RESEARCH ARTICLE

Losartan Has a Comparable Effect to Human Recombinant ACE2 in Reducing Interleukin-6 (IL-6) Levels on Human Adipocytes Exposed to SARS-CoV-2 Spike Protein

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Abstract

BACKGROUND: High angiotensin-converting enzyme 2 (ACE2) expression in adipocyte cells facilitates the initiation of SARS-CoV-2 infection and triggers a cytokine storm. This finding suggests that obesity is an independent risk factor for the severity of the symptoms caused by COVID-19. The use of cardiovascular medications that focus on ACE2, such as angiotensin II receptor blockers, remains controversial, and their effects on inflammatory cytokine production and ACE2 expression in cells, especially adipocytes, remain inconsistent.

METHODS: The human adipocytes were isolated from obese donor subcutaneous adipose tissue and infected with the subunit S1 spike protein from SARS-Cov-2. The adipocytes were later treated with either hrsACE2 or losartan. The levels of ACE2 and inflammatory cytokines interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α were measured using enzyme linked immunosorbent assay (ELISA). ACE2 and S1 spike protein binding assays were also performed.

RESULTS: ACE2, IL-6, and TNF-α levels were significantly increased in human adipocyte cells infected with SARS-Cov-2 but not IL-1β. There was a statistically significant positive correlation between ACE2 and IL-6 ($r=0.878$, $p<0.001$). Administration of losartan and hrsACE2 was shown to reduce ACE2 levels and its binding to the SARS-CoV-2 S1 spike protein, and IL-6 levels were statistically significant, but had no significant effect on IL-1β or TNF-α levels.

CONCLUSION: This study shows that the administration of losartan in COVID-19 may not be harmful, but instead has a protective effect similar to that of hrsACE2 in preventing a cytokine storm, especially IL-6.

KEYWORDS: obesity, SARS-CoV-2, losartan, IL-6, ACE2

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Introduction

The Coronavirus (COVID-19) pandemic has spread across the world becoming a global health issue, leading to Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) with morbidity and mortality that cannot be underestimated. Currently, various types of vaccines have been developed and are being used, which are key to reducing the transmission of COVID-19. However, several factors are thought to reduce the protective effect, one of which is obesity, which has many theories linking it to the immune system.(1) Obesity significantly increases the likelihood of developing a serious case of COVID-19 and of experiencing severe symptoms. People with obesity are strongly associated with a higher risk of requiring intensive care treatment and facing

a higher mortality risk in hospitals.(2–4) Obesity has long been associated with adipocyte dysfunction that affects not only the metabolic homeostasis system but also the body's overall homeostasis, particularly inflammation in immune system.(5)

Adipocytes are believed to play a significant role in the mechanism underlying SARS-CoV-2 infection by facilitating transmission, replication, and release of the virus. The infection process of SARS-CoV-2 begins with the binding of viral glycoprotein molecules to the angiotensin-converting enzyme 2 (ACE2) receptor on the membrane of host cells. It is important to note that SARS-CoV-2 can only infect cells that express the ACE2 receptor.(6,7) Adipocytes, which are known to express high levels of ACE2 receptors, have been found to exhibit elevated ACE2 gene expression in various tissues such as the small intestine, testis, kidneys, heart, thyroid, and adipose tissue. Surprisingly, gene bank data analysis revealed that ACE2 gene expression in adipose tissue surpassed that in the lungs. Furthermore, individuals with obesity tend to have adipocytes that produce higher levels of proinflammatory cytokines like interleukin (IL)-6 even without any external stimuli, in comparison to non-obese individuals.(8-10) IL-6 is known to be the cause of cytokine storms that led to multiple organ damage in COVID-19.(11,12) This evidence could be an explanation of how obesity can develop severer clinical manifestations of COVID-19.(13,14)

ACE2 is a glycoprotein that is firmly embedded in the cell membranes and is widely expressed in various tissues. It serves as a vital natural regulator of the renin–angiotensin system (RAS). One of its key functions is the conversion of angiotensin II (Ang II) into angiotensin-(1-7), as well as the conversion of angiotensin I (Ang I) into angiotensin (1-9).(15) Raising concern about the use of ACE inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs) have increased since the identification of ACE2 as a SARS-CoV-2 receptor. Several animal experiments have shown that the administration of ACEI and ARB causes ACE2 receptor overexpression, especially in the cardiovascular system. ACE2 overexpression is hypothesized to increase susceptibility to SARS-CoV-2 infection and aggravate the severity of COVID-19.(16,17)

However, the impact of ACEI and ARB on ACE2 receptor expression is not uniform across all types.(16) Additionally, no existing studies have examined this effect in cases where baseline ACE2 levels are already elevated in obese individuals. To address this, we conducted a previous study to evaluate the influence of losartan, a commonly used ARB. We specifically examined the effect of losartan on ACE2 expression and production of proinflammatory cytokines in SARS-CoV-2 infected adipocytes, which simulate obesity conditions in vitro.

Methods

Primary Culture of Adipocytes

Adipocytes were isolated from subcutaneous adipose tissue taken from a donor. The donor was an individual with obesity (BMI >30) without any significant history of disease indicated with normal blood count, normal renal and liver function, and without cardiac structure and function abnormality assessed by echocardiography. The donor also never tested positive for COVID-19 or received any COVID-19 vaccines. This study was conducted under the approval of the Health Research Ethic Committee Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia (No. 198/EC/KEPK/07/2021).

Adipose tissue was obtained from subcutaneous in the abdomen region by surgical resection. The donor was obtained from a person who had signed a consent form for a surgical resection of the abdominal area to remove subcutaneous adipose tissue for research purposes. Adipose tissue was then isolated enzymatically.(18) Briefly, adipocytes were finely minced and digested using type 1 Collagenase (Cat. #17018029, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min in a 37°C water bath with shaking at 100 rpm. Cells were passed through a nylon mesh filter and washed with culture media three times. Cells were resuspended and were cultured in growth media composed of Dulbecco's modified Eagle'smedium/F-12 (Cat. #11330032, Thermo Fisher Scientific) with the addition FBS (Cat. #F2442, MilliporeSigma, Burlington, MA, USA) and 1% penicillin-streptomycin (Cat. #15070-063, Thermo Fisher). Adipocyte culture was established in an incubator at 37°C and 5% CO_2 and then treated at 90%.

Adipocytes that have been cultured are divided into three groups: positive control, negative control, and treatment. The negative control consists of adipocytes that have not received any treatment. The positive control includes adipocytes exposed only to SARS-CoV-2 without any additional treatment. The treatment group consists of SARS-CoV-2-exposed adipocytes and further divided into two subgroups: one receiving Losartan, and the other receiving hrsACE2.

SARS-CoV-2 Subunit S1 Spike Protein Exposure to Adipocytes

Adipocytes were infected with the S1 subunit spike protein of SARS-Cov-2 (Cat. #230-30162-100, RayBiotech, Peachtree Corners, GA, USA,) using a modified direct exposure approach.(19) Adipocytes were streaked on 96-well plates at a density of 1×10^4 cells per well, followed by treatment with 10 nM SARS-CoV-2 subunit S1 spike protein and incubation at room temperature for 30 min.

Treatment with Losartan

As much as 0.7 µM losartan (Merck, Cat. #BP867) was added 30 minutes after SARS-CoV-2 S1 spike protein exposure to adipocytes.(20) The effect of losartan with human recombinant soluble ACE2 (hrsACE2) was also compared. One-hundred µg/mL hrsACE2 was added to another group of adipocytes culture treated with SARS-CoV-2 subunit S1 spike protein.(21)

ACE2-spike Protein Binding Assay

The impact of losartan and hrsACE2 on the interaction between the SARS-CoV-2 spike protein and ACE2 was evaluated using a binding assay kit (Cat. #CoV-SACE2-1, RayBiotech) following the manufacturer's instructions. To prepare the test reagents, losartan or hrsACE2 was mixed with 1.25 µL of 100x ACE2 protein concentrate, resulting in a final volume of 100 µL. Each test reagent was added to the appropriate wells coated with SARS-CoV-2 spike protein and incubated overnight at 4°C with gentle shaking. The wells were then washed four times using 300 µL of wash buffer and subsequently incubated with 100 µL of a detection antibody for 1 hour at room temperature. Following additional washing steps, 100 µL of HRP-Conjugated anti-IgG, 100 µL of TMB substrate, and 50 µL of stop solution were sequentially added to each well. The absorbance of the solution was immediately measured at 450 nm.

Measurement of ACE2 and Proinflammatory Cytokines Levels

ACE2, IL-6, Interleukin-1β, and tumor necrosis factor (TNF)-α levels were measured using ELISA kits according to the manufacturer's manual (Cat. #Ab235649, Abcam, Cambridge, UK; Cat. #E-EL-H0102, Elabscience, Houston, TX, USA; Cat. #E0143Hu, BT Lab, Birmingham, UK; Cat. #E0082Hu, BT Lab, respectively). Adipocyte culture supernatant was added to each primary antibody-coated well and incubated. After washing, secondary detection antibody was added to each well and incubated. Following additional washing, substrate was added, and the reaction was stopped using a stop solution. Each well's optical density was determined using a microplate reader.

Statistical Analysis

One-Way ANOVA and was followed by Tukey's Post-Hoc were used to analyze and compare the difference between groups. Data were first tested for normality with the Shapiro-Wilk test. Using 5% alpha, data were analyzed using SPSS version 26.0 (IBM Corporation, Armonk, NY, USA) and were considered significant if the $p<0.05$.

Results

SARS-CoV-2 S1 Spike Protein Exposure Increased ACE2, IL-6, and TNF-α Level

The effect of SARS-CoV-2 S1 spike protein on adipocytes was the first to be investigated. Significant elevation of ACE2 was seen after 30 minutes of incubation of spike protein compared to a negative control (90.22±4.72 *vs.* 13.33±1.51 ng/mL, $p<0.001$). Spike protein exposure also significantly increased the level of proinflammatory cytokines IL-6 (60.00±1.33 *vs.* 21.34±2.56 ng/mL, $p=0.000$) and TNF-α (284.91±34.82 *vs.* 138.00±55.92 ng/mL, $p=0.007$), but not in IL-1β (1171.66±198.10 *vs*. 895.33±46.23 pg/mL, $p=0.109$).

Losartan and hrsACE2 Reduced the Level of ACE2 and Inhibited the Binding of ACE2-spike Protein

The effect of losartan and hrsACE2 on ACE2 level and its consequences on the binding of ACE2 with SARS-CoV-2 spike protein was also evaluated. ACE2 levels significantly reduced following the addition of losartan (27.51±3.48 ng/mL) and hrsACE2 (17.33±0.18 ng/mL) into adipocyte culture exposed to SARS-CoV-2 spike compared to a positive control (90.22±4.72 ng/mL, $p<0.001$). ACE2 reduction level was slightly better in the hrsACE2 group (Figure 1). There was no ACE2-spike protein binding detected in losartan and hrsACE2 group, as was observed in the control group (31.23±3.53 ng/mL) (Figure 1).

Losartan and hrsACE2 Lowered IL-6 Levels

Measurement of proinflammatory cytokines levels showed that only IL-6 was significantly lowered after losartan (19.96±3.05 ng/mL) and hrsACE2 (36.11±0.53 ng/mL) treatment compared to a positive control (60.00±1.32 ng/mL, $p<0.001$). Losartan has a larger reduction of IL-6 than

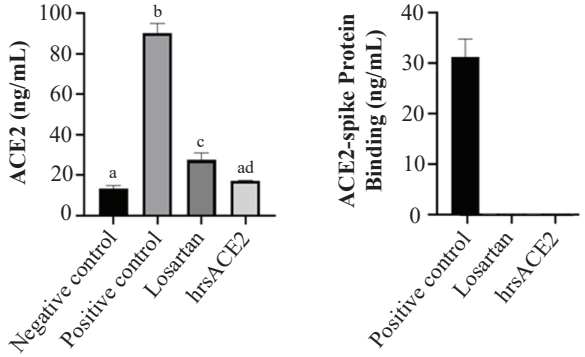

Figure 1. ACE2 levels and ACE2-spike protein binding levels in all experiment groups. [a,b,c,d]Different annotations indicate statistically significant differences between groups, tested with Post-Hoc Test.

hrsACE2 (Figure 2). The hrsACE2 also decreased IL-1β significantly compared to the positive control (611.00±38.43 pg/mL *vs.* 1171.66±198.10 pg/mL, $p<0.05$), but this finding was not observed in the losartan group (Figure 2). Both losartan and hrsACE2 had no significant effect on TNF-α levels (Figure 2). Pearson correlation measurement showed that IL-6 was positively correlated with ACE2 (r=0.878, $p<0.001$) (Figure 3).

ACE-2 Levels Correlated with IL-6 Levels but Not with IL-1β and TNF-α

In this study, the results also showed that ACE2 levels had a strong correlation with IL-6. This means that the higher the ACE2 levels in SARS-CoV-2 infection, the higher the IL-6 levels (r=0.878, p<0.001). However, there was no significant correlation between ACE2 with IL-1β and TNF-α (Figure 3, Figure 4).

Discussion

ACE2 plays a key role in developing SARS-CoV-2 infection (COVID-19) related cytokine storm, characterized by a surge of interleukin(IL)-6 and IL-1β.(6) It has been shown that in acute respiratory distress syndrome (ARDS), ACE2 is a significant regulator of inflammatory responses.(22) Recently, a phase II trial on hrsACE2 has shown promise in attenuating acute lung injury in ARDS while establishing a safety profile.(23) The hrsACE2 may also be beneficial in treating COVID-19 and its complication by acting as a decoy for circulating SARS-CoV-2 virus and converting Ang II to angiotensin-(1-7).(24)

This study successfully identified the presence of ACE2 expression in adipose tissue, which has supported previous literature that gene expression databases show ACE2 expression is present in subcutaneous adipose and human visceral adipose tissue, where levels are en higher than those in human lung tissue.(13) Hence, in obese patients, high levels of adipose tissue indicate high levels of ACE2, compared to patients without obesity. High levels of adipose tissues lead to an increase of pro-inflammatory cytokines in SARS-CoV-2 infection.

Two mechanisms have been proposed to explain this phenomenon. First, leptin, which is secreted by adipose tissue, is a pleiotropic molecule that functions to coordinate a person's immunity, specifically host innate immunity and adaptive responses and subsequently affects the increased secretion of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6.(25) However, in this study, the results showed that SARS-COV-2 infection in adipocyte cells only significantly increased TNF-α, IL-6, not IL-1β. This is because only 12% of those cytokines are produced by adipocytes. The primary source of these cytokines is non-fat cells in adipose tissue. In the context of IL-1β production, it is one of the cytokines produced the least by adipocytes, compared to TNF and IL-6.(26,27) Second mechanism is the fact that obese patients have higher levels of ACE2, which is the main route of entry of SARS-CoV-2 indirectly leads to increased viral replication and reproduction in the patient's body. This explains other studies that suggest that elevated

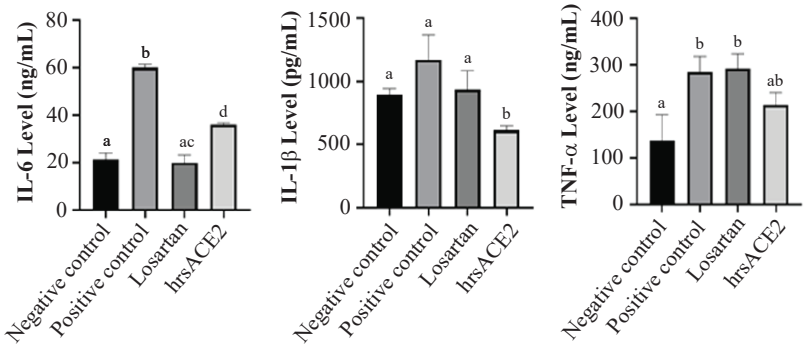

Figure 2. Proinflammatory cytokines (IL-6, IL-1β, and TNF-α) levels in all experiment groups. a,b,c,dDifferent annotations indicate statistically significant differences between groups, tested with Post-Hoc Test.

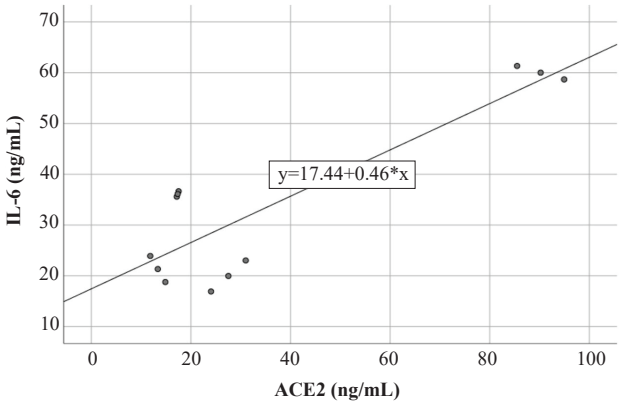

Figure 3. The scatterplot graph showed a strong positive correlation between ACE2 and IL-6 (r=0.878, *p*<0.001).

plasma ACE2 has been associated with poor outcomes in patients with COVID-19.(28)

This study also found that exposure to SARS-CoV-2 protein spikes increased ACE2 levels. The mechanism by which ACE2 is upregulated is thought to be at the transcriptional level by interferon which also appears to be elevated in SARS-CoV-2 infection.(29) This study also found that the SARS-Cov-2 protein spike increased the levels of pro-inflammatory cytokines.It is suspected that there is a disruption in this cytolytic activity, leading to the prolonged activation of innate immunity cells, which then many pro-inflammatory cytokines have increased secretion in undue pathways and cause cytokine storms.(30-32)

Losartan administration in this study showed the effect of reducing ACE2 levels in SARS-CoV-2 Infection. Losartan has a high affinity for ACE2, which results in its direct binding to ACE2, then prevents the virus from penetrating such that infection does not begin. This study showed that losartan also decreased the binding between the SARS-Cov-2 spike protein and ACE2. Previous *in silico* studies supported this finding, showing that losartan can reduce the affinity of the virus to ACE2 by distorting the receptor binding domain (RBD) on SARS-CoV2 to attach to ACE2.(33) This study also showed that losartan administration can reduce IL-6 levels in SARS-Cov-2 Infection. The results of this study showed that hrsACE2 administration had similar effects to losartan administration. Previous studies have indeed shown that hrsACE2 has therapeutic benefits in COVID-19. In addition to inhibiting the binding of SARS-CoV-2 with ACE2, hrsACE2 also minimizes multiple organ damage.(34) HrsACE2 has been shown to effectively protect mice from SARS-CoV-2 Infection as evidenced by reduced virus replication, histologic changes and decreased inflammation in the lungs.(35)

Thus, it can be concluded that losartan has a beneficial effect on SARS-CoV-2 infection, by reducing the binding of SARS-CoV-2 with ACE2, which directly and indirectly reduces pro-inflammatory cytokines, especially IL-6, which has been shown to cause various kinds of severe clinical manifestations of COVID-19. These findings provide additional support for the safety of losartan usage in obese patients with COVID-19 infections. Since inflammation is also a fundamental part of the pathophysiology of severe COVID-19, even in non-obese patients, the results may also be applicable to non-obese patients with severe COVID-19. Furthermore, additional discoveries, like the connection between losartan, ACE2, and IL-6, hold the potential to provide valuable insights into a range of medical conditions marked by involvement in the RAS system pathway and inflammation, including conditions like cardiovascular disease.

However, the specimens used in this study are only viral protein spikes, not whole viruses, which are expected to be sufficiently representative of the actual

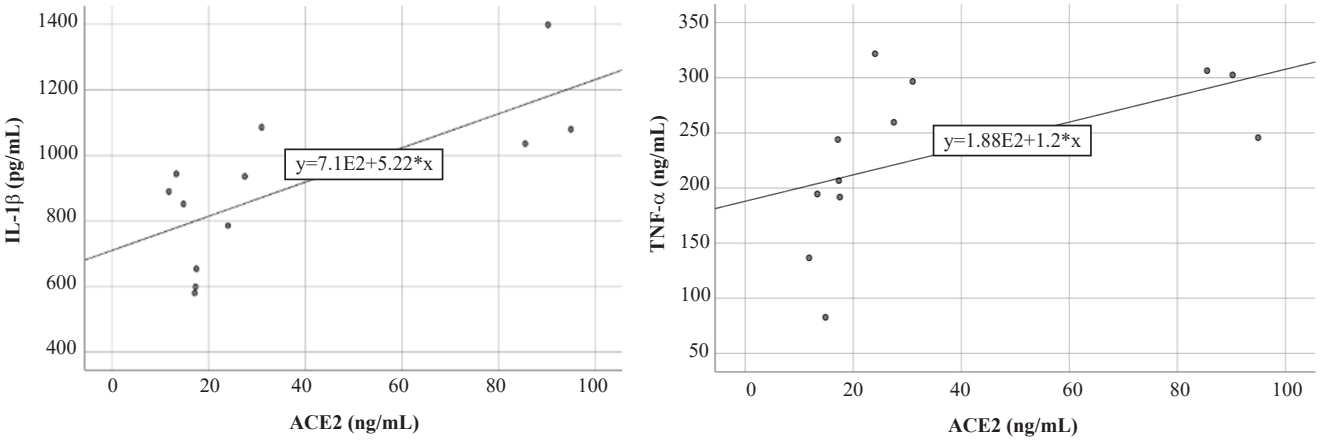

Figure 4. The Scatterplot graph showed no significant correlation between ACE2 with Il-1β and TNF-α (*p*>0.001).

condition of COVID-19 infection. In addition, this study also did not measure other parameters related to ACE2, such as Angiotensin-(1-7), and also did not measure other inflammatory pathways that may be related to ACE2 and adipokines. Therefore, further research is essential to conduct a thorough examination, leading to comprehensive results when evaluating the impact of losartan on the RAS system, encompassing both SARS-CoV-2 infection and various other scenarios.

Conclusion

This study provides evidence that losartan reduced ACE2 and IL-6 levels indicating that losartan might not be harmful when given to COVID-19 patients, especially in patients with obesity. Contrarily, losartan has a similar protective effect to human recombinant ACE2 in preventing cytokine storms, mainly due to IL-6.

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Authors Contribution

HOM, MA, IGRS, and PMH conceptualized and designed the research, collected data, analyzed and interpreted the results. HOM and MR developed data analysis and research results to prepare manuscripts, and revised manuscripts. All authors reviewed the results and approved the final version of the manuscript.

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