Initial Response of Endothelial Cells to Acute Stimulation with a Lipid Component: Increase Cyclooxygenase Activity by Induction of COX-2 through Activation of Tyrosine Kinase†

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Objective: *To study the initial response of endothelial cells acutely stimulated with a lipid component in the aspect of cyclooxygenase (COX) function which needed for prostacyclin synthesis, an endogenous antiatherogenic agent secreted from endothelial cells.*
Material and Method: *25 hydroxycholesterol (25OHC) was used as a representative lipid component for stimulating human umbilical vein endothelial cell (HUVEC) obtained from umbilical cords of healthy newborns with informed consent of their mothers. HUVEC were treated with 25OHC (0.1, 1 or 10 microgram/mL) at times 6, or 24 h. COX activity was measured from amount of 6-keto-PGF_{1alfa} production in the presence of exogenous arachidonic acids (10 micromolar; 10 min) by enzyme immunoassay. The amount of COX-1 and COX-2 protein were detected by Western blot. Cell viability was assessed by using MTT assay.*
Results: *25OHC induced COX-2 protein production with increasing the activity of COX enzyme in HUVEC without change in amount of COX-1 protein. The induction of COX-2 or increasing in COX activity depended on concentration of 25OHC and time to exposure which seemed to be inhibited by genistein, a specific tyrosine kinase inhibitor.*
Conclusion: *Acute stimulation of HUVEC with 25OHC, an atherosclerotic lipid component, increases the activity of COX by inducing COX-2 expression in a manner that depended on concentration and time. The induction of COX-2 expression might possibly mediated through activation protein tyrosine kinase. These responses may be an initial defensive mechanism of endothelial cells from lipid component attack.*

Keywords: *Cyclooxygenase 2, COX-2, Lipids, Oxidized LDL, 25-hydroxycholesterol, Human umbilical vein endothelial cell, HUVEC, Atherosclerosis, Tyrosine kinase*

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Cyclooxygenases (COX) are key rate-limiting enzymes in the synthesis of prostanoids from arachidonic acid[1]. At least three isoforms of COX have been described so far[2,3]. COX-1 is constitutively expressed in many cell types. In contrast, expression of COX-2 requires stimulation of cells with cell-specific extracellular signals such as autacoids, hormones, growth factors, and cytokines[4]. COX-3 is debated as a physiological and pathological defense which is waiting for elucidation[5]. COX-1 and COX-2 have been reported to be expressed in many cell types including human umbilical vein endothelial cells (HUVEC)[6]. The major

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prostanoid synthesized by vascular endothelium is prostacyclin (PGI_2)[7] which regulates various physiological processes occurring at the interphase between the blood and the vascular wall. Biological actions of PGI_2 include inhibition of platelet aggregation and adhesion, inhibition of vascular smooth muscle tone and growth, inhibition of leukocyte activation and adhesion, and reduction of cholesteryl ester accumulation in cells of the vessel wall[8]. These biological actions of PGI_2 and its efficacy in the treatment of complications of atherosclerosis such as peripheral vascular disease[9] suggest that PGI_2 is an endogenous antiatherogenic molecule. However, the mechanisms accounted for initiating atherosclerotic lesion have not been completely elucidated. Several lines of evidence suggest the role of 25-hydroxycholesterol (25OHC), produced from the oxidative modification of low density lipoprotein (LDL) that has been implicated in the early development of arteriosclerosis and inflammatory processes[10,11]. 25OHC induce atherosclerosis by impairing the barrier function of endothelial cells and promoting the attraction of monocyte to endothelial cells[12]. Blanco et al[13] and Hirai et al[14] have shown that the activation of protein-tyrosine kinase (PTKs) may be involved in the induction of COX-2 in endothelial cells. As atherosclerosis is associated with chronic inflammatory response of the intima of the vessel wall to the injured endothelial cells[15], therefore, we have investigated the effects of 25OHC on COX isoforms expressed in human umbilical vein endothelial cells (HUVECs). The molecular mechanism by which COX isoform is expressed in endothelial cells activated with 25OHC was also investigated by using the tyrosine kinase inhibitor, genistein.

Material and Method

Materials

Unless specified, all chemical reagents were obtained from Sigma, USA. Human endothelial-SFM basal growth medium and heat-activated fetal bovine serum (FBS) were purchased from Gibco (USA). M199 was obtained from Hyclone, USA COX-1 (ovine) electropholesis standard, COX-2 (ovine) electropholesis standard, COX-1 polyclonal antibody (rabbit antibody raised to purified sheep seminal vesicular COX-1), COX-2 (human) monoclonal antibody developed in mouse, 6-keto-PGF_{1alfa}, acetylcholinesterase tracer, 6-keto-PGF_{1alfa} rabbit antiserum, 6-keto-PGF_{1alfa} standard, precoated mouse anti-rabbit microtiter plate and Ellman's reagent were obtained from Cayman Chemical (Australia). Phosphatase-label antibody to mouse IgG was from Kirkegaard & Perry Laboratories (USA). Bio-Rad protein assay reagent, and nitrocellulose were purchased from Bio-Rad Laboratories (USA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords obtained from normal pregnant women after informed consent from their mother. They were cultured according to standard technique originally described by Jeffe et al[16] with some modifications[17]. Cells used in the studies were uniformly in the third passage. Confluent cultures of HUVEC were starved in FBS-free medium for 12 h before initiation the experiments.

Cell treatments

Control groups are starved cells treated with the medium only. In the treatment groups, the starved cells were stimulated with 25OHC (0.1, 1 or 10 microgram/mL) dissolved in the medium for the indicated periods of time. To study the molecular mechanism, genistein, an inhibitor of tyrosine kinase, was pre-incubated with cells 30 minutes prior to the addition of the stimulators. After treatment the COX activity was assessed from the supernatant and the remained cells were used to determine the COX proteins. At the end of each experiment, viability of cells was evaluated by using MTT assay[18].

Measurement of COX activity

The cells were washed once with PBS and treated with 10 micromolar arachidonic acid dissolved in the medium at 37°C for 10 minutes. Then, 6-keto-PGF_{1alfa} (a stable metabolite of PGI_2) was determined in cell supernatant using enzyme immunoassay as previously described[19].

Measurement of COX proteins

The treated cell were lysed in ice-cold exaction buffer (50 mM Tris base; 10 mM EDTA; 1% (vol/vol) TritonX-100; 0.57 mM phenylmethyl-sulphonyl fluoride (PMSF); 1.5 micromolar pepstatin A and 2 micromolar leupeptin) and measured the protein concentration by using Bio-Rad protein assay reagent[20]. An equal amount of total proteins in each sample was used to determine both isoforms of COX-1 and COX-2 proteins by immunoblotting using specific antibodies for COX-1 and COX-2 protein, as previously described[21]. The relative enzyme mass was estimated by densitometry using Image Master 1D software (Pharmacia Biotech) and expressed as densitometry unit per equal total

loading protein.

Measurement of cell viability

Cells respiration, and indicator of cell viability, was evaluated by mitochondrial-dependent reduction of 3-(4, 5-dimethyl-2yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan[18], with some modification as previously described[19]. Viability of cells in the treatment groups was assessed as percent of the control.

Statistical analysis

The results are shown as mean ± standard error of mean (SEM) of triplicate determinations (wells) from at least three separate experiments performed on different days. Statistical significance, as appropriate, was determined by one-way ANOVA or Student t-test. A $p < 0.05$ was considered significant. Scheff multiple comparison test was performed following a significant finding in the one-way ANOVA.

Results

The effects of 25OHC on COX activity

At 24 h (Fig. 1, black columns), untreated HUVEC in the presence of exogenous arachidonic acid (10 micromolar for 10 min) released low amount of 6-keto-PGF_{1alfa} (2.08 ± 0.39 ng/mL). The amount of 6-keto-PGF_{1alfa} was increased significantly in a dose dependent manner in HUVEC treated with 1 microgram/mL or 10 microgram/ml of 25OHC. However, at 6 h treatment (Fig. 1, white columns), the level of 6-keto-PGF_{1alfa} in control and treatments were not statistically significant difference.

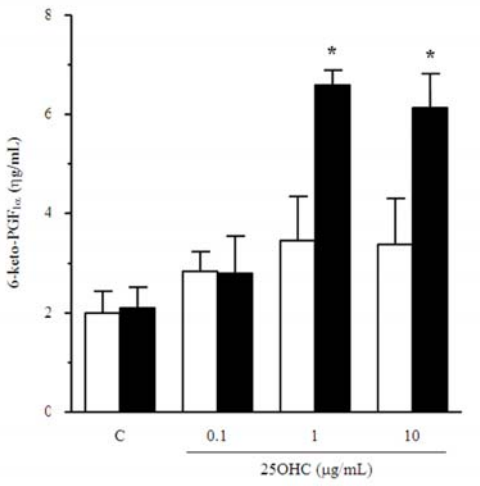

Fig. 1 The effects of 25OHC on COX activity in HUVEC as measured by 6-keto-PGF_{1a} production in the presence of exogenous arachidonic acid (10 mM, 10 min) after treatment for 6 h (white columns) and 24 h (black columns). The data represent Mean ± SEM of triplicate wells from at least 3 separate experiments performed on different days. *$p < 0.001$ when compared to untreated HUVEC (C) (Student t-test)

The effects of 25OHC on COX isoforms expressed in HUVEC

Untreated HUVEC expressed small amount of COX-2 protein (Fig. 2A). 6 h treatment of HUVEC with 25OHC did not induce COX-2 protein significantly from the control (Fig. 2A, white columns). However, at 24 h (Fig. 2A, black columns), 1 microgram/mL and 10 microgram/mL of 25OHC could significantly increase COX-2 protein (from 2.10 ± 0.24 to 3.67 ± 0.56 and 12.25 ± 1.50 densitometry units, respectively) without changed in the amount of COX-1 protein at both periods of time (Fig. 2B).

The effects of 25OHC on cell viability

Treatment of HUVEC with 0.1, 1 or 10 microgram/mL of 25OHC up to 24 h did not affect viability of cells except at 6 h treatment with 10 microgram/mL 25OHC, which seemed to increase cell viability ($p < 0.05$) (Fig. 3).

The effects of genistein on COX activity in HUVEC treated with 25OHC as measured by the production of 6-keto-PGF_1alfa

Fig. 4 showed the effects of genistein on COX activity in HUVEC treated with 25OHC. It was found that the increase in COX activity by 25OHC (10 microgram/mL for 24h) was inhibited by genistein (0.5, 5 and 10 microgram/mL). However, genistein alone, up to 10 microgramg/mL, did not affect the COX activity in untreated control HUVEC.

The effects of genistein on COX isoforms expressed in HUVEC treated with 25OHC

Fig. 5 showed the amount of COX-2 and COX-1 protein in HUVEC treated with medium only (C), genistein (10 microgram/mL), 25OHC (10 microgram/mL), 25OHC (10 microgram/mL) in the presence of genistein (0.5, 5 or 10 microgram/mL). Genistein alone up to 10 microgram/mL did not affect both isoforms of COX expression. Interestingly, the induction of COX-2 by 25OHC (10 microgram/mL for 24 h; Fig. 5A, black column) possibly inhibited by genistein in a dose dependent manner (Fig. 5A, hatch columns). However, the amount of COX-1 protein in all treatment groups

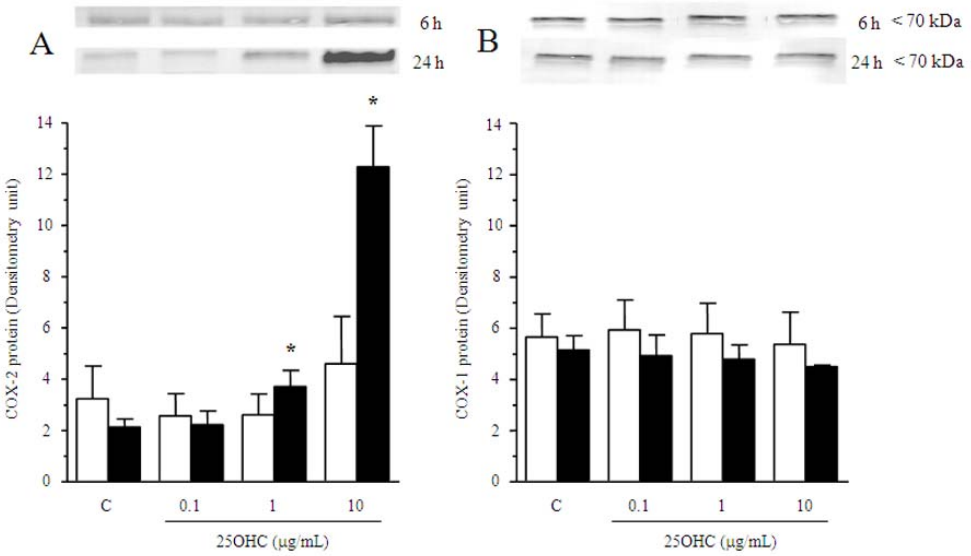

Fig. 2 The effects of 25OHC on COX isoforms expression in HUVEC after treatment for 6 h (white columns) and 24 h (black columns). Equal amounts of protein were loaded in all lanes. A) shows Mean ± SEM of COX-2 protein amount estimated by densitometry from three separate experiments. B) shows Mean ± SEM of COX-1 protein amount estimated by densitometry from three separate experiments. *p < 0.05 when compared to untreated HUVEC (C) (Student t-test)

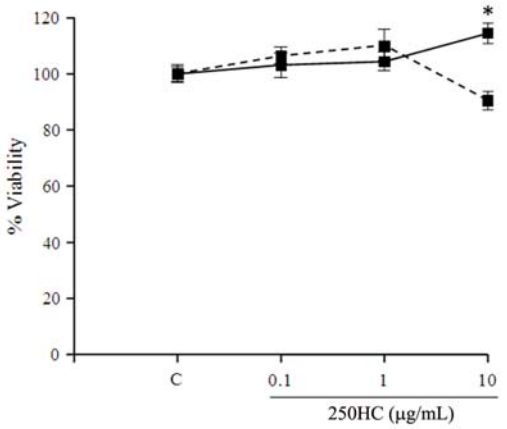

Fig. 3 The effects of 25-OHC on proliferation of HUVEC at 6 h (solid line) and 24 h (dash line). The percent proliferation was determined by the MTT assay of treatment groups with untreated HUVEC (C). The data represent Mean ± SEM of triplicate wells from at least 3 separate experiments performed on different days. *p < 0.05, when compared to untreated HUVEC (C).

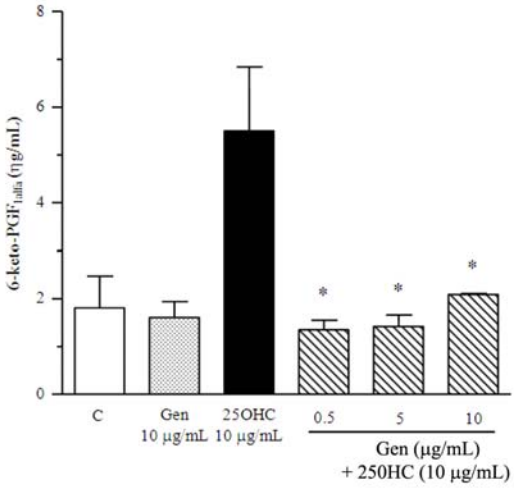

Fig. 4 The effects of genistein (Gen, 0.5, 5 or 10 mg/mL; hatch column) on COX activity induced by 25OHC (10 mg/mL for 24 h, black column) in HUVEC. COX activity was measured by the production of 6-keto-PGF_{1a} in the presence of exogenous arachidonic acids (10 mM; 10 min). Data are expressed as Mean ± SEM of 9 determinations from at least 3 separate experimental days. *p < 0.05 when compared to 25OHC treated group. (one-way ANOVA, Scheffe's multiple comparison test)

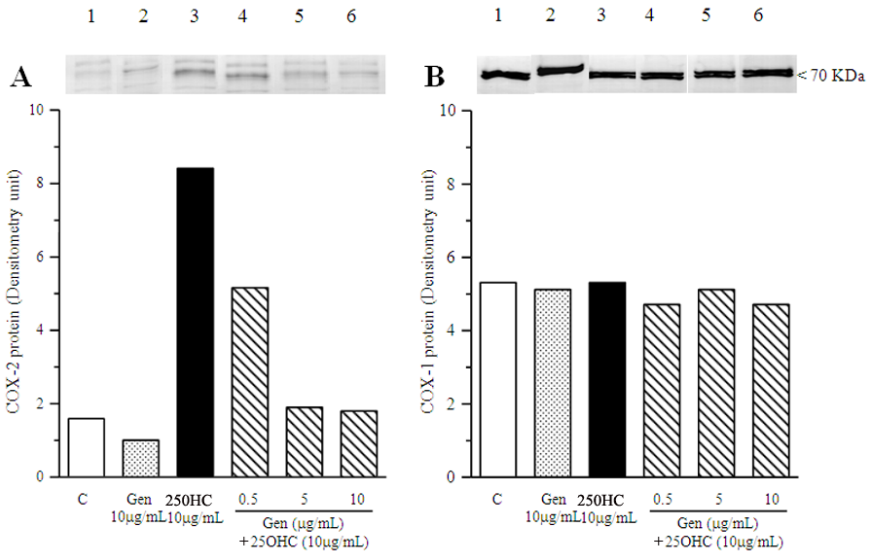

Fig. 5 The effect of genistein on COX isoform expressed in HUVEC treated with 25OHC. Genistein (0.5, 5 or 10ug/mL, hatch column) was pre-incubated with cells 30 minutes prior to the addition of 25OHC (10ug/mL). A) and B) show representative Western blots of COX-2 and COX-1 protein, respectively, from 2 experiments performed on different day. The cells were treated with no addition (lane 1), with genistein (10ug/mL) alone (lane 2), with 25OHC (10ug/mL) alone (lane 3), with 25OHC (10ug/mL) + genistein (0.5 ug/mL) (lane 4), with 25OHC (10ug/mL) + genistein (5 ug/mL) (lane 5) or with 25OHC (10ug/mL) + genistein (10 ug/mL) (lane 6). Equal amount of proteins were loaded in all lanes. Protein amount corresponding to each band was estimated by densitometry

were not changed when compared to untreated HUVEC (C) (Fig. 5B).

Discussion

Development and progression of atherosclerosis involve many factors[22,23]. The oxidized low density lipoprotein (oLDL) which is comprised of cholesterol oxides[11] is one of the predisposing factors that play a central role in atherogenesis[22,24]. Cholesterol oxides should be the atherogenic agents for the following four reasons. Firstly, they have been shown to be toxic to endothelial cells and caused endothelial cell injury[25] which initiate early events of atherosclerotic lesion by enhancing adhesion and infiltration of monocytes to the endothelial cells and promoting the migration and proliferation of the underlying smooth muscle cells[26]. Secondly, they impair barrier function of endothelial cells by reducing the activity of hydroxy methyl glutaryl coenzyme A (HMG CoA) reductase, the enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis[22,27]. Thirdly, they have been reported to decrease receptor-mediated LDL uptake by reducing LDL receptors without change in their affinities[28]. Finally, cholesterol oxides including 25OHC have been found to produce the increase in cellular content of cholesteryl ester by increasing the activity of acyl CoA cholesterol acyltransferase (ACAT)[29].

The role of lipid on producing atherosclerotic lesion leading to development to atherosclerosis has been widely studied and many mechanisms for them have been proposed. However, the effect of lipids on endothelial cells at early events was not well defined. Further study of the acute effects of lipids on endothelial cell will reflect the responses of endothelial cells to lipids at early events before atherogenesis. In this study 25OHC was chosen as representative lipid component for studying the acute effects of oLDL on endothelial cells because of the following reasons. Firstly, among cholesterol oxides, 25OHC is a much studied oxysterol that is both found endogenously and found in diet[30]. Secondly, it also presents in atheromatous plaque[31]. Thirdly, it is also most toxic and has been shown to produce endothelial damage with numerous balloon-like protrusions and crater-like defects as well as foam cell adherence on the luminal surfaces of rabbit aortae[12]. The results showed that early exposure of endothelial cells to 25OHC could enhance COX activity, which reflected by increasing 6-keto-PGF_{1alfa} production

from exogenous arachidonic acid. The increase in COX activity depended on concentration of 25OHC and incubation periods of time (Fig. 1). Interestingly, it was found that the increase in COX activity coincided with the increase in COX-2 protein without change in COX-1 protein (Fig. 1 and Fig. 2). Therefore, the increase in COX activity in 25OHC treated HUVEC was due to the induction of COX-2.

The concentration of 25OHC which gave the most potent effect was 10 microgram/mL which closed to the concentration of cholesterol oxides found in plasma of human with total cholesterol levels ranging from 160-210 mg/dL[25]. COX-2 has been reported to be involved in proliferation of many types of tumor cells which could be decreased by NSAIDs[32,33].

In this experiment, viability or cytotoxicity of HUVEC by 25OHC was also determined by using the MTT assay. Although viability of cells tend to be increased at a lower concentration of 25OHC and decreased at a higher concentration in 24 h treatment (Fig. 3), no significant change in viability of cells was observed at any concentration of 25OHC or at any time point except at 10 microgram/mL 25OHC of 6 h incubation period. At this point percent viability of cells was statistically increased ($p < 0.05$) which seemed to be some proliferation of endothelial cells (Fig. 3). Thus, our data suggested that besides COX-2 induction, initial exposure of endothelial cells with lipid components in oLDL at optimum concentration and time may also stimulate the endothelial cell proliferation.

Increased production of PGI_2 from COX-2 induction may be a protective response of endothelial cell that limits the progress of the atherosclerotic lesion by dis-persing platelet aggregation and white blood cells adhesion to the endothelial surface. However, increased production of PGI_2 may not be sufficient to overcome the toxic effect of lipids if chronic exposure is still going on. Recent evidences show diverse physiologic and pathologic roles of COX-2. Constitutive COX-2 mRNA and protein was found in kidney, brain, spinal cord, pancreatic islet cells, stromal tissue of iris and the ciliary body, as well as in utero during implantation, playing a role in their proper maturation and function[6]. COX-2 was also found to be up regulated in the brain with Alzheimer disease[6]. It also plays a role in development and progression of tumor cells in which some of them can be suppressed by selective COX-2 inhibitor[6]. In addition some researches found benefits of COX-2 expression in protective mechanism such as, up-regulated COX-2 expression by H. pylori infection can inhibit apoptosis of gastric epithelial cells[34].

These findings, including those in present work, have suggested a broad spectrum of biological activity of COX-2 and some benefits of COX-2 expression, especially as a defensive mechanism. With regard to atherosclerosis, several evidences support the position that atherosclerosis is a disease which involves many factors and blood components and is propagated from inflammation[26]. However, little is known about the cellular mechanisms involved in lipid induced alterations in cell functions. Changes in a number of intracellular signal transduction systems have been observed after exposure of cells to lipid components[35] and COX-2 induction in various cell types by extra cellular signal has been shown to be mediated via distinct receptors[36].

In our work we found that expression of COX-2 by 25OHC was possible to be suppressed by genistein (0.5-10 microgram/mL), a tyrosine kinase inhibitor (Fig. 5). Genistein was also found to exert anti-inflammatory effect and inhibit progression of cancer cells by reducing expression of COX-2 mRNA and protein in various cell types and rodent model[37-40]. Thus, acute exposure of endothelial cells with lipid components in oLDL (25OHC) caused the endothelial cells to defend themselves by up regulation of PGI_2 synthesis through the induction of COX-2. This induction might possibly meditated via tyrosine kinase. Signaling pathway of COX-2 expression should be further evaluated. MAPK may play a role on regulation of COX-2 induction[41].

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การตอบสนองเริ่มแรกของ endothelial cell ต่อการกระตุ้นอย่างเฉียบพลันด้วยองค์ประกอบของไขมัน: เพิ่มความสามารถในการทำงานของเอนไซม์ cyclooxygenase (COX) โดยเหนี่ยวนำการสร้าง COX-2 ผ่านการกระตุ้น tyrosine kinase

ดวงพร พลเสน, ประวิทย์ อัครเสรีนนท์, กิติรัตน์ เตชะไตรศักดิ์, ศิริกุล โชติวุฒากร, อธิวัฒน์ ถาวร

วัตถุประสงค์: *เพื่อศึกษาการตอบสนองเริ่มแรกของเซลล์เยื่อบุผนังหลอดเลือด ต่อการกระตุ้นอย่างเฉียบพลันด้วยองค์ประกอบไขมันในแง่การทำงานของเอนไซม์ cyclooxygenase (COX) ซึ่งจำเป็นต่อการสร้าง prostacyclin (สารต้านการแข็งตัวของหลอดเลือดชนิดหนึ่งที่หลั่งจากเซลล์เยื่อบุผนังหลอดเลือด)*

วัสดุและวิธีการ: *25-hydroxycholesterol (25OHC) ถูกใช้เป็นตัวแทนองค์ประกอบไขมันกระตุ้น human umbilical vein endothelial cell endothelial cell (HUVEC) ซึ่งได้จากเซลล์เยื่อบุผนังหลอดเลือดสายสะดือทารกแรกคลอดที่มีสุขภาพดีโดยความคำยินยอมของมารดา HUVEC ถูกนำมาทดสอบด้วย 25OHC (ความเข้มข้น 0.1, 1 หรือ 10 microgram/mL) ที่เวลา 6 หรือ 24 ชม. ความสามารถในการทำงานของเอนไซม์ COX ถูกวัดจากปริมาณ 6-keto-PGF_{1alfa} หลังให้ arachidonic acid (10 micromolar เป็นเวลา 10 นาที) โดยใช้ enzyme immunoassay ตรวจหาปริมาณของ COX-1 และ COX-2 โปรตีนโดยใช้ Werstern blot วัดการมีชีวิตของเซลล์ด้วยวิธี MTT assay*

ผลการศึกษา: *25OHC เหนี่ยวนำการสร้าง COX-2 โปรตีน พร้อมเพิ่มความสามารถการทำงานของเอนไซม์ COX ใน HUVEC โดยไม่มีการเปลี่ยนแปลงปริมาณ COX-1 โปรตีน การเหนี่ยวนำการสร้าง COX-2 หรือเพิ่มความสามารถการทำงานของเอนไซม์ COX ขึ้นกับความเข้มข้นของ 25OHC และเวลาที่ใช้ทดสอบซึ่งดูเหมือนจะถูกยับยั้งโดย Genestein (สารยับยั้งการทำงานของ protein tyrosine kinase อย่างจำเพาะเจาะจง)*

สรุป: *การกระตุ้น HUVEC อย่างเฉียบพลันด้วย 25OHC องค์ประกอบไขมันชนิดหนึ่งซึ่งเป็นสาเหตุภาวะหลอดเลือดแดงแข็งเพิ่มความสามารถการทำงานของเอนไซม์ COX โดยเหนี่ยวนำการแสดงออกของ COX-2 ที่ขึ้นกับความเข้มข้นของ 25OHC และเวลาที่ใช้ทดสอบการเหนี่ยวนำการแสดงออกของ COX-2 น่าจะเกิดผ่านการกระตุ้น protein tyrosine kinase การตอบสนองเหล่านี้อาจเป็นกลไกการป้องกันเริ่มแรกของ endothelial cell จากการถูกโจมตีด้วยองค์ประกอบไขมัน*