Anti-inflammatory Activity of Quercitrin on Hypoxia-induced EA.hy926

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Abstract. The evidence support the existence of oxygen deprivation involved in pregnancy disorder or preeclampsia. The intensive hypoxia also regulates production of placental pro-inflammatory cytokines. Quercitrin belongs to flavonoid group is known to have antioxidant and anti-inflammatory activity. This study aims to determine the potential of Quercitrin as anti-inflammatory in hypoxia-induced EA.hy926 as preeclampsia model. The cytotoxic assay of Quercitrin against EA.hy926 was conducted using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). The data were analyzed using SPSS ver 20.0 software. One-way analysis of variance (ANOVA) was conducted, followed Tukey HSD post-hoc test and p<0.05 was considered to be significant. The anti-inflammatory potential of Quercitrin (25 and 6.25 µg/ml) toward hypoxia-induced EA.hy926 was determined using ELISA to measure IL-10 and TNF-α. Quercitrin (3.13–25 µg/ml) were nontoxic to the EA.hy926 cells. Quercitrin (25 and 6.25 µg/ml) were capable to reduce TNF-α compared to positive control (2% O2 hypoxia-induced cells) but no significant increase in IL-10. Our study suggests that quercitrin possess anti-inflammatory properties through suppression of TNF-α in hypoxia-induced EA.hy926 cells.

1. Introduction

Preeclampsia is pregnancy disorder characterized by hypertension, proteinuria and increasing maternal systemic inflammatory response [1]. Preeclampsia affects 5% to 7% of pregnant women each year worldwide. Preeclampsia often ends with premature delivery of the fetus and also a major cause of fetal and perinatal morbidity and mortality [2].

TNF-α is one of the major proinflammatory cytokines involved in the pathogenesis of chronic inflammatory diseases and is modulated by oxidative stress [3]. A couple of evidence support that tumor necrosis factor-α (TNF-α) has direct contact with maternal endothelial cell in vivo. There was increasing concentration of TNF-α in pregnant women with preeclampsia as compared to normal pregnant women [1]. It is presumed that according to the recent studies, TNF-α is up-regulated during the preeclampsia’s symptomps. TNF-α is produced by variety of cell, including endothelial cells, on appropriate stimulation.
Hypoxia has been suggested to be leading factor of preeclampsia. Under hypoxic condition, numerous genes are activated such as HIF-α and HIF-2α which is essentials for the inflammation process. Hypoxia conditions activates NF-kB which in turn increases the production of TNF-α [4]. Furthermore, inflammation was also occured when cells undergo oxidative stress.

Several studies have been demonstrated that phytochemical compounds possess rich pharmacological properties that play beneficial roles in inflammation-related diseases. Flavonoids are perceived to posses good effects on health. Flavonoids have been shown to reduce TNF-α in human and animal studies and cell culture models. Among flavonoids, quercitin (3-rhamnosyl-quercetin) is mainly present in nature. Quercitin is glycossylated forms of Quercetin that can be found commonly in vegetables. In vitro studies have shown quercitin exerts down-regulation activity in inflammatory response [5]. Therefore, IL-10 plays an important anti-inflammatory role in the resolution of inflammation and has been shown to be upregulated by several flavonoids [6] including hesperidin, quercetin, and catechin. TNF-α and IL-10 are all regulated by the pro-inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and NF-κB has also been shown to be a target of several flavonoids including quercetin and cyanidin-3-glucoside (C3G) [6]. The aims of the present study was to evaluate the effects of Quercitrin toward TNF-α and IL-10 on hypoxia-induced EA.hy926.

2. Methods

2.1. Cell culture

EA.hy926 (ATCC ® CRL-2922™) cell line were cultured in Biomolecular and Biomedical Research Center, Aretha Medika Utama. The cell lines were grown in Dulbecco's Modified Eagle Medium DMEM high glucose (Biowest L0104) supplemented with 10% fetal bovine serum (FBS) (Biowest S181G), 1% antibiotic-antimycotic 100x (Biowest L0010), 1% Amphotericin B 100x (Biowest L0009). Cells were incubated at 37°C in a humidified atmosphere with 5% CO2. The cells were treated by 25 and 6.25 µg/ml of Quercitrin and incubated in 2% O2, 37°C for another 24 hours. The treatment of control, hypoxic (2% O2), control DMSO, Hypoxic (25 µg/ml), and Hypoxic (6.25 µg/ml) repeated at 3 times for each group.

2.2. Cytotoxic Assay

EA.hy926 (ATCC ® CRL-2922™) cell line were cultured in DMEM high glucose (Biowest L0104) supplemented with 10% fetal bovine serum (FBS) (Biowest S181G), 1% antibiotic-antimycotic 100x (Biowest L0010), 1% Amphotericin B 100x (Biowest L0009). Cells were incubated at 37°C in a humidified atmosphere with 5% CO2. After the cells reach 80% confluency, 1x104 cells were seeded in each well of 96-well plate. After 24 hours incubation, the cells were treated with various concentration (0-100µg/ml at final concentration) of Quercitrin (Chengdu BioPurify BP1192) and incubated in 37°C, 5% CO2 for 24 hours. The viability of the cells were determined using MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-(4-sulfophenyl)-2H-tetrazolium) (Abcam 197010). MTS was added to each well at a ration 1:10. The plate was incubated in 37°C, 5% CO2 for 3 hours. Absorbance was measured using spectrophotometer (Multiskan GO, Thermo) at 490 nm [7] [8] [9] [10].

2.3. Hypoxia-induced EA.hy926 (2% Oxygen)

Preeclampsia model was generated in vitro using hypoxia induced-EA.hy926. EA.hy926 was incubated in 2% O2 to mimic preeclampsia condition. The cells were plated in 6-well plate (5 x 105 cells/well) and incubated for 24 h at 37°C, 5% CO2. On the next day, the cells were treated by 25 and 6.25 µg/ml of Quercitrin and incubated in 2% O2, 37°C for another 24 hours. After incubation, the conditioned medium was taken for ELISA assays and centrifuged at 1600rpm for 10 minutes. The supernatant was collected and stored at -80°C for quantification of IL-10 and TNF-α [11] [12].
2.4. Measurement of TNF-α
TNF-α levels in uninduced, hypoxia-induced and Quercitrin treated and hypoxia-induced EA.hy926 were determined using ELISA assay according to the manufacture’s kit manual (BioLegend ELISA kit 421701). First, the plate was coated using capture antibody solution and incubated at 4°C overnight. The plate was washed four times using 300 μl of wash buffer, then incubated for one hour in orbital shaker. 50 μl of matrix C and 50 μl of assay buffer was added into each standard and sample well, respectively. Plates were shaken at room temperature and then washed for 4 times. Afterward, 100 μl of the detection antibody solution was added into each well and incubated at room temperature for 1 hr on the orbital shaker. The plate then was washed four times. Subsequently, 100 μl of diluted Avidin-HRP solution was added into each well, incubated at room temperature for 30 min in orbital shaker. The plate was washed again 5 times, then added with 100 μl of substrate solution, incubated for 10 min in the dark room. The reaction was stopped by adding 100 μl of stop solution briefly, and the absorbance was measured by Multiskan GO Microplate Reader at 450 nm [10] [13] [14].

2.5. Measurement of IL-10
IL-10 levels in uninduced, hypoxia-induced and Quercitrin treated and hypoxia-induced EA.hy926 were determined using ELISA assay according to the manufacture’s kit manual (BioLegend ELISA kit). The plate was coated using capture antibody solution and incubated at 4°C overnight. The plate was washed four times using 300 μl of wash buffer, then incubated for one hour in shaker. Around 50 μl of matrix C and 50 μl of assay buffer was added into each standard and sample well, respectively. Plates were shaken at room temperature and then washed for 4 times. Afterward, 100 μl of the detection antibody solution was added into each well, incubated at room temperature for 1 hr on the orbital shaker. The plate then was washed four times. Subsequently, 100 μl of diluted Avidin-HRP solution was added into each well, incubated at room temperature for 30 min in orbital shaker. The plate was washed again 5 times, then added with 100 μl of substrate solution, incubated for 10 min in the dark room. The reaction was stopped by adding 100 μl of stop solution briefly, and the absorbance was measured by Multiskan GO Microplate Reader at 450 nm [10] [13] [14].

2.6. Statistical Analysis
The data were analyzed using SPSS ver 20.0 software. One-way analysis of variance (ANOVA) was conducted, followed Tukey HSD post-hoc test and p<0.05 was considered to be significant.

3. Results
3.1. Cell Viability
Cell viability was measured using MTS assay based on the transformation of yellow tetrazolium salt to a purple formazan product. Figure 1. showed EA.hy926 viability that has been treated by 0-25 μg/ml Quercitrin were over 80% compared to the control (untreated EA.hy926), indicated that Quercitrin was less toxic to the cells and able for the next assay. Meanwhile, Quercitrin at 50 and 100 μg/ml given EA.hy926’s viability less than 80% indicated that the concentration given were quite toxic to the cells.
Figure 1. Cytotoxic of Quercitrin in various concentration (3.13-100 μg/ml) towards EA.hy926.

3.2. **TNF-α level**

A glance at the graphic bar provided reveals the level concentration of TNF-α on EA.hy926 cells which has been generated into preeclampsia model. Of the six Quercitrin’s concentration screened at the cytotoxic assays, then two concentrations were chosen. The highest concentrations given significantly reduced hypoxia-induced TNF-α protein levels compared to control (Figure 2.).

Figure 2. TNF-α levels on hypoxia-induced EA.hy926.

*The histograms are presented as mean ± standard deviation. The data were analyzed with ANOVA and continued with Tukey post hoc test. Different letters (a, b, c) indicate significant differences among treatment. Control represent uninduced cells. Hypoxia (2% O2) represent cell under hypoxia condition. Control DMSO represent uninduced cell treated by DMSO (1% at final concentration). Hypoxia + Quercitrin 6.25 or 25 µg/ml represent hypoxia-induced cell and treated by quercitrin.*

3.3. **IL-10 level**

IL-10 is anti-inflammatory cytokine that possess several benefits to reduce inflammation throughout preeclampsia symptoms. The concentrations given of Quercitrin (25 and 6.25 μg/ml) to preeclampsia model proved that it may increase IL-10 levels as compared to the control hypoxia (Figure 3.) yet it was not significant.
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Figure 3. IL-10 levels on hypoxia-induced EA.hy926.

* The histograms are presented as mean ± standard deviation. The data were analyzed with ANOVA and continued with Tukey post hoc test. Different letters (a,b) indicate significant differences among treatment. Control represent uninduced cells. Hypoxia (2% O2) represent cell under hypoxia condition. Control DMSO represent uninduced cell treated by DMSO (1% at final concentration). Hypoxia + Quercitrin 6.25 or 25 µg/ml represent hypoxia-induced cell and treated by quercitrin.

4. Discussion

Preeclampsia is a systemic syndrome characterized by an antiangiogenic and inflammatory status [15]. In this study, EA.hy926 cell line was cultured under hypoxia condition (2% O2) to mimic placental hypoxia and treated with Quercitrin. According to Zhou et al. (2013), persistence of low oxygen tension may cause development of preeclampsia [16]. Therefore, hypoxia and inflammation share an interdependent relationship [17].

Hypoxia condition (2% O2) caused an increase TNF-α and decrease IL-10 which was principally involved in inflammation process during preeclampsia [1]. Recent study has proved that TNF-α have been implicated in the pathogenesis of preeclampsia because of endotelial effect [18]. While preeclampsia is associated with increased levels of proinflammatory cytokines, it is also associated with decreased placental production of the anti-inflammatory cytokine IL-10 [19] [20]. IL-10 is essentials during pregnancy as of its ability to inhibit secretion of TH1 inflammatory cytokines and thus provide an important counterbalance for controlled inflammation at the fetal-maternal interface [21].

In line with Hung et al., (2004), tumor necrosis factor alfa (TNF-α) in EA.hy926 which cultured in hypoxia condition was significantly increased [1]. Albeit the explanation behind the increasing production of TNF-α is remain debatable but the generation of reactive oxygen species (ROS) as a result of hypoxia condition may play an important role. According to Qutub & Popel (2008) and Azimi et al. (2017), under hypoxic conditions ROS are highly generated by mitochondrial complex III at the Q0 via electron transfer from ubisemiquinone to molecular oxygen. Increases in cellular ROS production can result in an increase in the expression of NF-kB, leading to the upregulation of factors involved in inflammation [22] [23]. Moreover, ROS contributes to intracellular signaling cascade leading to placental production of TNF-α. In addition, the balance between HIF-α and HIF-2α involved in the process of inflammation in the pathogenesis of preeclampsia [24].

Moreover, Quercitrin that belongs to polyphenols group decreased TNF-α. The mechanisms how Quercitrin exert anti-inflammatory effects is by inhibiting the endogenous production of the proinflammatory cytokine TNF-α. According to Yahfoufi et al. (2018), polyphenols undoubtedly contribute as anti-inflammatory by interrupting the ROS-inflammation cycle. Polyphenols are known for its ability to scavenge a wide-range of free radicals and chelate metal ions [25] [26].
Quercitrin increased IL-10 cytokine that are linked to decrease inflammatory cytokines e.g. TNF-α. IL-10 is crucial due to its ability to improve characteristics of PE. IL-10 also significantly lower blood pressure in preeclampsia [20]. According to Leyva-Lopez et al. (2016), Quercetin down-regulated NF-kB expression that linked to up-regulation of IL-10. In this regard, the author proposed a hypothetical mode that quercitrin possess anti-inflammatory activity similar to Quercetin [27].

In other words, certain polyphenols such as Quercitrin exert their effects on the balance between pro- and anti-inflammatory cytokines production, they enhance IL-10 release while they inhibit the secretion of TNF-α [25] [28] [29].

5. Conclusion
Quercitrin possess anti-inflammatory activity in hypoxia-induced EA.hy926 showed by decreasing production of pro-inflammatory cytokines TNF-α but no significant increase in IL-10.

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7. Conflict of Interest
All contributing authors declare no conflicts of interest.

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