Expression of anti-inflammatory mediator lipoxin A4 and inflammation responsive transcriptive factors NF-kappa B in patients with preeclampsia

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Summary

Objective: To evaluate the role of lipoxin A4 (LXA4), NF-κB p65 in preeclampsia. *Materials and Methods:* LXA4 in blood serum and the lipoxin A4 receptor (ALX-R), NF-κB p65 mRNA, protein expressions in placenta-specific tissues were obtained from patients with preeclampsia and normal pregnancy. *Results:* Levels of lipoxin A4 in women with mild preeclampsia was significantly high ($p < 0.05$). There was no significant statistical difference between normal pregnancy and severe preeclampsia ($p > 0.05$). The mRNA expression of ALX-R was significantly low in women with preeclampsia than in control group ($p < 0.01$) and mRNA expression of NF-κB p65 was significantly high in preeclampsia ($p < 0.01$). The immunohistochemical staining of NF-κB p65 protein was stronger in severe preeclampsia group whereas staining of ALX-R in placental tissue was weaker than in control group ($p < 0.01$). ALX-R mRNA was negatively correlated with NF-κB ($p < 0.0001$), but there was no correlation between LXA4 and ALX-R mRNA. *Conclusion:* There was an excessive maternal inflammatory response in preeclampsia. LXA4, ALX-R, and NF-κB p65 may be involved in the disease process of preeclampsia.

Key words: Preeclampsia; Lipoxin A4; Lipoxin A4 receptor; NF-κB p65; Inflammatory reaction.

Introduction

Preeclampsia is one of the common complications seen in pregnancy, and its severity can contribute significantly to maternal and perinatal morbidity and mortality. It is a multisystem disorder of unknown etiology. Studies have shown that preeclampsia is due to an excessive inflammatory response to pregnancy and the basic pathology is vascular endothelial dysfunction [1]. Vascular endothelial injury in eclampsia is due to involvement of factors like leukocytes, coagulation system, the complement system leading to excessive intravascular inflammatory response [2, 3], resulting in multiple organ dysfunction and in a variety of other clinical manifestations of eclampsia [1].

LXs are derived from arachidonic acid, a member of eicosanoid family made of 20 carbon fatty acids [4]. In the process of inflammation or other diseases, lipoxins are synthesized after stimulation of inflammatory cytokines like lipopolysaccharide, through transcellular biosynthesis pathway which involves catalytic action of different lipoxygenase (1ipoxygenases, LOX) [5, 6]. According to the molecule hydroxyl position and different conformation, other members includes lipoxin A_4 (LX A_4), lipoxin B_4(LXB4), aspirin-triggered LXs (15- epi-LXA4 and 15-epi-LXB4), which promote the resolution of inflammatory reaction, and is thought to inhibit the signal of endogenous inflammation [5]. LXA4 binds with its specific receptor (lipoxin A4 receptor, belongs to G protein-coupled receptor super family ALX-R) to exert its biological effects [7]. This receptor is widely distributed in bone marrow-derived cells, such as neutrophil, monocytes / macrophages; they are also expressed in endothelial cells, lymphocytes, and also in different tissues, such as lung, kidney, and placenta [7, 8]. Nuclear transcription factor NF-κB/Rel family consists of five members: Rel-A (p65), RelB, C-Rel, p50 (NF-κB1) and p52 (NF-κB2). They are widely present in different types of cells to regulate immune response, apoptosis, and inflammation responsive transcriptive factors. In inflammatory diseases, such as systemic inflammation, infectious diseases, NF-κB activity is significantly increased [9]. LX when combined with its specific receptor, can pass through PI3K/Akt pathway, through inhibition of mitogen-activated protein kinase 3/6 (MAPK kinase 3/6), and p38-MAPK phosphorylation level, inhibiting the activation of NF-κb [10].

In this experiment, preeclamptic patient is the study subject; in preeclampsia group and normal control group, the authors detected blood LXA4 levels and level of ALX-R, NF-κB p65 mRNA in placenta tissue and protein expression, to determine the relation of LXA4 and its receptor along with NF-κb p65 in development and occurrence of preeclampsia and to investigate its role in pathogenesis of preeclampsia.

Materials and Methods

Patients and samples

Serum specimen and placenta tissue samples were obtained by 30 preeclampsia patients and 20 normal late pregnant women who consulted in the present center. All the patients with preeclampsia

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fulfilled the criteria of the American College of Obstetricians and Gynecologists [11, 12]. Placental tissue samples were obtained by cesarean section. Serum specimens and placental tissue samples were matched-pairs. All patients gave informed consent for the use of their samples in research. In both groups, the clinical characteristics of the patients (age, maternal BMI, gestational age, mean arterial blood pressure, serum C-reactive protein) were collected.

Supernatants of serum were obtained from the centrifugate of venous blood samples (3,000 g for 20 minutes at -4°C) and stored at –80°C until use. Placental tissue samples were fixed in 4% paraformaldehyde within 30 minutes of placental expulsion and were paraffin imbedding. Other fresh placental tissue were conserved in -80°C freezer.

Measurement of LXA4 in serum

The LXA4 level in the supernatants of serum specimens was determined with an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions, which was specific for LXA4.

Assessment of human LXA4 receptor (ALX) and NF-κB p65 mRNA expression in placental tissue was via reverse transcription-PCR (RT-PCR).

Total RNA was extracted from the placenta using trizol reagent method, and cDNA was synthesized from three μg of total RNA using M-MVcl2 reverse transcriptase and oligo (dT) primers. According to the sequence of Genebank, gene was designed and synthesized.

The RT-PCR primers for human ALX-R were 5'- GAG TCT GCT GGC TAC ACT GTT C -3' (sense: bp) and 5'- GAG GTT GAT GTC CAC CAC GAT -3' (antisense bp), and the PCR product obtained with these primers was 276 bp in size. The primers for human NF-κB p65 were 5'- TCA ATG GCT ACA CAG GAC CA -3' and 5'- CAC TGT ACA CTG GAA GCA GA -3', and the PCR product had a size of 308 bp. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control, with two primers (5'- ACC ACA GTC CAT GCC ATC AC -3' and 5'- TCA CCA CCC TGT TGC TGT A -3') yielding an expected PCR product of 450 bp. For amplification, an initial reverse transcription step was followed by denaturing step (94°C for five minutes) and then by 30 cycles of denaturing (94°C for 30 seconds), annealing (61°C for 30 seconds for ALX-R, 62°C for 45 seconds for NF-κB p65), and extending (72°C for 60 seconds), followed by ten minutes at 72°C for elongation. The PCR products produced were separated by electrophoresis on 2% agarose gel. Results were photographed, scanned. Analysis was carried out with a by a gel imaging analysis system scanning.

Immunohistochemistry for ALX-R, NF-κB p65 The 4-μm histologic sections from the placenta glands were routinely deparaffinized, rehydrated, and incubated with a solution of methanol and hydrogen peroxide (3%) for 10 minutes. antigen retrieval was accomplished by boiling tissue slides in a citrate buffer solution. Endogenous peroxidase was quenched with 3% hydrogen peroxide for ten minutes. The slides were blocked with 5% bovine serum albumin (BSA) in 0.1 M phosphate-buffered saline (PBS) for 30 minutes, followed by incubation overnight with primary antibodies against ALX-R(1:30), NF-κB p65(1:25), and then rinse by PBS, five minutes each time for three times. Anti-rabbit detection reagent was incubated with supervision for ten minutes at home temperature. Immunoreactivity was visualized with DAB kit according to the kit's instructions. The sections were counterstained with Harris hematoxylin, rinsed with deionized water, dehydrated, transparency, and mounted using resinene. Negative controls omitted the primary antibody in a histologic section while performing all the other immunostaining steps.

Evaluation of immunostaining.

Immunohistochemical results were evaluated for cytoplasm ALX-R, NF-κB p65-specific staining only. The immunohistochemical expression for ALX-R, NF-κB p65 was evaluated by using the scoring method ImmunoReactivity Score (IRS) [13]. Fields were at x10, x 40 magnifications. Four high-power fields in each section, were randomly selected, with a x400 magnification. The sections were examined by two independent researchers. Fields were at x400 magnification and the staining intensity in the Trophoblast and vascular endothelial cell was scored as 0, 1, 2, or 3 corresponding to the presence of negative, weak, intermediate, and strong brown staining, respectively. The percentage of positively stained cells (PP) was assessed in each field as: 0 (< 10;% stained cells), 1 (≥ 10%), 2 (≥ 25%), 3 (≥ 50%), and 4 (≥ 75%). The score of the staining intensity (SI) for each field was multiplied by the score of the percentage of stained cells (PP) to provide a combined immunoreactivity score value (IRS) (IRS: PP×SI). The mean of the four fields was the final IRS score for the sample.

Statistical analysis

The experimental data were analyzed by statistical software SPSS13.0. Quantitative data are expressed as mean ± standard deviation (SD). Mean values were compared by using unpaired t test (for parametric data). Non-parametric test with Kruskal-Wallis H test; correlation with Pearson correlation test, $p < 0.05$ was considered statistically significant differences.

Results

Clinical data in the pregnancy women with preeclampsia and control group showed that there was significant difference in patient's clinical characteristics, such as gestational age, mean arterial blood pressure, and serum C-reactive protein level, but no difference in age and maternal BMI was seen (Table 1). Gestational age of severe preeclampsia is significantly less than control group (250.5 ± 12.38 vs 260.7 ± 8.65, $p = 0.045$). Mean arterial blood pressure was higher in the mild (114.14 ± 2.79, $p = 0.000$) and severe (125.05 ± 9.75, $p = 0.000$) preeclampsia in comparison with the control group (88.00 ± 3.59). In patients with preeclampsia, a tendency of high C-reactive protein was detected in mild (12.04 ± 9.09) and severe (10.31 ± 9.96) preeclampsia, and difference was statistically significant ($p = 0.000$ and $p = 0.019$, respectively).

LXA4 in serum of pregnancy women with preeclampsia and control: serum LXA4 level of the pregnancy women was measured by ELISA method (Figure 1A). The mean concentration of LXA4 in mild preeclampsia was 180.84 ± 65.69 ng/ml, which was significantly higher than in control group (91.58 ± 23.94, $p = 0.046$). In the severe preeclampsia group LXA4 level was in lower concentration than control group but there was no significant difference (92.76 ± 16.11, $p = 0.085$).

ALX-R and NF-κB p65 mRNA expression in placenta tissues of preeclampsia and control group: the authors examined the expression of ALX mRNA in placenta tissues of ten patients with mild preeclampsia, 20 patients with severe preeclampsia, and 20 cases with control group. The

Table 1. — *Clinical characteristic of subject and control groups.*

Variables	Mild preeclampsia (n = 10)	Severe preeclampsia (n = 20)	Control group (n = 20)
Maternal age (year)	31.14 ± 3.57	29.15 ± 3.99	28.70 ± 3.24
Maternal BMI	19.27 ± 2.30	20.33 ± 2.43	18.12 ± 3.09
Gestational age (days)	257.57 ± 9.11	250.50 ± 12.38*	267.70 ± 8.65
MAP (mmHg)	114.14 ± 2.79*	125.05 ± 9.75*	88.00 ± 3.59
CRP (mg/L)	12.04 ± 9.09*	10.31 ± 9.96*	4.25 ± 3.24

*Significant difference compared to the control group.

expression of ALX-R and NF-κB p65 mRNA in placenta tissue was detected by RT-PCR. Figure 2A shows weaker expression of ALX-R mRNA signals in mild and severe preeclampsia group compare to control. On the contrary, NF-κB p65 mRNA signals were more strongly expressed in the mild and severe preeclampsia compare to control group (Figure 2C).

RT-PCR revealed statistically significant difference in expression of ALX-R mRNA between the three groups, (p = 0.008, highly expressed by control group 1.62 ± 0.45, followed by mild preeclampsia 0.86 ± 0.96 and severe preeclampsia 0.49 ± 0.17, Figure 2B). There was statistically significant difference in expression of NF-κB p65 mRNA between the three groups, (p = 0.001, lowly expressed by control group 0.55 ± 0.14, followed by mild preeclampsia 0.73 ± 0.19 and severe pre-eclampsia (1.20 ± 0.40, Figure 2D).

To assess the presence of ALX-R and NF-κB p65 in patients with severe preeclampsia, concentrations of ALX-R mRNA were significantly negatively correlated with those of NF-κB (Figure 1B; Pearson r = 0.8464, $p < 0.0001$). In control group, concentrations of ALX-R mRNA showed significant negative correlation with NF-κB (Figure 1C; Pearson r = 0.7931, $p < 0.0001$). There were no correlations between LXA4 and ALX-R mRNA, however, these indices decrease together with severe preeclampsia.

The immunohistochemical staining of the ALX-R and NF-κB p65 was observed in the cytoplasm of villous trophoblastic cells and blood vessel endothelial. The present results indicate that the staining intensity of ALX-R in placental tissue of the mild and severe preeclamptic group was weaker than the control group. (Figure 2A-C). However, the NF-κB showed the opposite pattern, with greater staining in the severe preeclamptic group compared to the control group (Figures 2D-F). Table 2 and Table 3 summarize the results which were categorized by the intensity of immunostaining. The staining intensity of ALX-R in the placental tissue of the severe preeclamptic group was weaker than the mild and normal controls ($\chi 2$ =17.107; $p < 0.001$), while the staining intensity of NF-κB p65 was greater in the severe preeclamptic group ($\chi 2$ =10.093; p = 0.001, Figures 3A-C).

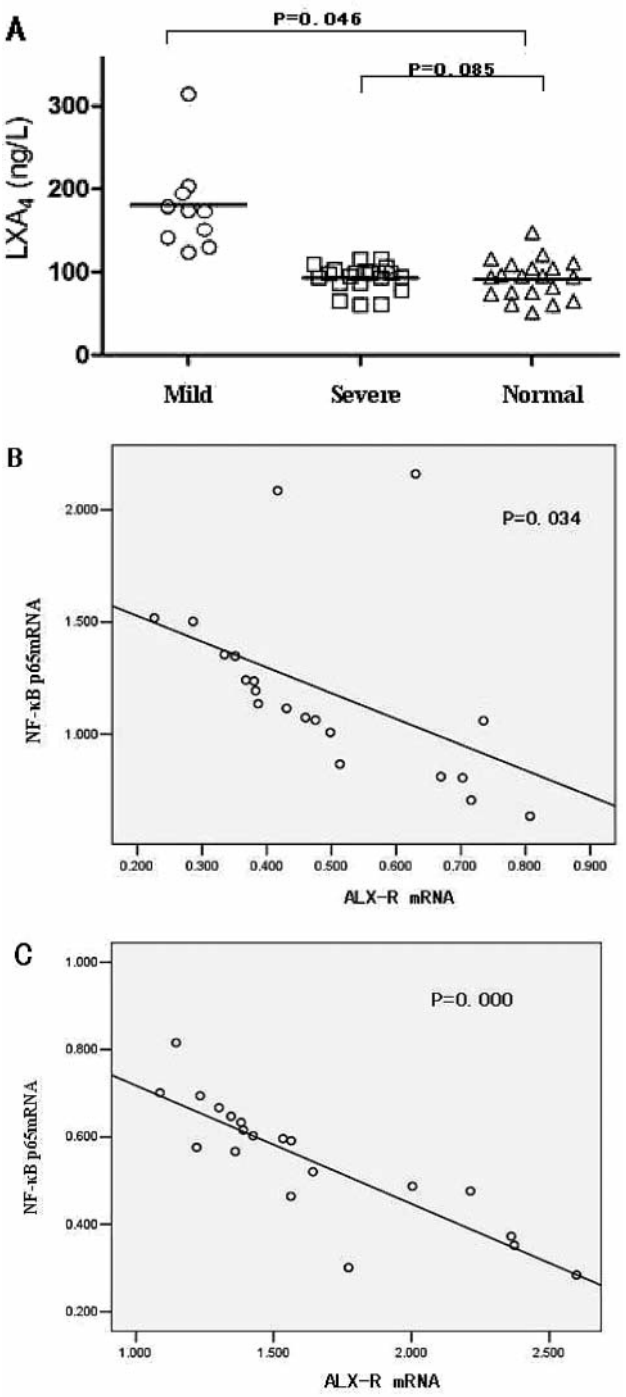

Figure 1. — A) Concentration of serum LXA4 in pregnant women with preeclampsia and control group. LXA4 level (180.84 ± 65.69 ng/mL) in serum of pregnant women with mild group was significantly higher than control group (91.58 ± 23.94, p = 0.046). The mean concentration of LXA4 in severe preeclampsia was 92.76±16.11ng/ml, which was not significantly different compared with the control group. B) Plots show a significant negative correlation between concentrations of ALX-R mRNA with NF-κB p65 mRNA in severe preeclampsia. C) Plots show a significant negative correlation between concentrations of ALX-R mRNA with NF-κB p65 mRNA in normal group.

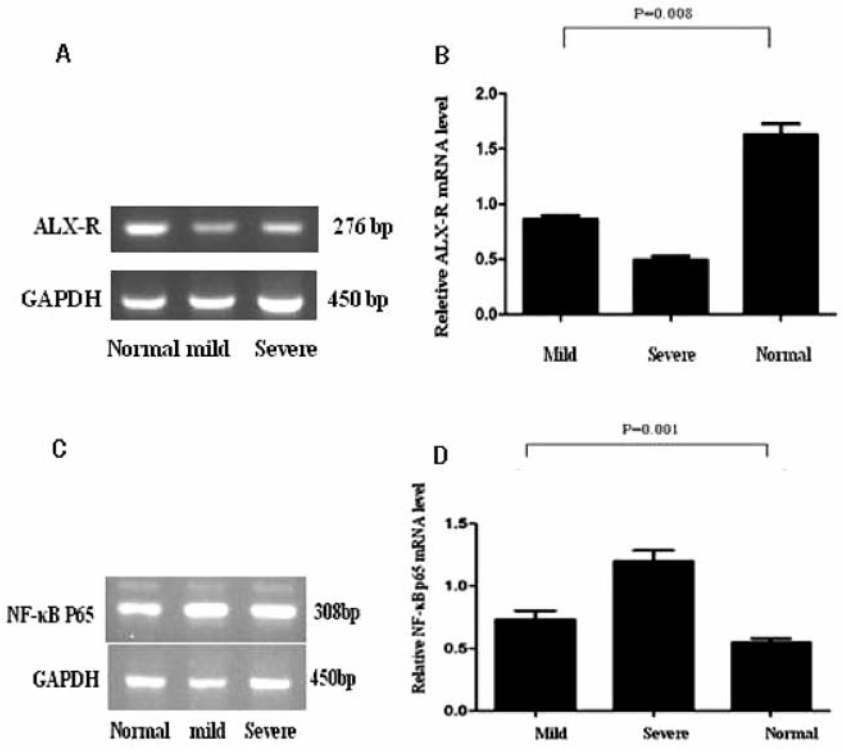

Figure 2. — A) ALX-R mRNA expression in placenta tissues of preeclampsia and control cases. ALX-R mRNA was detected by RT-PCR in all placenta tissue samples. B) Comparison of the expression of ALX-R mRNA among placenta tissues of patients with preeclampsia and control cases. ALX-R mRNA expression was significantly stronger in control group (1.62 ± 0.45) than in mild preeclampsia (0.86 ± 0.96, p <0.05) and severe preeclampsia (0.49 ± 0.17, p < 0.0165). C) NF-κB p65 mRNA expression in placenta tissues of the preeclampsia and control cases. NF-κB p65 mRNA was detected by RT-PCR in all placenta tissue samples. D) Comparison of the expression of NF-κB p65 mRNA among placenta tissues of patients with preeclampsia and control cases. NF-κB p65 mRNA expression was significantly weaker in control group (0.55 ± 0.14) than in mild preeclampsia (0.73 ± 0.19) and severe preeclampsia (1.2 ± 0.40, p < 0.0165). Results represent the mean ± standard deviation (n = 20 for control, n = 10 for mild preeclampsia, and n = 20 for severe preeclampsia). mRNA levels are shown relative to the mean value for internal control.

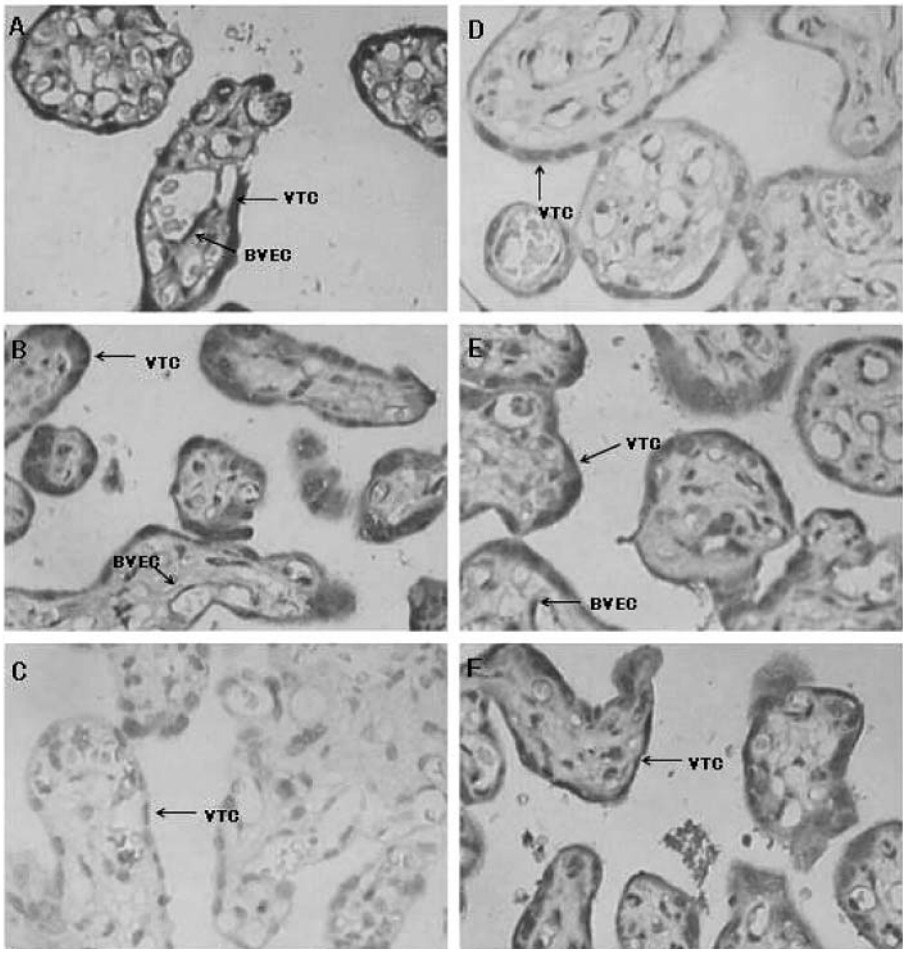

Figure 3. — Immunohistochemical analysis of ALX-R and NF-κB protein in the placenta-specific tissue. A), B), and C) expression of ALX-R in normal, mild preeclampsia, ad severe preeclampsia placenta tissue. The staining intensity of ALX-R in the placental tissue of the severe preeclamptic group (C) was weaker than the mild and control group (B and A). C), D), and E) expression of NF-κB p65 in normal, mild preeclampsia, and severe preeclampsia placenta tissue. The staining intensity of NF-κB p65 in the placental tissue of the severe PE group (C) was greater than the mild and control group (B and A). VTC = villous trophoblastic cell, BVEC = blood vessel endothelial cell. Original magnification: ×400.

Table 2. — *Immunostaining of ALX-R in mild and severe preeclamptic group and control group.*

	Immunostaining of ALX-R			
	-	+	++	+++
mPE	10	10	30	0
sPE	0	65	80	5
C	0	15	30	55
sum	10	90	140	60
the average rank	25.50	135.22	130.86	240.08

Mild preeclampsia group (mPE), severe preeclampsia group (sPE), control group (C). $\chi^2 = 113.665$ $p = 0.000$

Table 3. — *Immunostaining of NF-κB p65 in mild and severe preeclamptic group and control group*

	Immunostaining of NF-κB p65			
	-	+	++	+++
mPE	15	10	25	0
sPE	20	25	55	0
C	70	30	0	0
sum	105	65	80	0
the average rank	148.75	129.88	67.50	0

Mild preeclampsia group (mPE), severe preeclampsia group (sPE), control group(C). $\chi^2 = 65.138$, $p = 0.000$

Discussion

More and more researches reveal [14, 15] that pathological changes of preeclampsia are similar to uncontrolled inflammatory response of acute lung injury and acute respiratory distress syndrome. Moderate inflammatory reaction is physiological changes of the maternal fetus body which is well-adapted [16], and excessive inflammatory responses are likely to lead to pathological changes during pregnancy. CRP is a nonspecific and sensitive inflammatory marker [17]. In this experiment, serum CRP level of preeclamptic patient was higher than control group, similar to other reports [18, 19], suggesting that excessive inflammation is the basic pathophysiological changes in preeclampsia.

In this experiment maternal LXA serum concentration of both groups were detected (Table 1). In mild preeclampsia, serum LXA4 was higher than control group whereas in severe preeclampsia group there was decreasing tendency of serum LXA4 expression. The aforementioned results show that mild pre-eclampsia patients were in LXA4 activated state, and mechanism may be due to inflammation, local hypoxia, and cell toxins [20, 21]. Increased level of LXA4 plays important role in balancing the inflammatory response, protecting body tissue and organs. In severe preeclampsia, decreased level of LXA4 may be due to the imbalanced biosynthesis and loss of braking signal of LXA4 which may lead to persistence and/or deterioration of patient condition. It theorizes the important role of LXA4 level in normal and preeclampsia patient.

LXA4 through combining with ALX-R, exert biological effect [6], ALX-R protein, ad ALX-R mRNA were expressed in placental tissue (Table 1). In this experiment the authors found that placental tissue of preeclampsia patient expressed lesser ALX-R protein and ALX-R mRNA than control group. ALX-R is a specific receptor of LXA4 and decreased expression of LXA4 leads to weaker response of cells to LXA4, leading to development and progression of disease. Although there is a increased expression of LXA4 level during stress condition of mild preeclampsia, however receptor is not sufficient, making the biological activity of LXA4 limited; this explains why the disease is in a state of progression.

In this experiment both preeclampsia and control group expressed NF-κB p65 mRNA and protein. Expression of NF-κB p65 mRNA and protein were significantly higher in placental trophoblastic cell and vascular endothelial cells of preeclampsia patient, especially in severe patients. It confirms the role of NF-κB in pathophysiological mechanism of preeclampsia; its increased activity is related with the oxidation stress and imbalance of cytokines in preeclampsia patient. It has been reported that, in inflammatory disease like generalized inflammatory response, infective diseases etc., NF-κB activity is significantly increased [9]. NF-κB along with cytokine network, oxidase system participates in pathogenesis of preeclampsia. It includes activation of inflammatory cells through NF-κB signaling pathway to promote their adhesion and chemotaxis, and the release of inflammatory mediators leading to vascular system damage [22], placental ischemia–reperfusion results in oxidative stress, activate NF-κB signaling pathway leading to activation of inflammatory mediator, and endothelial dysfunction [23]. NF-κB activates expression of growth factor causing [24] revascularization, arteriolar wall thickening, atherosclerosis, and increase the mean arterial pressure. Above reaction, which begins from utero-placental lesion, eventually leads to symptoms of preeclampsia as in systemic hypertension and proteinuria.

LXA4 exert extensive regulatory role in a various type of inflammatory cell function and inflammatory related gene. Gewirtz *et al*. [25] using cDNA microarray method found that 50% of downregulation action of LXA4 to pro-inflammatory gene was through the NF-κB signaling pathway. In vitro cell culture and animal model research also shows that LXA4 inhibits lipopolysaccharide induced intestinal epithelial cell expression of TNF-α mRNA. LXA4 can also inhibit LPS induced production of IL-1β, IL-6 and IL-8 from pulmonary microvascular endothelial cells. The aforementioned mechanism is one of the antagonistic mechanisms belonging to inhibition of NF-κB nuclear translocation and other signaling pathways [10, 26]. Recently from animal model rat's LXA4 receptor, amino acid sequences were cloned, which have 74% homology with human ALX-R [27]. Rat model research shows that LXA4 combines with receptor and exerts anti-inflammatory response through inhibiting the NF-κB activity,

which is similar to humans. In this experiment, severe preeclampsia group shows decreasing expression of LXA4 and ALX-RmRNA, whereas NF-κB p65 mRNA and protein were significantly higher than control and mild preeclampsia group. There was a negative correlation in expression of ALX-R mRNA and NF-κB p65mRNA. Insufficient synthesis of LXA4 and ALX-R, attenuated activity of NF-κB p65 together accelerate the progression of disease in severe preeclampsia patient.

In summary, this research illustrates, LXA4 and ALX-R, NF-κB p65 all participate in disease process of preeclampsia. In severe preeclampsia, lack of LXA4 and ALX-R may have weakened downregulation action of pro-inflammatory gene through decreased NF-κB signal pathway, causing deterioration of patient's condition. Preeclampsia patients showed excessive inflammatory response and further study about the expression and regulation of LXA4, ALX-R protein, NF-κB is needed. Through activation of ALX-R, inhibition of activity of NF-κB, it is hoped to control the inflammatory disease and lead to new direction in treatment of preeclampsia.

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