Ca^{2+} signals in human umbilical endothelial cells derived from pregnancy with fetal growth restriction associated with hypertensive disorder

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Abstract. Fetal growth restriction associated with hypertensive disorders of pregnancy (FGR-HDP) is a prevalent pathology with a higher risk of perinatal morbimortality. In this condition, placental insufficiency and endothelial dysfunction serve key roles. The present prospective cohort study monitored 11 patients with an FGR-HDP and 15 with full-term normotensive pregnancies and studied post-natal intracellular calcium concentration ($[Ca^{2+}]_i$) signals in human umbilical vein endothelial cells (HUVECs). Small fetuses with placental insufficiency were identified using fetal biometry with Doppler velocimetry. Mean gestational age and birth weight were 31.8±4.1 weeks and 1,260±646 g for FGR-HDP and 39.2±0.8 weeks and 3,320±336 g for normal births, respectively. Abnormal umbilical artery Doppler waveforms were found in 64% of neonates with FGR-HDP. A significant percentage (86%) of FGR newborns were admitted to the neonatal intensive care unit at Gustavo Fricke hospital, Viña del Mar, Chile, with one case of death after birth. $[Ca^{2+}]_i$ signals were measured by microfluorimetry in Fluo-3-loaded HUVECs from primary cultures. Altered $[Ca^{2+}]_i$ signals were observed in HUVECs from FGR-HDP, where the sustained phase of ATP-induced $[Ca^{2+}]_i$ responses was significantly reduced compared with the normotensive group. Also, the $[Ca^{2+}]_i$ signals induced with 10 mM Ca^{2+} after depletion of internal Ca^{2+} stores were significantly higher. The present study provides a better comprehension of the role of altered cytosolic Ca^{2+} dynamics in endothelial dysfunction and an *in vitro* model to assess novel therapeutic approaches for decreasing or preventing complications in FGR-HDP.

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Abbreviations: AC, abdominal circumference; $[Ca^{2+}]_i$, intracellular calcium concentration; EFW, estimated fetal weight; FGR, fetal growth restriction; HDP, hypertensive disorders of pregnancy; HUVEC, human umbilical vein endothelial cell; IP_3, inositol triphosphate; SOCE, store-operated Ca^{2+} entry; UA, umbilical artery

Key words: ATP, calcium, fetal growth restriction, hypertensive disorders of pregnancy, P2Y2 receptor, store-operated Ca^{2+} entry

Introduction

Fetal growth restriction (FGR) is a pregnancy complication associated not only with adverse perinatal outcomes but also with increased risk of cardiovascular diseases in adult life of offspring (1,2). The unborn baby has an estimated fetal weight (EFW) below the 10th percentile by gestational age as determined by prenatal ultrasound evaluation (3).

The etiology of FGR is multifactorial. However, one of the most common contributing factors is placental insufficiency (4), a condition that also serves a role in the pathogenesis of hypertensive disorders of pregnancy (HDP), including preeclampsia (5). In a recent study, it was demonstrated that FGR-HDP, a condition with significant obstetric morbidity and mortality, exhibits maternal vascular malperfusion of the placental bed, abnormal feto-placental Doppler parameters and signs of oxidative stress of the syncytiotrophoblast (6). Other recent studies show that extracellular vesicles derived from placental tissue influence endothelial cell function, explaining the relationship between placental insufficiency and hypertensive disorder (7,8).

There is widespread evidence for endothelial dysregulation of the fetoplacental vascular tone in FGR, which compensates for restricted blood flow (9-12). In this regard, nitric oxide production by the endothelium, which is key in maintenance of blood flow in the fetal placental bed during normotensive pregnancy (10) may be disordered in pregnancy with FGR and/or preeclampsia (11,12). Nitric oxide is produced by nitric oxide synthases, including endothelial nitric oxide synthase isozyme whose activity is markedly increased when intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) rises (13). The activation of this endothelial isozyme depends on conformational changes induced by its interaction with the Ca^{2+}-calmodulin complex (14). The vascular endothelium responds to agonists by increasing $[Ca^{2+}]_i$. This response depends on different elements, including the type of receptor activated, release of Ca^{2+} from intracellular stores and store-operated Ca^{2+} entry (SOCE). The latter mechanism is activated by depletion of internal Ca^{2+} stores (15).

Our previous studies demonstrated that bradykinin, histamine, ATP and α-7 nicotinic acetylcholine receptors agonists increase $[Ca^{2+}]_i$ in endothelial cells (16-18). In human umbilical vein endothelial cells (HUVECs), ATP-induced Ca^{2+} signal typically consists of an initial transient phase followed by a prolonged sustained phase (18). ATP is an important autacoid and paracrine molecule that exerts dual control of vascular tones by being released from perivascular nerves and endothelial cells in response to changes in blood flow (shear stress) and hypoxia (19). Among purinergic receptors reportedly involved in ATP-induced Ca^{2+} responses in HUVECs are the metabotropic P2Y2 (20) and ionotropic P2X4 receptors (21). The P2Y2 receptor, in addition to inducing rapid Ca^{2+} response, promotes nitric oxide production and appears to be the purinergic receptor that contributes most to the ATP-induced Ca^{2+} signal (20). P2X4 receptor seems to be overexpressed in pathological conditions and involved in production of reactive oxygen species and pro-inflammatory activators but not in nitric oxide synthesis (22).

The present study aimed to record clinical data of newborns and mothers from normotensive and pathologic pregnancies and investigated Ca^{2+} signals induced by ATP and SOCE in HUVECs.

Subjects and methods

Study design and patients. The present study was a prospective cohort study of single pregnancies complicated by FGR-HDP and healthy pregnancies (controls). Data and samples were collected from January to December of 2006 from Dr Gustavo Fricke Hospital, Viña Mar, Chile. All patients (13-39 years, n=26) gave informed written consent according to the Declaration of Helsinki.

Inclusion criteria for full-term normal pregnancies (control) were as follows: i) No medical or obstetrical complications during pregnancy, labor, or puerperium and ii) pregnancies with EFW between the 10th and 90th percentiles adjusted for gestational age within the local population (23,24) Exclusion criteria were as follows: i) Chronic pathologies, such as chronic hypertension or gestational diabetes; ii) patients taking aspirin or nitric oxide donor agents; iii) consumption of alcohol or any illicit drugs during pregnancy and iv) fetuses with chromosomal abnormality, congenital infection or malformation.

Inclusion criteria for FGR-HDP were as follows: i) Patients diagnosed with HDP, such as pregnancy-induced hypertension (preeclampsia and eclampsia), chronic hypertension in the presence or absence of superimposed preeclampsia and transient hypertension, as outlined in the Perinatal Guide of the Ministry of Health, Chile (24); ii) EFW and abdominal circumference (AC) <10th percentile for their gestational age (validated locally) (24,25), combined with Doppler-defined intrauterine hypoxia or oligohydramnios and iii) EFW and/or AC <5th percentile for gestational age (validated locally), regardless of other parameters (Doppler or oligohydramnios). Exclusion criteria, determined according to the recommendations of the International Federation of Gynecology and Obstetrics (FIGO) (23) were as follows: i) Anemia, preexisting high blood pressure or maternal chronic disease; ii) patients taking aspirin or nitric oxide donor agents; iii) consumption of alcohol or any illicit drugs during pregnancy; iv) fetuses with genetic disorder, structural anomalies, congenital infections, or exposure to teratogens and v) multiple pregnancies.

For all patients, maternal age, parity, hemoglobin levels in the blood, mode of delivery, gestational age at delivery, birth weight, neonate sex, Apgar score and neonatal intensive care unit admission were recorded. The gestational age was calculated with respect to the last menstrual period or estimated by ultrasonography before the 12th week of pregnancy (24). Apgar score is a method for reporting clinical status of the newborn at 1 and 5 min of life, is useful for response to resuscitation, and considers heart rate; 2) respiratory effort; 3) muscle tone; 4) reflex response or irritability; 5) skin color; each of these components is given score of 0, 1, or 2 (26).

For neonates with FGR-HDP, AC, oligohydroamnios (27,28), Doppler velocimetry (29,30), biophysical profile score, fetal heart rate monitoring and neonatal death were also analyzed. Doppler velocimetry reflects the resistance to flow produced by the vascular bed (29,30). The biophysical profile score to quantify fetal behavior uses dynamic variables such as fetal tone, breathing movement, gross body movement, amniotic fluid volume and fetal heart rate analysis (31). Other profile is the non-stress test that measures fetal heart rate in responses to spontaneous fetal movement (32).

Endothelial cell culture and cytosolic Ca^{2+} measurement. The umbilical cords were collected after delivery. The endothelial cells were isolated as described by Jaffe *et al* (33) according to the validated methodologies developed in the Cellular and Molecular Biochemistry laboratory of the Pharmacy Faculty at the University of Valparaíso (17). In summary, endothelial cells was isolated by collagenase-I (0.5 mg/ml; Gibco; Thermo Fisher Scientific, Inc.) digestion from human umbilical veins a 37˚C for 15 min. After this, dissociated cells were cultured in 199 medium (cat. no. M199; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 2.5 mM L-glutamine, 14 mM HEPES acid, 200 IU/l penicillin, 400 IU/l streptomycin, 10% fetal bovine serum and 10% newborn calf serum (Gibco, Thermo Fisher Scientific, Inc.), pH 7.42 at 37˚C Experiments were performed on confluent primary cultures (80% confluence) 2-5 days after seeding.

Table I. Clinical characteristics of pregnant patients and newborns.

Characteristic	Healthy control (n=15)	FGR-HDP (n=11)	P-value
Mean age (range), years	22.0±4.0 (13.0-28.0)	26.0±7.9 (17.0-39.0)	0.145[a]
First pregnancy, %	53.3	72.7	0.428[b]
Hemoglobin, g/dl	11.6±0.8	13.1±1.1	<0.001[c]
Delivery, cesarean/vaginal	0/15	11/0	<0.001[d]
Mean gestational age (range), weeks	39.0±0.8 (38.0-40.5)	32.1±4.1 (24.5-38.2)	<0.001[a]
Mean birth weight (range), g	3,320±336 (2,800-3,940)	1,260±646 (560-2,520)	<0.001[a]
Female, %	73.3	54.5	0.419[b]
Apgar score <7 at 5 min	0	1	0.428[d]
NICU admission	0	9	<0.001[a]

[a]Mann-Whitney rank sum, [b]Fisher's exact, [c]Student's t and [d]Yates' χ^2 test. FGR-HDP, fetal growth restriction associated with hypertensive disorders of pregnancy; NICU, neonatal intensive care unit.

$[Ca^{2+}]_i$ was measured using the fluorescent indicator Fluo-3 AM, as previously reported (17,18). Briefly, confluent HUVECs grown in coverslips and incubated in Locke's solution (NaCl, 135.0; KCl, 5.6; $CaCl_2{\cdot}2H_2O$, 2.5; HEPES-acid, 10.0; $MgCl_2{\cdot}6H_2O$, 1.2 and D-glucose, 5.5 mM) were mounted in a perfusion chamber on the stage of an epifluorescence microscope (Nikon Eclipse E600FN) implemented with 490 nm excitation and 530 nm emission filters. The fluorescence signals were measured using a photomultiplier (Hamamatsu Photonics K.K.), digitalized at 3 Hz using an analogue converter board (Data Translation) and collected using Axotape software (version 2.0; Axon Instruments). The amplitude of the fluorescent signal was expressed as $\Delta F_t/F_b=(F_t-F_b)/F_b$, where F_t is the fluorescence at time t and F_b is basal fluorescence (34).

Cytosolic Ca^{2+} response to ATP was evaluated in HUVECs, as described in a previous study (18). In the present study, the parameters analyzed were time to initial peak (t_P), amplitude of initial peak, amplitude of sustained phase, and return to the baseline $[Ca^{2+}]_i$.

To isolate the initial phase (Ca^{2+} release from ternal stores) without contributing P2X4 receptors and SOCE, HUVECs were stimulated with 100 μM ATP for 3 min in a Ca^{2+}-free Locke's solution (0 Ca^{2+}). When fluorescence returned to levels close to the baseline (~50 sec), cells were perfused with 10 mM Ca^{2+} in Locke's solution for 180 sec. This latter $[Ca^{2+}]_i$ rise corresponds to the sustained phase associated with SOCE (35). After that, HUVECs were returned to the 0 Ca^{2+} Locke's solution, and fluorescence declined. These experiments were performed in HUVECs from 13 healthy and nine FGR-HDP umbilical cords. HUVEC cultures from two healthy and two FGR-HDP umbilical cords were not included because of technical problems. The pathological umbilical cords from neonates with FGR of gestational ages of 31 and 33 weeks with severe pre-eclampsia were not evaluated.

Statistical analysis. Clinical data are expressed as mean ± standard deviation for continuous numerical variables and median and ranges for discrete numerical variables. $[Ca^{2+}]_i$ signal data are expressed as means ± standard error of 1-3 experimental replicates. The Kolmogorov-Smirnov test was used to verify normality of the distribution of numerical variables. Results were compared using unpaired Student's t test for data with normal distribution and Mann Whitney U-test for non-normal distribution. Differences between proportions of nominal variables were compared using Fisher's exact or Yates' χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference. The data were analyzed using the software Stata/SE 18.0 (Universidad de Valparaíso).

Results

Clinical parameters of patients. A total of 26 patients were enrolled (11 with FGR-HDP and 15 controls; Table I). No significant differences were found in age and parity between patients and healthy controls, but hemoglobin levels of FGR-HDP patients were significantly higher than healthy controls. There was a significant difference in delivery mode between the groups due to fetal compromise and clinical decision (24). Gestational age and birth weight of the FGR-HDP group were significantly lower than in the healthy group (24). There was no significant difference in Apgar score at 5 min between both groups. However, 82% FGR-HDP neonates were admitted to the neonatal intensive care unit, while no healthy control neonates were admitted.

The FGR-HDP neonate group exhibited 82% AC <3rd percentile and a 73% EFW ≤5th percentile for gestational age, which was confirmed in all cases at birth (Table II). The highest gestational age in this group was 38 weeks; this neonate had EFW in the 2nd percentile for gestational age without alteration in biophysical profile score, fetal heart rate monitoring, and UA Doppler; and without oligohydroamnios. On the other hand, one of the three neonates with EFW >5th percentile (considered low severity), had EFW at the 10th percentile and a gestational age of 24 weeks. This neonate experienced severe asphyxia and died on the second day after birth.

Abnormal (pulsatility index >95th percentile) umbilical artery (UA) Doppler was found in 64% of neonates with FGR. Abnormal UA Doppler, especially if end-diastolic flow velocities are absent, is a predictor of fetal compromise (29,30). A total of five patients showed absent end-diastolic flow (Table II). In

Table II. Obstetric and neonatal characteristics from pregnancies with fetal growth restriction associated with HDP.

	Patient no.										
Characteristic	1	2	3	4	5	6	7	8	9	10	11
HDP	SPE	SPE	MPE	SPE	CrHT	SPE	SPE	CrHT + PE	SPE	SPE	HDPw
GA at birth, weeks	24	27	28	31	31	32	33	34	34	37	38
EFW, percentile	10	2-5	5	2	2-5	5-10	5	2	10	2-5	2
AC <3rd percentile	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
OHA	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
UA Doppler	A-AEDF	A-AEDF	A-AEDF	N	A	N	A	A-AEDF	A	N	N
BPS	ND	N	N	N	N	N	N	A	N	N	N
FHRM	ND	ND	R	R	NR	NR	ND	NR	NR	R	R
NICU admission, days	2	88	>60	63	50	>25	38	31	21	0	0
Neonatal death	Yes	No	No	No	No	No	No	No	No	No	No

Pulsatility index >95th percentile or AEDF were considered to indicate A UA Doppler results. AEDF, absent end-diastolic flow; CrHT, chronic hypertension; HDPw, hypertensive disorders of pregnancy without proteinuria analysis; MPE, moderate preeclampsia; SPE, severe preeclampsia; FHRM, fetal heart rate monitoring; R, reactive; NR, non-reactive; GA, gestational age; EFW, estimated fetal weight; AC, abdominal circumference; OHA, oligohydroamnios; BPS, biophysical profile score; NICU, neonatal intensive care unit; A, abnormal; N, normal; ND, not determined; UA, umbilical artery.

addition, four neonates did not react during the basal recording of the non-stress test (low or absent accelerations of heart rate responses to spontaneous fetal movement) (32). Only a neonate from the pathological group, who had abnormal UA Doppler as well as oligohydroamnios, exhibited an abnormal biophysical profile score.

Calcium signals in HUVECs from healthy and FGR-HDP pregnancies. Our previous study demonstrated that $[Ca^{2+}]_i$ rise induced by ATP is time- and concentration-dependent, with a biphasic process typically consisting of an initial transient phase (initial peak) followed by a sustained phase (18). To obtain a maximum response and observe both phases, Fluo-3-loaded HUVECs in confluent primary cultures were treated with 100 μM ATP in Locke's solution for 3 min. Fig. 1A and B show representative ATP-induced $[Ca^{2+}]_i$ signals in control and FGR-HDP HUVECs, respectively. Similar numbers of cells/field were recorded for each group, with 16±2 cells in 23 coverslips from control and 17±1 cells in 34 coverslips from FGR-HDP groups (Fig. 1C). The scatter plot displays the association between ATP-induced maximum fluorescence and the number of cells of each coverslip (Fig. 1D). Mean maximum fluorescence intensity (measured as ΔFt/Fb) was 2.60±0.20 and 1.50±0.16 for control and FGR-HDP HUVECs (P<0.0001), suggesting altered $[Ca^{2+}]_i$ responses in the pathological condition.

Parameters of the ATP-induced $[Ca^{2+}]_i$ signals were analyzed (Fig. 2A). Time to peak (t_P) was significantly increased in HUVECs from FGR-HDP (Fig. 2B); t_P values were 2.0±0.7 and 4.5±0.9 sec (P<0.05) for control and FGR-HDP HUVECs, respectively. On the other hand, no statically significant difference was found between the control and FGR-HDP groups in amplitude of the initial peak ($\Delta F_t/F_b$, 2.3±0.2 and 1.7±0.3, respectively; P>0.05; Fig. 2C), but delayed phase of the $[Ca^{2+}]_i$ signal was significantly lower in FGR-HDP compared with control, with $\Delta F_t/F_b$ of 1.7±0.1 and 1.3±0.2 for control and FGR-HDP cells, respectively (P<0.05; Fig. 2D). Finally, no significant difference was found between the control and FGR-HDP groups in the return to the baseline $[Ca^{2+}]_i$ following termination of the stimulus with ATP ($\Delta F_t/F_b$, 0.40±0.08 and 0.50±0.08 for control and FGR-HDP cells, respectively; P>0.05; Fig. 2E).

Fig. 3A and B show $[Ca^{2+}]_i$ signals induced in HUVECs from control and FGR-HDP groups, respectively. The maximum amplitudes of the ATP-induced Ca^{2+} responses in the absence and presence of extracellular Ca^{2+} are shown in Fig. 3C and D. The analysis of the maximum amplitude of ATP-induced Ca^{2+} signals in the absence of extracellular Ca^{2+} showed no significant difference with ΔFt/Fb values of 2.4±0.3 and 1.9±0.4 (P>0.05), control and FGR-HDP respectively. Conversely, the amplitude of Ca^{2+} responses to 10 mM Ca^{2+} were significantly higher in HUVECs from the FGR-HDP group, with ΔFt/Fb values of 1.8±0.2 in control and 3.1±0.3 in FGR-HDP cells (P<0.005), respectively. These results suggested that SOCE-mediated Ca^{2+} influx, but not ATP-induced Ca^{2+} release from internal stores, was altered in FGR-HDP HUVECs.

Discussion

During gestation, there is an active metabolic exchange between the fetus and mother, a process in which the efficiency

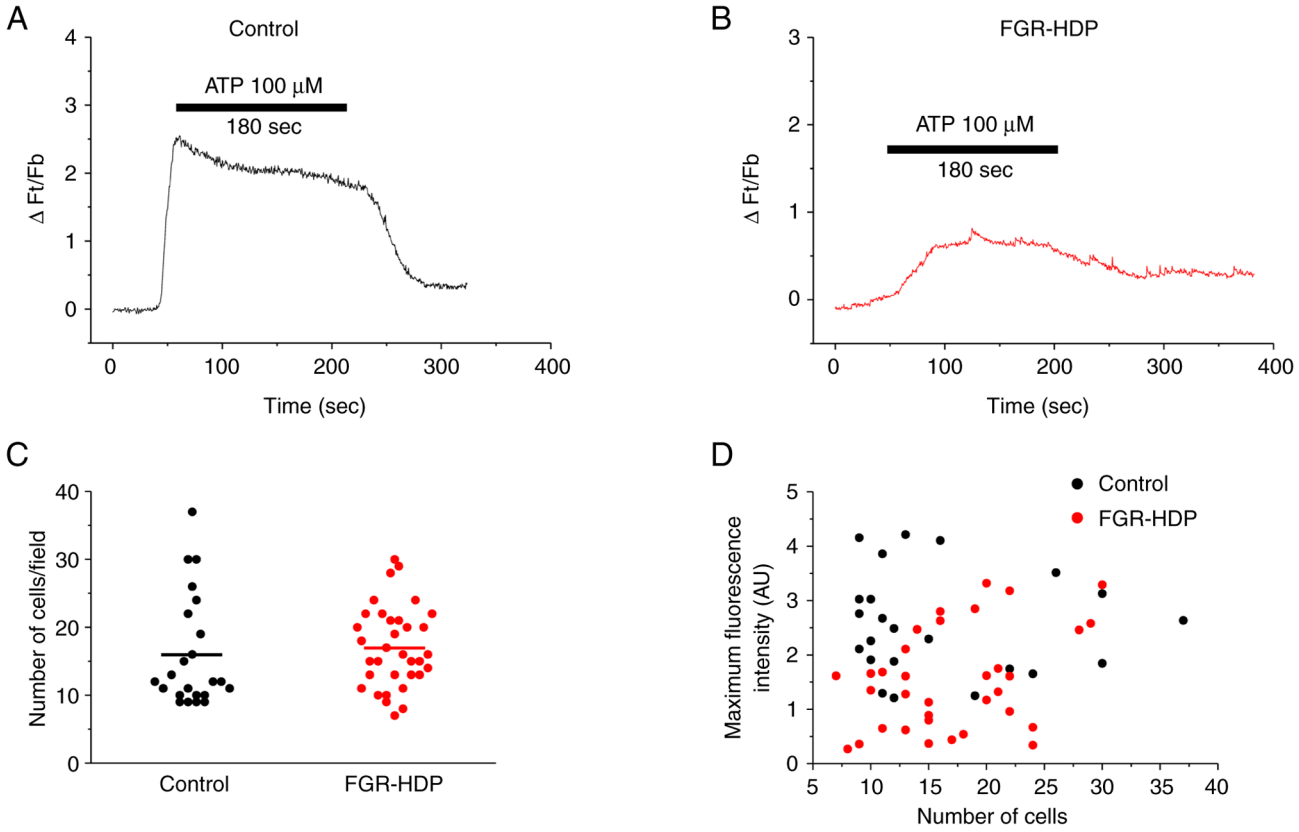

Figure 1. Cytosolic Ca^{2+} response to ATP in HUVECs from control and FGR-HDP groups. Fluo-3-loaded HUVEC from 15 control and 11 FGR-HDP umbilical cords were stimulated for 3 min with 100 μM ATP and $[Ca^{2+}]_i$ signals were measured by microfluorometry. ATP-induced $[Ca^{2+}]_i$ signals in HUVEC from (A) control and (B) FGR-HDP. (C) Numbers of cells per field. (D) ATP-induced maximum fluorescence intensity. HUVEC, human umbilical vein endothelial cell; FGR-HDP, fetal growth restriction associated with hypertensive disorders of pregnancy; Ft, fluorescence at time t; Fb, basal fluorescence.

depends on adequate development of the fetoplacental unit. Alterations in the efficacy of this exchange trigger hypoxia, which is associated with fetal distress, perinatal mortality and a potential risk of cardiovascular diseases in offspring (1,2,36). Both FGR and HDP are associated with placental insufficiency and are aggravated by placental ischemia, a condition accompanied by oxidative stress, wherein nitric oxide is unable to compensate for this impairment (37). Recent studies show that during hypoxia, placenta releases extracellular vesicles, which carry cytokines and microRNA that alter the function of endothelial cells (7,8,38). This phenomenon worsens pregnancy pathologies such as FGR and HDP (39).

The present study analyzed clinical characteristics of 11 single pregnant patients with FGR-HDP, a complication that has the highest rates of obstetric morbidity and mortality and elevated incidences of low gestational age at delivery, cesarean section and neonatal death compared with other types of pregnancy disorder (6). Despite attending to ~3,000 pregnant patients annually at Gustavo Fricke Hospital, only 11 met the inclusion criteria FGR-HDP and consented to participate. Given the small sample size, it is difficult to generalize the present findings to the broader population. Future research should employ larger and more representative samples to ensure generalizability of results.

The present study excluded cases of multiple pregnancies as this constitutes an independent risk factor for HDP (40), regardless of chorionicity or zygosity (41-43). In terms of fetal outcomes, moderately elevated blood pressure in multiple pregnancies increases blood flow to the placenta, contributing to decreased risk of preterm birth and low birth weight (43). Multiple pregnancies have other risks, the major one being prematurity, which is associated with adverse outcomes such as respiratory morbidity, intraventricular hemorrhage, necrotizing enterocolitis, and metabolic disorders (23,44). However, the risk of future cardiovascular disease is not increased (45), which contrasts with single pregnancies, where HDP is a risk factor for future cardiovascular disease (23,46,47).

In addition, other maternal, placental or fetal risk factors for abnormal placentation may result in placenta-mediated FGR. Therefore, in the present study these other co-existing factors were excluded due to their potential interference with normal fetal growth and effect on outcomes (23). To the best of our knowledge, however, there is no evidence that the combination of risk factors predicts the presence FGR (48,49). FIGO does not recommend using multiparameter algorithms (combining ultrasound and biochemical markers) for universal screening. This recommendation is based on the lack of sufficient validation of the effectiveness of these models in predicting FGR (23).

Here, gestational age and birth weight were significantly lower and neonatal intensive care admission was higher in the FGR-HDP group compared with the healthy group. The death of a newborn in the FGR-HDP group with an EFW in the 10th percentile and a 5-min Apgar score of 3 suggests that factors beyond these metrics play a critical role in determining the risk of neonatal mortality (50). This case highlights the

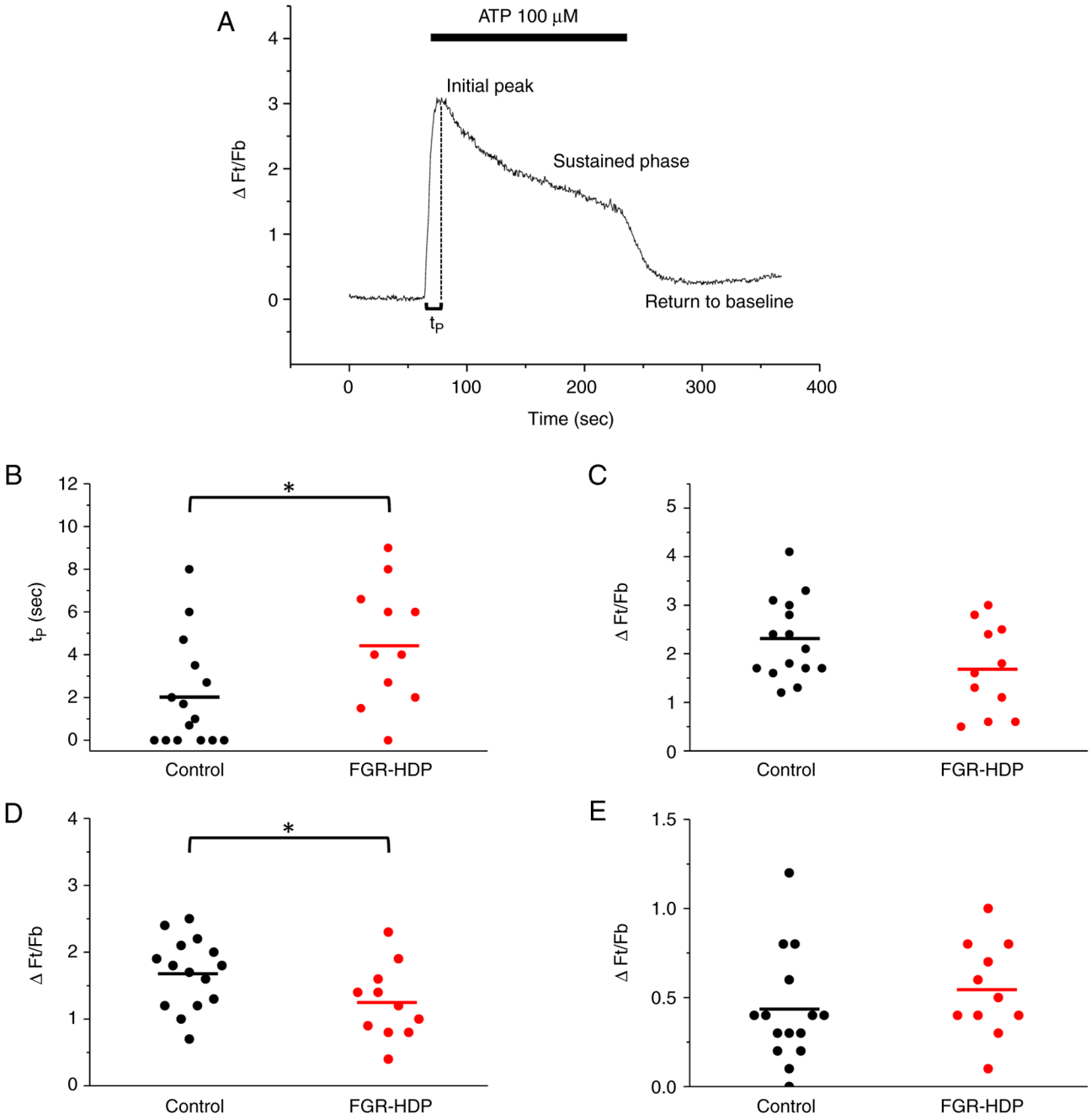

Figure 2. Characteristics of ATP-induced $[Ca^{2+}]_i$ signals in HUVECs from control and FGR/HDP groups. Fluo-3-loaded HUVEC from 15 control and 11 FGR-HDP umbilical cords were stimulated for 3 min with 100 μM ATP and $[Ca^{2+}]_i$ signals were measured by microfluorometry. (A) Typical $[Ca^{2+}]_i$ signal in HUVECs. (B) t_P. Amplitudes of (C) initial peak and (D) sustained and (E) return phase. *P<0.05 (t-test). HUVEC, human umbilical vein endothelial cell; FGR-HDP, fetal growth restriction associated with hypertensive disorders of pregnancy; t_p, time to peak; Ft, fluorescence at time t; Fb, basal fluorescence; t-test, unpaired Student's t test.

greater prognostic value of the 5-min Apgar score compared to the 1-min score in predicting neonatal morbidity and mortality (26).

A total of 64% (7/11) of neonates from FGR-HDP pregnancies had abnormal UA Doppler, with 57% (4/7) exhibiting absent end diastolic flow; of these 75% (3/4) had oligohydroamnios. The use of Doppler velocimetry evaluation, especially of the UA, has been studied and reviewed in cases of FGR (30,31,51). A recent study showed that compared with other pregnancy complications, FGR-HDP has higher values of UA Doppler velocimetry, and maternal vascular malperfusion (6). A progressively increasing pulsatility index in UA corresponds to increased fetal artery resistance, which generates a progressive decrease of the placental area available for gas and nutrient exchange (52,53). FGR is associated with a dysfunction of the feto-placental vasculature involving endothelial cells. Compensatory upregulation of the nitric oxide system in feto-placental endothelial cells has been observed in FGR (12).

In accordance with the recommendations outlined by FIGO (23) for managing FGR, pregnant patients affected by FGR should be monitored using biophysical assessments

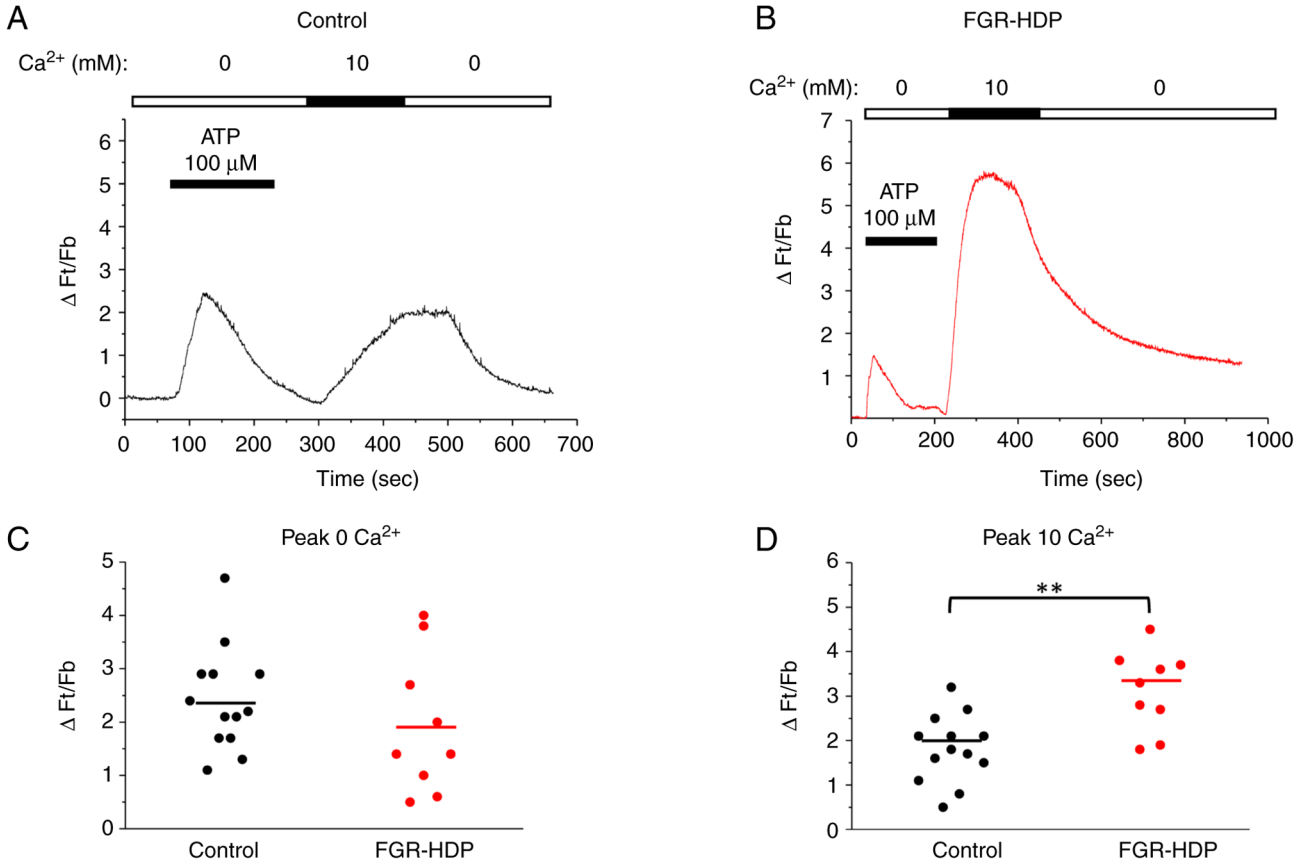

Figure 3. Isolated phases from ATP-induced $[Ca^{2+}]_i$ response in HUVECs. Cells were stimulated with 100 μM ATP in a Ca^{2+}-free Locke solution for 180 sec (peak 0 Ca^{2+}) and then exposed to extracellular 10 mM Ca^{2+} solution for 180 sec (peak 10 Ca^{2+}). Ca^{+2} signals induced in HUVECs from (A) 13 control and (B) 9 FGR-HDP umbilical cords. Peak (C) 0 and (D) 10 Ca^{2+} correspond to maximum amplitude of the isolated initial and delayed phase, respectively. **P<0.005 (t-test). HUVEC, human umbilical vein endothelial cell; FGR-HDP, fetal growth restriction associated with hypertensive disorders of pregnancy Ft, fluorescence at time t; Fb, basal fluorescence; t-test, unpaired Student's t test.

and cardiovascular tests, to determine the timing of delivery. Many studies have focused on the prevention and treatment of HDP and/or FGR (23,54). To the best of our knowledge, however, there is currently no effective treatment to reverse the course of FGR and improve fetal growth (54). For future pregnancies, patients with a history of FGR should be counseled on risk of recurrence, considering the timing of onset, severity of FGR and placental histopathological findings (23). If the placenta is available, histopathological examination may provide valuable insights for counseling in future pregnancies (23).

HUVECs are used as a model to study cardiovascular diseases (55). They are also considered as potential predictors of cardiovascular risk in offspring of pregnancies involving preeclampsia (56). In this regard, HUVECs from preeclampsia pregnancies reportedly display impaired functional capacity, such as migration and tubule formation (57,58). As $[Ca^{2+}]_i$ signals are involved in these processes, the present study investigated how they were altered in FGR-HDP.

ATP-induced $[Ca^{2+}]_i$ signals were altered in HUVECs from the FGR-HDP group, including slower t_p and lower sustained phase. The initial phase of the ATP-$[Ca^{2+}]_i$ signal is primarily mediated by P2Y2 receptors (20), whose activation generates inositol triphosphate (IP_3) and Ca^{2+} release from Ca^{2+} stores, whereas the sustained phase of the $[Ca^{2+}]_i$ signals is determined by SOCE (59). The present result suggest that the kinetics of the IP_3-induced Ca^{2+} release and SOCE-mediated Ca^{2+} influx are diminished in FGR-HDP cells. The sustained phase of histamine-induced $[Ca^{2+}]_i$ signal is decreased in preeclampsia HUVECs (60,61), which agrees with the present results in FGR-HDP HUVECs, where the delayed phase of the ATP-induced $[Ca^{2+}]_i$ signal was also diminished, suggesting that HDP influences endothelial dysfunction.

To understand how Ca^{2+} dynamics is altered by FGR-HDP, components of the ATP-induced Ca^{2+} signals were separated (18). There were no significant changes in the Ca^{2+} signal induced by ATP in the absence of extracellular Ca^{2+}, suggesting that the P2Y2 receptor mediated response was not affected in HUVECs from FGR-HDP group. However, the Ca^{2+} signal induced in presence of 10 mM Ca^{2+} was significantly higher in HUVECs from FGR-HDP compared with control HUVECs. This Ca^{2+} signal is associated with SOCE, a mechanism that depends on the Ca^{2+} sensor stromal interaction molecule-1, which senses Ca^{2+} depletion in the endoplasmic reticulum and activates Ca^{2+} release-activated Ca^{2+} channel protein 1 at the plasma membrane (15). This mechanism involves transient receptor potential and connexin channels and mitochondria (59,62). A recent study demonstrate that mitochondrial Ca^{2+} uniporter regulates SOCE at different levels, including Ca^{2+} store replenishment and cytosolic Ca^{2+} buffering systems, and that deletion of the mitochondrial uniporter increases SOCE-mediated $[Ca^{2+}]$

$_i$ signals (63). Mitochondrial Ca^{2+} uniporter is impaired in hypertension and cardiovascular disease generating high cytosolic $[Ca^{2+}]_i$ levels (64,65).

An unexpected finding of the present study was that whereas the sustained phase of ATP-induced $[Ca^{2+}]_i$ signals was diminished in HUVECs from the FGR-HDP group, Ca^{2+} peak amplitude induced with 10 mM Ca^{2+} following depletion of internal Ca^{2+} stores was significantly higher. This result was also different from that observed by Steinert *et al* (60) using a similar protocol but applying a lower extracellular Ca^{2+} concentration (1 mM) to induce the second peak. High extracellular Ca^{2+} concentration (10 mM) may saturate the Ca^{2+} buffering mechanisms and induce dysfunction. In physiological conditions, the sustained phase of the response induced by agonists such ATP or histamine stimulates the synthesis of nitric oxide, which favors vasodilation (66). However, dysfunction of Ca^{2+} buffering mechanisms might cause overload of cytosolic Ca^{2+} levels, resulting in deleterious cellular effects such as oxidative stress, which contribute to FGR (67).

In conclusion, the present study found that FGR-HDP resulted in impaired UA resistance and altered Ca^{2+} responses to ATP regulated by SOCE in HUVECs. The present results provide better understanding of the mechanisms that regulate $[Ca^{2+}]_i$ dynamics in fetal endothelial cells and how they are altered in FGR-HDP. As dysfunction of HUVECs is a potential predictor of cardiovascular risk in offspring (56), the present study also provides an *in vitro* model to assess novel therapeutic approaches for decreasing or preventing cardiovascular disease in adulthood.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MPC and AMC conceived the study and wrote the manuscript. MPC, and CA performed the experiments and wrote the manuscript. LMS and KVC performed statistical analysis and confirm the authenticity of all the raw data. RV and TFBC interpreted data. RV, KVC, LMS and TFBC critically revised and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate.

The present study was approved (approval no. 9395.06) by the Scientific Ethics Committee of Dr. Gustavo Fricke Hospital (Viña Mar, Chile). All patients provided written informed consent prior to data and umbilical cord collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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