

**Fig. 4** Impairment in the endothelial nitric oxide synthase activation mediated by $A_{2A}$ adenosine receptor in early- and late-onset preeclampsia. **a** Representative images of Western blots for endothelial nitric oxide synthase (*eNOS*, 130 kDa) and β-actin (43 kDa) in the absence (*negative sign*) or presence (*positive sign*) of CGS-21680 (100 nM) and/or ZM-241385 (100 μM) for 12 h. **b** Densitometry of the VEGF/β-actin ratio as shown in (**a**). **c** Representative images of Western blots for phosphorylated (ser$^{1177}$) endothelial nitric oxide synthase (*p-eNOS*, 135 kDa) and eNOS (130 kDa) after 30 min of incubation with CGS-21680 and/or ZM-241385. **d** Densitometry of the p-eNOS/eNOS ratio as shown in (**c**). **e** Nitrite concentration in the homogenate of cell after incubation (30 min) with CGS-21680. **f** *Upper panel* Representative images of Western blots for the nitration of tyrosine residues (*Nitro-Y*) in protein(s) at 55 kDa and β-actin (43 kDa). *Bottom panel* Densitometry of the nitrotyrosine/β-actin ratio in the analyzed groups as shown in the *upper panel*. In (**b**), $*p<0.05$ and $^{‡}p<0.05$ vs. the respective values in normal pregnancy and LOPE, respectively. In (**d**–**f**), $*p<0.05$ vs. the respective value in normal pregnancy; $^{†}p<0.05$ vs. the respective value in their own basal conditions; $^{‡}p<0.05$ vs. the respective value in LOPE. Values are the mean ± SEM. $n$=3–5 different umbilical cords in each group. Exact $n$ is included in the respective bars of basal conditions. All experiments were performed in duplicate

migration. This idea is reinforced by the fact that ZM-241385, the $A_{2A}$AR antagonist, reverts the high NO synthesis and VEGF expression observed basally in LOPE. On the other hand, in EOPE, stimulation of $A_{2A}$AR by CGS-21680 recovers the reduced cell proliferation/migration, a phenomenon associated with increasing NO and VEGF synthesis. Nevertheless, at basal condition, preterm delivery was associated with high cell proliferation mediated, at least partially, by the $A_{2A}$AR/NO signaling pathway since both ZM-241385 and L-NAME reverted these phenomena. These results reinforce the concept that impaired feto-placental blood flow observed in EOPE is associated with low placental angiogenesis, whereas normal or enhanced feto-placental blood flow in LOPE might correspond to an increased angiogenesis process. These alterations would be related with preeclampsia itself rather than changes in gestational age. Adenosine seems to coordinate these processes in both preeclamptic conditions.

Initial evidence has described high adenosine levels in the feto-placental circulation during preeclampsia [16, 17]. Moreover, using primary culture of hPMEC, we have confirmed the elevation in adenosine extracellular levels as a response linked to changes in adenosine uptake via the human equilibrative nucleoside transporters type 1 (hENT1) and type 2 (hENT2) [20]. Thus, we found that hPMEC isolated from preeclampsia exhibited an increased total (i.e., hENT1 + hENT2) and hENT2 uptake, but reduced hENT1-mediated adenosine transport. Considering that adenosine at high concentrations could have deleterious effects, and the fact that intracellular uptake is one of the main mechanisms for controlling the extracellular level of adenosine, those results were interpreted as a compensatory response, present in preeclampsia, to address the recovery of the physiological extracellular adenosine concentration in the feto-placental circulation. Compatible with this idea, Karabulut et al. [29] have shown that elevation of adenosine levels triggers a compensatory elevation of adenosine deaminase activity in the umbilical circulation. Despite this latest evidence, the causes or consequences of high adenosine levels in the feto-placental circulation during preeclampsia are not well understood. We have proposed that adenosine might regulate vascular tone in the placental bed and also placental angiogenesis [6, 19]. Nonetheless, it has also been shown that ATP infusion (the precursor of adenosine) induced a preeclampsia-like syndrome in animal models [30], which might indicate that purines, and adenosine in particular, take part in the pathophysiology of preeclampsia and/or in the feto-placental adaptation to this disease [6, 16].

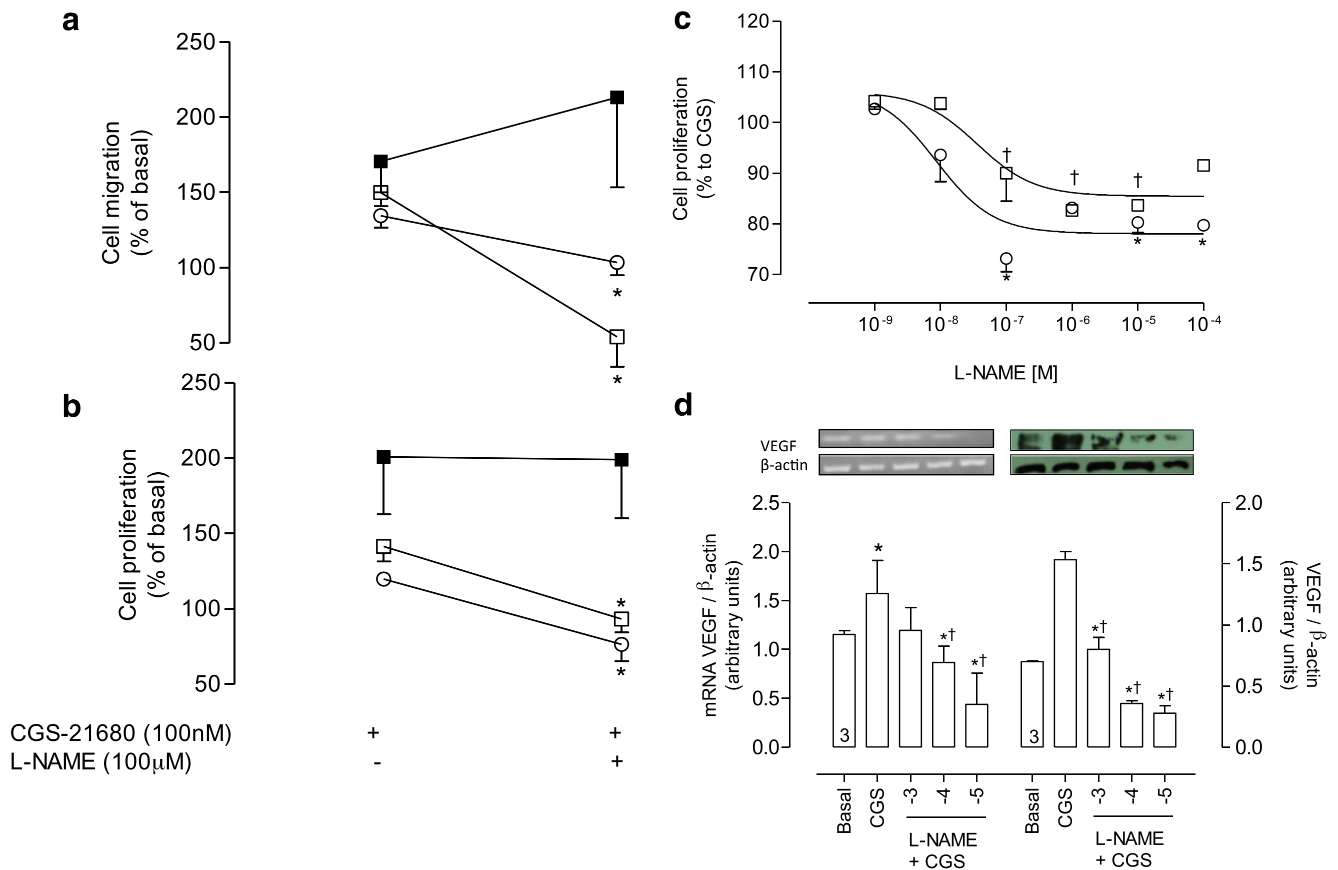


**Fig. 5** Effect of nitric oxide inhibition in the cell migration and proliferation induced by $A_{2A}$AR stimulation in early- and late-onset preeclampsia. **a** Percentage of cell migration considering respective basal condition (i.e., without agonist, *negative sign*) or in the presence (*positive sign*) of CGS-21680 (*CGS*, 100 nM) or L-NAME for 24 h in normal (*NP*, *white circle*), late-onset preeclampsia (*LOPE*, *white square*), and early-onset preeclamptic pregnancies (*EOPE*, *black squares*). **b** Percentage of cell proliferation as in (**a**). **c** L-NAME dose–response curve in cells co-incubated with CGS-21680 for 24 h. **d** *Upper panel* Representative images of semiquantitative PCR and Western blots of VEGF (55 kDa) and β-actin (43 kDa) in cells derived from normal pregnancies in similar conditions as the respective bars in (**d**). *Bottom panel* Densitometry of the VEGF/β-actin ratio in the mRNA and protein levels, respectively. In (**a**–**c**), *line* represents tendency. *$p<0.05$ vs. the respective value in CGS-21680 by itself in the respective group. In (**c**), †$p<0.05$ vs. CGS-21680 by itself in LOPE. In (**d**), *$p<0.05$ vs. basal and †$p<0.05$ vs. CGS-21680 by itself. Values are the mean ± SEM. In (**a**–**c**), $n$=3–9 different umbilical cords in each group. In (**d**), $n$=3 is included in the respective bars of basal conditions. All experiments were performed in duplicate

Endothelial proliferation/migration are the initial steps in the angiogenesis process. Basally, we have found that HUVECs from EOPE exhibit low proliferation/migration, whereas cells from LOPE present high cell proliferation/migration compared to normal pregnancy. These results might indicate a differential capacity for feto-placental angiogenesis in both pathological conditions. Supporting this idea, it has been described that placental histological changes related to reduced perfusion [10–13] and reduced placental expression of several pro-angiogenic factors [14, 15] are mainly observed in EOPE rather than LOPE. The underlying causes for this behavior are unclear, but considering that adenosine is a well-characterized pro-angiogenic molecule, its level is increased in preeclampsia and the inhibitory ZM-21680-mediated effect on NO and VEGF observed in this study, we propose that adenosine via $A_{2A}$AR is conducting the proliferation/migration of HUVEC in LOPE. On the other hand, exaggerated extracellular adenosine levels are associated with deleterious effects; therefore, the reduced cell proliferation/migration observed in EOPE could be associated with extremely high levels. More studies are needed to clarify this issue.

Another possibility to explain the differences in cell proliferation/migration observed in LOPE and EOPE is the deregulation in the expression and activity of the adenosine receptors. In this study, we have found a reduced amount of $A_{2A}$AR protein in cells from EOPE, without changes in cells from LOPE, compared to normal pregnancies. These results are linked with our previous results [20] where a low expression of $A_{2A}$AR was reported in hPMEC from preeclampsia, but disagree with previous analysis using total placental homogenate from preeclampsia, where high expressions of all adenosine receptors have been shown [31]. Methodological differences may explain this

controversy. Moreover, even though the expressions of $A_{2A}AR$ and $A_{2B}AR$ in HUVECs are well described [23, 32, 33], our data reinforce the importance of classifying into EOPE and LOPE when studying preeclampsia, as in this analysis; they presented important pathophysiological differences which seem to not have a relationship with gestational age. In this regard, and despite we have not studied the mechanisms linked with those differences, but considering that hypoxia upregulates $A_{2A}AR$ expression in placental explants [31] and epithelial cells [34, 35], it is interesting to speculate that the differences observed in the expression of $A_{2A}AR$ in EOPE and LOPE might suggest different capacities to respond to hypoxia in those conditions. This hypothesis is reinforced by recent evidence [36] showing that villous explants extracted from EOPE and cultured under varying oxygen tensions exhibited reduced levels of hydroxylated hypoxia-inducible factor type 1 alpha, a phenomenon that was absent in LOPE. Alternatively, adenosine by itself may regulate the expression of its own receptors as a mechanism of desensitization [37].

Moreover, it has been well described that the activation of $A_{2A}AR$, using CGS-21680, promotes endothelial cell proliferation, migration, and synthesis of VEGF [32, 33, 38–40]. Those results were confirmed in our study, where the proportion of increase mediated by $A_{2A}AR$ in those parameters tended to be higher in EOPE than LOPE or normal cells. On the other hand, the stimulatory effect of CGS-21680 was blocked by ZM-241385 (a selective antagonist) in all the studied groups, but the $IC_{50}$ was significantly high in EOPE compared to LOPE or normal cells (2- to 4-fold, respectively). Although we did not analyze changes in receptor binding, these results might indicate a reduction in the affinity of ZM-241385 to $A_{2A}AR$. Another explanation would be that high adenosine levels in the culture medium of those cells [20] might act as a competitive molecule for both CGS-21680 and ZM-241385. This last hypothesis is supported by the fact that we used concentrations for both molecules at least ten times higher than reported in other cells [41].

Our study found an increase in VEGF protein levels in LOPE, but a decrease in EOPE. These results support evidence that there are high levels of VEGF protein [42, 43], but reduced levels of mRNA [44], in placentas from preeclampsia. In this regard, although we found low protein levels of VEGF in placenta from EOPE, there were no significant changes in LOPE or preterm delivery compared to normal pregnancy. Moreover, we confirm previous evidence [21, 33, 38, 40] showing that stimulation of $A_{2A}AR$ increases the concentration of VEGF in HUVECs from normal pregnancies, an effect that was also observed in EOPE. Furthermore, basal production of VEGF in normal or preeclamptic pregnancies seems to be dependent on $A_{2A}AR$ because the inhibition of this receptor (by ZM-241385) reduces the amount of this protein. Therefore, this evidence suggests that adenosine would be regulating the VEGF-dependent angiogenesis in the feto-placental circulation.

Nitric oxide has pro- [45] or anti-proliferative [46] effects in HUVECs from normal pregnancies. In agreement with previous results [47], we found a correlative relationship between NO availability and proliferation in HUVECs in both LOPE and EOPE. This evidence agrees with previous reports where $A_{2A}AR$ triggers an intracellular pathway dependent on NO synthesis [23, 41]. Specifically, our results indicate that $A_{2A}AR$ stimulation enhances the activation of eNOS (i.e., serine$^{1177}$ phosphorylation) as well as nitrite and nitrotyrosine formation in both normal pregnancy and EOPE, but not in LOPE. Moreover, incubation with ZM-241389 reduced the eNOS phosphorylation in LOPE, suggesting that this pathologic condition would be characterized by a maximal activity of the $A_{2A}AR$/NO pathway. Therefore, it is feasible that continuous stimulation of NO synthesis mediated by $A_{2A}AR$ in LOPE triggers an enhancement in HUVEC proliferation, whereas in EOPE this mechanism would be reduced or exhausted.

In addition, proliferation and migration induced by CGS-21680 were blocked by L-NAME only in normal and LOPE, showing in this last group a 4-fold increase in the $IC_{50}$ for L-NAME compared to normal pregnancy. In the same way, L-NAME blocked the high cell proliferation observed in preterm delivery, suggesting participation of NO in this fact. This reinforces the participation of the $A_{2A}AR$/NO signaling pathway in cell proliferation/migration during normal pregnancy, preterm delivery, and LOPE. Nevertheless, despite the activation of $A_{2A}AR$ being associated with NO synthesis in EOPE, it is intriguing that L-NAME does not affect the stimulatory effect on cell proliferation/migration of CGS-21680, suggesting that basal impairment of NO synthesis observed in EOPE might generate a deregulation in the downstream signaling of NO by itself. More studies are needed to elucidate this. Co-incubation (12 h) of CGS-21680 and L-NAME reduced the mRNA and protein levels of VEGF in normal cells, suggesting that VEGF expression is downstream of NO synthesis mediated by $A_{2A}AR$. The molecular mechanisms associated with VEGF expression mediated by NO are unclear, but since HIF is controlling the expression of VEGF, it is feasible that nitration of HIF is occurring in the human placenta, as has been described in jejunum extracted from rats [48].

One of the weaknesses in our study is the sample size, in particular in preterm delivery. We are trying to overcome this limitation through the inclusion of detailed clinical characteristics of the included patients. In fact, we have suggested [3] that it is necessary to include as much clinical data as possible in order to facilitate the interpretation of the results presented in studies including preeclamptic patients.

In addition, the general concern about the inclusion of "preterm normal deliveries" is the fact that most of the time (if not all), they are actually a pathological condition.

In conclusion, elevation of the activity of the $A_{2A}AR$/NO/VEGF signaling pathway in LOPE is involved in the high proliferation/migration of HUVECs, which may indicate that vessel formation in the feto-placental circulation could be increased for ensuring the delivery of nutrients toward the fetus. On the other hand, EOPE is characterized by a decreased expression of $A_{2A}AR$, which is responsible for reducing NO, VEGF, cell proliferation, and migration, which in turn could be associated with diminished angiogenesis, feto-placental blood flow, and perhaps abnormal fetal growth. These differences between LOPE and EOPE seem not to be related to gestational age. More studies are needed in order to understand differential cell proliferation/migration regulation by adenosine in EOPE and LOPE pregnancies.

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