

Different Effects of Testosterone to the Expression of Endothelial COX-2 in Normal and High Glucose Environment

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Abstract—Male is one of the risk factors for the development of cardiovascular diseases. It is suggested that testosterone (T) may contribute to cardiovascular events, which is initiated by platelet adhesion, activation, and aggregation. Endothelial cells (EC) prevent platelet activation by synthesizing and releasing thromboregulator, such as prostacyclin (PGI₂). The activity of cyclooxygenase (COX) enzyme is necessary for the conversion of arachidonic acid into PGI₂. COX exists in two isomers: COX-1 and COX-2. This study aimed to examine the influence of T on the expression of endothelial COX-2 in either normal glucose (NG) or high glucose (HG) environment. An *in vitro* study using human umbilical vein endothelial cells culture (HUVEC) was performed in this study. With the 2x4 factorial designs, HUVEC was exposed to T in incremental doses: 0, 1, 10 and 10² nM in either NG (5.6 mM) or HG (22.4 mM) medium. Expression of COX-2 was measured using immunocytochemistry. Data were analyzed using analysis of variance for 2x4 factorial designs. *P*-value <0.05 was considered statistically significant. There was a main effect of either T or glucose medium to the percentage of EC that positively stained with the anti-COX-2 antibody. Moreover, there was an interaction between T and glucose medium to the percentage of EC that positively stained with the anti-COX-2 antibody. In conclusion, testosterone increases the expression of COX-2 enzyme in resting endothelial cells (normal glucose environment) but decreases significantly the expression of COX-2 enzyme in activated endothelial cells (high glucose environment).

Keywords— *testosterone, high glucose, HUVEC, COX-2*

I. INTRODUCTION

It has been known since the release of the results of the Framingham study that male gender is an unmodified risk factor for cardiovascular diseases (CVD). Most of the following epidemiological studies confirm that CVD occurs more frequently in male subjects as compared to age-matched female subjects during reproductive years [1].

The higher concentration of testosterone (T) in the plasma in male subjects compared to female subjects leads to

the suggestion that T and/ other androgen hormones may play an important role in the development of CVD [2-3]. Evidence shows that T has indeed exerted influence on the cardiovascular system, including endothelium [1].

Endothelial cells (EC) contain androgen receptors (AR) as well as estrogen receptors (ER). Thus, endothelial cells are subjected to the effects of either T or estrogen. Moreover, the 5 α -reductase enzyme, as well as aromatase enzyme, is detected in EC. The 5 α -reductase enzyme catalyzes the conversion of T to become dihydrotestosterone (DHT), while aromatase enzyme catalyzes the cleavage of one carbon (C) molecule from T (C19) to form estradiol (C18). Thus, T does not only influence EC but also is metabolized inside the EC. As a consequence, the effect of T to EC can be exerted through AR directly or via ER indirectly as well as non-genomic pathway [4-5].

EC has the capacity to prevent cardiovascular events in normal condition by synthesizing and releasing thromboregulator factors, i.e. nitric oxide (NO) and prostacyclin (PGI₂). PGI₂ is synthesized from arachidonic acid, which in turn is derived from the phospholipid cell membrane. PGI₂ released from EC, in turn, increases the cAMP level in platelets and prevents calcium influx and inhibits platelets aggregation [6-8].

Cyclooxygenase (COX) enzyme activity contributes to the conversion of arachidonic acid to become PGI₂. COX enzymes have two isomers, namely COX-1 and COX-2. Fortunately, EC contains both isomers. However, they play a role in a different situation. COX-1 is a constitutive enzyme meaning that it is produced in resting EC. On the other hand, COX-2 is more abundant in stimulated or activated EC [7-8].

EC is activated by several factors, such as shear stress, high glucose, hypercholesterolemia, lipopolysaccharide (LPS), pro-inflammatory cytokines (interleukin-1 β , tumor necrosis factor- α , interferon- γ) [7-8]. High glucose is defined as glucose concentration in plasma (*in vivo*) or medium used

in EC culture (in vitro) >10 mM. At this level, glucose disturbs EC metabolism [9].

The researcher hypothesized that T influences the expression of COX-2 enzyme either in resting (normal glucose concentration) and stimulated (high glucose concentration) EC. To examine the effect of T to EC, endothelial cells culture derived from a human umbilical vein (HUVEC) was used. This in vitro model has been acknowledged as an approach to observe EC behavior.

II. METHODS

A. Study design

The study design was an in vitro laboratory experimental study with randomized-block factorial design and post-test only control group comparison. HUVEC was established according to the technique that have been previously described [10-12]. The HUVEC protocol used in this study had been previously reported [13-14]. The donors of umbilical cords were healthy delivered mothers with a healthy full-term newborn baby (Apgar score was >10). They have been assessed for not having high blood pressure based on JNC 7 criteria, high blood glucose, and pre-eclampsia/eclampsia. Informed consent was signed by the umbilical cord and platelets donors. The Institutional Review Board of the Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada (FMPHN UGM) had approved this study.

B. Reagents

Medium 199 (M199) was used as the growth medium both in primary and secondary primary culture, which was prepared from the powdered M199 (Gibco) with additional 2 g of sodium bicarbonate and 2 g of HEPES sodium salts. M199 solution was supplemented with 10% fetal bovine serum (FBS) (Caisson), 100 IU/mL of penicillin and 100 µg/mL of streptomycin (Sigma), 0.5% fungizone (Gibco), and 2 mM of L-glutamine (Sigma) to make complete M199. The buffer solution was prepared from 9.6 g of Dulbecco's phosphate buffered saline (PBS) powder, which was dissolved with distilled water to make 1000 cm³ total volume. The PBS solution was sterilized using autoclave at 121°C for 15 minutes.

C. Umbilical cord collection

The umbilical cord was taken from the placenta of the mothers by aseptic technique. The umbilical cord with 10-20 cm length was put in PBS solution supplemented with antibiotic penicillin and streptomycin and kept at 4°C until it was processed in Cell Culture Laboratory of Department of Physiology FMPHN UGM for less than 12 hours post sample collection.

D. Primary culture of endothelial cells

The primary culture was carefully done under the sterile condition in class II biosafety cabinet (Delta series, Labconco corp., USA). The researcher used enzymatic disaggregation method using 0.25% trypsin-EDTA (Gibco) to detach endothelial cells from the basal membrane.

For endothelial cells collection, the inlet of the umbilical vein of the cord was identified and swapped using povidone iodine. Then a sterile cannula was inserted into the umbilical vein and firmed with a sterile clamp. A ringer lactate solution was flushed into the lumen of the umbilical vein to

dislodge the lumen from a blood clot. Trypsin-EDTA solution was injected into the lumen of the umbilical vein and another end of the lumen was clamped. It needed another 3-5 minutes to incubate the cord in warm PBS solution already supplemented with antibiotic penicillin and streptomycin. After that, the clamp was taken off and the enzyme solution containing disaggregated endothelial cells was collected in a sterile bottle supplemented with 1 cm³ of FBS.

The cells effluent was centrifuged at 2000 rpm for 10 minutes. The supernatant was removed and the cells pellet was washed with M199. Then, the cells were supplemented with the growth medium and inoculated on a gelatin-coated 60 mm-diameter tissue culture dish (Iwaki). The dish was incubated overnight at 37°C and 5% CO₂ condition. The growth medium was changed in alternate days until the HUVEC reached a subconfluent state of 80%. The cells were observed by an inverted microscope (Eclipse, Nikon, Japan). To identify HUVEC, the researcher used morphological and immunological approach. Morphologically, HUVEC has a cobblestone appearance in confluent condition. Meanwhile, immunologically, HUVEC expresses von-Willebrand factor (vWF) in their membrane.

E. Secondary culture of endothelial cell

After HUVEC primary culture reached confluence on the day 7th-9th, the cells were detached from the bottom of the dish using trypsin-EDTA solution (trypsinization). Cell counting was carried using the improved Neubauer chamber. The number of endothelial cells that could be subcultured was dependent on the counting results and calculation. Finally, the subculture (1st passage) of endothelial cells were done with 3x10⁵ endothelial cells/ well in gelatin coating-24 wells microplate (Iwaki) supplemented with similar growth medium used in primary culture.

F. Treatment

The medium for HUVEC subculture was replaced after overnight incubation with either normogluucose (NG) (5.6 nM glucose) or high glucose (HG) medium (22.4nM glucose). T in incremental doses started from 0, 1, 10, and 10² nM was added to each medium. The treatment groups were arranged in 2x4 factorial designs for T and glucose medium factors (Table 1).

TABLE I. 2x4 FACTORIAL DESIGNS

Testosterone (T)	Normogluucose medium (NG)	High glucose medium (HG)
0	G1	G5
1	G2	G6
10	G3	G7
100	G4	G8

Note. NG = 5.6 mM glucose. HG = 22.4 mM glucose. T was in nM. G = group.

The following detail of the treatment groups:

- G1 = 0,45 cm³ complete M199 with NG + 0,05 cm³ DMSO 0,1%.
- G2 = 0,45 cm³ complete M199 with NG + 0,05 cm³ 1 nM T.
- G3 = 0,45 cm³ complete M199 with NG + 0,05 cm³ 10 nM T.
- G4 = 0,45 cm³ complete M199 with NG + 0,05 cm³ 10² nM T.

- G5 = 0,45 cm³ complete M199 with HG + 0,05 cm³ DMSO 0,1%.
- G6 = 0,45 cm³ complete M199 with HG + 0,05 cm³ 1 nM T.
- G7 = 0,45 cm³ complete M199 with HG + 0,05 cm³ 10 nM T.
- G8 = 0,45 cm³ complete M199 with HG + 0,05 cm³ 10² nM T.

After 24 hours of incubation, the treatment solution was removed. Then, it was followed by immunocytochemistry procedure to measure endothelial expression of COX-2.

G. Immunocytochemistry

Cells were undergone fixation with absolute methanol for 15 minutes. Then fixation solution out was poured off and 3% of H₂O₂ solution was pipetted to the monolayer of endothelial cells on the gelatin-coated coverslip at the bottom of wells. Incubation for H₂O₂ solution was done for 20 minutes to inhibit the activity of endogenous peroxidase. For washing steps, the monolayer of endothelial cells was washed with tapping water as long as 3 minutes, aquadest once and finally PBS solution twice as long as 3-5 minutes. After that, the monolayer was incubated with blocking serum for 15 minutes and followed by SP21 clone anti-COX-2 primary antibody - β (Lab Vision, ThermoScientific, USA) with 1:200 dilution for 1 hour. Then the wells were washed with PBS solution twice for 3-5 minutes and the monolayer was incubated with trekie universal link as the secondary antibody for 20 minutes. It was followed by washing the wells with PBS solution twice for 3-5 minutes and incubating with trekie avidin horse reddish peroxidase (HRP) for 10 minutes. It was followed by washing the wells with PBS solution twice for 3-5 minutes. Then chromogen DAB (1:50) was dripped into the monolayer and underwent incubation for 2 minutes. The wells were washed with tapping water. Counterstaining with Mayer's hematoxylin was performed for 2 minutes. The wells were washed with tapping water and then added graded alcohol from 70%, 96% and finally 100%. Lastly, the coverslip was lifted from the wells and underwent mounting with Canada balsam.

H. Cell counting

The coverslips or slides were observed with an optic microscope (Olympus, Japan) with 10x magnification. Endothelial cells that positively stained with anti-COX-2 antibody were looked brown in the cytoplasm. The observation and counting were performed for 5 optical fields/slide.

I. Statistical analysis

The unpaired t-test was used to analyze the influence of each dose of T to the presence of endothelial COX-2 compared to the negative control in either NG or HG medium. One-way ANOVA was used to analyze the influence of T in the incremental doses to the presence of endothelial COX-2 in either NG or HG medium. Analysis of variance was used to analyze the 2x4 factorial design of the influence of T and glucose medium to the presence of endothelial COX-2. P-value <0.05 was considered as statistically significance.

III. RESULTS

Primary HUVEC was successfully performed using enzymatic disaggregation method. The morphological cobblestone of the confluence cells and the expression of von Willebrand factor on endothelial cell membrane have been reported in the researcher's previous reports [13-14].

The proportion of EC that positively stained with anti-COX-2 antibody in incremental doses in NG or HG medium can be seen in the Fig. 1.

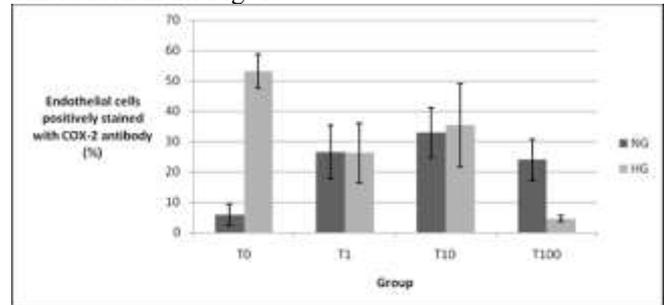


Figure 1 Percentage of endothelial cells positively stained with COX-2 antibody in incremental doses in normoglycose or high glucose medium.

Note: Σ endothelial cells = 1×10^5 / group; COX-2 = cyclooxygenase-2; NG = normoglycose (5.6 mM); HG = high glucose (22.4 mM); T0 = without testosterone; T1 = 1 nM testosterone; T10 = 10 nM testosterone; T100 = 10² nM testosterone.

The results of statistical analysis were presented as follows:

1. Unpaired t-test:

- Percentage of EC that positively stained with anti COX-2 antibody in NG medium without T was $5.82 \pm 3.62\%$. It was significantly lower than percentage of EC that positively stained with anti COX-2 antibody in NG medium with 1 nM T ($26.47 \pm 8.78\%$; $P = 0.001$), 10 nM T ($32.93 \pm 8.15\%$; $P = 0.0001$), and 10² nM T ($23.97 \pm 6.81\%$; $P = 0.001$).
- Percentage of EC that positively stained with anti COX-2 antibody in HG medium without T was $53.2 \pm 5.64\%$. It was significantly higher than percentage of EC that positively stained with anti COX-2 antibody in HG medium with 1 nM T ($26.23 \pm 9.86\%$; $P = 0.001$), 10 nM T ($35.34 \pm 13.79\%$; $P = 0.028$), and 10² nM T ($4.54 \pm 0.96\%$; $P = 0.0001$).

2. One-way ANOVA:

- Exposure of T in incremental doses in NG medium significantly influenced the percentage of EC that positively stained with anti-COX-2 antibody ($P = 0.0001$).
- Exposure of T in incremental doses in HG medium significantly influenced the percentage of EC that positively stained with anti-COX-2 antibody ($P = 0.0001$).

3. Analysis of variance for 2x4 factorial design:

- There was a main effect of glucose medium to the percentage of EC that positively stained with anti-COX-2 antibody ($P = 0.0001$).

- b. There was a main effect of T to the percentage of EC that positively stained with anti-COX-2 antibody ($P = 0.006$).
- c. There was an interaction between T and glucose medium to the percentage of EC that positively stained with anti-COX-2 antibody ($P = 0.0001$).

IV. DISCUSSION

To examine the influence of T to resting and activated EC, the researcher exposed HUVEC with T in different doses (0, 1, 10 and 10^2 nM) in either normal (5.6 mM) or high glucose (22.4 mM) concentration in growth medium. This study showed that T induced endothelial COX-2 expression in normal glucose environment. However, this study revealed that T inhibited endothelial COX-2 expression in a high glucose environment. To date, these results are novel findings regarding the role of T in cardiovascular events.

The results of this study underscore that male gender may no longer be an unmodified cardiovascular risk factor. It is already identified that EC is one of the target cells for testosterone outside the reproductive system [15]. The influence of T to EC is through the genomic and non-genomic pathway [16]. A part of bioavailable T that diffuses into EC can also be metabolized by the aromatase enzyme, which catalyzes the cleavage of T to become estrogen [17]. Thus, the genomic pathway of the influence of T to endothelial COX-2 expression can be mediated through AR or indirectly via ER. The researcher recently reported that exposure of T to HUVEC increases expression of the endothelial ER- β in normal glucose medium [14]. It is possible that the expression of COX-2 can be modified by the ER since it is known as a transcription factor. Several studies had reported that COX-2 expression and PGI₂ synthesis decrease in ER- β knocked down mice [18-19]. However, other studies showed the opposite evidence in which the induction of ER by the metabolite of DHT decreases COX-2 expression in mouse EC culture and human brain vessels [20-22], it was revealed that castrated male rats, in which the concentration of T has decreased, show significantly higher COX-2 expression in the mesenteric artery [23].

The endothelium can be regarded as an interface between the vascular system and the blood. Any changes in terms of mechanic (pressure) or chemical (including glucose level) in the blood will exert influence to EC. For example, it was reported that high glucose medium depresses the capacity of EC to prevent platelet aggregation [13]. Regarding the influence of high glucose environment to the expression of endothelial COX-2, it still opens to be discussed. It was reported that high glucose medium increases the expression of COX-2 but not COX-1 in HUVEC [24]. However, castrated male rats with a high fructose diet have shown significantly lower COX-2 expression in a mesenteric artery as compared to the castrated only group [23].

PGI₂ is well known as an inflammatory mediator [25]. For decades, anti-inflammatory non-steroidal (AINS) drugs have been widely used to combat inflammatory diseases, such as rheumatoid arthritis [26]. However, PGI₂ also has beneficial role in the cardiovascular system. Therefore, it is hypothesized that the use of AINS that inhibits the activity of COX enzyme may exert negative impact to endothelial

function [25]. Not supporting this deduction, it was reported that selective COX-2 inhibition has beneficial effect in the repair of endothelial dysfunction in patients with peripheral vascular diseases (PAD). In the group receiving the selective COX-2 inhibitor celecoxib for 1 week, brachial artery flow-mediated dilatation increased significantly, whereas the level of endothelin, high sensitive C-reactive protein (hsCRP) and low-density lipoprotein cholesterol decreased significantly [27].

The roles played by PGI₂ and, of course, COX-2, to exert either positive or negative effects to the cardiovascular system (dual capacity) is like the concept of Yin and Yang in the Chinese philosophy [28]. In normal condition, the differential influences of COX-2 that are contrary to each other will produce balance.

The results of this study support the concept that the phenotype of the endothelial cells is influenced by glucose level, in which high glucose is correlated with endothelial dysfunction [9, 29]. Further studies are needed in this field, either in vitro or in vivo, to elucidate other conditions that can change the normal capacity of the endothelial cells. Moreover, regarding high glucose environment, the results of this study can be elaborated to clinical setting in which plasma glucose level may interact with hormonal condition of the patients with endothelial dysfunction, such as those with hypertension and PAD. Therefore, glucose environment will contribute to determine to the treatment efficacy.

V. CONCLUSION

The influence of testosterone to the expression of COX-2 enzyme in endothelial cells is modified by glucose level in the environment.

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