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Placental expression of AChE, α7nAChR and NF-κB in patients with preeclampsia

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ABSTRACT

Objectives: This study aimed to investigate placental expression of AChE, α7nAChR and NF-κB in patients with preeclampsia and discuss about its clinical significance.

Material and methods: mRNA expression levels of acetylcholine (AChE), alpha-7 nicotinic acetylcholine receptor (α7nAChR) and nuclear factor-kB (NF-κB) in placenta were detected by qRT-PCR, and protein levels were determined by immunohistological analysis and Western Blot in 35 women with preeclampsia (including 20 cases of mild preeclampsia and 15 cases of severe preeclampsia) and 30 cases in control group, respectively.

Results: The expression of AChE mRNA and protein in placenta increased significantly in patients with preeclampsia compared with the control group ($p < 0.01$). It was lower in patients with severe preeclampsia than in patients with mild preeclampsia ($p < 0.05$). The expression of α7nAChR mRNA and protein in placenta decreased significantly in patients with preeclampsia compared with the control group ($p < 0.01$). However, the expression of α7nAChR mRNA and protein in patients with severe preeclampsia was higher than that in patients with mild preeclampsia, without significant difference ($p > 0.05$). The expression of NF-κB protein in placenta decreased significantly in patients with preeclampsia compared with the control group ($p < 0.01$). It was higher in patients with severe preeclampsia than in patients with mild preeclampsia ($p < 0.05$), but there was no significant difference between preeclampsia group and control group in the expression of NF-κB mRNA in placenta ($p > 0.05$). The results of Western blotting assay were consistent with those of immunohistochemistry.

Conclusions: Abnormal expression of AChE, α7nAChR and NF-κB in placenta may be associated with preeclampsia. Cholinergic anti-inflammatory pathway may play an important role in the pathogenesis of preeclampsia.

Key words: preeclampsia; cholinergic anti-inflammatory pathway; AChE; α7nAChR; NF-κB

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INTRODUCTION

Preeclampsia (PE) is a specific and common disease during pregnancy and poses great threat to the health of the mother and infant [1]. The pathogenesis of PE remains unclear. It is generally believed that PE is a systemic inflammatory response of the mother, presenting as inflammatory response in the maternal-fetal interface, activation of inflammatory cells and dysfunction and injury of vascular endothelial cells. Cholinergic anti-inflammatory pathway has attracted increasing attention in recent years. Nervous system has intrinsic connections with the immune system, with the former regulating the immune response and controlling the inflammatory response [2–4]. It has been shown that acetylcholine (ACh) levels increase in PE patients during late pregnancy compared with normal pregnant women. It is inferred that cholinergic anti-inflammatory pathway may be associated with the pathogenesis of PE [5]. But what a specific role cholinergic anti-inflammatory pathway plays in this process is largely unknown. This study determined the expression of acetylcholinesterase (AChE), alpha-7 nicotinic acetylcholine receptor (α7nAChR) and nuclear factor-kB (NF-kB) in the placenta of PE patients and normal pregnant

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women, and discussed about its clinical implications. The relationship between cholinergic anti-inflammatory pathway and PE was investigated. The findings shed new light on the pathogenesis of PE and the prevention and treatment strategies.

MATERIAL AND METHODS

Thirty-five PE patients who gave birth at department of obstetrics and gynecology of Hainan Provincial People's Hospital from January 2013 to January 2014 were included. PE was diagnosed and classified according to Obstetrics and Gynaecology (8th Version) edited by Xin Xie [6]. Mild PE was defined as new onset of both hypertension (blood pressure ≥ 140/90 mm Hg) and proteinuria [≥ 300 mg/24 hours urine collection or random urine protein (+)] after 20 weeks of gestation. Indicators of severity of preeclampsia were blood pressure ≥ 160/110 mm Hg, plus severe proteinuria (5.0 g/24 h or greater than 2+ by dipstick) and clinical or laboratory findings indicative of organ dysfunction. There were 20 cases with mild PE and 15 cases with severe PE. Among the cases, there were 10 women (8 with severe and 2 with mild preeclampsia) with early onset preeclampsia (before 34 weeks gestation) and 25 cases (7 with severe and 18 with mild preeclampsia) with late-onset preeclampsia (after 34 weeks gestation). The gestation-matched placentas of normal pregnancy were chosen as normal control with no history of hypertension or proteinuria during 35–40 weeks of pregnancy who delivered healthy neonates via cesarean section. The baseline blood pressure in the control group was 121.6 ± 0.6/78 ± 0.8 mm Hg. None of the cases had other obstetric, internal medicine and surgical complications. This study was approved by the ethics committees of Hainan People's Hospital (Haikou, China). Written consent was obtained from each patient.

Sample collection

Placental tissues were collected within 5min after delivery of placenta. A block of placental tissues of about 3 cm × 3 cm × 3 cm was taken at the center of the maternal side of the placenta away from the calcified region. The sample was washed with normal saline, fixed in 10% neutral formalin and made into paraffin-embedded sections.

Immunohistochemical detection of AChE, α7nAChR and NF-κB protein in the placenta

Paraffin-embedded sections (3–4 μm thick) were deparaffinized in xylene three times (10 min each at room temperature) and rehydrated through a gradient of ethanol. Antigen retrieval was performed with microwave treatment in 0.01 M citrate buffer for 30 min. After quenching endogenous peroxidase activity and blocking with normal goat serum, sections were incubated with primary antibodies against human α7nAChR (1:100; ProteinTech Group, Inc.Chicago, USA), AChE and NF-κB (1:200, Biotechnology Inc, CA, USA) for 1 h. Washed sections were incubated for 10min with secondary goat anti-rabbit IgG biotin. The slides were then rinsed with PBS-Tween-20 (three times, 3 min each), and visualized with DAB chromogenic agent. Sections were counterstained with hematoxylin and then observed with a microscope system (Olympus LX70; Olympus, Middlesex, UK). The sections were sealed with neutral balsam. Appearance of pale brown and brown particles in the nuclei and cytoplasm indicated positive expression of AChE, α7nAChR and NF-κB. Different percentages of positive cells were given different scores: 0 — 5%; 1 — 5–20%; 2 — 20–50%; 3 — 50–75%; 4 — 75%. Different staining intensity was also given different scores: 0 — no staining; 1 — light yellow; 2 — yellow; 3 — deep yellow. The two scores were multiplied together. A final score of ≤ 4 was assigned to the negative expression group, while a final score of ≥ 5 was assigned to the positive expression group. Two senior pathologists determined the scores

Detection of AChE, α7nAChR and NF-κB expression in placenta using Western Blot

The placenta sample was weighed and crushed in a mortar. Protein sample was collected. Total protein extraction from the placenta was performed according to kit instructions. Coomassie R-250 was used to stain protein gels (Bradford assay). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Thereafter, the primary antibodies including AChE (diluted at 1:400; Beyotime Biotechnology), α7nAChR (diluted at 1:400; Beyotime Biotechnology), NF-κB (diluted at 1:400; Beyotime Biotechnology) and β-actin (diluted at 1:1,000, Abcam) were dissolved in TBST and used to incubate membranes at 4°C overnight. After a cleaning in TBST, membranes were incubated with the appropriate HRP-conjugated secondary antibody (diluted at 1:2000; Beyotime Biotechnology) for 2 hours at room temperature. The membrane was air dried and photographed using a gel imaging system. The results were analyzed using Quality One software. The relative expression of the target band was calculated based on the optical density ratio of target band to β-actin.

qRT-PCR detection of AChE, α7nAChR and NF-κB mRNA expression

The qRT-PCR was performed on a 7500 fast Real Time PCR system (Applied Biosystems, USA) with a final volume of 20 μL per reaction. Total RNA was isolated from the placental tissue with TRIzol reagent (Invitrogen). cDNA was synthesized from total RNA using RevertaidTM First Strand cDNA Synthesis Kit (Thermo, USA), according to the manufacturer's instructions. Each 20 μL reaction mixture contained 2 μL of diluted cDNA, 10 μL of TaqMan® Universal PCR Master Mix

Table 1. Detail information on the selection of primers for real-time RT-PCR experiments

Name	Primers sequence (sense)	Primers sequence (antisense)
AChE	5′-TAC TTC TCC CAC ACC TGT CCT CA-3′	5′-ATA GAT ACC AAC ACG GTT CCC TC-3′
α7nAChR	5′-GGT CGT ATG TGG CCG TTT G-3′	5′-TGC GGT TGG CGA TGT AGC G-3′
NF-κB	5′-CAC AGA TAC CAC TAA GAC GCA CC-3′	5′-GAC CGC ATT CAA GTC ATA GTC C-3′
β-actin	5′-CCC ATC TAT GAG GGT TAC GC-3′	5′-TTT AAT GTC ACG CAC GAT TTC-3′

(Applied Biosystems, USA) 1 μL of each primer, and 7 μL of sterile distilled water. The thermal cycling program was 95° C for 30 s, followed by 40 cycles of 95° C for 5 s, 60° C for 30 s, and 72° C for 15 s. Melt-curve analyses were performed using a program with constant heating from 70° C to 95° C, followed by 95° C for 15 s. Each reaction was carried out for three technical replicates. The sequences of the qRT-PCR primers used in this study were designed and synthesized by Sangon Biotech Co., Ltd (Sanghai, China) and listed in Table 1. The qRT-PCR data were analyzed using the ΔΔCt method.

Statistical method

Statistical analysis was conducted using SPSS 21.0 software. Comparisons of multiple groups were performed using one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) post hoc test or Dunnett's test as appropriate. When homogeneity test of variance was neat, an appropriate LSD post hoc test was employed. Also, Chi-square test was used for data analysis. Data are expressed as the mean ± standard deviation (SD). $P < 0.05$ indicated significant difference. Assays were performed at least three times independently.

RESULTS

Immunohistochemical detection of AChE, α7nAChR and NF-κB in placenta

The rate of strongly positive expression of AChE was 45.0% (9/20) in the mild PE group and 33.3% (5/15) in the severe PE group. Both rats were lower compared with 73.3% (22/30) in the normal group. There was significant difference between PE group (mild and severe group) and control group. The rate of strongly positive expression of α7nAChR was 65.0% (13/20) in the mild PE group and 53.3% (8/15) in the severe PE group. Both rats were higher compared with 20.0% (6/30) in the normal group. There was significant difference between PE group (mild and severe group) and control group. The rate of strongly positive expression of NF-κB was 75.0% (15/20) in the mild PE group and 86.7% (13/15) in the severe PE group. Both rats were higher compared with 26.7% (8/30) in the normal group. There was significant difference between PE group (mild and severe group) and control group (Fig. 1 and Tab. 2).

Expression levels of AChE, α7nAChR and NF-κB protein in the placenta were detected by Western Blot, with the results shown in Figure 2 and Table 3.

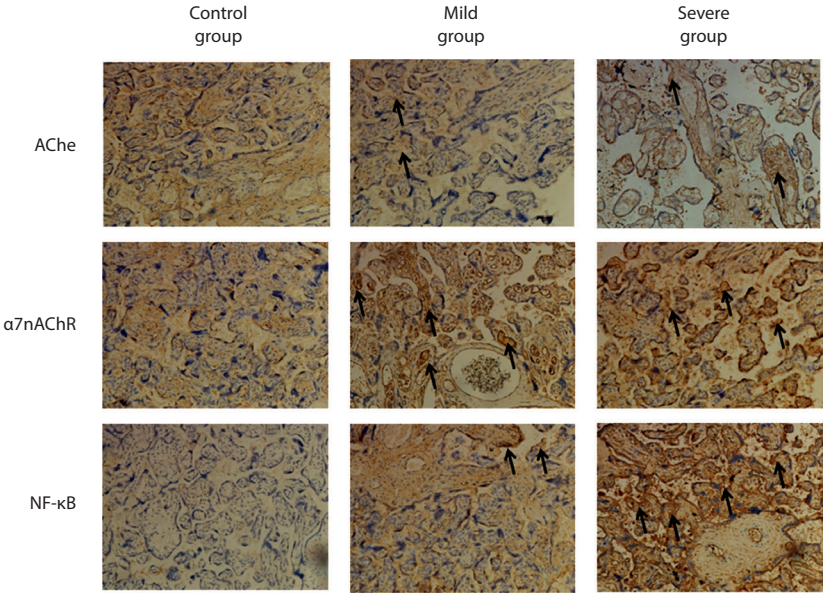

Figure 1. Expression of AChE, α7nAChR and NF-κB in placenta: Immunohistochemistry analysis of AChE, α7nAChR and NF-κB from placenta in preeclampsia (including mild preeclampsia and severe preeclampsia) or control group. (SP, × 400)

Table 2. Differential expression of AChE, α7nAChR and NF-κB in distinct histological placental tissue

Group	n	AChE		α7nAChR		NF-κB	
		–	+	–	+	–	+
Control	30	8	22	24	6	22	8
Mild	20	11	9	7	13	5	15
Severe	15	10	5	7	8	2	13
χ2值		4.09 [a]		10.31 [a]		11.29 [a]	
		6.67 [b]		5.18 [b]		14.46 [b]	
P值		< 0.05 [a]		< 0.01 [a]		< 0.01 [a]	
		< 0.05 [b]		< 0.05 [b]		< 0.01 [b]	

[a]vs. mild; [b]vs. severe; (+) — positive; (–) — negative

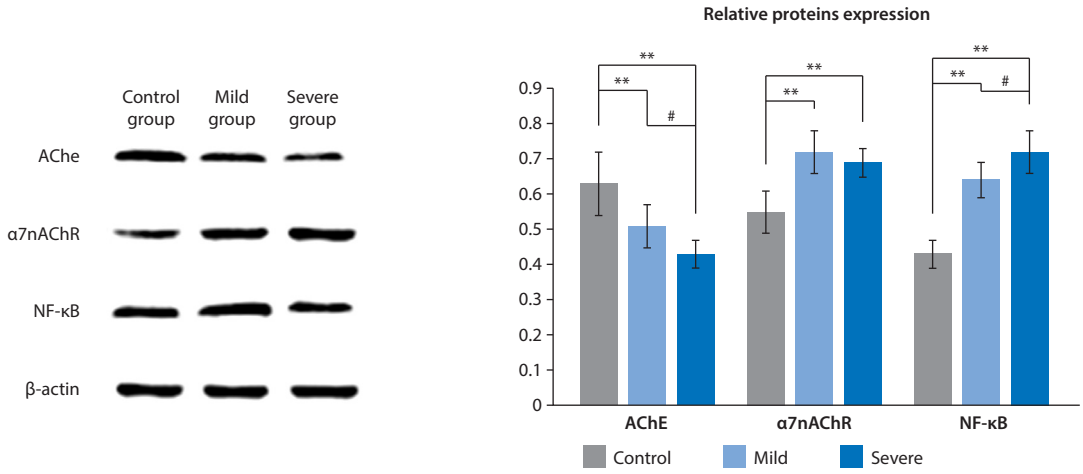

Figure 2. Expression of AChE, α7nAChR and NF-κB protein in placental tissues of pregnant women with preeclampsia (including mild preeclampsia and severe preeclampsia) and normal tissues detected by Western blot
*p < 0.05 vs. control; **p < 0.01 vs. control; #p < 0.01 vs. mild; ##p < 0.05 vs. mild

Table 3. Comparison of protein expressions of AChE, α7nAChR and NF-κB in placental tissues

Group	AChE	α7nAChR	NF-κB
Control group	0.63 ± 0.09	0.55 ± 0.06	0.43 ± 0.04
Mild group	0.51 ± 0.06**	0.73 ± 0.06**	0.64 ± 0.05**
Severe group	0.43 ± 0.04**/#	0.69 ± 0.04**	0.73 ± 0.06**/#
F	31.00	56.38	177.90

*$p < 0.05$ vs. control; **$p < 0.01$ vs. control, #$p < 0.05$ vs. mild, ##$p < 0.01$ vs. mild

The AChE expression in the placenta of PE patients was significantly lower compared with the normal pregnant women (p < 0.01). Moreover, the AChE expression was lower in the severe PE cases than in the mild PE cases (p < 0.05). The α7nAChR expression in the placenta of PE patients was significantly higher compared with the normal pregnant women (p < 0.01). Moreover, the α7nAChR expression was lower in the severe PE cases than in the mild PE cases, but without significant difference. The NF-κB expression in the placenta of PE patients was significantly higher than that in the normal pregnant women (p < 0.01). Moreover, the NF-κB expression was considerably higher in the severe PE cases than in the mild PE cases (p < 0.05).

Changes in mRNA expression levels of AChE, α7nAChR and NF-κB in the placenta are shown in Figure 3 and Table 4.

The mRNA expression of AChE in PE patients was significantly lower compared with the normal pregnant women (p < 0.01). Moreover, the expression was considerably lower in severe PE cases than in mild PE cases (p < 0.05). The mRNA expression of α7nAChR in PE patients was significantly higher

Table 4. Comparison of mRNA expressions of AChE, α7nAChR and NF-κB in placental tissues

Group	AChE	α7nAChR	NF-κB
Control group	1.55 ± 0.12	1.68 ± 0.19	1.87 ± 0.20
Mild group	0.96 ± 0.11**	2.04 ± 0.33**	1.95 ± 0.25
Severe group	0.64 ± 0.09**/#	1.92 ± 0.15**	2.02 ± 0.22
F	28.9	13.28	1.75

*$p < 0.05$ vs. control, **$p < 0.01$ vs. control, #$p < 0.05$ vs. mild, ##$p < 0.01$ vs. mild

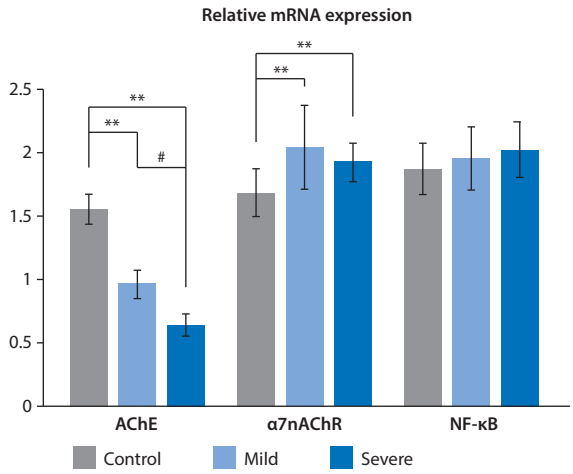

Figure 3. Expression of AChE, α7nAChR and NF-κB mRNA in placental tissues of pregnant women with preeclampsia (including mild preeclampsia and severe preeclampsia) and normal tissues detected by qRT-PCR
*$p < 0.05$ vs. control; **$p < 0.01$ vs. control; #$p < 0.05$ vs. mild; ##$p < 0.01$ vs. mild

compared with the normal pregnant women ($p < 0.01$). Moreover, the expression was considerably reduced in severe PE cases than in mild PE cases, but without significant difference ($p > 0.05$). The two groups showed no significant difference in mRNA expression of NF-κB ($p > 0.05$).

DISCUSSION

Preeclampsia (PE) usually affects multiple systems during pregnancy, causing death of mothers and perinatal infants [7]. Recent study has shown that excess inflammatory response to pregnancy in PE can cause immune imbalance in maternal-fetal interface, placental vascular lesions, vascular endothelial injury, limited migration of trophocytes and shallow placental implantation [8]. PE is associated with an upregulation of proinflammatory cytokines and chemokines [9]. Many scholars believe that inflammatory response plays an important role in pathological processes of PE and IUGR and HELLP syndrome. Excess inflammatory response is harmful to the organisms, which in turn produce complex compensatory anti-inflammatory response.

In 2000 Borovikova et al. [10] proposed the concept of cholinergic anti-inflammatory pathway as a possible neural anti-inflammatory mechanism. Specifically, the brain recognizes the signals transmitted by the afferent vagus nerve, which stimulates the release of ACh by the vagus nerve endings. ACh then binds to the α7nAChR on the surface of macrophages and other types of cells, inhibiting the synthesis and release of inflammatory cytokines, including tumor necrosis factor (TNF), interleukin (IL) –1 and high-mobility group box 1 (HMGB1), and hence suppressing the anti-inflammatory effect [11, 12]. In 1986 Satyanarayana [13] reported a significant reduction in the synthesis of ACh in syncytiotrophoblasts with moderate generation (e.g., PE, eclampsia and intrauterine death). Murthy et al. [14] also reported similar reduction of ACh release in the placenta of PE patients. Our preliminary study indicated variability of the expression of cholinesterase (ChE) and cytokines in PE of different severity [5]. From this we inferred that the cholinergic anti-inflammatory pathway may play a role in it.

AChE is a key enzyme in biological nerve conduction that can degrade ACh, terminate the excitation of the postsynaptic membrane caused by neurotransmitters and ensure the normal transmission of nerve impulses. AChE is an important regulatory factor in cholinergic transmission. Some mechanisms must work to regulate the activity of AChE to ensure the normal functioning of ACh. Study has shown that serum AChE activity decreased in severe trauma, burn injury and sepsis [15, 16]. Cholinergic anti-inflammatory pathway that regulates neural and immune system may play a role by downregulating AChE to generate the compensatory anti-inflammatory response, thus promoting further release of ACh by vagus nerve. Our results indicated that the mRNA and protein expression levels of AChE in the placenta of PE patients were considerably lower compared with the normal pregnant women. In addition, the expression levels were significantly lower in severe PE than in mild PE. However, it remains unclear whether the decreased AChE activity was caused by activation of the cholinergic anti-inflammatory pathway or by liver injury associated with PE.

Accumulating evidence has pointed out that a7nAChR plays an essential role in Cholinergic anti-inflammatory pathway [17, 18]. In vitro study has shown that α7nAChR is most highly expressed in human umbilical vein endothelial cells (HUVECs) and microvascular endothelial cells.

Hypoxia and nicotine can cause a significant upregulation of α7nAChR in HUVECs [19]. However, contradictory results have been reported as well, which found a downregulation of α7nAChR under hypoxia [20, 21]. Our results showed that α7nAChR was expressed in the placenta of both normal pregnant women and PE patients. The expression in PE patients was much higher than that in the control group, and it was reduced in severe PE compared with mild PE. qRT-PCR confirmed the above findings. Thus, it was inferred that ACh-bound α7nAChR may play a role in the pathogenesis of PE. However, the divergence from the result by Rita Machaalani et al. may be due to the variation in severity and duration of hypoxia exposure in PE, patients' age and number of patients [22, 23].

Activation of NF-κB nuclear translocation is a key element to upregulate many inflammatory mediators such as TNF-α, IL-6 and IL-1β. There is increasing evidence that cholinergic stimulation can inhibit cytokines by inhibiting the NF-kB pathway through an a7nAChR-dependent anti-inflammatory pathway [24, 25]. An early study showed that the plasma from pregnant women with hypertension could activate NF-κB and the activity of NF-κB was increased by 2.5 times compared with the normal pregnant women [26]. In our study, qRT-PCR was performed to detect NF-κB in the placenta of PE patients. The results indicated no significant difference in mRNA expression of NF-κB between PE patients and normal pregnant women. Immunohistochemistry indicated a stronger staining of NF-κB in the placenta of PE patients than in normal pregnant women. Moreover, the NF-κB expression in the placenta of severe PE patients was higher than that in mild PE patients. These results implied the pathological role of NF-κB in PE, whose expression intensity reflects the severity of PE. This agrees with the findings in many other studies [27–29]. The pathogenesis of PE may be related to the activation of NF-κB, which in turn participates in microvascular wall remodeling and placental vascular inflammation. Interestingly, our study found no significant difference in mRNA expression of NF-κB in the placenta between PE patients and normal pregnant women. It can be inferred that the anti-inflammatory target of ACh is the protein. The discordance between transcript and protein expression levels may be due to differential rates of transcription and translation and/or in vivo and post mortem degradation rates of transcripts and proteins [30–32]. While the stability of transcripts and proteins vary according to their functional characteristics [33, 34], the rate of translation has been found to be the most important factor in predicting protein expression [35].

The present study showed that in mild PE cholinergic anti-inflammatory pathway can be activated as a compensatory response by inhibiting the nuclear factor-kB pathway through an a7nAChR-dependent pathway. However, the above processes induce abnormalities of the cholinergic anti-inflammatory pathway in severe PE. As AChE activity decreases and less ACh is synthesized and released, α7nAChR expression will decrease or even become zero. In contrast, as NF-κB release increases, a large number of toxic factors such as inflammatory mediators will be released, leading to vascular stenosis and disorder of placental vascular remodeling.

In conclusion, we found that AChE, α7nAChR and NF-κB were involved in the pathophysiology of preeclampsia. The results demonstrate that α7nAChR-mediated CAP is a neuro-physiological mechanism and its disruption may be due to the impairment of placental function in preeclampsia. Further, these results imply that targeting α7nAChR may be a promising interventional strategy for preeclampsia.

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