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Treatment with Genistein Decreases AT1R Expression and ERK1/2 Phosphorylation in the Aorta of Hypoestrogenic *Rattus norvegicus.*

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ABSTRACT

The prevalence of cardiovascular disease (CVD) has increased among menopausal women. This could be caused by low estrogen levels, upregulation of the renin angiotensin system (RAS), and aging. However, a clinical trial of estrogen replacement therapy in menopausal women did not show any benefit for their cardiovascular profile. The present study explored the possibility at genistein, a phytoestrogen, may decrease expression of the angiotensin II type 1 receptor (AT1R) and downstream signaling of the extracellular signal regulated kinase 1/2 (ERK1/2) in female *Rattus norvegicus* in the hypoestrogenic state. Rats (*Rattus norvegicus*) aged 9 months were ovariectomized. Twenty-eight days later, genistein was administered at doses of 2.5, 5, and 10 mg/kg/d, subcutaneously for 4 weeks. At the end of this period, the animals were sacrificed. Sections of the aorta were stained with AT1R and ERK1/2 antibodies, and then counterstained with rhodamine and fluorescein isothiocyanate (FITC), respectively. These sections were quantified using confocal laser scanning microscopy. There were significant decreases in AT1R expression ($p<0.05$) and ERK1/2 phosphorylation ($p<0.05$) in hypoestrogenic rats treated with genistein in a dose-dependent manner. Genistein treatment decreases AT1R expression and ERK1/2 phosphorylation in a rat-based model of menopause.
Keywords: Genistein, Hypoestrogen, ERK 1/2 phosphorylation, AT1R expression.

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INTRODUCTION

The risk of cardiovascular disease is lower in pre-menopausal women but is increased in post-menopausal women (1). Estrogen depletion, the renin angiotensin system (RAS), and aging have been suggested as contributing factors for the development of cardiovascular disease (CVD) in post-menopausal woman. The RAS is a key system for regulating blood pressure and body fluid volume (2). Vasoconstriction, protein synthesis, and vascular remodeling induced by angiotensin II (Ang II) occur through the binding of Ang II to the Ang II receptor type I (AT1R). Upregulation of the vasoconstrictor/proliferative arm of RAS (ACE, Ang II, and AT1R) have been implicated in the adverse effects of CVD pathophysiology (3, 4).

The adverse effect of estrogen depletion, as well as the beneficial effects of both estrogen and genistein, a phytoestrogen, on ovariectomized rats have been proven experimentally. Ovariectomy in rats increased blood pressure (5) and AT1R expression (6), while estrogen (5,6) and genistein (6) administration decreased both. This suggests that RAS activation contributes to CVD in ovariectomized rats and treatment with either estrogen or genistein protects against the process. Genistein is widely used as a phytoestrogen in menopausal experiments. It is considered an alternative for hormone replacement therapy. Previous reports have shown that hormone replacement therapy did not demonstrate beneficial cardiovascular effects as studied by the Heart Estrogen/Progestin Replacement Study (HERS) I, II (9), Women's Health Initiative (WHI) (10), and Women's Health in the Lund Area (WHILA) (11).

One of the cellular responses responsible for the RAS effect on vasoconstriction and vascular smooth muscle cell (VSMC) growth is mediated by extracellular signal regulated kinase 1/2 (ERK1/2) phosphorylation (12). The attachment of Ang II to the AT1R causes a number of complex intracellular signals that activate protein kinase cascades synergistically to increase protein synthesis. An Ang II-mediated signaling event activates ERK 1/2-dependent pathways. Downstream signaling of Ang II ultimately influences cell differentiation, migration, and adhesion by binding to gene promoter sequences. The present study explored the possibility that treatment with the phytoestrogen genistein may decrease AT1R expression and its downstream ERK1/2 signaling in hypoestrogenic female *Rattus norvegicus*.

METHOD

Animal and Experimental Group

Experiments followed our institutional guidelines for the ethical care of animals (Faculty of Medicine, Brawijaya University, Malang, Indonesia). Female *Rattus norvegicus* (9–10 months old, 250–350 g) were obtained from the Pharmacology Laboratory, Brawijaya University, Malang, Indonesia. Rats were ovariectomized using 40 mg/kg ketamine as anesthesia. Twenty-eight days after surgery, rats were divided into five groups, i.e. control, bilateral ovariectomy (OVX), OVX + genistein 2.5 mg/kg/d, OVX + genistein 5 mg/kg/d, and OVX + genistein 10 mg/kg/d. Genistein was administered subcutaneously for four weeks. At the end of treatment, rats were sacrified.

AT1R Expression and ERK1/2 Phosphorylation Analysis

The aorta was quickly excised and immediately placed in a10 % formalin solution. The organ tissue was removed and fixed in Bouin's fluid for 24 hours. After fixation, the tissue was dehydrated through treatment with ascending grades of ethanol. Thereafter, it was cleared in xylene and finally embedded in paraffin wax. Specimens were sectioned into 5-µm thick slices and these sections were mounted on clean slides. Slides were stained with AT1R and ERK1/2 antibodies (p44/p42 MAPK (T202/Y204)) (Bioss Antibodies™) and then counterstained with rhodamine and FITC (Rockland), respectively. Rhodamine and FITC were incubated with the slides in PBS for 24 hours at 4°C (13). AT1R expression and phosphorylated ERK1/2 were quantified using a confocal laser scanning microscope (CLSM) Olympus type FV1000.

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Analysis

Statistical Analysis

Results are displayed as mean ± standard deviation. Data were first tested to determine whether they were normally distributed. Once all requirements were fulfilled, data were tested using one-way ANOVA test followed by post hoc Tukey multi-comparison test. SPSS (version 16) software program was used for these analyses.

RESULT

Effect of Genistein on AT1R Expression and ERK1/2 Phosphorylation

AT1R expression and ERK1/2 phosphorylation levels in OVX rats were higher compared to those in the controls. Treatment with genistein was able to decrease both AT1R expression and ERK1/2 phosphorylation in a dose-dependent manner. AT1R expression was observed as red fluorescence (Figure 1.1) while ERK1/2 phosphorylation was observed as green fluorescence (Figure 1.2).

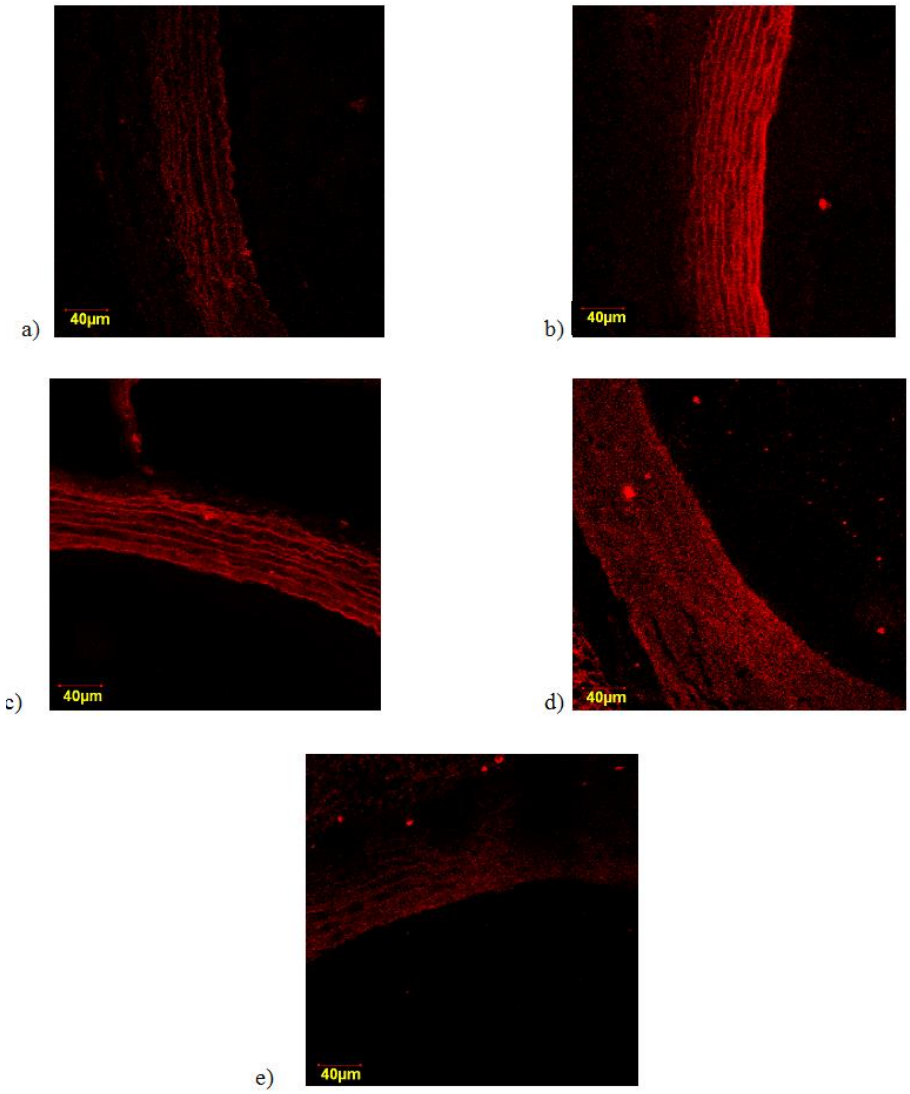

Figure 1.1. Immunofluorescent AT1R Expressioninthe Aorta (400× magnification) a) control b) OVX c) OVX + 2.5 mg/kg/d genistein d) OVX + 5 mg/kg/d genistein e) OVX + 10 mg/kg/d genistein. Fig 1.1.a shows weak fluorescence intensity in control rats. This is in contrast with Fig 1.1.b that shows significantly stronger fluorescence,which gradually lessens with the dose-dependent administration of genistein (Fig 1.1.c–1.1.e).

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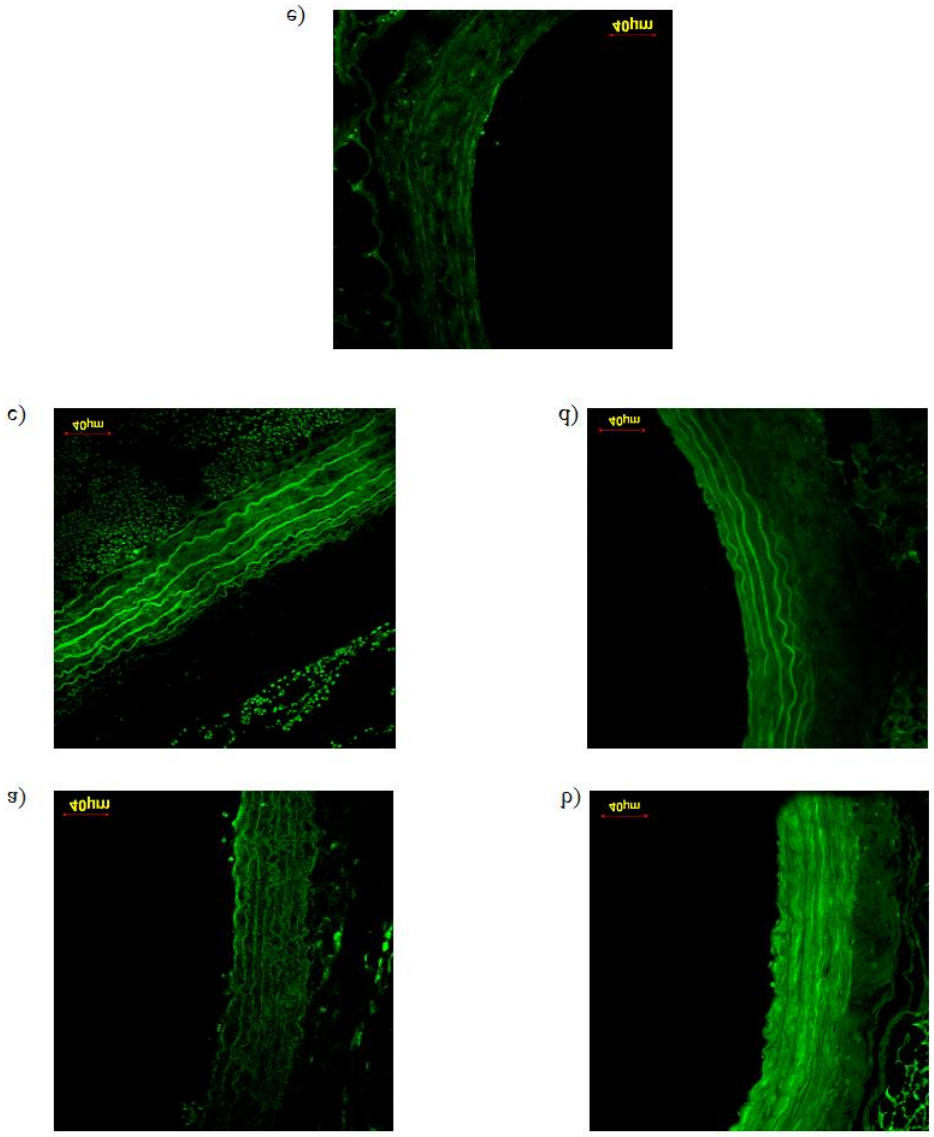

Figure 1.2 Immunofluorescent ERK1/2 in the Aorta (400× magnification) a) control b) OVX c) OVX + 2.5 mg/kg/d genistein d) OVX + 5 mg/kg/d genistein e) OVX + 10 mg/kg/d genistein. Fig 1.2.a shows weak fluorescence intensity in control rats. This is in contrast with Fig 1.2.b that has significantly stronger fluorescence, which gradually lessens with the dose-dependent administration of genistein (Fig 1.2.c-1.2.e).

Table 1.1 Effect of Genistein on AT1R Expression and ERK1/2 phosphorylation (data expressed as mean ± SD)

Treatment	N	AT1R Expression (arbitrary units)	ERK1/2 Phosphorylation (arbitrary units)
Control	4	495.2±51	631.6±49
Ovariectomized	4	1945±195	1269.7±97
Ovariectomized + Genistein 2.5 mg/kg/d	4	1346±226	1109.3±95
Ovariectomized + Genistein 5 mg/kg/d	4	1009.6±14	1069.2±32
Ovariectomized + Genistein 10 mg/kg/d	4	724.2±23	902.7±58

AT1R expression and ERK1/2 phosphorylation levels in OVX rats were higher compared to those in the controls. Treatment with genistein was able to decrease both AT1R expression and ERK1/2 phosphorylation in a dose-dependent manner.

DISCUSSION

We have previously shown that ovariectomy of adult (9 months) normotensive female rats resulted in a hypoestrogenic state and higher follicle stimulating hormone levels (14). Hence, ovariectomy in rats is a useful model for menopausal hormone loss accelerating the aging process. This study in 9 month old adult rats was designed to determine if ovariectomy upregulates AT1R expression and its downstream ERK1/2 signaling in the aorta, as well as to investigate whether genistein could attenuate this upregulation.

This experiment established that the hypoestrogenic state induced by ovariectomy significantly increased the expression of AT1R and its downstream phosphorylation of ERK 1/2 compared to the expression in the control group. It is known that estrogen destabilizes AT1R mRNA (15). The estrogen-dependent destabilization of AT1R at the mRNA and protein level is mediated by estrogen receptor (ER) activation and might also be through several nitric oxide (NO)-mediated pathways (16). As evidence for other possible mechanisms, studies observed that estrogen also increased the protein levels of the MAP kinase phosphatase (MKP) family, including MKP-1,14 in VSMCs (17) and rat cardiomyocites (18). Moreover, MKP family activation inactivated/dephosphorylated the ERK cascade (17). This result explained the molecular mechanism of the attenuation of ERK 1/2 phosphorylation by estrogen. Presumably, the increased AT1R and its downstream phosphorylation of ERK1/2 in the ovariectomized rats was due to hypoestrogen state.

This research showed that ovariectomy-induced increases in AT1R expression and ERK1/2 phosphorylation were abolished by genistein treatment in a dose-dependent manner. Genistein is a phytoestrogen widely used in menopausal model experiments. Vera *et al* observed that ovariectomy of spontaneously hypertensive rats (SHRs) increased aortic AT1R expression and the administration of either estrogen or genistein was able to decrease expression to control levels (6). Matrogui *et al* (2010) observed that genistein was able to inhibit Ang II-induced ERK1/2 phosphorylation in isolated mesenteric resistance arteries. Moreover, they determined that Ang II increased ERK1/2 phosphorylation via AT1R (19). Ishida *et al* (1998) also established that genistein decreased ERK1/2 phosphorylation in Ang II-exposed smooth muscle cell culture (20). Additionally, Xiang *et al* (2010) found that genistein inhibited angiotensin II-induced VSMC proliferation as well as decreasing the phosphorylation of ERK1/2 (21). Nevertheless, the mechanism utilized by genistein to reduce AT1R and ERK1/2 expression still needs to be elucidated.

Genistein reduced AT1R and phosphorylated ERK1/2 expression in a dose-dependent manner. However, data dose of 10 mg/kg/d, the reduction in expression of AT1R was not in accord with that of pERK1/2. This is likely due to other pathways used by Ang II to phosphorylate ERK1/2, aside from AT1R activation. Ang II also transactivates epidermal growth factor receptor (EGFR), leading to ERK1/2 phosphorylation and resulting in an increase of protein synthesis and smooth muscle cell migration (22).

CONCLUSION

In conclusion, genistein is able to decrease AT1R expression and diminish ERK1/2 phosphorylation in hypoestrogenic female *Rattus norvegicus*.

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