THE EFFECTS OF EXOGENOUS ARACHIDONIC ACID ON CYCLOOXYGENASE ACTIVITY AND ISOFORMS EXPRESSED IN CULTURED ENDOTHELIAL CELLS

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ABSTRACT

Cyclooxygenase (COX) which exist as COX-1 and COX-2 isoform, is the first enzyme in the pathway in which arachidonic acid (AA) is converted to several prostaglandins (PGs) such as prostacyclin (PGI₂), PGE₂ and thromboxane (TX) A₂. AA is released from cell membrane (endogenous AA) by several agonists such as histamine, bradykinins, angiotensin, cytokines and growth factors, including shear stress. However, exogenous AA can directly activate PG synthesis via COX enzyme (COX activity). Here, we have investigated the effects of AA on COX activity and isoform expression in human umbilical vein endothelial cell (HUVEC). HUVEC was obtained from babies born to normal pregnancy and grown as standard technique. The cells were grown to confluent and replaced with fresh medium containing AA (0.1, 1, 10 and 20 µM). Cells were then incubated at 37°C under 5% CO₂ concentration in the CO₂-incubator for variable periods of times (5, 10, 20, and 30 minutes). After which time, the release of 6-keto-PGF₁₀ (a stable metabolite of PGI₂) in supernatant medium was measured by using enzyme immunoassay (EIA). The remained cells were extracted to detect COX protein expression using specific antibody to COX-I and COX-2. The effects of AA on cell viability were also investigated using MTT assay. Neither various concentrations (0.1-20 µM) nor variable periods of times (5-30 min) of AA had any effects on cell viability. Control HUVEC without AA released undetectable amount of 6-keto-PGF₁₀ (< 3 pg/ml). The incubation of exogenous AA (0.1-20 µM) in HUVEC can release significantly higher amount of 6-keto-PGF₁₀ which could be referred to activity of the COX enzyme. However, the release of 6-keto-PGF₁₀ in AA activated HUVEC did not depend on incubation of AA when cells were incubated with AA up to 24 h. In our model study we found that the incubation of AA 10 µM for 10 min is the most appropriate concentration and time to determine COX activity in HUVEC. Moreover, AA did not effect on COX-1 protein which was constitutively expressed in HUVEC while COX-2 protein was undetectable in AA (10 µM) treated HUVEC for up to 24 h. Thus the increase release of 6-keto-PGF₁₀ from exogenous AA (10µM) was presumably due to the increase in COX activity but not from the increase amount of COX-1 or the induction of COX-2.

Key words: COX, PGs, endothelium, arachidonic acid

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INTRODUCTION

Prostaglandins (PGs) have numerous cardiovascular and inflammatory effects. They are not stored by cells but are rapidly synthesized prior to release by a variety of physiological and pharmacological stimuli. Prostaglandin H synthase (PGHS) or cyclooxygenase (COX) is the key enzyme in the synthesis of PGs from arachidonic acid (AA). It is a membrane-bound, bifunctional enzyme that exhibits both cyclooxygenase and peroxidase activity. AA is released from cell membrane (endogenous AA) by several agonists such as histamine, bradykinin, cytokines, and growth factors, including shear stress. However, exogenous AA can directly activate PG synthesis via COX enzyme (COX activity). Recently, two isoforms of COX that are encoded by different genes have been identified. Type 1 PGHS (PGHS-1) or COX-1 is constitutively expressed in various mammalian cells and tissues, whereas type 2 is an immediate-early gene induced by a wide variety of stimuli including hormones, growth factors and cytokines. COX-2 is the major COX metabolite released from endothelial cells which participate in inflammation, atherosclerosis, thrombosis, etc. Endothelial cells can be activated by AA and its metabolites resulting in changed PG release. PGs inhibit platelet aggregation and thrombus formation as well as a potent vasodilator by causing relaxation in vascular smooth muscle cells. It was shown that PGs are mainly regulated by the activity of cyclooxygenase. However, it is not known whether AA can regulate the isoforms of COX and their function in endothelial cells. The study of the effect of AA on COX isoform expression and function will be tool to study the regulation and signaling pathway of COX enzyme. This study will show such effect of AA on cultured endothelial cell by using human umbilical vein endothelial cell (HUVEC).

MATERIALS AND METHODS

Materials

Unless otherwise indicated, all chemical reagents were obtained from Sigma. Human endothelium SFM medium and heat-activated fetal bovine serum (FBS) were purchased from GibCo (USA). COX-1 (ovine) electrophoresis standard, COX-2 (ovine) electrophoresis 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-PGF1α acetylcholinesterase tracer, 6-keto-PGF1α rabbit antiserum, 6-keto-PGF1α standard, pre-coated 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. Bio-Rad protein assay reagent, and nitrocellulose were purchased from Bio-Rad Laboratories.

Human umbilical vein endothelial cells (HUVEC) culture

Human umbilical vein endothelial cells were harvested from umbilical cords obtained at normal vaginal deliveries or Cesarian sections. The method originally described by Jeffe et al. with some modifications. Briefly, a sterile technique was utilized in all manipulations of the cord. Firstly, untraumatized umbilical cord was severed from placenta at least 20 cm length presently after birth, placed in a sterile container filled with normal saline and kept at 4°C. Within 24 h after storage, the umbilical vein was cannulated and perfused with phosphate buffer saline (PBS) (138 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4 and 1.46 mM KH2PO4) pH 7.4 until all traces of blood was removed. The vein was then filled with trypsin-EDTA (0.5 % trypsin, 5.3 mM EDTA in PBS). After 10 min incubation at 37°C, the content of the vein were gently flushed out with an equal volume of M199 (Sigma) and collected in a 50 ml falcon tube filled with FBS (1 ml of FBS for 10 ml of trypsin-EDTA). The tube was centrifuged at 1400 g for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in EC medium (Human endothelial SFM supplemented with 15 % FBS in addition of 100 μg / ml streptomycin and 100 IU of penicillin G). Cells were grown in T75 plastic flasks (Sarstedt), fed twice a week and maintained at 37°C in a water-saturated atmosphere at 95 % air/5 % CO2 until confluence. The confluent cells, then, were subcultured and grown in EC medium supplemented with 10 % FBS, 100 μg/ml streptomycin and 100 IU of penicillin G. Cells used in the studies were uniformly in the third passage.
The second passage of confluent monolayer HUVEC in 6-well culture plate (1.2 x 10^6 cells/well) was used in this experiment. The cells were incubated with 1.5 ml of EC medium containing 0.02 % ethanol (vehicle) or 10 µM of AA at 37°C in water-saturated, 95 % air/ 5 % CO2 incubation for 10 min, 3, 6, 12, 18 and 24 hrs. After which time the medium was discarded and cells were washed once with 2 ml of PBS, pH 7.4. Then 100 µl of extraction buffer (50mM Tris base; 10 mM EDTA; 1 % (V/V) TritonX-100; 0.57 mM phenylmethysulphonyl fluoride (PMSF); 1.5µM pepstatin A and 2 µM leupeptin) was added to each well with gentle shaking. The crude cell lysate was then pooled and kept in an Eppendorf tube at -40°C until further analysis for total protein and COX protein. The protein concentration in the cell lysate was measured using Bio-Rad protein assay reagent. Bovine serum albumin at concentration of 10-100 µg / ml in distilled water was used as standard. Ten µl of standard, diluted sample, or distilled water (blank) were pipetted into 96-well plate. Then, 200 µl of Bradford reagent diluted with distilled water at 1:4 were added. The plate was kept at room temperature for 5 min and measured for OD at 595 nm. All samples or standards were determined in triplicate and the average values were used for calculation. After protein concentration of cell were calculated, immunoblotting for COX protein was done by some modification of original method. The crude cell lysate was boiled for 10 min in a ratio of 1:1 with gel loading buffer (Tris, 123.9 mM; SDS 4 % w/v, glycerol 20 % V/V; 2-mercaptoethanol, 10 % v/v and bromphenol blue, 0.2 %). Samples were centrifuged at 3,200 g for 1 min before being loaded at equal amount of total proteins on SDS-PAGE (separating gel, 7.5 %; stacking gel, 4 %) and subjected to electrophoresis (1.5 h at 70 V) with separating buffer (Tris base, 0.25 M; glycine, 0.192 M; SDS, 0.1 %). Following electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (1 h at 408 A) using a transfer buffer consisting of 0.025 M Tris base, 0.192 M glycine and 20 % (V/V) methanol. After transferring to nitrocellulose, the blots were washed 6 times with wash buffer (10 mM Tris base, 100 mM NaCl and 0.1 % Tween 20) and blocked in blocking solution (5 % low-fat dry milk in wash buffer) for 1.5 h. After washing with the
wash buffer for 6 times, the blots were primed (1.5 h) with a selective antibody raised to ovine COX-1 developed in rabbits (primary antibody, dilution 1:1000 in wash buffer) or monoclonal antibody raised to COX-2 developed in mouse (primary antibody, dilution 1:1000 in wash buffer). The membranes were then washed 6 times with blocking solution and alkaline phosphatase conjugated goat anti-rabbit antibody (secondary antibody for COX-1 detection; dilution 1:1000 in blocking solution) or phosphatase conjugated goat anti-mouse antibody (secondary antibody for COX-2 detection; dilution 1:5000 in blocking solution) were added. After 1.5h incubation at room temperature, the blots were washed 4 times with the wash buffer. Blots were visualizes using premixed solution containing 5-bromo-4-chloro-3 indolyl phosphate (BCIP), 0.89 mM; nitro blue tetrazolium (NBT), 0.4 mM; Tris base, 100 mM; NaCl, 100 mM; and MgCl₂, 5 mM; (pH 9.5). The relative enzyme mass was estimated by densitometry using Image Master ID software (Pharmacia Biotech) and expressed as densitometry unit per equal total loading proteins.

Measurement of cell viability

Cell respiration in mitochondria is one of indication of cell viability. To evaluate viability of cells 200 µL of 0.2 mg/ml 3-(4, 5 dimethylthiazol-2-yl)2, 5 diphenyl tetrazolium bromide (MTT) in EC medium was added to each well and incubated at 37°C for 1 h under water-saturated atmosphere with 95 % air/5% CO₂. The medium was then removed and 100 µL of DMSO was added at each well to solubilize the cells. The extent of reduction of MTT to formazan in cells was quantitated by measurement of optical density at 595 nm using a microplate reader (BIO-RAD).

Statistical analysis

Triplicate wells were done at each experiment and at least three experiments were performed in the same manner on consecutive days. Statistical significance was determined by ANOVA or unpaired t-test. A p value of less than 0.05 was selected to denote statistical significance.

RESULTS

Effect of exogenous AA on 6-keto-PGF₁α release from HUVEC

Incubation of HUVEC with EC medium up to 30 min produced slight release of PGJ₂ (<3 pg/ml) which could not be precisely detected by EIA technique in this method. When AA at various concentrations were exogenous given and incubated at 37 °C for variable periods of time from 5 min to 30 min, it was found that the lowest concentration of AA used (0.1 µM) could increase 6-keto-PGF₁α significantly from the control (Figure 1). However, 0.1 and 1 µM of AA could not produce significant difference in the release of 6-keto-PGF₁α (p>0.05; Figure 1). 10 µM AA could increase the release of 6-keto-PGF₁α significantly from 1 µM AA (p<0.05; Figure 1), and further increased concentration of AA to 20 µM could augment the release of 6-keto-PGF₁α which was only significantly at 5min and 30-min incubation period (p<0.05; Figure 1A and 1D). Moreover, 10 min or 20 min incubation of AA did not produce significant difference in 6-keto-PGF₁α level between the 10 µM AA and 20 µM AA groups (p>0.1; Figure 1B and 1C).

The incubation times were also compared with each other at the equal dose of AA. It appears that the incubation periods for 5 min up to 30 min could not produce significant difference in 6-keto-PGF₁α release at 0.1 and 1 µM AA (p>0.05; Figure 2A and 2B). At 10 µM AA, 10 and 20 min incubation period gave higher release of 6-keto-PGF₁α which were significant difference from 5 min (p<0.05; Figure 2C). However, when the time was increased to 30 min the release was significantly decreased (p<0.01; Figure 2C). At 20 µM AA, 10 min incubation period increase 6-keto-PGF₁α, not quite significantly when compare with the 5-min group (p = 0.05; Figure 2D). Further increase the incubation time to 20 min or 30 min did not produce significant difference in 6-keto-PGF₁α level from 10 min (p>0.05; Figure 2D).

Effect of exogenous AA on isoform of COX protein expressed in HUVEC

Untreated control HUVEC contained COX-1 protein but not COX-2 protein (Figure 3 and 4). However, very small amount of COX-2 protein could be detected in some batch of untreated HUVEC when compared to
Figure 1  Dose dependent effects of exogenous arachidonic acid (AA; 0.1, 1, 10, 20 µM) on 6-keto-PGF<sub>1α</sub> production in HUVEC after 5, 10, 20, or 30 min incubation of AA (panel A, B, C and D, respectively). A) The release was significant increase in dose dependent manner from 1-20 µM AA. 0.1 and 1 µM AA could not produce significant difference in 6-keto-PGF<sub>1α</sub> production. B) The release was significant increase in dose dependent manner from 1-10 µM AA. 0.1 and 1 µM AA or 10 and 20 µM AA could not produce significant difference in 6-keto-PGF<sub>1α</sub>. C) The release was significant increase in dose dependent manner from 1-10 µM AA. 0.1 and 1 µM AA or 10 and 20 µM AA could not produce significant difference in 6-keto-PGF<sub>1α</sub> production. D) The release was significant increase in dose dependent manner from 1-20 µM AA. 0.1 and 1 µM AA could not produce significant difference in 6-keto-PGF<sub>1α</sub>. Data are expressed as Mean ± SEM of triplicate wells from 3 separate experiments performed on different days. * p < 0.05 when compared to control untreated cells (C)
The effects of incubation times (5, 10, 20 or 30 min) of arachidonic acid (AA) on 6-keto-PGF$_{1α}$ production in HUVEC after treatment with 0.1, 1, 10 or 20 µM AA (panel A, B, C and D respectively). A) Incubation of AA 0.1 µM for 5 min up to 30 min could not produce significant difference in 6-keto-PGF$_{1α}$ release. B) Incubation of AA 1 µM for 5 min up to 30 min could not produce significant difference in 6-keto-PGF$_{1α}$ release. C) 10 min incubation of AA 10 µM increase 6-keto-PGF$_{1α}$ significantly from 5 min. 20 min incubation of AA 10 µM did not produce significant difference in 6-keto-PGF$_{1α}$ production from 10 min incubation of AA 10 µM. 30 min incubation of AA 10 µM significantly decrease 6-keto-PGF$_{1α}$ production from 20 min incubation of AA 10 µM. D) 10 min incubation of AA 20 µM increase 6-keto-PGF$_{1α}$ which was not quite significantly from 5 min incubation of AA 20 µM (p=0.05). 20 min or 30 min incubation of AA 20 µM could not increase 6-keto-PGF$_{1α}$ production from 10 min. Data are expressed as Mean ± SEM of triplicate wells from 3 separate experiments performed on different days. * p < 0.05 when compared to control untreated cells (C).
Figure 3  The effects of AA (10 μM) on COX-1 protein expressed in HUVEC at 10 min up to 24h incubation. A) shows representative Western blot from three independent experiments (lane 1 to 7). B) shows Mean ± SEM of COX-1 protein amount estimated by densitometry from three separate experiments. Equal amounts of protein (10 μg/lane) were loaded in all lanes. Untreated control HUVEC (C); lane 1 expressed COX-1 protein at 23.9 ± 3.2 densitometry unit/10 μg total loading protein. The amounts of COX-1 protein were not change significantly in 10 μM AA treatment for 10 min, 3, 6, 12, 18 or 24 h (lane 2 to 7).
The effect of AA (10 µM) on COX-2 protein expressed in HUVEC at 10 min up to 24 h. A) shows representative Western blot from three independent experiments (lane 1 to 7). B) shows Mean ± SEM of COX-2 protein amount estimated by densitometry from the three separate experiments. Equal amounts of protein (10 µg/lane) were loaded in all lanes. Untreated control HUVEC (C); lane 7 contained no COX-2 protein but could be detected very small amount of COX-2 protein in some batch (lane 1) which was very low when compared to COX-1 in Figure 3 (4.97 ± 0.78 and 23.88 ± 3.2 densitometry unit/10 µg total loading protein respectively). Although COX-2 expression trend to increase after 3 h treatment (lane 3 and 4) and decline to control level within 24 h (lane 5 to 7), the difference between the control group and all of the treatment groups as shown were not significant difference (p > 0.05).
COX-1 protein (4.97 ± 0.78 and 23.88 ± 3.2 densitometry unit/10 µg total loading protein). The expression of COX-2 protein in small amount of untreated HUVEC was not changed when compared to the batch of undetectable COX-2 protein in untreated HUVEC. In AA (10 µM) treated HUVEC for up to 24 h, the amount of COX-1 protein was not changed significantly when compared to untreated HUVEC (Figure 3). For the batch of detectable small amount of COX-2 in untreated HUVEC, the amount of COX-2 protein was also not changed significantly when compared to untreated HUVEC (4.97 ± 0.78 densitometry unit/10 µg total loading protein in untreated HUVEC and 4.85 ± 0.15, 9.45 ± 2.22, 9.15 ± 1.84, 6.81 ± 0.47, 5.65 ± 0.88, 4.94 ± 0.91 densitometry unit/10 µg total loading protein at times 10 min, 3, 6, 12, 18 and 24 h respectively).

**Effect of exogenous AA on cell viability**

To assess whether AA was toxic to cell, cell respiration, which was an indicator of cell viability, was determined by mitochondrial dependent reduction of 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to formazan.

The incubation of AA in HUVEC up to 20 µM for 30 min and up to 24 h for 10 µM gave the high percentage of MTT reduction, which was not statistical difference from untreated control HUVEC (Table 1 and 2).

**Table 1**

<table>
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<th>Time (mins)</th>
<th>Control (medium only)</th>
<th>Arachidonic acid (µM)</th>
<th>% reduction</th>
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<tr>
<td></td>
<td></td>
<td>0.1</td>
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<tr>
<td>5</td>
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<td>97.9 ± 4.6</td>
<td>96.1 ± 3.3</td>
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<td>10</td>
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<td>20</td>
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<td>94.7 ± 2.9</td>
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<tr>
<td>30</td>
<td>100.0 ± 2.7</td>
<td>99.6 ± 3.3</td>
<td>106.6 ± 6.7</td>
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</table>

**Table 2**

Cell viability of HUVEC assessed by the ability of cells to reduce MTT to formazan when cells were treated with AA 10 µM at times 10 mins, 3, 6, 12, 18, and 24 hrs. Data are expressed as MEAN ± SEM from triplicate wells performed on 3 separate experimental days (n=9).

<table>
<thead>
<tr>
<th>Time</th>
<th>Control (%)</th>
<th>AA treated (%)</th>
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<tr>
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<td>100.0 ± 1.9</td>
<td>92.4 ± 3.0</td>
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<tr>
<td>3 hrs</td>
<td>100.1 ± 2.4</td>
<td>92.0 ± 2.6</td>
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<tr>
<td>6 hrs</td>
<td>100.0 ± 1.2</td>
<td>107.0 ± 2.5</td>
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<tr>
<td>12 hrs</td>
<td>100.1 ± 1.0</td>
<td>93.4 ± 2.9</td>
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<td>18 hrs</td>
<td>99.9 ± 1.0</td>
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<tr>
<td>24 hrs</td>
<td>99.9 ± 2.0</td>
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DISCUSSION

PGI₂ synthesis in human vascular tissue is mainly regulated by the cyclooxygenase enzyme. Here we show that HUVEC in growth medium expresses COX-1, which is the constitutive isoform, while COX-2 protein was undetectable. However, very small amount of COX-2 can also be detected in some batches of the control group by this specific antibody. There is similar to previous reports which Wohlfeil E.R. and Campbell W.B. also found that untreated bovine coronary artery endothelial cells (BCAECs) and HUVEC can express very low when compared to the amount of COX-2 protein was undetectable. However, HUVEC in growth medium expresses COX-2 protein was undetectable. However, HUVEC in growth medium expresses COX-2 protein. Although COX-2 expression appeared in some batch of control HUVEC, the amount of protein was very low when compared to the COX-1 protein (4.97 ± 0.78 vs 23.89 ± 3.20 densitometry unit/10 µg total loading protein). Moreover, COX activity was not significantly different between the batch of undetectable and detectable small amount of COX-2 protein.

Incubation of HUVEC with EC medium up to 30 min released undetectable amount of 6-keto-PGF₁α (< 3 pg/ml). From our results shown in Figure 1 and Figure 2 we found that exogenous AA at the concentration of 10 µM with 10 min incubation time is the most appropriate to study COX activity in HUVEC because (i) 10 µM AA produced adequate amount of 6-keto-PGF₁α which could be precisely detected by this EIA technique and the increased concentration of AA to 20 µM did not produce significant difference in 6-keto-PGF₁α for all periods of incubation time except at 30 min (Figure 1). (ii) At lower level of AA (0.1 and 1 µM), incubation of AA for 5, 10, 20 or 30 min did not produce significant difference in 6-keto-PGF₁α production from 5 min but further increase in time also produced significant difference in 6-keto-PGF₁α level (Figure 2D).

Metabolism of AA in the vascular wall proceeds mainly by the cyclooxygenase pathway to PGs which a major product is PGI₂ (6-keto-PGF₁α is a stable hydrolyzed form of PGI₂). It was shown that activators, e.g. IL-1, histamine, thrombin, calcium ionophore or phorbol myristate acetate (PMA) can increase PGI₂ synthesis in endothelial cells. Histamine, thrombin and calcium ionophore rapidly initiate PGI₂ production by stimulating release of AA, whereas, IL-1 and PMA is accompanied by elevated AA concentration and increased the expression of cyclooxygenase. It is unclear that elevated AA can contribute to the expression of COX-2 protein in HUVEC. Barry OP. et al also found that AA in platelet microparticle can induce expression of COX-2 and prostacyclin production in HUVEC. Therefore, we have examined whether arachidonic acid (10 µM) altered COX protein expression when cells were incubated for up to 24 h. Figure 3 and 4 shows that AA (10 µM) treated HUVEC for up to 24 h neither changed COX-1 protein nor induced COX-2 protein expression. These suggested that elevated endogenous AA in some states could not contributed to the expression of either COX-1 or COX-2 protein. Thus, the increase in 6-keto-PGF₁α production after incubation of HUVEC with 10 µM AA for 10 min was due to stimulation of COX activity (especially COX-1) without upregulation of either COX-1 or COX-2 protein.

The experiment was also evaluated the effect of AA at various concentrations and times of incubation on the viability of HUVEC. It was shown that AA at concentration up to 20 µM for 30 min and up to 24 h for 10 µM did not influence on respiration of cell when compared to the control. Thus, our study showed that incubation of HUVEC with AA 10 µM for 10 min was the most appropriate concentration and time to study COX activity in this cell type when EIA was used as measurable method.
REFERENCES

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