Original article | DOI: 10.26402/jpp.2023.3.03

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PERINDOPRIL AND LOSARTAN ATTENUATE PRO-COAGULATION FACTORS IN HUMAN ADIPOCYTES EXPOSED TO SARS-COV-2 SPIKE PROTEIN

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Thrombotic events are highly prevalent in coronavirus disease 2019 (COVID-19), especially in patients presenting with risk factors of adverse outcomes such as obesity. Recently, the associations between the angiotensin converting enzyme 2 (ACE2) pathway and thrombosis have been reported. Angiotensin-converting enzyme inhibitors (ACEI) and angiotensin II receptor blockers (ARBs) are widely used cardiovascular pharmacologic agents that upregulate ACE2 levels. An observation of the alterations in pro-coagulation factors after exposure to ACEIs and ARBs may provide valuable insight into the thrombosis mechanism and how it may relate to ACE2. This study use adipose tissue harvested from an obese male donor was isolated and exposed to perindopril, losartan, and ACE2 recombinant as binding assay, following exposure with 10 nm of SARS-CoV-2 S1 spike protein. After 48 hours, tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) as pro-coagulation factors as well as ACE2 levels and binding evaluated. The results shows TF level was significantly reduced in Perindopril group compared to control (4.834; p=0.005), while a non-significant reduction was observed in Losartan group (5.624; p=0.111). However, Losartan group showed a better reduction of PAI-1 levels (2.633; p≤0.001) than Perindopril group (3.484; p=0.001). These findings were consistent with the observations in ACE2 recombinant group, suggesting that both drugs lowered the bindings of ACE2 and SARS-CoV-2 spike proteins. This study indicated that both perindopril and losartan may attenuate pro-coagulation factors in human adipocytes exposed to SARS-CoV-2 spike proteins, and therefore showcased a potential role of ACE2 in the mechanism of COVID-19-related thrombosis. Further investigation in non-COVID-19 populations should commence and may be of value to expanding this potential in general cardiovascular diseases.

Key words: *coronavirus disease 2019, perindopril, losartan, angiotensin converting enzyme 2, SARS-CoV-2 S1 spike protein, thrombosis, angiotensin II receptor blockers, adipocytes, tissue factor, plasminogen activator inhibitor-1*

INTRODUCTION

The pandemic coronavirus disease 2019 (COVID-19) has caused high mortality and morbidity throughout the world, especially in COVID-19 infections with severe symptoms (1). Endothelial dysfunction increases the risk of thromboembolic complications in COVID-19 patients (2, 3). An increase in coagulation and platelets (tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1)) was observed in earlier trials by Al-Tamimi *et al.*, particularly in COVID-19 patients with moderate to severe symptoms (4). Additionally, tissue plasminogen activator (TPA) and PAI-1 promote spontaneous fibrinolysis, which is substantially correlated with mortality in certain COVID-19 patients, according to Zuo *et al.* (5). Furthermore, endothelial cells can release proinflammatory cytokines, like tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), which in turn increase PAI-1, as well as TF expression in the endothelial layer (6).

The use of cardiovascular medications like ACEIs and ARBs in cases of COVID-19 is controversial whether they have a therapeutic effect or the exact opposite. Some early studies suggested that renin-angiotensin-system (RAS) inhibitors might increase the risk of severe COVID-19 infection. This was based on the observation that the virus uses the ACE2 receptor to enter cells, and RAS inhibitors can increase the expression of ACE2. Baral's meta-analysis found no evidence to support the advice of medical societies that using ACEIs, or ARBs increased the risk of multivariable-adjusted mortality and serious adverse events in COVID-19 patients who also had hypertension or numerous comorbidities (7). The latter occurs because not all ARBs or ACEIs consistently affect the expression of ACE2 (8). On the other hand, the prevention of cytokine storms and increased ACE2 is one of the effective approaches to prevent severe symptoms of COVID-19 (9). Perindopril, an ACEI, and losartan, an ARB, have demonstrated to promote ACE2 expression (10). However, no studies have been conducted on the effects of these medications on coagulation and platelet factors. Therefore, it is necessary to determine the role of these drugs on coagulation and platelets in adipocyte exposed to SARS-CoV-2.

MATERIALS AND METHODS

Primary culture of adipocyte

This research is an experimental *in vitro* study, with a post-test only Control group design, conducted in the biosafety level 2 (BSL 2) laboratory at the Department of Physiology of the Faculty of Medicine, Brawijaya University in Malang City, Indonesia.

This study used adipose tissue from visceral abdomen obtained from an obese male donor of around 30–50 years of age. The adipose tissue was isolated enzymatically, according to Carswell *et al.* (11). The adipocytes were isolated using collagenase type 1 and grown in a media of alpha-minimum essential medium (a-mem) with the addition of supplements like penicillin, streptomycin, and platelet rich plasma (PRP). Incubation was performed at 37°C in a 5% CO_2 environment.

SARS-CoV-2 subunit S1 spike protein exposure to adipocyte

This study used the SARS-CoV-2 S1 spike protein 10 nm subunit, based on previous studies that tested the effect of SARS-CoV-2 protein on the stimulation of human immune cells (12). 10 nm subunit of SARS-CoV-2 S1 spike protein was added into 100 µL of cells suspension per well and incubated overnight.

Administration of losartan and perindopril

Adipocyte cells are divided into 5 groups. The first group is a Negative Control group. The second group is a group which exposes to SARS-CoV-2 S1 spike protein as a Positive Control group. The third group is the Losartan group which given 0.7 µM of losartan (13). The fourth group is Perindopril group which given 0.5 µM of perindopril (14). The fifth group is given 100 µg/mL of ACE2 recombinant (15).

Angiotensin converting enzyme 2-spike protein binding assay

A binding test kit was used to gauge how losartan, perindopril and ACE2 recombinant affected the interaction between the SARS-CoV-2 spike protein and ACE2. Reagents were made by combining 100 mL of 100 × ACE2 protein concentrate with 1.25 µL of losartan, perindopril or ACE2 recombinant. The relevant SARS-CoV-2 spike protein-coated wells were filled with each test substance, and they were then incubated at 4°C overnight. Each well was washed four times with 300 mL of wash buffer before being incubated with 100 mL of detection antibody for an hour at room temperature. Following more washing, 50 mL of stop solution, 100 mL of TMB substrate, and 100 mL of HRP-conjugated anti-IgG were progressively added to each well. At 450 nm, the solution absorbance was immediately measured.

Measurement of tissue factor and plasminogen activator inhibitor-1

ACE2 levels was carried out 48 h after administration of perindopril and losartan (13, 14). The sample was then given a human tissue factor antibody (BTLAB/E1195Hu) from the ELISA kit (BT LAB, Shanghai, China) and then incubated for 48 h. The results were evaluated with an ELISA Reader at λ 450 nm. The same procedure was followed during the PAI-1 examination, but a human plasminogen activator inhibitor antibody (BTLAB/E1159Hu) was used instead. Each group was run in triplicate to maintain data validity.

Statistical analysis

Data obtained and analyzed using SPSS 23.0. One-way ANOVA was used to compare the groups. The difference was considered to be significant if $p<0.05$.

RESULTS

We assessed the impact of SARS-CoV-2 spike protein on adipocyte cell ACE2 level *in vitro*. SARS-CoV-2 S1 spike protein exposure was successfully carried out on adipocyte culture after 48 hours of incubation. The results showed that SARS-CoV-2 S1 spike protein exposure could increase ACE2 levels (80.31) compared to baseline (14.48) ($p<0.001$). We used perindopril and losartan to define the role of ACEIs/ARBs on ACE2 levels in infected adipocyte cells. Different results were shown by the Perindopril and Losartan groups. Adipocytes with losartan admission showed a higher ACE2 level (150.98) than the Control groups ($p<0.001$). In contrast, the Perindopril group demonstrated significantly lower ACE2 levels (47.54) than the Control groups ($p<0.001$). If we compare the Perindopril and Losartan groups, both have significant differences ($p<0.001$). To determine the binding of the SARS-CoV-2 spike protein to the ACE2 receptor, we used the recombinant ACE2 protein as a competitor. The results showed that the ACE2 protein recombinant had lower ACE2 levels than the Positive Control ($p<0.001$) and was not significantly different from the baseline ($p=0.516$). Next, we assessed the differences in ACE2 levels between Perindopril and Losartan groups compared to the recombinant ACE2 protein. The ACE2 protein recombinant differed significantly from the Perindopril or Losartan group ($p<0.001$). The mean of each variable is shown in *Table 1*.

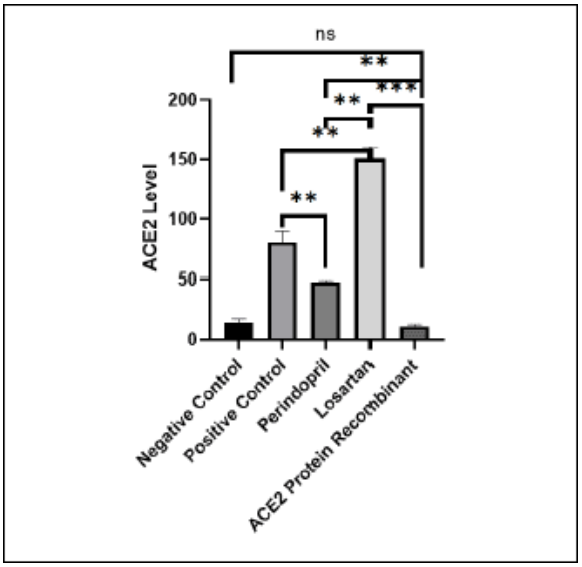

*Fig. 1.*Effect of administration of perindopril, losartan and ACE2 protein recombinant in ACE2 levels.

Table 1. Mean and standart deviation of angiotensin-converting enzyme 2 (ACE2), tissue factor (TF), and plasminogen activator inhibitor-1 (PAI-1) levels in each group.

Groups	Replication (n)	ACE2 (ng/mL)		Tissue factor (ng/mL)		PAI-1 (ng/mL)	
		Mean ±SD	Min–Max	Mean ±SD	Min–Max	Mean ±SD	Min–Max
Negative control (baseline)	3	14.48 ±2.756	11.73–17.24	2.993 ± 0.641	2.43–3.69	1.956 ±0.076	1.88–2.03
Positive control	3	80.312 ±9.312	71.00–89.62	6.857 ±0.228	6.60–7.02	4.865 ±0.115	4.75–4.98
Perindopril	3	47.54 ±1.36	46.05–48.71	4.843 ±0.396	4.43–5.22	3.484 ±0.252	3.22–3.72
Losartan	3	150.98 ±9.47	141.37–1160.32	5.624 ±0.606	5.01–6.22	2.633 ±0.269	2.40–2.93
ACE2 protein recombinant	3	11.107 ±1.577	10.13–12.93	4.121 ±0.437	3.76–4.61	3.375 ±0.443	3.10–3.89

The study showed that SARS-CoV-2 S1 protein spike exposure can also increase TF levels (6.857) compared to baselines (2.993) ($p<0.001$). The Perindopril group was able to reduce the TF value (4.843) significantly compared to the control ($p=0.005$). Conversely, losartan was able to decrease the value of TF (5.624) compared to the Positive Control, but the difference was not particularly significant ($p=0.111$). There was no discernible difference in TF values between the Perindopril and Losartan groups ($p=0.772$). To determine the impact of binding between the SARS-CoV-2 spike protein and the ACE2 receptor on pro-coagulation factors, we used the recombinant protein ACE2 that competes for binding to the ACE2 receptor. The results showed that the ACE2 protein recombinant had lower TF levels (4.121) than the positive control ($p<0.001$). Next, we assessed the difference in the ability of the Perindopril and Losartan groups to the recombinant protein ACE2 in reducing TF levels. The results showed that the Perindopril group had TF levels that were not much different from the recombinant ACE2 protein group ($p=0.09$). In contrast, the Losartan group had higher TF levels than the recombinant ACE2 protein group with a significant difference ($p=0.004$).

Table 2. Summary of p-values of ACE-2, TF, PAI-1 in all groups.

Comparison Test	ACE-2	TF	PAI-1
	p value	p value	p value
Negative Control vs. Positive Control	<0.0001	<0.0001	<0.0001
Negative Control vs. Perindopril	0.0005	0.0061	0.0003
Negative Control vs. Losartan	<0.0001	0.0004	0.0654
Negative Control vs. ACE2 Protein Recombinant	0.9578	0.0983	0.0005
Positive Control vs. Perindopril	0.0005	0.0033	0.0006
Positive Control vs. Losartan	<0.0001	0.065	<0.0001
Positive Control vs. ACE2 Protein Recombinant	<0.0001	0.0003	0.0003
Perindopril vs. Losartan	<0.0001	0.345	0.019
Perindopril vs. ACE2 Protein Recombinant	0.0002	0.4153	0.9864
Losartan vs. ACE2 Protein Recombinant	<0.0001	0.0232	0.0406

Other results show that SARS-CoV-2 S1 protein spike exposure can increase PAI-1 levels (4.865) compared to baseline (1.956) ($p<0.001$). The Perindopril-administered groups were able to

lower PAI-1 levels (3.484) compared to the Control group (p=0.001), but the Losartan-administered groups did so more effectively (2.633) (p<0.001). Compared to Perindopril group, the Losartan group was proven to significantly reduce PAI-1 levels (p=0.028). Compared with the recombinant ACE2 protein group, the Perindopril group had PAI-1 levels that were not much different (P=0.624). In contrast, the Losartan group had lower PAI-1 levels than the recombinant ACE protein, with a significant difference (0.006). The comparison of each group describes in *Fig. 2*.

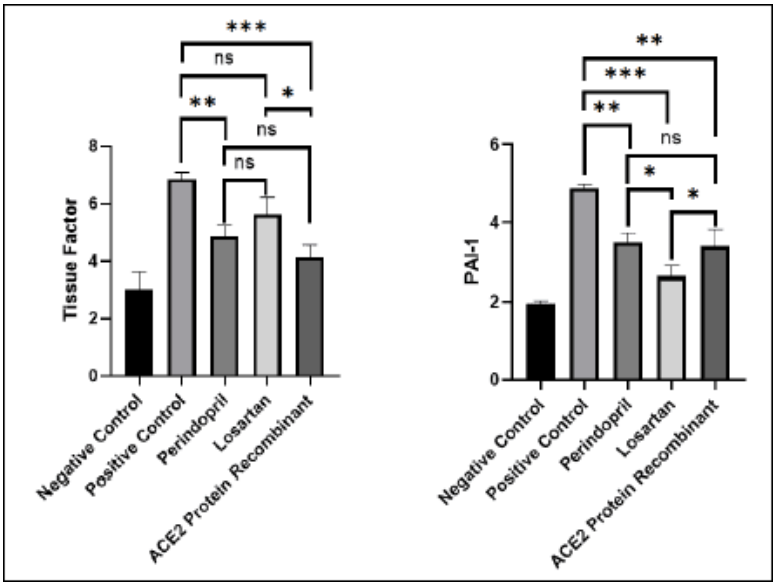

Fig. 2.Effect of administration of perindopril, losartan and ACE2 protein recombinant in TF and PAI-1 levels.

To prove the role of ACE2 on tissue factor and PAI-1 levels, in our study examines the correlation between ACE2, tissue factor and PAI-1 levels. The results show there is a strong significant correlation between ACE2 levels with tissue factor (p=0.01), but the other results show that there is no correlation between ACE2 levels with PAI-1 (p=0.735). The correlation of ACE2, TF, and PAI-1 described in *Table 3*.

Table 3. Pearson correlation between ACE2 levels to TF and PAI-1.

		Dependent variable	
		Tissue factor	PAI-1
ACE2 level	Pearson correlation	0.640	0.096
	Sig (2-tailed)	**0.01***	0.735
	N	15	15

*Correlation is significant at the 0,05 level (2-tailed) with strong correlation

DISCUSSION

Our research showed that the adipocyte cells given the SARS-CoV-2 S1 protein spike have a significant increase in ACE2 levels compared to baseline. Patel *et al.* showed the same evidence in their research on ACE2 plasma activity in COVID-19 patients; ACE2 activity was proven to increase during SARS-CoV-2 exposure and to continue increasing afterwards (16). Increased ACE2 levels are related to an increase in shedding from infected cells and are associated with the downregulation of membrane bound ACE2 (17).

Perindopril seem to take on opposite roles in our study, previous studies found that the administration of a single dose of Lisinopril caused a significant increase in ACE2 compared to the positive control (18), but in our study the ACE2 expression is increase in Perindopril administration group but not as high as positive control. Another study of human alveolar cells showed a sharp increase in the expression of the ACE2 gene, 24 h after giving Captopril (19). While on Losartan there is a significant increase and higher than the positive control. The difference between the effects of perindopril and losartan in our research is allegedly related to the way each therapy modulates ACE2. Although both ACEIs and ARBs directly affect ACE2, they may have different impacts on ATII. ACEIs work by reducing circulating ATII, while ARBs increase free ATII levels in the circulation (20, 21). The three mechanisms by which ATII affects ATII type 1 receptors to reduce ACE2 regulation are as follows: 1) ATII decreases ACE2 expression by inducing ERK1/2 or p38 MAPK; 2) ATII causes ACE2 shedding from the cell surface by disintegrin and metalloprotease ADAM17; 3) ATII causes the internalization of ACE2 to enter the cell (22).

ACE2 plays an important role in preventing increased TF exposure and PAI-1 levels, thereby inhibiting an increase in extrinsic path activation and decreased fibrinolysis. Both are responsible for

the incidence of thrombotic complications in COVID-19 patients (23). TF activation causes a disruption of fibrin disposal, disseminated intravascular coagulation (DIC), consumption of fibrin, and a decreased platelet count (24). A prothrombotic condition is linked to the inhibition of the ACE2/AT1-7/MasR axis because ACE2 stimulates tissue plasminogen activator (tPA). The endothelium and smooth muscle cells produce and secrete PAI-1 in response to ATII via the AT1R (25). However, there was no appreciable difference between losartan and the positive control in terms of lowering TF levels. We presume that the functional distinctions between ACEIs and ARBs also affects TF regulation via the function of ATII (20, 21). Nevertheless, in our research, there was no significant difference in effect between the ACEI and the ARB in reducing TF levels.

Endothelial PAI-1 is produced by the molecules that cause endothelial cell damage, including TNF, TGF, ATII, and thrombin (26). Adipose tissue is the major source of circulating PAI-1 (27). This clarifies how obesity raises the risk of thromboembolism, hospitalization, and COVID-19-related death (28). Our results also showed that PAI-1 levels significantly increase in the positive control groups compared to baseline. The drugs perindopril and losartan were successful in significantly lowering PAI-1 levels. These findings indicate that ACEI/ARBs can help prevent thrombotic problems, particularly in COVID-19 patients. ACEI decreases the amount of PAI-1 through the suppression pathway of extracellular matrix (ECM) (29). While losartan managed to reduce PAI-1 levels more significantly, in Rosselli's study (30) it was also proven that losartan reduced PAI-1 better than other drugs in the ARB group (Telmisartan). This is due to the mechanism of action of the drug, it was demonstrated that both losartan and telmisartan produce a rightward shift of the AngII dose-response curve; however, the maximal response is unaffected by surmountable antagonists, such as losartan, whereas it is reduced by insurmountable antagonists, such as Telmisartan, leading to a nonparallel displacement of the AngII response curve.

Our study simultaneously responds to previous cohort research queries by examining the potential influence of ACE2 and ARB therapy on the occurrence of thrombotic events in COVID-19 patients (31). We looked at the difference between the effects of perindopril and losartan, finding that losartan is actually more effective at reducing PAI-1 than perindopril, with a significant difference. Our study is the first to compare an ACEI and ARB in adipocyte cells that have been exposed to the SARS-CoV-2 S1 protein spike. Some speculation exists regarding the relationship between ARBs and an increase in free ATII, which is considered capable of causing fibrinolytic instability and plaque rupture (32). Further research is needed to establish how far the role of ARBs can contribute to the potential prevention of thromboembolism complications.

ACEIs and ARBs have different pathways in modulating ACE2. Losartan and perindopril both have the ability to lower TF levels, but only Perindopril shows significant differences to the control. Additionally, perindopril and losartan were successful in significantly lowering PAI-1 levels when compared to positive controls, but losartan reduced PAI-1 levels more extensively compared to perindopril. This reduction in PAI owing to ACEI/ARBs may have an effect on the lowering of thrombotic complications, notably in COVID-19 infection.

Acknowledgement: This work was supported by the Ministry of Research, Technology, and Higher Education of Indonesia to I Gde Rurus Suryawan (279/UN3.15/PT/2021).

Conflict of interests: None declared.

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R e c e i v e d : February 5, 2023
A c c e p t e d : June 30, 2023

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