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Estradiol Down-Regulates Gene Expression of Angiotensin-II Receptor Type One in Ovariectomized Rats

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Abstract:

Both estrogen deficiency and activation of renin-angiotensin- aldosterone system (RAAS) are implicated in the development of cardiovascular diseases in postmenopausal women, therefore this study aimed to clarify the influence of estradiol (E2) on expression of angiotensin receptor type one (AT1R) in target tissues of ovariectomized rats and whether it can add to the antihypertensive effects of angiotensin receptor blockers (ARBs). Fifty adult female rats were equally divided into 5 groups: Control group, ovariectomized (OVX) group not receiving any treatment, OVX group treated with conjugated estrogen (premarin), OVX group treated with valsartan and OVX group treated with premarin and valsartan. Treatments started 4 weeks after ovariectomy and continued for 8 weeks. By the end of experiment serum levels of renin, angiotensin-II, aldosterone, Na+ and K+ were measured. Hearts, adrenal glands, and kidneys were removed to quantify the gene expression of AT1R. Ovariectomy resulted in a significant increase in serum concentrations of renin, angiotensin-II, aldosterone, Na+ and AT1R gene expression, as well as a significant decrease in K+ concentration compared to control group. These findings were improved in all groups received either estrogen and/or valsartan. The best results were achieved when both drugs were used together. In conclusion concomitant use of estrogen with ARBs may provide a more effective form of RAAS blockade than the monotherapy of either of them.

Keywords: ***Menopause, Hypertension, Estrogen, RAAS, Valsartan and AT1R.***

1. Introduction:

Cardiovascular disease is a leading cause of death in postmenopausal women worldwide.[1] It is well known that estrogen deficiency increases the risk of developing hypertension, coronary atherosclerosis, and myocardial infarction in postmenopausal women[2],[3]

In addition to estrogen deficiency, another recognized factor implicated in the pathogenesis of hypertension, atherosclerosis, and congestive heart failure is activation of the renin-angiotensin- aldosterone system (RAAS).[4] Angiotensin-II (Ang II) is the principle effector of the renin-angiotensin system that makes critical contributions to the pathogenesis of hypertension, atherosclerosis, vascular and myocardial remodeling, and congestive heart failure.[5]

Ang II is also, a potent stimulator of NADPH oxidase, which is the major source and primary trigger for reactive oxygen species (ROS) generation in various tissues. Recently, the importance of oxidative stress in Ang II-induced heart diseases has been confirmed.[4]

Recent accumulating evidence has demonstrated that angiotensin receptor type one (AT1R) is the angiotensin receptor involved in most of the classical effects of Ang II such as oxidative stress generation and vasoconstriction. Via these effects ANG-II could induce blood pressure elevation and cardiovascular injury.[6] Accordingly, specific AT1R blockers (ARBs) dramatically lower blood pressure and improve vascular and myocardial function in patients with cardiovascular disease.[7]

Therefore, this study was conducted to investigate the influence of estrogen on expression of AT1R in target tissues (heart, kidney and adrenal glands) of ovariectomized rats and whether it can add to the antihypertensive effects of angiotensin receptor blocker.

2. Material and methods

Drugs:

- **Conjugated equine estrogen (Premarin):** Premarin tablets (each one containing 0.625 mg of conjugated estrogen) were obtained from Wyeth pharmaceuticals Inc., now a part of Pfizer

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Philadelphia, Canada, USA. The tablets were grounded and dissolved in distilled water just before administration.

- **Valsartan (Vasotec):** Vasotec tablets (each one containing 80 mg of valsartan) were obtained from EIPICO pharmaceuticals company, Egypt. The tablets were grounded and dissolved in distilled water just before administration.

Location and duration of study:

Fifty adult female albino Wister rats, weighing 140–160 gm, were used in this study. The animals were housed in plastic cages (45 x 30 x 20 per 5 rats) in the laboratory of pharmacology department, National Research Center, El Buhouth St., Dokki, Cairo. Rats were maintained on commercial rat chow and water ad libitum, under the prevailing atmospheric conditions all over the experimental period. The experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals. After two weeks of acclimatization, the rats were randomized into 5 equal groups; ten rats each then 4 groups were subjected to ovariectomy. Treatments started 4 weeks after ovariectomy and continued for 8 weeks as follows.

Experimental design and grouping:

Group 1(Control): Rats subjected to sham surgery and served as control - I group.
Group 2 (OVX): Rats exposed to ovariectomy and served as control - II group.
Group 3 (Estrogen group): Rats treated with conjugated estrogen (50µg/kg BW /day).[8]
Group 4: (Valsartan group): Rats, treated with Valsartan (10 mg/kg BW /day)[9]
Group 5 (Estrogen/ Valsartan group): Rats treated with conjugated estrogen (50µg/kg BW /day) and Valsartan (10 mg/kg BW /day).

Experimental Procedures:

Ovariectomy: Bilateral ovariectomy was performed as described by Khajuria et al.,[10]
Measurement of the arterial blood pressure (ABP): ABP was measured (before and two weeks after ovariectomy, after one month and two months of treatment) by the non-invasive blood pressure monitor (Ugo, Basile-Italy) by the tail cuff technique for which all animals were trained before the measurement to minimize any stress.[11]
Sample collection: The animals were anesthetized at the end of the experiments and blood samples were obtained from the orbital sinus of overnight fasted rats. Blood was immediately centrifuged at 3000 rpm for 10 minutes. Sera were separated and stored at -80◦C till used for measurement of renin, angiotensin-II, aldosterone, Na^+ and K^+. Animals were sacrificed by over dose of anesthetic ether and tissues (kidneys, adrenal glands and hearts) were removed and stored at -70◦C in lysis buffer till used to quantify AT1R number by Quantitative Real Time PCR.

Determination of biochemical parameters:

1. Measurement of renin: Serum renin was estimated by Quantitative Sandwich ELISA kit, supplied by My Bio Source, Inc., California, USA.[12]
2. Measurement of angiotensin-II: Angiotensin-II level was estimated by the Enzyme Immunoassay kit, supplied from DRG International, Inc.,[13]
3. Measurement of aldosterone: Serum aldosterone was estimated by using aldosterone (ALD) Elisa kit supplied by MyBioSource, Inc., California, USA,[14]
4. Measurement of Na^+: Serum Na^+ was estimated by Sodium Enzymatic Assay Kit, manufactured by Gentaur molecular products, Kampenhout, Belgium.[15]
5. Measurement of K^+: Serum K^+ was estimated by Potassium Enzymatic Assay Kit, manufactured by BioSupply UK Ltd,[16]

Detection of tissue angiotensin-II receptor type 1 (AT1R) gene expression with real time-polymerase chain reaction (RT-PCR): Total RNA was extracted from frozen tissue samples using the RNeasy Mini Kit (Qiagen Inc) following the manufacturer's protocol. Real-time RT-PCR for quantitative assessment of mRNA expression was performed on step one plus (Applied Biosystems, USA).[17]

Statistical analysis: The data are expressed as mean ± SD for each group. Results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey Kramer test for multiple comparisons, $P < 0.05$ was considered as being significant in all types of statistical test.

3. Results:

Biochemical findings and AT1R gene expression:

Ovariectomy (OVX) resulted in a significant increase in serum concentrations of renin, angiotensin-II, aldosterone, Na^+ and AT1R gene expression in heart, adrenal glands and kidney, as well as a significant decrease in K^+ concentration compared to control group.

Treatment with conjugated estrogen resulted in improvement in all parameters as manifested by a significant decrease in serum concentrations of renin,

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angiotensin-II, aldosterone, Na^+ and AT1R gene expression in heart, adrenal glands and kidney, as well as a significant increase in K^+ concentration compared to OVX group. However, none of these returns to normal level.

Treatment with Valsartan improved all parameters as manifested by a significant decrease in serum concentrations of renin, angiotensin-II, aldosterone, Na^+ and AT1R gene expression in heart, adrenal glands and kidney, as well as a significant increase in K^+ concentration compared to OVX group. However, only serum levels of renin and AT1R gene expression in heart returned to normal levels. Valsartan induced significant changes in all parameters (a significant decrease in serum concentrations of angiotensin-II, aldosterone, Na^+ and AT1R gene expression in heart, adrenal glands and kidney, as well as a significant increase in K^+ concentration) compared to estrogen group except for renin.

Treatment with both conjugated estrogen and Valsartan resulted in much more improvement in all parameters compared to OVX, estrogen and Valsartan groups with returning of renin, angiotensin-II, aldosterone and AT1R gene expression in heart to their normal levels **(Table 1)**.

Table 1: Serum levels of renin, angiotensin-II, aldosterone, Na^+, K^+ and AT1R gene expression in various groups at the end of the treatment period (Mean ± SD)

Groups / *Parameters*	*Control*	*OVX*	*Estrogen*	*Valsartan*	*Estrogen/ Valsartan*
Renin (ng/ml)	8.7±1.6	30.4±6.5^{a}	14.8±2.7^{ab}	13.2±1.9^{b}	10.5±0.31^{bcd}
Angiotensin-II (ng/l)	7.5±1.2	52.1±6.7^{a}	27.6±3.6^{ab}	14.6±3.9^{abc}	12.9±1.6^{bcd}
Aldosterone (pmol/l)	11.7±0.96	69.6±3.9^{a}	48.3±2.4^{ab}	18.9±1.5^{abc}	16.6±2.1^{bcd}
Na^+ (mEq/l)	140.5±2.6	678.4±58.6^{a}	359.3±38.7^{ab}	233.3±43^{abc}	209.9±11.6^{abcd}
K^+ (mEq/l)	5.02±0.29	2.7±0.4^{a}	3.2±0.56^{ab}	3.97±0.42^{abc}	4.2±0.48^{abcd}
AT1R (IU/ml) in heart	1.01±0.02	9.6±1.3^{a}	3.9±1.1^{ab}	1.1±0.12^{bc}	1.07±0.54^{bc}
AT1R (IU/ml) in adrenal gland	1.02±0.01	13±1.7^{a}	5.6±1^{ab}	2.7±0.5^{abc}	1.4±0.3^{abcd}
AT1R (IU/ml) in kidney	1.03±0.3	13.6±0.98^{a}	6.8±0.35^{ab}	2.4±0.66^{abc}	1.2±0.61^{abcd}

(a) Significant values versus control group.
(b) Significant values versus OVX group.
(c) Significant values versus estrogen group.
(d) Significant values versus valsartan group.

Changes in systolic blood pressure:

No significant changes were found in the level of SBP of control rats throughout the experimental period (12 weeks). OVX induced significant increase in SBP after 4 weeks compared to pre-OVX value. Also, the 8 weeks value was significantly increased compared to pre-OVX value.

After 8 weeks of treatment, estrogen induced significant decrease of SBP. Similar results were found in valsartan treated group with non-significant changed compared to estrogen treated group. Treatment with both estrogen and valsartan returned the SBP to its normal level **(Table 2)**.

Table 2: Changes in systolic blood pressure (SBP) in various groups at the end of the treatment period (Mean ± SD)

Measurements / *Groups*	*Systolic blood pressure (mm Hg)*		
	Before ovariectomy	*After 4 weeks of ovariectomy*	*After 8 weeks of treatment*
Control	72.6±1.4	72.5±3.8	73.3±3.3
OVX		92.3±3.9^{a}	115.7±3.6^{a}
Estrogen			79.5±4.8^{ab}
Valsartan			80.5±3.9^{ab}
Estrogen/ Valsartan			73.4±3.7^{bcd}

(a)Significant values versus control group.
(b) Significant values versus OVX group.
(c)Significant values versus estrogen group
(d) Significant values versus valsartan group.

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4. Discussion:

In the present study, ovariectomy resulted in activation of the RAAS as manifested by a significant increase in the serum levels of renin, aldosterone and angiotensin-II and also induced significant increase in the serum levels of sodium and a significant decrease in serum potassium levels. The examination of AT1R gene in ovariectomized rats showed a significant increase in its expression in all examined tissues (heart, adrenal glands and kidney) together with increased systolic blood pressure.

These results are consistent with several experimental animal studies.[18],[2] Also, others reported that two weeks following ovariectomy the mRNA expression of renin gene was up-regulated resulting in high levels of renin, suggesting that estrogen deficiency affected the regulation of the renin-angiotensin-aldosterone system.[19] The ovariectomy of diabetic rats resulted in increased plasma levels of renin and increased ACE activity which led to an increase in ANG-II levels.[20] Moreover, in premenopausal women, plasma aldosterone levels were lower than in men but, the difference in plasma aldosterone levels disappeared after menopause.[21]

A previous study suggested that estrogen loss caused by OVX can amplify the activity of the RAAS by increasing AT1R density in ANG-II target tissues including the kidney and adrenal gland and by increasing angiotensin converting enzyme activity in several tissues that would increase ANG-II levels and aldosterone secretion.[22]

Similar to our results, it was reported that ovariectomy increases catecholamines levels and increases reabsorption of Na^+ and Cl^- in the kidneys through increasing AT1R expression that modulate glomerular blood flow and transport processes within the renal tubules.[23]

Moreover, the blockade of basal NO synthesis by OVX has been shown to decrease renal blood flow and sodium excretion.[24] In addition, OVX result in detectable increases in plasma aldosterone levels.[25] Also renal AT1R densities were higher in the glomeruli and proximal tubules of OVX rats compared with intact ones. AT1Rs act in the kidney to regulate the expression of ion channels and transporters, and thus increased AT1R activity modulates the ability of aldosterone to regulate potassium excretion, resulting in a decrease in the K^+ levels.[26]

In agreement with the results of this work, others reported that estrogen deficiency led to up regulation of vascular AT1R expression accompanied by an increased effect of ANG-II in heart tissues,[27] kidney and adrenal glands.[28]

The results of the present study showed that ovariectomy resulted in a significant increase in the systolic blood pressure after 2 weeks, 4 weeks and 8 weeks of ovariectomy. Similarly, other studies demonstrated that after ovariectomy of normotensive rats it had an elevation in systolic blood pressure. Deficiency in estradiol directly results in the development of hypertension by increasing the production of vasoconstrictor factors and by stimulating the activities of renin–angiotensin-aldosterone system as well as sympathetic nervous system.[29],[30]

Ovariectomy also increased constrictor responses to ANG-II and decreased dilatory responses to ANG-(1-7).[31]

The results of the current study, revealed that estrogen (premarin) treatment resulted in a significant decrease in the serum levels of renin, aldosterone, angiotensin-II and sodium while, there was a significant increase in the serum levels of potassium. Also, there was a significant decrease in the gene expression of angiotensin-II type I receptors in the heart tissue, kidney and adrenal glands with reduction of the systolic blood pressure.

In ovariectomized female rats, estrogen decreases plasma angiotensin-II levels, renin and ACE activities thereby, leading to a decreased conversion of ANG-I to ANG-II.[32] Estrogen also reduces tissue responsiveness to ANG-II.[33] Estrogen-induced reduction of the levels of aldosterone secretion may contribute to the cardiovascular benefits associated with estrogen by decreasing the overall mineralocorticoid activity in OVX mice.[34]

In accordance to our findings, a study carried out by Stachenfeld and Taylor found that estrogen treatment in ovariectomized rats decreased serum sodium levels. Estrogen may increase the release of arginine vasopressin.[35] Furthermore, reduced Na^+-K^+ ATPase pump activity by estrogens was observed in female rats.[2] Moreover, estrogen treatment in OVX female rats increased serum levels of K^+ through inhibiting the effects of aldosterone on plasma electrolytes.[36]

Our present study demonstrated that OVX rats treated with premarin showed a significant decrease in the gene expression of angiotensin-II type I receptors in the kidney tissue. Our results are in agreement with others, who reported that in the adrenal gland, kidney and heart of ovariectomized rats, subcutaneous administration of estrogen decreases AT1R density.[37],[27] Also, estrogen down regulates AT1R gene expression in OVX rats treated with estrogen, through estrogen sensitive binding proteins which are thought to post-transcriptionally regulate AT1R mRNA.[38],[31]

In contrast to our results, a study showed that regulation of AT1R by estrogen might depend on the specific animal model, as well as the existence of other regulatory influences such as a high salt intake.[30]

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Our results demonstrated that in OVX rats treated with premarin there was no change in systolic blood pressure after 4 weeks of treatment but at the end of experimental period there was a significant decrease in systolic blood pressure. Our results are in line with a study done by Luo et al., who reported that treatment with estrogen decreased systolic blood pressure, and indicates a potential benefit of estrogen therapy through a mechanism that involves dampening of the protective counter-regulatory axis of RAAS. Estrogen has also been shown to reduce ANG-II levels by decreasing the tissue and serum ACE activity, thereby leading to a decreased conversion of ANG-I to ANG-II.[39]

The results of the present study demonstrated that OVX rats treated with Valsartan showed a significant decrease in the serum levels of renin, aldosterone, angiotensin-II and sodium while, there were a significant increase in the serum levels of potassium. Also, there was a significant decrease in the gene expression of angiotensin-II type I receptors in the heart tissue, kidney and adrenal glands. Also, the systolic blood pressure was lowered. However, only serum levels of renin and AT1R gene expression in heart returned to normal levels.

The angiotensin- II (ANG- II) receptor antagonist valsartan acts competitively at, and is selective for, the angiotensin-II AT1R subtype, which is responsible for most of the known effects of angiotensin- II, preventing its actions. Also the level of ANG- II may cause a negative feedback on renin release.[40] In post-myocardial infarction rats receiving valsartan, the levels of plasma aldosterone were significantly reduced simultaneously with AT1R blockade.[41]

In contrast to our results Nagasawa et al., found no change in plasma renin activity in five-week-old male inbred Dahl salt-sensitive rats fed on 8% NaCl diet and were treated with valsartan. However, the age, the species and the model used are different from the current study.[42]

Our data showed that OVX rats treated with valsartan had a significant decrease in the gene expression of angiotensin-II type I receptors in various tissues. ARBs inhibit the binding of sensitive transcription factors such as stimulator protein 1 (SP1) and inhibition of the binding of SP1 would be predicted to decrease transcription of the gene.[43]

Akazawa et al., suggested that angiotensin receptor blockers (ARBs) could be of clinical advantage in inhibition of both ANG-II activation and overexpression of AT1 receptor, as valsartan, irbesartan and losartan can reduce the constitutive GTPase stimulating activity of AT1Rs.[44]

Our present study demonstrated that OVX rats treated with valsartan showed a significant decrease in systolic blood pressure after 4 weeks of treatment and at the end of experimental period. ARBs reduce blood pressure by decreasing systemic vascular resistance; results from a combination of inhibition of ANG-II-mediated vasoconstriction, reduced sympathetic nervous system activity, and reduced extracellular volume (by direct inhibition of proximal sodium reabsorption and by inhibition of aldosterone release).[45],[42]

Our results demonstrated that the lowest levels of renin, aldosterone, angiotensin-II and gene expression of angiotensin- II type I receptors in various tissues were among OVX rats treated with both Premarin and Valsartan after control group and the highest levels among were in OVX group. Our data go with the study of Mirza et al., who suggest additive actions of both drugs on RAAS.[33]

Moreover, the results of the present study demonstrated that the combination of premarin and valsartan showed a significance decrease in serum Na^+ levels with a significance increase in serum K^+ levels. This was possibly due to additive action of both drugs on lowering aldosterone levels that affect potassium excretion.[46] Our results demonstrated that rats treated with both Premarin and Valsartan having the nearest levels to normal control group in most of the parameters, so we could consider it the best combination group in our study.

In a vascular injury mice model, olmesartan (AT1R blocker) and E2 co-administration decreased blood pressure and inhibited neointima formation (a marker of vascular injury) suggesting an augmentation of the hypotensive protective effect of olmesartan by E2.[47] Others found that, concomitant administration of estrogen and AT1R blockers might be effective for protection of the heart and the kidney in OVX rats with chronic heart failure.[48]

5. Conclusion:

Concomitant use of estrogen with ARBs may provide a more effective form of RAAS blockade than the monotherapy of either of them. Further studies on estrogens are needed to find biological mechanisms through which estrogen confers its beneficial effects on the cardiovascular system, independent of the hormone's actions on cancer.

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