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Short Communication

Perindopril decreases angiotensin-converting enzyme 2 (ACE2) expression in human adipocytes exposed to SARS-CoV-2 S1 spike protein

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Abstract

The expression of angiotensin-converting enzyme 2 (ACE2) in the adipose tissues of obese patients needs further study, as it may aid infection and serve as a viral reservoir. There has been controversy over whether to use ACE inhibitors to prevent coronavirus disease 2019 (COVID-19) severity. Perindopril, an ACE2 inhibitor, has been proposed; however, its relationship with COVID-19 has not yet been clear. The aim of this study was to investigate the effect of perindopril to reduce the expression of ACE2 and pro-inflammatory cytokine in adipocytes exposed to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Enzymatic isolation of adipose tissues was performed from obese male donor patients aged 30–50 years, then exposed it with SARS-CoV-2 S1 spike protein. This study also included human recombinant ACE2 (hrsACE2) as a comparison to perindopril. The expression of ACE2 was evaluated using ELISA. Our data indicated that SARS-CoV-2 Spike protein exposure increased ACE2 expression significantly. Administration of perindopril decreased ACE2 expression (43.37 µg/mL) significantly compared to the positive group (80.31 µg/mL) ($p<0.001$). Perindopril administration also decreased IL-6 levels significantly compared to positive group ($p<0.001$). This study highlights that perindopril could reduce the ACE2 expression and pro-inflammatory cytokine levels in adipocytes exposed to SARS-CoV-2 S1 spike protein.

Keywords: SARS-CoV-2, obesity, ACE2, IL-6, perindopril

Introduction

In coronavirus disease 2019 (COVID-19), obesity is associated with unfavorable outcomes and as an independent risk factor, obesity is associated with severe disease and death [1]. There have been some theories put forth as to how obesity affects COVID-19 severity. Despite certain inflammatory cytokines, like tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6, are expressed, one important pathogenic pathway is the production of angiotensin-converting enzyme 2 (ACE2). The cell membranes such as the kidney, heart, and lungs have ACE2 receptors; however, the levels of ACE2 in adipose tissues are significantly greater than in lung tissues. Moreover, adipocytes from people with type 2 diabetes and obesity have higher levels of ACE2 [2]. These adipocytes, additionally, overexpress ACE2 receptors, which could facilitate infection and act as a reservoir for viruses [3]. In line with the mentioned pathogenic pathway, SARS-CoV-2 exclusively infects cells that have ACE2 receptors. SARS-CoV-2 enters host cells through the

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ACE2 receptor in adipocytes. The ACE2 receptor is bound by the transmembrane spike (S) glycoprotein of SARS-CoV-2, mediating receptor recognition and membrane fusion. This clarifies why COVID-19 individuals experiencing severe symptoms have higher ACE2 levels [4].

Adipocytes in obese individuals trigger the production of cytokines such as interleukin-6 (IL-6) and IL-6 also increased due to the SARS-CoV-2 infection [5,6]. One of its pathogenic routes involves the Spike protein attaching to angiotensin-converting enzyme 2 (ACE2) receptors, which activates the mitogen-activated protein kinase-nuclear factor kappa B (MAPK-NF-κB) pathway [7]. It is suggested that the elevated of IL-6 is associated with to ACE2 activation and is responsible for the severe symptoms experienced by obese COVID-19 patients.

Angiotensin I (Ang I) and angiotensin II (Ang II) are converted by ACE2 to angiotensin-(1-9) and angiotensin-(1–7). Angiotensin II regulates the equilibrium between vasoconstriction and vasodilation by acting on angiotensin type 1 (AT1R) and angiotensin type 2 (AT2R) receptors [8]. ACE2's main purpose is to counteract Ang II's effects on the RAS system [9]. Increasing ACE2 levels has been demonstrated in earlier research to help prevent diabetes, hypertension, and heart failure [10]. As a result, ACE inhibitors (ACEi) and angiotensin II receptor blockers (ARBs) are widely available and regularly used as a routine treatment for hypertension and heart failure.

Since ACE2 was identified as the SARS-CoV-2 receptor, there has been debate on the usefulness of cardiovascular medications, such as ACEIs, to lessen the severity of COVID-19. ARBs, aminoglycosides, and ACEIs are a few examples of optional COVID-19 therapies. The goal of this treatment is to stop ACE2 elevation and cytokine storms before the condition gets worse. The usage of ACEi in COVID-19 patients and its association with up-regulation of ACE2 expression have been studied previously [11]. A previous study showed that ACE inhibitors are a double-edged sword that may increase viral binding, while also decreasing lung injury [12]. An umbrella review of 1,351,633 people with COVID-19 treated with ACEi found that it reduced the risk of hospitalizations and intubations or deaths [13]. In COVID-19, only a small number of drugs from the ARB, ACEi, and aminoglycoside classes consistently affect ACE2 expression [14]. Perindopril and losartan, two ACEi and ARB drugs, have been shown in several studies to upregulate ACE2 expression [14-16]. However, other studies with perindopril have been inconclusive findings [11,12]. The aim of this study was to assess the effect of the ACE-inhibitor perindopril on ACE2 expression and pro-inflammatory cytokines production in SARS-CoV-2-infected adipocytes mimicking obesity conditions in vitro.

Methods

Study design and setting

This was an experimental *in vitro* study with a post-test-only control group design carried out in the biosafety level 2 (BSL 2) laboratory of the Department of Physiology at the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia. The donor who provided the adipose tissue samples was a 45-year-old male obese with no history of acute myocardial infarction, valvular heart disease, peripheral arterial disease, heart failure, malignant arrhythmias, transient ischemic attack, stroke, diabetes mellitus, or kidney failure. Echocardiography was conducted to ensure their heart structure was normal. The donor also had no history of COVID-19 (confirmed by history taking and a negative PCR swab result) and never received any COVID-19 vaccines.

Adipocytes collection and preparation

Using the skin incision (elliptical) technique, subcutaneous fat was collected in accordance with the method described previously [17]. After enzymatically isolating the adipose tissues with 0.1% collagenase type 1, they were cultured in alpha-minimum essential medium (α-MEM) supplemented with 10% platelet-rich plasma (PRP) and 100 µg/mL of streptomycin, gentamycin, and penicillin (100 U/I) [17]. For seven days, the cells were cultured at 37°C with 5% CO_2. After an incubation time, 1×10^6 cells were stained with oil red O on each 10 cm culture dish to detect viable isolated adipocytes [18].

Study groups and SARS-CoV-2 S1 spike protein exposure to adipocytes

This study used 10 µM of SARS-CoV-2 S1 spike protein, which was based on a previous study [19]. The cultured medium of adipocytes was divided into four groups: (1) negative control group,

untreated cells consisting of 1 mL medium with 1.66×10^5 adipocytes; (2) positive control group, consisted of 1 mL of cultured medium of adipocytes added with 1 mL of 10 µM subunit of the SARS-CoV-2 S1 spike protein; (3) perindopril group, consisted 1 mL cultured medium of adipocytes exposed with 1 mL of 10 µM of SARS-CoV-2 S1 spike and treated with 0.5 µM perindopril; and (4) hrsACE2 group, consisted 1 mL cultured medium of adipocytes exposed with 10 µM of SARS-CoV-2 S1 spike and treated with 100 µg/mL of hrsACE2. After exposure with SARS-CoV-2 S1 spike protein, the cultures were incubated for 30 min at room temperature, then washed using PBS.

Perindopril and hrsACE2 treatment

A total of 1 mL perindopril (0.5 µM) and 100 µg/mL hrsACE2 were added to the perindopril group and hrsACE2 group, respectively, one time only. Then, the cultures were incubated at room temperature for two hours before the ACE2 and cytokines were measured.

Measurement of ACE2 and pro-inflammatory cytokines levels

ACE2, interleukin (IL)-6, interleukin (IL)-1β, and tumor necrosis factor-α (TNF-α) levels were measured using ELISA kits according to the manufacturer's manual (Abcam, Cat. ab235649; Elabscience, Cat. E-EL-H0102; BT Lab, Cat. E0143Hu; BT Lab Cat. E0082Hu, respectively). After coating each well with a primary antibody, adipocyte culture supernatant was added, and the mixture was incubated. Each well was filled with a secondary detection antibody and allowed to incubate after washing. Subsequent washing was done, and then a stop solution was used to end the reaction. The microplate reader was utilized to ascertain the optical density of every well. ACE2 and other cytokines were measured in µg/mL unit.

Statistical analysis

Data analysis was performed using one-way ANOVA with post-hoc Tukey's test to see if the data were normally distributed and the Kruskal-Wallis test if the data were abnormally distributed. The level of significance was set at $p<0.05$. The statistical analysis was carried out using SPSS Statistics for Windows, Version 25.0 (IBM Corp, Armonk, NY, USA).

Results

The combination of SARS-CoV-2 spike protein in adipocyte cells changed the expression of ACE2 and pro-inflammatory cytokines. As for confirming the over-expression of ACE2 and pro-inflammatory cytokines, we assessed the expression of ACE2 and pro-inflammatory cytokine levels in the positive control group compared to the negative control group.

Effect of perindopril and human recombinant ACE2 (hrsACE2) on ACE2 level

We analyzed the effect of perindopril on ACE2 expression using ELISA. The exposure of perindopril on adipocytes caused a change in the expression of ACE2. In the first 24 hours, ACE2 expression increased significantly in the perindopril (113.52 µg/mL) and positive control group (90.22 µg/mL) compared to the negative group (13.34 µg/mL) ($p<0.001$). However, in the next 48 hours, the perindopril group had reduced ACE2 expression (47.37 µg/mL) compared to the positive control group (80.31 µg/mL) significantly ($p<0.001$) (**Table 1**).

Table 1. Comparisons of angiotensin-converting enzyme 2 (ACE2) levels between groups within the first 24 hours and 48 hours of observation

Groups	n	Angiotensin-converting enzyme 2 (ACE2) level, µg/mL					
		24 hours			48 hours		
		Mean±SD	ANOVA	Post-hoc	Mean±SD	ANOVA	Post-hoc
Negative control	3	13.34±1.51	<0.001	<0.001	14.48±2.75	<0.001	<0.001
Positive control	3	90.22±4.72		Ref	80.31±9.31		Ref
Perindopril	3	113.52±0.59		<0.001	47.37±1.33		<0.001
hrsACE2	3	17.33±0.18		<0.001	11.59±1.33		<0.001

In addition, hrsACE2 administration also caused changes in ACE2 expression. The number of ACE2 expressions decreased significantly (17.33 µg/mL) compared to the positive group (90.22 µg/mL) in 24 hours and also decreased ACE2 expression in 48 hours after treatment (11.59 vs 80.31 µg/mL, $p<0.001$) (**Table 1**).

Effect of perindopril and human recombinant ACE2 (hrsACE2) on IL-6 levels

The effect of perindopril on inhibition of IL-6 production was determined by using ELISA. In the 48 hours, the perindopril group and hrsACE2 group have similar effects in lowering IL-6. As indicated by the sample tables provided in **Table 2**, the percentage of IL-6 for each group in 48 hours was significantly decreased compared to the positive control 90.93 µg/mL (p<0.001). Next, we assess how differently the hrsACE2 protein and the perindopril group reduce the expression of IL-6. The outcomes demonstrated that the hrsACE2 also lowered IL-6 levels compared to the positive control group (p<0.001).

Table 2. Comparisons of interleukin-6 (IL-6) levels in each group within the first 24 hours and 48 hours of observation

Groups	n	Interleukin-6 level, µg/mL					
		24 hours			48 hours		
		Mean±SD	ANOVA	Post-hoc	Mean±SD	ANOVA	Post-hoc
Negative control	3	21.33±2.56	<0.001	<0.001	19.92±0.53	<0.001	<0.001
Positive control	3	60.00±1.32		Ref	90.93±4.29		Ref
Perindopril	3	64.65±0.22		<0.001	42.66±3.36		<0.001
hrsACE2	3	36.11±0.53		<0.001	22.62±0.92		<0.001

Effect of perindopril and human recombinant ACE2 (hrsACE2) on IL-1β level

We assessed the expression of IL-1β in 24 hours and 48 hours using ELISA. In the 24 hours, the perindopril group (1435.66 µg/mL) had no significant difference compared to the positive control group (**Table 3**). However, the hrsACE2 group had lower IL-1β significantly (p=0.013) compared to the positive control group. In the setting of 48 hours, compared with the positive group (919.00 µg/mL), the perindopril group (1011.33 µg/mL) and hrsACE2 group (811.57 µg/mL) did not have lower IL-1β levels compared to positive control (**Table 3**).

Table 3. Comparisons of interleukin-1β (IL-1β) levels in each group within the first 24 hours and 48 hours of observation

Groups	n	Interleukin-1β level, µg/mL					
		24 hours			48 hours		
		Mean±SD	ANOVA	Post-hoc	Mean±SD	ANOVA	Post-hoc
Negative control	3	895.33±46.23	<0.001	0.243	726.66±103.00	<0.001	0.437
Positive control	3	1171.66±198.10		Ref	919.00±99.00		Ref
Perindopril	3	1435.66±254.19		0.274	1011.33±17.01		0.870
hrsACE2	3	611.00±38.43		0.013	881.57±260.02		0.813

Effect of perindopril and human recombinant ACE2 (hrsACE2) on TNF-α levels

In 24 hours, there are no significant differences in TNF-α between the group treated with SARS-CoV-2 (284.91 µg/mL), perindopril (130.80 µg/mL) (p=0.866) and hrsACE2 protein (214.16 µg/mL) (p>0.231) (**Table 4**). However, based on TNF-α expression in the 48 hours after treatment, was decreased in hrsACE2 protein (128.55 µg/mL) (p=0.018) compared with perindopril (403.28 µg/mL) (p=0.243) and positive group (307.95 µg/mL), respectively. The treatment with hrsACE2 protein can significantly decrease TNF-α concentration.

Table 4. Comparisons of tumor necrosis factor-α (TNF-α) levels in each group within the first 24 hours and 48 hours of observation

Groups	n	Tumor necrosis factor-α level, µg/mL					
		24 hours			48 hours		
		Mean±SD	ANOVA	Post-hoc	Mean±SD	ANOVA	Post-hoc
Negative control	3	138.00±55.92	0.004	0.010	126.78±52.54	0.001	0.017
Positive control	3	284.91±34.02		Ref	307.95±57.34		Ref
Perindopril	3	310.80±42.38		0.866	403.28±75.80		0.234
hrsACE2	3	214.16±26.87		0.231	128.55±25.89		0.018

Discussion

Increased ACE2 expression in adipocyte tissue is linked to a number of unfavorable cardiometabolic health indices, all of which are risk factors for severe COVID-19. Previous studies

have demonstrated that increased susceptibility to thrombotic events and extensive microvascular damage may affect the severity of COVID-19, with ACE2 and the RAS system potentially playing a crucial role [19,20]. ACE2 has been shown to facilitate viral cellular invasion and replication and is characterized by enhanced ACE2 expression in the microcirculatory and tissue level, which is correlated with an imbalance of paracrine action of Ang compounds and local depletion of Ang 1–7 leading to vasoconstriction and inflammation [21].

In this in vitro experimental study, we examined the impact of perindopril on ACE2 expression reduction in the adipocyte cells of SARS-CoV-2 obese patients. Treatment with ACEi and ARB has been demonstrated to control ACE2 expression in the in vivo experiment [12]. There is reason to believe that ACE inhibitors or ARB medications increase the risk of COVID-19 hospitalization, severity, or death. Nonetheless, perindopril has been found to have a beneficial effect in reducing ACE expression in the adipocyte cells of individuals with SARS-CoV-2 obesity. This result was consistent with some other research suggesting ACE medications, such as perindopril, may protect against severe COVID-19 caused by ACE2 expression [22]. A previous umbrella review of 1,351,633 people with COVID-19 has shown that ACE inhibitors reduce the risk of hospitalizations, intubations, or deaths, suggesting the benefit of ACE Inhibitors. Remarkably, a 2019 study by South *et al*. demonstrated that angiotensin receptor blockers (ARBs) and ACE inhibitors may be helpful in lowering Ang-II and restoring balance between Ang 1–7 [22].

When ACE inhibitors or ARBs were administered to COVID-19 patients instead of calcium channel blockers (CCB), the likelihood of hospitalization was shown to be lower. A study found that in comparison to patients receiving non-ACEi or ARB therapy, exposure to ACEi or ARB was linked to a lower risk of mortality and was not related with a high risk of COVID-19 infection [23]. Additionally, for COVID-19 patients, the American Heart Association (AHA) and the European Society of Cardiology (ESC) advise continuing ACEi or ARB treatment [24].

Nevertheless, little is known about the biological mechanism behind ACE inhibitors' beneficial benefits. Perindopril and ACE2 activity were found to be linked to lower angiotensin II production, which was followed by down-regulation of ADAM17, but higher plasma levels of Ang 1–7. Although the blockade of Ang II by perindopril is decreasing Ang 1–7, Ang I is also converted to Ang 1–7 mediated by neprilysin [23,25,26]. Ang 1–7 has potentially beneficial effects on vasodilation, anti-inflammatory, antifibrotic, antiangiogenic, and antihypertensive actions [27].

In COVID-19 patients, circulating pro-inflammatory cytokines are increased. Additionally, we saw elevated concentrations of pro-inflammatory cytokines, such as IL-6, TNF-α, and IL-1β. Our findings show that perindopril has significantly lower IL-6 levels compared to the positive control group. COVID-19 exacerbates inflammation and coagulopathy in obese patients through upregulation of Ang II and ADAM 17-mediated activation of IL-6, TNF-α, prothrombotic, and STAT-3 pathways [28,29].

Previous studies have demonstrated that treatment of COVID-19 patients with ACEi can result in reduced production of Ang-II and effectively downregulate the production of inflammatory cytokines mediated by Ang 1–7 [30]. Ang 1–7 binding to the Mas receptor (MasR) induces vasodilatation via nitric oxide (NO), thereby decreasing inflammatory response. Perindopril has also been shown to have a direct effect in lowering IL-6 expression by inhibiting the STAT3 pathway [31].

In our experimental study, we did not find the concentration of IL-1β and TNF-α in the ACE-inhibitor group to be significantly different compared to the positive group and hrsACE2 group, suggesting that ACE-inhibitor treatment alone or hrsACE2 treatment may not be efficient in modulating the production of these cytokines. In patients who are obese, there is an expansion of white adipose tissue, which in turn triggers the release of cytokines and chemotactic factors. These include platelet-derived growth factor, IL-1β, IL-6, TGF-β, and TNF-α. These elements draw in and stimulate immune system cells, preadipocytes, and endothelial precursor cells [32]. Nevertheless, it has been demonstrated that only IL-6 is connected to ACE2 through the activation of the mitogen-activated protein kinase-nuclear factor kappa B (MAPK-NF-κB) pathway [7].

Furthermore, treatment with both perindopril and hrsACE2 lowers the ACE2 expression in COVID-19 infection. A report mentioned that treatment with ACE2 (0.4 mg/kg) in COVID-19

patients resulted in a reduction of viral load in plasma [33]. HrsACE2 effectively lowers Ang II levels and increases Ang 1–7 levels by binding to the spike glycoprotein of SARS-CoV-2 [33]. A clinical study with infusion of hrsACE2 in COVID-19 patients was associated with a rapid and marked reduction in serum Ang II concentration, leading to increased Ang 1–7 concentration and a reduction in IL-6 production [34]. This finding suggests that perindopril may have similar therapeutic effects as hrsACE2 in the setting of COVID-19 infection, which is characterized by a cytokine storm. Interestingly, both perindopril and hrsACE2 were able to decrease IL-6 levels, but not TNF-alpha and IL-1β levels, suggesting that pathways other than ACE-2 SARS-CoV-2 Spike protein binding are involved. Therefore, there is a lack of data and experimental studies on the correlation between hrsACE2 and the expression of TNF-α and IL-1β. However, IL-6 remains the major cytokine involved in a cytokine storm, and thus, perindopril may still be beneficial in reducing cytokine storm.

Conclusion

Perindopril reduced ACE2 and pro-inflammatory cytokine levels in adipocytes exposed to SARS-CoV-2 S1 spike protein. These findings are in line with the severity and mortality of COVID-19 and are linked to a substantial number of adipocytes in obese people.

Ethics approval

This study has been approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia (No. 198/EC/KEPK/07/2021).

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

How to cite

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