The Induction of Cyclooxygenase-2 (COX-2) in Cultured Endothelial Cells Treated with Serum from Preeclampsia is Mediated by Interleukin-6

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Abstract

COX-2 protein, but not COX-1 protein, was induced in HUVEC from women with a normal pregnancy (nHUVEC) treated with serum from patients with preeclampsia (pSerum), but not with serum from women with a normal pregnancy (nSerum). COX activity in pSerum treated nHUVEC was less than in nSerum treated nHUVEC. Interestingly, the induction of COX-2 protein in nHUVEC treated with pSerum was inhibited by antiIL-6 antibody. The decreased COX activity in nHUVEC treated with pSerum plus antiIL-6 antibody was also reversed in a dose dependent manner. Thus, the induction of COX-2 in pSerum treated nHUVEC was mediated by IL-6. Therefore, the development of selective inhibitors of COX-2 or of IL-6 antagonists may have a potential role in the prevention and treatment of preeclampsia.

Key word : Preeclampsia, Serum, HUVEC, COX-2

Preeclampsia is a multisystem disorder of pregnancy of unkown cause. This syndrome is characterized by increased blood pressure, edema, proteinuria and abnormal clotting, liver and renal function, all of which may result from generalized vascular endothelial cell dysfunction[1-4]. The cause of this endothelial dysfunction is unknown, but there is evidence for a circulating endothelial cell "toxic" factor in preeclampsia. Sera from preeclamptic women are reported to be cytotoxic to endothelial cells[5,6], and endothelial cells cultured in preeclampsia sera show an increased release of platelet-derived growth factor[7], procoagulant protein[8], an increased triglyceride content, and a decreased prostacyclin (PGI_2) release[9]. The diffuse nature of preeclampsia and the important role of endothelial cells in the regulation of vascular tone and the coagulation system imply that the dis-

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order may be characterized by structural or functional damage of maternal endothelial cells. Several studies have suggested that the production of prostacyclin (PGI_2) is reduced in uteroplacental tissues of women with preeclampsia[10-13].

PGI_2 is one of several prostaglandins (PGs) which have numerous cardiovascular and inflammatory effects[14]. Cyclooxygenase (COX) is the first enzyme in the pathway in which arachidonic acid is converted to PGs including PGI_2[15,16]. COX exists in at least two isoforms. One is the constitutive enzyme, COX-1, producing regulatory prostanoids under physiological conditions[17], whereas the other, COX-2, is induced by mitogens[18,19] and proinflammatory cytokines[20,21] during pathological states such as inflammation. Recently, we have shown that COX-2 is expressed in human umbilical vein endothelial cells from patients with preeclampsia (pHUVEC), but not from those with normal pregnancy (nHUVEC)[22]. Moreover, pHUVEC released significantly less PGI_2 than nHUVEC[23]. The signalling pathway by which COX-2 is expressed in pHUVEC is not known. Interleukin-6 (IL-6) has been reported to be elevated in preeclampsia[24]. In this paper, we have used preeclamptic-serum (pSerum) or normal pregnancy serum (nSerum) treated with nHUVEC and coincubated with or without human polyclonal antiIL-6 antibody as a pharmacological tool to investigate the signalling mechanism of COX-2 expressed in preeclampsia.

MATERIAL AND METHOD

Collection of blood

Blood was collected from subjects at room temperature into 10 ml disposable syringes (Terumo) containing no additives. Blood was allowed to clot at room temperature and was then centrifuged for 10 minutes at 1,000 g. Aliquots of the serum (under sterile condition) were then stored at -70°C until they were required for the experiments.

Subjects

The study included 12 normal pregnant and 12 preeclamptic women. Gestational age at the time of study did not differ significantly, 35 weeks (range 33 to 36 weeks) in the normal pregnant group and 36 weeks (range 34 to 38 weeks) in the preeclamptic group.

Preeclampsia was defined on the basis of the following criteria: no prior history of hypertension or renal disease, a blood pressure of at least 140 mmHg systolic or 90 mmHg diastolic (manifested on two readings at least 6 h apart) or a rise in blood pressure of at least 30 mmHg systolic or 15 mmHg diastolic, and proteinuria of $\geq 1^+$ urine protein[25].

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were obtained from babies born to normal pregnant women (nHUVEC) as previously described[26] and cultured in 96-well plates with Human Endothelial-SFM Basal Growth Medium (Gibco) containing 10 per cent foetal calf serum (Gibco), 100 units/ml penicillin G sodium (M & H, Thailand) and 100 μg/ml streptomycin (M & H, Thailand). Cells were incubated at 37°C in a humidified incubator and grown to confluence before use.

Measurement of COX activity

Confluent nHUVEC samples were gently washed two times with PBS and incubated with Human Endothelial-SFM Basal Growth Medium (200 μl/well) with and without human polyclonal antiIL-6 antibody (R & D; 10 μg/ml; control groups), 10 per cent nSerum with and without human polyclonal antiIL-6 antibody (0.1 to 10 μg/ml) and 10 per cent pSerum with and without human polyclonal antiIL-6 antibody (0.1 to 10 μg/ml) for 24 h. After 24 h, the medium was removed and washed twice with PBS. COX activity was measured by the production of 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2, which is the major COX metabolite in endothelial cells) in the replaced fresh medium containing exogenous arachidonic acid (Sigma; 10 μm for 10 min) using enzymeimmunoassay (EIA). Briefly, 50 μl of standard 6-keto-$PGF_{1\alpha}$ (Sigma) or samples were added to pre-coated mouse anti-rabbit IgG microtitre plates (Clayman; 96-well). Then, 6-keto-$PGF_{1\alpha}$ acetylcholinesterase tracer (Clayman; 50 μl) and rabbit antiserum of 6-keto-$PGF_{1\alpha}$ (Clayman) were added. The plate was covered with plastic film and incubated for 18 h at 4°C, after which time the wells were emptied and rinsed five times with wash buffer (PBS containing 0.05% Tween). Ellman's reagent (Clayman; 200 μl) was added to each well and the plates were shaken on a microtitre plate shaker. The reaction took about 90 min. A yellow colour develops which can be read using a microplate reader (BIORAD; OD 415 nM).

Immunoblot (Western blot) Analysis

nHUVEC samples which were untreated with and without human polyclonal antiIL-6 antibody (10 μg/ml; control groups), treated with 10% nSerum with and without human polyclonal antiIL-6 antibody (10 μg/ml) and treated with 10 per cent pSerum with and without human polyclonal antiIL-6 antibody (10 μg/ml) were cultured in 6-well culture plates (37°C; for 24 h). After incubation, cells were extracted and analysed by immunoblotting using specific antibodies for COX-1 and COX-2 protein as previously described[17].

Measurement of cell viability

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan[27]. At the end of each experiment, cells in 96-well plates were incubated (37°C; 1 h) with MTT (0.2 mg/ml) dissolved in culture medium, after which time the medium was removed by aspiration and cells were solubilized in DMSO (200 μl). The extent of reduction of MTT to formazan within cells was quantitated by the measurement of optical density at 650

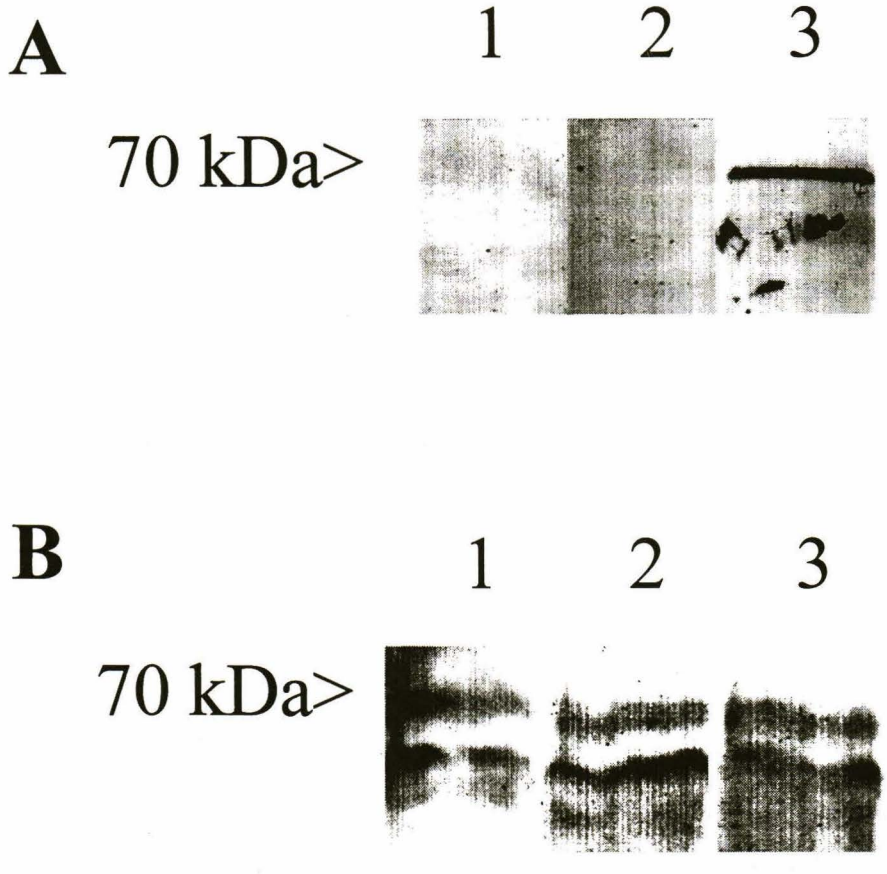

Fig. 1. Western blots using polyclonal antibodies to COX-2 (panel A) and COX-1 (panel B) in cell extracts of HUVEC from normal pregnancy (nHUVEC) treated with serum from normal pregnancy (nSerum) or preeclampsia (pSerum). (A) Untreated nHUVEC (lane 1) or nHUVEC treated with 10% nSerum (lane 2) contain no COX-2 protein. In contrast, nHUVEC treated with 10% pSerum (lane 3) contain COX-2 protein. (B) Untreated nHUVEC (lane 1), nHUVEC treated with 10% nSerum (lane 2) or 10% pSerum (lane 3) contain equal amounts of COX-1 protein. Equal amounts of protein (10 μg/lane) were loaded in each lanes. Similar results were obtained with cell extracts from 12 separate batches of cells.

nm (OD_{650}) using a microplate reader (BIORAD, USA).

Statistical analysis

The results are expressed as mean ± SEM of triplicate determinations (wells) from at least four separate experimental days (n=12). Student's paired or unpaired *t*-test, as appropriate, were used for the determination of significance of differences between means and a p -value of less than 0.05 was taken as statistically significant.

RESULTS

The effect of serum on COX protein expressed in endothelial cells (HUVEC)

Untreated nHUVEC contained no COX-2 protein. COX-2 protein was expressed in nHUVEC treated with 10 per cent pSerum, but not in nHUVEC treated with 10 per cent nSerum (Fig. 1A). The amount of COX-1 protein expressed in untreated nHUVEC was not changed when compared to nHUVEC treated with 10 per cent nSerum or 10 per cent pSerum (Fig. 1B).

The effect of serum on COX activity in endothelial cells (HUVEC)

In nHUVEC treated with 10 per cent nSerum, COX activity did not change significantly when compared to untreated nHUVEC (3.73±0.13 and 3.51±0.07 ng/ml; n=12, respectively). Interestingly, COX activity was decreased significantly in nHUVEC treated with 10 per cent pSerum when compared to untreated and 10 per cent nSerum treated nHUVEC (2.25±0.11, 3.51±0.07 and 3.73± 0.13 ng/ml; n=12, respectively), as shown in Fig. 2.

Effect of antiIL-6 antibody on COX protein expressed in serum treated HUVEC

In 10 per cent pSerum treated nHUVEC, COX-2 protein, but not COX-1 protein, was also inhibited by co-incubation with antiIL-6 antibody (10 μg/ml; Fig. 3A and 3B; lane 6). In untreated and 10 per cent nSerum treated nHUVEC, COX-1 protein was not affected by antiIL-6 antibody (10 μg/ml; Fig. 3B; lane 2 and 4, respectively).

Effect of antiIL-6 antibody on COX activity in serum treated HUVEC

Interestingly, the decreased COX activity in 10 per cent pSerum treated nHUVEC could be restored to the level of untreated or 10 per cent nSerum treated HUVEC when cells were co-incubated with antiIL-6 antibody (0.1 to 10 μg/ml; Fig. 4). This reversal effect is dose dependent (Fig. 5).

DISCUSSION

Our studies have demonstrated that serum from women with a normal pregnancy did not reveal any increase in COX activity (as measured by PGI_2 production in endothelial cells) or COX protein in endothelial cells, but serum from patients with pre-eclampsia significantly decreased COX activity and induced COX-2 protein. Recently, we have shown that COX-2 is expressed in human umbilical vein endothelial cells from patients with preeclampsia, but not from women with a normal pregnancy[22]. Moreover, HUVEC from patients with preeclampsia released significantly fewer amounts of 6-keto-

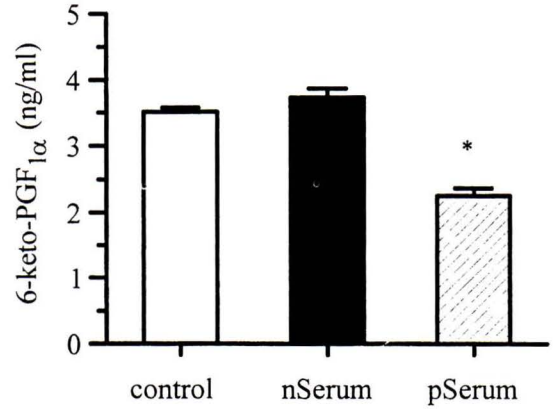

Fig. 2. COX activity in HUVEC from normal pregnancy (nHUVEC) treated with 10% normal pregnant serum (nSerum) or 10% preeclamptic serum (pSerum) for 24 h measured by the formation of the 6-keto-$PGF_{1\alpha}$ in the presence of exogenous arachidonic acid (10 μM; 10 min). COX activity was shown to be decreased significantly in nHUVEC treated with 10% pSerum (hatch column) when compared to nHUVEC treated with no addition (white column) or 10% nSerum (black column). Data are expressed as mean ± SEM of twelve determinations from at least four separate experimental days. *p < 0.05 when compared to untreated nHUVEC at 24 h.

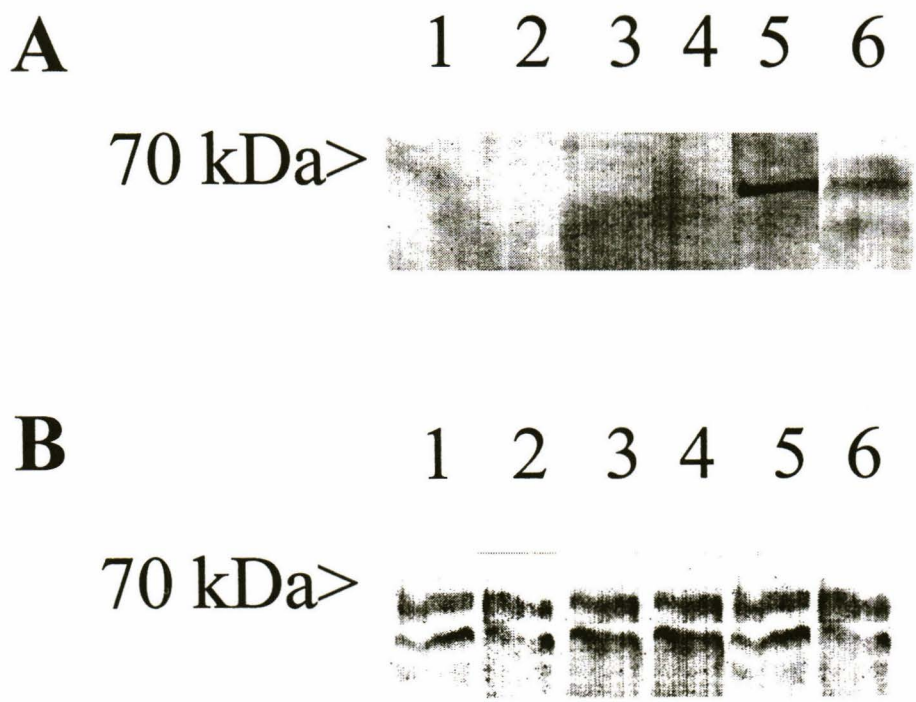

Fig. 3. The effects of human polyclonal antiIL-6 antibody (hAntiIL-6; 10 µg/ml for 24 h) on COX-2 (panel A) and COX-1 (panel B) protein in cell extracts of HUVEC from normal pregnancy (nHUVEC) treated with serum from normal pregnancy (nSerum) or preeclampsia (pSerum). (A) COX-2 proteins are not detected in nHUVEC treated with no addition (lane 1), hAntiIL-6 (lane 2), 10% nSerum (lane 3) or 10% nSerum plus hAntiIL-6 (lane 4). Interestingly, COX-2 expression in nHUVEC treated with 10% pSerum (lane 5) are inhibited when cells are co-incubated with hAntiIL-6 (lane 6). (B) COX-1 proteins are detected in equal amounts of nHUVEC treated with no addition (lane 1), hAntiIL-6 (lane 2), 10% nSerum (lane 3), 10% nSerum plus hAntiIL-6 (lane 4), 10% pSerum (lane 5), 10% pSerum plus hAntiIL-6 (lane 6). Equal amounts of protein were loaded in all lanes (10 µg/lane). Similar results were obtained using cell extracts from 12 separate batches of cells.

$PGF_{1\alpha}$ (a stable metabolite of PGI_2) than HUVEC from women with a normal pregnancy[23]. Extrapolating from *in vitro* to *in vivo* events, this implies that some factor(s) in serum from preeclampsia can increase COX-2 protein and decreases COX activity resulting in decreased PGI_2 in endothelial cells. The decreased PGI_2 in endothelial cells causes the imbalance in vascular tone which is one of the pathogenetic factors in preeclampsia[28,29].

Previous studies have shown the effects of serum from preeclampsia on PGI_2 production (also referred to COX activity)[9,30,31]. Branch et al[30] and Zammit et al[31] showed that serum from either normal pregnancy or preeclampsia stimulates the production of PGI_2 by HUVEC. These findings conflict with our results, but this discrepancy may be due to the model used to detect PGI_2 production as used by Branch et al[30] and Zammit et al[31] which included the activity of both COX and phospholipase A_2 (PLA_2) to produce PGI_2 in endothelial cells[32]. Moreover, it was found in our study that 1 to 10 per cent diluted serum from normal pregnancy did not affect COX activity (3.51±0.07, 3.52±0.04 and 3.73±0.13 ng/ml; n=12, $p > 0.05$, for untreated, 1% nSerum and 10% nSerum, respectively) but 15 per cent and 20 per cent diluted serum from normal

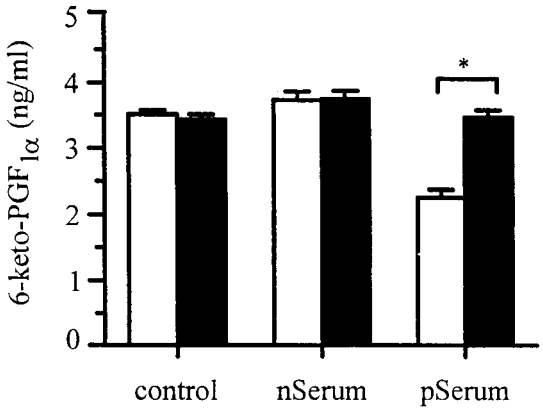

Fig. 4. The effects of human polyclonal antiIL-6 antibody (hAntiIL-6; 10 μg/ml for 24 h) on COX activity in HUVEC from normal pregnancy (nHUVEC) treated with 10% normal pregnant serum (nSerum) and 10% preeclamptic serum (pSerum) for 24 h. COX activity was measured by the formation of the 6-keto-$PGF_{1\alpha}$ in the presence of exogenous arachidonic acid (10 μM for 10 min). COX activity in nHUVEC treated with no addition (control; white column) or 10% nSerum (nSerum; white column) is not affected when cells were coinoculated with hAntiIL-6 (control or nSerum; black column). Interestingly, the decreased COX activity in nHUVEC treated with 10% pSerum (pSerum; white column) is reversed when cells are coincubated with hAntiIL-6 (pSerum; black column). Data are expressed as mean ± SEM of twelve determinations from at least four separate experimental days. * $p < 0.05$ when compared to pSerum treated cells at 24 h.

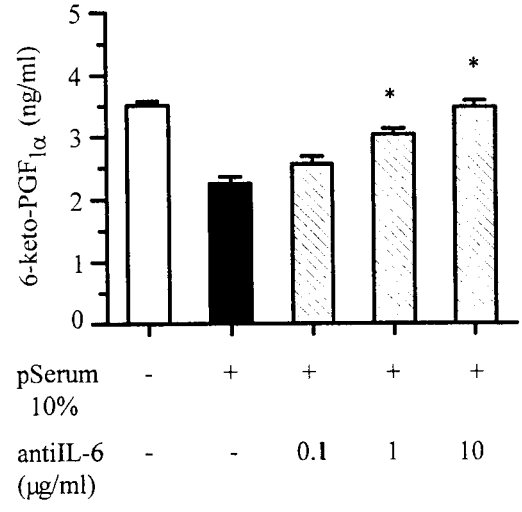

Fig. 5. Dose dependent effects of human polyclonal antiIL-6 antibody (hAntiIL-6; 0.1, 1 and 10 μg/ml for 24 h) on COX activity in HUVEC from normal pregnancy (nHUVEC) treated with 10% preeclamptic serum (pSerum) for 24 h. COX activity was measured by the formation of the 6-keto-$PGF_{1\alpha}$ in the presence of exogenous arachidonic acid (10 μM for 10 min). COX activity is decreased in pSerum treated nHUVEC (black column) when compared to untreated nHUVEC (white column). Interestingly, hAntiIL-6 can reverse the decreased COX activity in pSerum treated nHUVEC in a dose dependent manner (hatch column). Data are expressed as mean ± SEM of twelve determinations from at least four separate experimental days. * $p < 0.05$ when compared to pSerum treated cells at 24 h.

pregnancy could increase COX activity (3.51±0.07, 4.50±0.05 and 4.77±0.10 ng/ml; n=12, $p < 0.05$), for untreated, 15% nSerum and 20% nSerum, respectively). These findings may explain the different results of Branch et al[30] and Zammit et al[31] in the production of PGI_2 in serum treated HUVEC using 20 per cent diluted serum. In agreement with the present study using the same model as Branch et al[30] and Zammit et al[31], Lorentzen et al showed that serum from preeclampsia reduced the release of PGI_2 in HUVEC.

What are the factor(s) in serum from preeclampsia which cause decreased COX activity and induce COX-2 protein in HUVEC? There are several inflammatory mediators involved in the pathogenesis of preeclampsia such as IL-1, IL-6 and TNF-α [24,33,34]. IL-1 and TNF-α, but not IL-6, have been shown to increase COX activity and COX-2 protein in endothelial cells[21]. IL-6 has been reported to be elevated in preeclampsia[24] and shown to inhibit PGI_2 release in human myometrial[33] and pulmonary artery smooth muscle cells[34]. Therefore,

it was decided to use human polyclonal antiIL-6 antibody as a pharmacological tool to investigate the signalling mechanism of COX-2 expression in preeclampsia. Our study showed that human polyclonal antiIL-6 antibody could reverse the effect of serum from preeclampsia treated HUVEC on COX activity and inhibit COX-2 protein induction by serum from preeclampsia treated HUVEC while COX activity and COX-1 protein in untreated and serum from normal pregnancy treated HUVEC were not affected by human polyclonal antiIL-6 antibody. Moreover, the inhibition of COX-2 or reversed COX activity in serum from preeclampsia treated HUVEC by antiIL-6 antibody was not complete, suggesting there might be other mediators besides IL-6 involved in this process. Thus, IL-6 is proposed as one of mediators involved in the induction of COX-2 and the decreased PGI_2 released from endothelial cells in preeclampsia. Therefore, the development of selective inhibitors of COX-2 and antiIL-6 antibody therapy may have a potential role in the prevention and treatment of preeclampsia.

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ซีรั่มจากหญิงตั้งครรภ์เป็นพิษ (preeclampsia) กระตุ้นการสร้าง cyclooxygenase-2 (COX-2) ในเซลล์เพาะเลี้ยงจากผนังหลอดเลือด (endothelial cells) โดยผ่านทาง interleukin-6

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เซลล์จากผนังหลอดเลือดของสายสะดือเด็กที่คลอดจากหญิงตั้งครรภ์ปกติ (HUVEC) ถูกนำมาเพาะเลี้ยง เมื่อเซลล์โตเต็มที่จึงนำมาเลี้ยงในน้ำเลี้ยงเซลล์ที่ไม่มีซีรั่ม (กลุ่มควบคุม) น้ำเลี้ยงเซลล์ที่มีซีรั่มของหญิงตั้งครรภ์เป็นพิษ (pSerum) น้ำเลี้ยงเซลล์ที่มีซีรั่มของหญิงตั้งครรภ์ปกติ (nSerum) น้ำเลี้ยงเซลล์ที่มี pSerum ร่วมกับ antibody ต่อ interleukin-6 (anti-IL-6 antibody) และน้ำเลี้ยงเซลล์ที่มี nSerum ร่วมกับ anti-IL-6 antibody นาน 24 ชั่วโมง จากนั้นจึงสกัดเซลล์มาวิเคราะห์หาการปรากฏของโปรตีน COX-1 และ COX-2 โดยวิธี Western blot หน้าที่ของโปรตีน COX (COX activity) จะถูกวัดโดยดูจากปริมาณ 6-keto-$PGF_{1\alpha}$ (stable metabolite ของ prostacyclin ซึ่งเป็น major COX metabolites ใน HUVEC) ในน้ำเลี้ยงเซลล์ โดยวิธี enzyme immunoassay (EIA) พบว่า HUVEC ในน้ำเลี้ยงเซลล์ที่มี pSerum (12 ราย) หลั่ง 6-keto-$PGF_{1\alpha}$ ลดลงเมื่อเทียบกับกลุ่มควบคุมและกลุ่มที่เลี้ยงในน้ำเลี้ยงเซลล์ที่มี nSerum ร่วมกับการปรากฏของโปรตีน COX-2 ซึ่งไม่พบในกลุ่มควบคุมและกลุ่มที่เลี้ยงในน้ำเลี้ยงเซลล์ที่มี nSerum ขณะที่การปรากฏของโปรตีน COX-1 พบมีปริมาณใกล้เคียงกันทั้ง 3 กลุ่ม นอกจากนี้ยังพบว่าการปรากฏของโปรตีน OOX-2 ในกลุ่มที่เลี้ยงในน้ำเลี้ยงเซลล์ที่มี pSerum จะมีปริมาณลดลงเมื่อเลี้ยงในน้ำเลี้ยงเซลล์ที่มี pSerum ร่วมกับ anti-IL-6 antibody ขณะที่การหลั่ง 6-keto-$PGF_{1\alpha}$ ในน้ำเลี้ยงเซลล์กลับมีปริมาณเพิ่มขึ้นใกล้เคียงกับกลุ่มควบคุมและกลุ่มที่เลี้ยงในน้ำเลี้ยงเซลล์ที่มี nSerum จากการศึกษาวิจัยบ่งชี้ว่า COX-2 มีบทบาทเกี่ยวข้องกับกลไกการเกิดโรคความดันเลือดสูงในหญิงตั้งครรภ์เป็นพิษ โดยมี interleukin-6 เป็น mediator ดังนั้นการใช้สารยับยั้งหน้าที่หรือการสร้างโปรตีนของ COX-2 หรือ interleukin-6 อาจจะมีส่วนช่วยในการป้องกันและรักษาโรคความดันเลือดสูงในหญิงตั้งครรภ์เป็นพิษได้

คำสำคัญ : หญิงตั้งครรภ์เป็นพิษ, คอกซ์-2, ซีรั่ม, เซลล์จากผนังหลอดเลือดสายสะดือเด็กครรภ์ปกติ

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