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Contractility of Placental Vascular Smooth Muscle Cells in Response to Stimuli Produced by the Placenta:

Roles of ACE vs. Non-ACE and AT1 vs. AT2 in Placental Vessel Cells

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Abstract

Our previously published work has shown that non-ACE angiotensin II (Ang II) generating system is dominate in the placenta and may play a critical role in regulation of placental vascular contractile function. In the present study, using a collagen gel contraction assay we further studied contractility of placental vascular smooth muscle cells (VSMCs) in response to factors produced by preeclamptic (PE) placentas. Placental VSMCs/type-1 collagen gels were incubated with PE placental conditioned medium in the presence or absence of inhibitors or receptor blockers. Captopril (an ACE inhibitor), chymostatin (a non-ACE chymase inhibitor), losartan (an AT1 receptor blocker) and PD123,319 (an AT2 receptor blocker) were used to study the specific ACE vs. non-ACE and AT1 vs. AT2 effects on placental VSMC contractility, respectively. Our results showed that chymostatin, but not captopril, and PD123,319, but not losartan, significantly attenuated placental VSMC/collagen gel contraction, $p < 0.01$, respectively. The inhibitory effects of chymostatin and PD123,319 were dose-dependent. Our results suggest that chymase, a non-ACE Ang II generating enzyme, may contribute significantly to Ang II generated in the placenta vascular tissue and that the AT2 receptor may play an important role in the regulation of Ang II induced contractility of placental VSMCs. These results provide new insights into Ang II generation and Ang II receptor regulation of vessel contractile function in the placental vasculature. These results also suggest the potential role of increased chymase activity and altered AT2 receptor function in placental related pregnancy disorders such as preeclampsia and IUGR.

Keywords

Placenta; Ang II; Chymase; AT1; AT2; Vasoconstriction

1. Introduction

During pregnancy, fetal development and growth largely depend on placental vascular function. The low resistance and high blood flow of well-developed maternal decidual spiral arteries ensure adequate maternal blood perfusion to the placenta and provide sufficient oxygen and nutrition supplies to the fetus. Furthermore, an appropriate regulation of vasomotion in the placental vasculature is not only important in maintaining a constant blood flow for the fetal

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circulation, but is also critical to the well being of the maternal vascular system during pregnancy. It is believed that inadequate blood flow and increased vasoconstriction of the placental vasculature contribute to pregnancy-associated disorders such as intrauterine growth restriction (IUGR) and preeclampsia (PE). Because placental vessels lack autonomic innervations, locally hormonal effects of the placenta must play a dominant role in the regulation of fetal-placental vascular contractility.

Placental tissues are not only able to produce various vasoactive agents, like angiotensin II (Ang II) [1-3], thromboxane (TX) [4-6], and endothelin (ET) [7,8], but also possess receptors for each of these vasoactivators, which ensure that the placental vasomotor activity is controlled in both the autocrine and/or the paracrine fashions. Using an organ bath perfusion model, we recently demonstrated that vasoconstrictors produced by preeclamptic placentas could induce constriction of chorionic plate arteries [9]. We further noticed that chymostatin, a specific inhibitor of chymase (chymotrypsin-like protease), exerted a stronger inhibitory effect than captopril, a specific inhibitor of angiotensin converting enzyme (ACE), in attenuating increased vasoactivity of the chorionic plate artery rings induced by stimuli in the preeclamptic placental conditioned medium [9].

Chymase is a potent non-ACE angiotensin generating enzyme which is responsible for approximately 70-80% Ang II generated in the human heart [10,11]. We found that the chymase gene was active in the placenta [12]. Strikingly, the open reading frame of trophoblast chymase gene is 100% homologous to that reported in the human heart [10,12], suggesting chymase may play an important role in Ang II generation and regulation of placental vascular reactivity during pregnancy. Ang I and Ang II are present in the placenta, and the uteroplacental unit possesses all necessary components of the renin-angiotensin-system (RAS) [1,3]. However, the specificities of ACE and non-ACE Ang II generating enzymes and Ang II receptors in regulation of placental vessel cell contractility have not been well studied.

In the present study, using the collagen gel contraction assay as a model, we further evaluated the contractile responsiveness of the placental vascular smooth muscle cells (VSMCs) in response to stimuli produced by preeclamptic placentas. Captopril and chymostatin were used to study possible ACE and non-ACE generation of Ang II induced cell contractility. Losartan (an AT1 receptor blocker) and PD123,319 (an AT2 receptor blocker) were used to study differential regulatory effects of AT1 and AT2 receptor on the placental VSMC contractile function. Our results showed that chymostatin, but not captopril, and PD123,319, but not losartan, significantly attenuated placental vessel cells/collagen gel contraction. These results provide new insights into control of Ang II generation and Ang II receptor regulation in the placental vasculature.

2. Materials and methods

2.1. Placental vessel smooth muscle cell preparation

Placental tertiary chorionic plate vessels from normal placentas were used to generate placental vessel smooth muscle cells (VSMCs). Placentas were obtained immediately after delivery from normal term pregnant women ($n = 4$ with gestation age between 37 and 40 weeks). Normal pregnancy is defined as mothers with normal blood pressure ($<140/90$ mmHg), absence of proteinuria, and without medical and obstetrical complications. This study was approved by the Institutional Review Board for Human Research at LSUHSC-Shreveport, LA. Briefly, chorionic plate vessels were dissected under sterile conditions, then opened longitudinally and placed in Dulbecco's Modified Eagles Medium (DMEM, Sigma Chemical Inc., St Louis, MO). The vessels were denuded of endothelium and cut into approximately $0.5\text{-}1\text{ mm}^2$ pieces. The vessel pieces were placed into serum coated 6 well/cluster cell culture plate and cultured with DMEM containing 10% FBS and penicillin/streptomycin in a humidified incubator at 37°C

with 95% air and 5% CO₂. In general, cell clones would start to grow out of the vessel pieces 5-7 days after implantation. VSMCs were then subcultured into 25 cm² flask. Confluent cells showed their spindle shape and “hill-valley” appearance under a phase contrast microscope. VSMCs exhibit positive staining of α -smooth muscle actin and vimentin with negative staining of CD31.

2.2. Preparation of rat-tail type-1 collagen

Rat-tail type-1 collagen was prepared by our laboratory as previously described [13]. Rat-tail tendons were extracted and digested with 1% acetic acid for two days at 4 °C under constant vortexing. The collagen solution was then filtered with 200micron mesh filters and centrifuged at 3000 rpm to get rid of undigested tendon pieces. The collagen solution was then aliquoted, dried via an Integrated Speedvac system (Savant, Holbrook, NY), and then stored at -80 °C.

2.3. Preparation of VSMC/type-1 collagen gel

All cells were serum-deprived 24 h prior to each experiment. At the day of experiment, the dried collagen was re-solubilized with 0.012 M HCl. Briefly, VSMCs were washed 2× with phosphate buffer saline (PBS) and then harvested with trypsin-EDTA. The harvested cells were centrifuged at 1500 rpm for 3 min and resuspended in 2.5× DMEM at a volume required to match the desired cell density. The pH of the collagen was quickly titrated by 0.5 M NaOH to 7.35-7.45, as monitored by pH strips. The final collagen concentration was 1.25 mg/ml as described in previously published works [13,14]. To determine an appropriate experimental condition, we performed a series of preliminary experiments with different cell number per gel and duration of culture. Cell numbers at 1×10^5 , 5×10^4 , and 2.5×10^4 /ml in collagen gel mixture were cultured at different time courses for 12 h, 24 h, and 36 h. We observed that increased cell number in gels increases the rate of contraction. Fig. 1 shows that the cell contraction in culture is time- and cell number-dependent. Based on these results, we selected 5×10^4 cells in 1.25 mg/ml of collagen as our final working condition and cultured for 24 h for all subsequent experiments.

2.4. Preparation of placental conditioned medium

Placentas delivered by women complicated with severe preeclamptic patients were used for preparing placental conditioned medium. Preeclampsia is defined as a maternal blood pressure of 140/90 mmHg of higher on two separate readings at least six hours apart with proteinuria >300 mg/24 h after 20 weeks of gestation. Preeclampsia is defined severe if one or more of the following criteria is present: maternal blood pressure $\geq 160/110$ mmHg; proteinuria >3+ or >5 g/24 h; oliguria of less than 500 ml in 24 h; intrauterine growth restriction, and presence of persistent headache or visual disturbances. Preeclamptic placental conditioned medium was prepared as previously described [9,15,16]. Briefly, villous tissue, excluding basal plates and fetal membranes, was incubated with serum free Dulbecco's Modified Eagles Medium (DMEM, Sigma Chemical Inc., St Louis, MO) for 48 h. At the end of incubation, the culture medium was collected and stored at -70 °C until use.

2.5. Collagen gel contraction assay

An aliquot of 500 μ l of the VSMCs/collagen mixture at 5×10^4 cells/ml was added to each well (24 well/plate). The plates were allowed to polymerize at 37 °C for 30 min and then 1.0 ml of DMEM or placental conditioned media (CM) were added to each well. Once DMEM, PE-CM, or PE-CM plus inhibitors (captopril or chymostatin) or blockers (losartan or PD-123,319) were added to the well, the gels were lifted off the bottom of the wells with a L-shaped blunted glass pipette tip. The VSMC/collagen gel was allowed to float freely. The plates were then returned to the incubator with 5% CO₂ and 95% air. All cultures were performed in triplicate for 24 h. At the end of each experiment, the plates were scanned via a Canon flatbed

scanner. For data analysis the area of the gel in each well was analyzed by NIH Image J Imaging analysis program. The image was first calibrated to the area of empty wells and then the edges of the gels were outlined and measured by the software. The data was calculated as area in mm². Captopril was purchased from ICN Biochemicals (Costa Mesa, CA); losartan, a generous gift from Dr. Neil Granger (LSUHSC-S); chymostatin and PD-123,319 from Sigma Chemical Inc. (St. Louis, MO), respectively.

2.6. Protein expression

VSMCs were grown to confluent in 75 cm² flasks. At the end of experiment, cells were lysed with lysis buffer containing 10 mM HEPES pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 1 M DTT, 100 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 20 mM NA orthovanadate, Triton X-100, SDS 0.5%, and IGEPAL CA-360. Total cellular protein 20 µg of each sample was subject for electrophoresis (Mini-cell protein-3 gel running system, Bio-Rad, Hercules, CA) and then transferred to nitrocellulose membrane. The membranes were probed with a primary AT1 antibody (Santa Cruz Biotechnology; rabbit), and AT2 antibody (Santa Cruz biotechnology; rabbit). Actin (Sigma) expression was used to normalize the protein expression. The secondary antibody was horseradish peroxidase-linked anti-mouse antibody. The bound antibodies were visualized with an enhanced chemiluminescent (ECL) detection Kit (Amersham Corp, Arlington Heights, IL). Nitrocellulose membranes were stripped and blocked before they were probed again with actin primary antibody. The density was scanned and analyzed by NIH Image J imaging analysis program.

2.7. Statistical analysis

Data are presented as mean ± standard error (SE). Statistical analyses were performed with paired *t*-test for the data shown in Fig. 2 and analysis of variance (ANOVA) with Student-Newman-Keuls test as post hoc test for the data shown in Figs. 4 and 5. A computer software program StatView (Cary, NC) was used. A probability level of *p* < 0.05 was considered statistically significant.

3. Results

3.1. Collagen gel contraction-induced by native vasoconstrictors produced by the placenta

To study effects of native vasoconstrictors produced by the placenta on placental vessel cells, placental VSMC/collagen gels were incubated with PE placental CM. The use of PE-CM is based on several previous published studies showing that preeclamptic placental tissues produce more vasoactivators such as thromboxane and Ang II [2,4,6,17]. Placental VSMCs/collagen gels were incubated with serum free DMEM as a control. We performed 12 independent experiments with triplicate gels in each. Our results showed that gel size was significantly reduced when incubated with PE-CM, compared with DMEM alone. The mean gel area shows about 40% reduction when VSMC/collagen gels were incubated with PE-CM compared to the controls (Fig. 2), indicating that placental vascular cells respond to the native vasoconstrictors produced by the placenta.

3.2. Effects of ACE and non-ACE Ang II generation on placental VSMC contractility

To specifically test ACE vs. non-ACE Ang II generation on placental vessel cell contractility, the ACE inhibitor captopril and the non-ACE chymase inhibitor chymostatin were added both to the collagen gel and in the PE-CM medium. Three test concentrations were used; 10⁻⁴ M, 10⁻⁵ M and 10⁻⁶ M for captopril and 10⁻³ M, 10⁻⁴ M and 10⁻⁵ M for chymostatin. These concentrations are comparable with previously published dose strategies [18]. VSMC/collagen gels were then incubated for 24 h. As shown in Fig. 3, chymostatin dose-dependently inhibited PE-CM-mediated collagen gel contraction. The collagen gel contraction was completely

inhibited by chymostatin at a concentration of 1×10^{-3} M (Fig. 3). Fig. 4 shows effects of captopril (upper panel) and chymostatin (lower panel) on collagen gel contraction. In contrast to chymostatin, captopril had no inhibitory effects on placental vessel cells/collagen gel contraction induced by either stimuli produced by preeclamptic placentas and/or Ang II being generated upon conditioned medium stimulation by VSMCs in culture. These results suggest that the non-ACE Ang II generating enzyme chymase, but not ACE, is responsible for the Ang II induced vasoconstriction in placental vessel cells.

3.3. Roles of AT1 and AT2 receptors in placental VSMC contractility

The effects of Ang II receptors, AT1 and AT2, on placental vessel cell contractility were next examined. Losartan, an AT1 receptor blocker, at concentrations of 10^{-3} M, 10^{-4} M and 10^{-5} M and PD123,319, a specific AT2 blocker, at concentrations of 10^{-4} M, 10^{-5} M and 10^{-6} M were used. Same as captopril and chymostatin, losartan and PD123,319 were added to both the collagen gel and culture medium. As shown in Fig. 5, losartan had no significant inhibitory effects on conditioned medium induced collagen gel contraction (upper panel). Surprisingly, collagen gel contraction was attenuated when PD123,319 was present in the culture and this inhibitory effect was concentration dependent (Fig. 5, lower panel). These results imply that the AT2 receptor, but not the AT1 receptor, may be dominant in controlling placental VSMC contractility induced by stimuli from PE placentas.

3.4. AT2, but not AT1, receptor expression is inducible in placental vessel cells

It has been reported that both AT1 and AT2 receptors are present in placental tissues [19,20]. To further examine AT1 and AT2 receptor expression on placental vessel cells, VSMCs were exposed to placental conditioned medium for 30 min, 2 h and 4 h. Total cellular protein was extracted and protein expressions were then determined by Western blot analysis. Fig. 6A shows AT1 and AT2 expressions in VSMCs exposed to placental conditioned medium. Our results revealed that AT1 receptor was strongly expressed in VSMCs. However, the AT1 receptor expression level was not affected when cells were exposed to conditioned medium compared to the cells incubated with DMEM alone. In contrast, AT2 expression was undetectable in control cells but the expression was observed in cells, which had been treated with conditioned medium. These data indicate that the AT1 receptor is constitutively expressed in placental VSMCs, whereas AT2 receptor expression is inducible upon stimulation. We further noticed that chymostatin, PD123,319 and losartan, but not captopril, could reduce placental conditioned medium induced up-regulation of AT2 receptor expression, Fig. 6B.

4. Discussion

Since the placenta lacks autonomic innervations, it is believed that the placental vascular tone must be regulated by circulating and/or locally released vasoactive substances. In the present study, using the collagen gel contraction assay we investigated the possible role of ACE and non-ACE Ang II generation, and effects of AT1 and AT2 on placenta-derived vasoconstrictors induced VSMC contraction. Our results showed that placental products could induce placental vessel cell contraction. This finding agrees with our previously published work using an organ bath perfusion model to study placental chorionic plate artery contractility [9]. We found that the ACE inhibitor captopril had little effects on placental VSMC contraction. However, the non-ACE chymase inhibitor chymostatin dose-dependently attenuated VSMC contraction. Furthermore, AT2 receptor blocker PD123,319, but not AT1 receptor blocker losartan, significantly inhibited placental VSMC/collagen gel contraction. These findings suggest that non-ACE Ang II convertase like chymase might be more responsible than ACE for Ang II generation in the placental tissue during pregnancy and that the AT2 receptor, rather than AT1 receptor, may play a dominant role in control of VSMC contractility in response to stimuli produced within the placenta.

Ang I is converted to Ang II by both ACE and non-ACE such as chymase. ACE is a membrane-associated enzyme that, after cleavage of its membrane anchor, can be found in a soluble form in extracellular fluid [21]. ACE is expressed highly in endothelial cells, but is detectable in low levels in non-endothelial cell types including smooth muscle cells (SMCs). It has been shown that in non-placental tissues such as in heart tissue and arteries, chymase contributes to Ang II production to a much greater degree than ACE [21,22]. Urata et al. demonstrated that chymase is responsible for approximately 80% Ang II generated in the human heart [23]. However, Ang II regulation of VSMC contractility in the placental tissue is largely unknown. In the present study using the collagen gel contraction model, we used captopril and chymostatin to differentiate possible ACE from non-ACE Ang II generation induced placental VSMC contractility. We observed that captopril had no effect on VSMC contraction, but it could attenuate chorionic plate artery contractility observed in our previous organ bath perfusion study, in which placental chorionic plate vessel rings were used [9]. This discrepancy in response to captopril could be due to an intact vessel with endothelium contact [9] versus no endothelial interaction in cultured VSMCs in the present collagen gel contraction study. On the other hand, chymostatin, a specific chymase inhibitor, produced dose dependent inhibitory effects on placental vessel cell contraction. These observations are highly relevant to the placental vasculature. We previously reported that placental trophoblast cells release chymotrypsin-like protease/chymase [12], which could affect placental VSMC contractile activity by regulation Ang II generation.

We did not examine Ang I, angiotensinogen and renin levels in the culture medium. However, early studies have shown that cultured human arterial smooth muscle cells produced an immunologically specific renin-like enzyme [24] and aortic smooth muscle cells were also able to produce angiotensinogen in culture [25]. Furthermore, studies have also shown that the uteroplacental unit contains all the components of renin-angiotensin system including angiotensinogen, renin, and Ang I to generate Ang II [1,26], which suggests that VSMCs might be able to generate Ang II intracellularly independent of the extracellular Ang I levels.

Another important finding of the present study is the role of Ang II receptor AT2 in placental vessel cells. Although AT1 receptor is dominantly expressed in placental VSMCs (as shown in our Western blot results), AT2 receptors may be more significant than AT1 receptors in controlling Ang II induced contractility of placental VSMCs. This conclusion is based on our observations that AT2 is inducible in these cells, and that the AT2 receptor antagonist PD123,319 blocks placental conditioned medium induced collagen gel contraction. The inhibitory effect is dose-dependent. In contrast to AT2, no change in AT1 expression was observed when cells were challenged with conditioned medium, and AT1 receptor blocker losartan lacked a significantly inhibitory effect on VSMC contraction. The different blocking effect suggests that AT2 receptor may play important roles in placental vessel contractile activity. Another explanation could be that AT2 expression is dominant in the fetal tissue [27]. VSMCs from the placental vasculature belong to fetal tissues. Further studies of investigating the difference of contractile function between maternal (adult) and fetal VSMCs would provide an answer. In addition, AbdAlla et al. found AT2 receptor could bind directly to AT1 receptor in PC-12 cells and fetal fibroblasts thereby antagonize the function of the AT1 receptor [28]. Whether this accounts for the phenomenon observed in our study is not known.

Chymase functions as ACE to convert Ang I to Ang II. Chymase also has an ability to convert big endothelin to endothelin-1 (1-31) [29], a strong vasoconstrictor in the vascular tissue. It is possible that activation of chymase-Ang II pathway in VSMCs induced by placental conditioned medium results in not only in Ang II generation but also in endothelin production, which may contribute to the greater inhibitory effect of chymostatin than PD123,319 on VSMC contraction, since PD123,319 only acts on the receptor level.

It is known that most of Ang II function is regulated via AT1 receptor. AT1 is constitutively expressed in placental VSMCs as shown in the Western blot data. AT1 plays important roles in angiogenic, inflammatory and oxidative stress responses in the placental vasculature [30-33]. A recent study by Herse et al. showed that AT1 receptor mRNA expression was 10-fold higher in the placenta than in the decidua tissues in both normal and preeclamptic pregnancies, but no difference was noticed for AT1 mRNA expression between normal and preeclamptic placental tissues [34]. In addition, AT1 autoantibody was also found in fetal serum in AT1 autoantibody positive mother complicated with preeclampsia [34]. However, whether AT1 autoantibody exerts any effects on AT2 receptor in VSMCs is not known. Results from our study suggest that a possible divergent regulatory mechanism of AT1 and AT2 receptors may exist among the different compartments and cell types within the placenta, i.e. endothelial cells, VSMCs, stromal cells and trophoblasts, which warrants further study.

In summary, the unique collagen gel model enabled us to directly study the VSMC contractile function without the contribution from or interaction with endothelium. We demonstrated the non-ACE angiotensin generating enzyme chymase may significantly account for Ang II generated in the placental tissue and that AT2 receptors may play an important role in regulating Ang II induced vasoconstriction in placental VSMCs. These results provide new insights related to Ang II generation and Ang II receptor regulation in the placental vasculature. Future studies are needed to better understand the nature of chymase mediated Ang II generation and to explore the pathways and mechanisms of Ang II receptor regulation in the placenta and their potential roles in clinical application such as preeclampsia and IUGR.

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Fig. 1.

Placental VSMC-mediated collagen gel contraction. VSMCs/type-1 collagen gels were embedded with different cell numbers per gel and incubated with serum free DMEM at 37 °C for 12 h, 24 h and 36 h. Left column indicates cell numbers per gel. These results indicate that the contraction of VSMC/collagen gels is cell number - and time - dependent.

Fig. 2.

The mean area of VSMCs/collagen gels incubated with DMEM alone or with preeclamptic placental conditioned medium (CM) at 24 h of culture. The area of gels is significantly smaller in gels cultured with PE-CM than that in DMEM alone, $**p < 0.01$. Data are presented as mean $\pm$ SE from 12 independent experiments with triplicate well in each. Inserts: photographs of contracted collagen gels. A: gels in DMEM alone and B: gels in CM.

Fig. 3.

A representative photograph of VSMC/collagen gels incubated with conditioned medium alone or in the presence of different concentrations of chymostatin. Chymostatin attenuates VSMC/collagen gel contraction in a concentration dependent manner.

Fig. 4.

Effects of captopril and chymostatin on VSMC/collagen gel contraction. The area of collagen gels incubated with CM alone was set as 100%. Results showed that chymostatin (lower panel), but not captopril (upper panel) significantly attenuated collagen gel contraction in a concentration dependent manner, ** $p < 0.01$. Data are presented as mean $\pm$ SE from 3 independent experiments with triplicate well in each.

Fig. 5.

Effects of losartan and PD123,319 on VSMC/collagen gel contraction. The area of collagen gels incubated with CM alone was set as 100%. The data showed that PD123,319 (lower panel), but not losartan (upper panel), effectively attenuated collagen gel contraction in a concentration dependent manner, $**p < 0.01$. Data are presented as mean $\pm$ SE from 3 independent experiments with triplicate well in each.

Fig. 6.

Ang II receptor AT1 and AT2 expression. A: VSMCs were treated with CM for 30 min, 2 h and 4 h and protein expression for AT1 and AT2 were examined by Western blot analysis. No change for AT1 expression in control cells and cells treated with CM. In contrast, AT2 is undetectable in control cells, but its expression is up-regulated when cells were challenged with placental CM. B: Up-regulation of AT2 expression induced by placental CM could be reduced by pretreatment of cells with chymostatin (Ch), PD123,319 (PD) and losartan (Lo), but not captopril (Cp). Representative blots from 3 independent experiments are shown.