

Research Article

## Evaluation of sST-2 role in LVH Regression Obtained in Hypertensive Mice Models After Blocking Renin-Angiotensin System

Lilik Yusetyani <sup>1,2</sup>, Siti Rofida <sup>2</sup>, Dian Yuliartha Lestari <sup>3</sup>, Wawan Kurniawan <sup>2</sup>, Nursela Hijriani <sup>2</sup>, Ilham Niawan Saputra <sup>2</sup>, Setyawati Soeharto <sup>4</sup>, Mohammad Saifur Rahman <sup>5,6</sup>

<sup>1</sup> Faculty of Medicine, Universitas Brawijaya, Malang 65145, Indonesia

<sup>2</sup> Pharmacy Study Program, Faculty of Health Sciences, Universitas Muhammadiyah, Malang 65145, Indonesia

<sup>3</sup> Department of Pathology, Faculty of Medicine, Universitas Muhammadiyah, Malang 65145, Indonesia

<sup>4</sup> Department of Pharmacology, Faculty of Medicine Universitas Brawijaya, Malang 65145, Indonesia

<sup>5</sup> Department of Cardiology and Vascular Diseases, Faculty of Medicine, Universitas Brawijaya, Malang 65145, Indonesia

<sup>6</sup> Dr. Saiful Anwar Public Hospital, Malang 65112, Indonesia

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*\*Corresponding author:*

E-mail: [lilik\\_yy@yahoo.co.id](mailto:lilik_yy@yahoo.co.id)

**ABSTRACT**

Soluble ST2, is a protein which acts as a decoy receptor for interleukin-33, and served as biomarker associated with left ventricular hypertrophy (LVH). Few data exist in evaluating the effects of anti-hypertensive agents on the role-played form ST2 on regression of LVH. This study was designed to compare the effects of captopril and valsartan on blood pressures, plasma renin and soluble ST2 levels and regression of LVH in hypertensive mice models. Twenty-four male mice (*Mus musculus* L), were divided into four groups, namely aquadest/control, L-NAME, L-NAME + captopril and L-NAME + valsartan groups respectively. Mice blood pressures were measured on day 14th after induction with L-NAME extract 1.75 mg/25g BW/day (pretreatment) and day 14th post treatment. Levels of plasma renin, sST2, and ventricular wall thicknesses reflecting LVHs, were measured on day 14th post treatment. Administration of L-NAME within 14 days resulted in making mice models to be hypertensive paralleled by an increase of Ventricular wall thickness. Treatment with captopril and valsartan lowered the blood pressures to normal level within the next 14 days. Valsartan and captopril treatment induced a significant decrease of plasma renin level. Valsartan, but not for captopril treatment prevented wall thickness increase ( $p < 0.05$ ), while plasma sST2 was not able to mirroring this effect. Captopril and valsartan had similar effect in lowering plasma renin level and blood pressure, but sST2 seems to be not involved in LVH regression obtained in hypertensive mice models after blocking renin-angiotensin system.

*Keywords: LVH-regression, soluble-ST2, renin, ACEi, ARBs, mice-models*

### Introduction

Hypertension (HT) remains a major public health problem associated with the leading risk factor for morbidity and mortality [1]. The prevalence of this disease has been estimated to increase up to 29% of entire population in 2025 [2, 3]. Based on Health Research (Riset Kesehatan Dasar/Riskesdas) 2013 data, there are 25.8% of Indonesian people suffering of hypertension [4]. If the total number of Indonesian people now are

252,123,456, means that 65,048,110 people are hypertensives in Indonesia.

Meta-analysis studies suggested that reducing systolic blood pressure to levels below currently guidelines recommended targets could reach to significantly reduction of the risk of cardiovascular disease and all-cause mortality [5, 6]. The most frequent organ damage complication in hypertensive patients is left ventricle hypertrophy (LVH) which may result in heart failure (HF) [7, 8].

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Therefore, intensive treatment for lowering blood pressure to the current guidelines recommended levels would lead to prevent LVH.

Many studies, aimed to compare the effectiveness of common anti-hypertensive drugs used in hypertensive patients, such as diuretics, beta-blockers, calcium-channel blockers (CCBs), angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs); already demonstrated a reduction in both cardiovascular mortality and morbidity with all of these drug classes [9, 10, 11]. Soluble ST2 is a blood protein that has confirmed to act as a decoy receptor for interleukin-33. Soluble ST2 is also known to be markedly involved into the process of mechanically overloaded cardiac myocytes [14, 15], and could represent a cardiac stress biomarker associated with LVH [16]. Moreover, in a meta-analysis plasma sST2 has been demonstrated to have a prognostic value with respect to all-cause and cardiovascular death as well as the composite outcome of all-cause death or HF hospitalization [33]. Previous studies already also have shown that plasma sST2 was not only useful biomarker for predicting development of stress cardiomyopathy [34] but also has prognostic role in patients with sepsis [35, 36] and correlated with left ventricular structure and function in patients with metabolic syndrome [37]. Renin is the well-known enzyme of the Renin Angiotensin Aldosterone System, which plays central role in the regulation of blood pressure and the development of LVH [12,13].

This study was conducted in order to measure, in hypertensive mice model, the level of plasma renin and Soluble Suppression of Tumorigenicity-2 (sST2) as chemical potential effectors of left ventricular hypertrophy regression obtained as consequence of blood pressure reduction after ACE-inhibitor (Captopril) or ARB (Valsartan) treatment.

## Material and Methods

This true experimental study using randomized posttest only control group design was conducted at the laboratory of Animal Model Faculty of Medicine - Universitas Muhammadiyah Malang, Laboratory of Formulative Preparation and Laboratory of Integrated Chemistry, Pharmacy Study Program Faculty of Health Sciences – Universitas Muhammadiyah Malang, from January to April 2015. The proposal of this study had been

approved by Health Research Ethics Committee Faculty of Medicine, Universitas Brawijaya No. 370/EC/KEPK/07/2015.

## Hypertensive mice models

This study was done within 28 days, the first 14 days were used for induction of hypertensive mice model and the next 14 days were used for treatment. As many of 24 male mice (*Mus musculus* L), 10 – 12 weeks old and 20-30 grams of body weights were included in this study. After one-week acclimatization, the mice were randomly divided into four groups, consisted of six mice in each group. Mice in group (A) were given aquadest only and used as negative control, while the group (B), (C) and (D) were given L-NAME extract 1.75 mg/25g BW/day diluted in drinking water for two weeks [17, 18, 19]. For the next 14 days, the mice in group (B) were still given L-NAME only, served as positive control group. At the same period of the next 14 days, mice in group (C) were given L-NAME plus captopril 0.0488 mg/30g BW, and mice in group (D) were given L-NAME plus valsartan 0.312 mg/30g BW orally after multiplied with 0.0026 as conversion factor from human dose to mouse [20]. Both of captopril and valsartan in those doses were diluted in 0.5 cc aquadest and given orally per sonde to each mouse by an expert technician. The mice blood pressures were examined on the 14 days (pretreatment) and the next 14 days (post treatment), while intracardial blood sampling for measurement of plasma renin and sST2 levels and isolation of heart for histological measurement of ventricular wall thickness were conducted on the day 14th post treatment.

## Measurement of mice blood pressures and mean arterial pressures (MAPs)

Blood pressures (BP) of all mice were measured in mmHg. using the Blood Pressure Recorder. The 58500 BP RECORDER which is basically a recording Riva Rocci sphygmo-manometer, has been conceived to provide an objective precise recording of the systolic and diastolic BP of every processed rodent. The BP recorder combines three main systems, those are: 1) pressure generation-pressure monitoring system; 2) a pulse amplifier and 3) a thermal-array analog and digital recording unit [21]. Blood pressures were measured after the mice acclimatized in quite condition

for 10 minutes in the animal holder. The measurements for each mouse were repeated three times to ensure the results obtained (systolic and diastolic values) constantly. The mean arterial pressures (MAPs) values were found by calculation using the formula of:  $MAP = (Systolic\ BP + 2 \times Diastolic\ BP) / 3$  [22]. Those procedures were done individually on the day 14<sup>th</sup> of study (pretreatment), and on the next 14<sup>th</sup> day after treatment, before the mice were sacrificed.

#### **Measurement of plasma renin and sST2 level**

After the mice had been deep anesthetized using chloroform, cardiac puncture was done upon each mouse to take the blood and collect it into EDTA tubes, centrifuge at 5000 rpm at room temperature for 15 minutes. Plasma samples were collected and transferred to separate tubes and kept freeze at -20°C. Plasma renin level was analyzed by ELISA techniques by following the procedure which described on the kit leaflets and on Abcam's IL-1 R4 ELISA Kit (ST2) protocol [23,24]. The value of plasma renin levels was showed in ng/mL and sST2 were showed in pg/mL

#### **Measurement of ventricular wall thicknesses**

After the mice had been anesthetized and taken the blood, their hearts were removed and kept individually. In the preparation of histological slides, in order to get homogenous parts of ventricular wall myocards, the hearts were cut transversally at the middle level part of cardiac tissues were fixed with 4% formaldehyde. Furthermore, the heart tissue specimens were dehydrated in graded alcohol and embedded in paraffin. Ten serial sections were made transversally (3 µm thickness). These section then were deparaffinized, rehydrated, dehydrated, and stained with hematoxylin-eosin (H&E) according to standard method [25], and then observed under light microscope. Examination of wall thickness on three slides of each samples were done under 40× magnifications and measured in micrometer (µm).

#### **Statistical analysis**

All of the collected data were tabulated and analyzed statistically using SPSS-21 software (*IBM SPSS Statistic Processor*). The differences of means among measured variables were analyzed with ANOVA, then continued by Post Hoc and Pearson Correlation test. A value of  $p < 0.05$  was

considered statistically significant.

#### **Results and Discussion**

In order to check the homogeneity of animal models, after one-week acclimatization and randomly divided into four groups, all of the 24 mice were weighted individually. The body weights of mice (mean ± 1SD) were 30.25 ± 2.22 grams for aquadest group, 29.50 ± 0.58 grams for L-NAME group, 28.75 ± 0.957 grams for captopril group and 28.00 ± 2.16 grams for valsartan group respectively. Statistical analysis showed that the body weights of mice among groups were not significant different ( $p = 0.265$ , ANOVA test). In this study administration of L-NAME extract 1.75 mg/25g BW/day diluted in drinking water for 14 days, had significantly increased the mice blood pressures and successfully made the mice to be hypertensive. The systolic blood pressure (SBP) of L-NAME group (208 ± 8.04 mmHg); Captopril group (192.25 ± 13.52 mmHg) and Valsartan group (199.75 ± 13.47 mmHg) are significantly higher than normal/aquadest group (144.75 ± 4.99 mmHg). It is also shown by the differences of MAP of L-NAME group (122.5 ± 2.5 mmHg), Captopril group (116.75 ± 4.88 mmHg) and valsartan group (119.58 ± 4.77 mmHg) with normal/aquadest group (99.41 ± 2.34mmHg) ( $p < 0.05$ , ANOVA test). The differences of SBPs and MAPs among groups after 14 days induction of L-NAME extract (pretreatment) are presented on Table 1.

On day 14<sup>th</sup> post treatment, the SBP mean of L-NAME group is still high (224.75 ± 18.8 mmHg) and significantly higher than normal group (151.25 ± 2.21mmHg); Captopril (156.50 ± 8.34 mmHg) and valsartan group (167 ± 15.13 mmHg) ( $p < 0.05$ ). Indeed, the MAP of L-NAME group (128.08 ± 6.33mmHg) is also significantly higher than normal group (101.41 ± 2.50mmHg); captopril group (105.17 ± 2.99 mmHg) and valsartan group (108.75 ± 4.77 mmHg) ( $p < 0.05$ ). The treatment of captopril and valsartan within 14 days are able to significantly decrease blood pressures compared with L-NAME group ( $p < 0.05$ ), to be as low as normal ( $p > 0.05$ ). There was no difference between effects of captopril and valsartan in lowering blood pressure ( $p > 0.05$ ). The level of SBPs and MAPs post 14 days treatment are shown on Table 1.

Plasma renin and sST2 levels were examined

Table 1. Comparisons of blood pressures, level of plasma renin and sST2, and ventricular wall thickness among the four groups of study

Variables	Groups			
	Aquadest (mean ± 1 SD)	L-NAME (mean ± 1 SD)	Captopril (mean ± 1 SD)	Valsartan (mean ± 1 SD)
Systolic Blood Pressures Pre treatment (mmHg)	145.60 ± 4.72 <sup>a</sup>	208.00 ± 6.96 <sup>b</sup>	191.00 ± 12.04 <sup>b</sup>	204.60 ± 15.93 <sup>b</sup>
Mean Arterial Pressures Pre treatment (mmHg)	98.93 ± 2.30 <sup>a</sup>	123.20 ± 2.75 <sup>b</sup>	116.47 ± 4.28 <sup>b</sup>	119.40 ± 4.15 <sup>b</sup>
Systolic Blood Pressures Post treatment (mmHg)	150.00 ± 3.39 <sup>a</sup>	223.40 ± 16.59 <sup>b</sup>	157.80 ± 7.79 <sup>a</sup>	168.00 ± 13.11 <sup>a</sup>
Mean Arterial Pressures Post treatment (mmHg)	99.73 ± 4.34 <sup>a</sup>	128.33 ± 5.51 <sup>b</sup>	105.40 ± 2.64 <sup>a</sup>	108.9 ± 4.15 <sup>a</sup>
Level of Plasma Renin (ng/mL)	36.16 ± 10.7 <sup>a</sup>	59.06 ± 5.46 <sup>b</sup>	29.01 ± 10.42 <sup>a</sup>	24.66 ± 5.21 <sup>a</sup>
Level of Plasma sST-2 (pg/mL)	1.40 ± 0.60 <sup>a</sup>	3.04 ± 0.30 <sup>a</sup>	2.26 ± 0.52 <sup>a</sup>	1.90 ± 0.43 <sup>a</sup>
Ventricular wall thickness (µm)	853.1 ± 232.74 <sup>a</sup>	1480.1 ± 281.6 <sup>b</sup>	1169.7 ± 273.2 <sup>a,b</sup>	1033.2 ± 217.9 <sup>a</sup>

Note: different notation means there is significant different ( $p < 0.05$  ANOVA)

post treatment only. Plasma renin level of hypertensive mice in L-NAME group ( $59.44 \pm 6.23$  ng/mL) is the highest among others, and significantly higher than normal/aquadest group ( $36.37 \pm 1.38$  ng/mL) ( $p < 0.05$ ). The level of plasma renin in captopril ( $29.54 \pm 11.95$  ng/mL) and valsartan group ( $23.33 \pm 4.94$  ng/mL), are significantly lower than L-NAME group ( $p < 0.05$ ) (Table 1), but there is no difference between captopril and valsartan group them self. In contrast there are no differences of plasma level of sST2 among all four groups ( $p > 0.05$ , ANOVA), as also presented on Table 1. Pearson Correlation Analysis with 2-tail significance shows that there are significant correlations between plasma renin level post treatment with systolic blood pressure ( $r = 0.700$ ;  $p = 0.003$ ) and MAP ( $r = 0.669$ ;  $p = 0.005$ ), but not for sST2 level, either with SBP ( $r = -0.067$ ;  $p = 0.805$ ), MAP ( $r = -0.03$ ;  $p = 0.91$ ) level of plasma renin ( $r = 0.189$ ;  $p = 0.48$ ) and regression of LVH ( $r = 0.047$ ;  $p = 0.862$ ).

There are various types of preclinical models of HT, such as surgically induced HT, endocrine HT, dietary HT, neurogenic HT, and chemically induced HT. The L-NAME induced HT is an example of chemically induced HT. In L-NAME hypertensive animal model, blood pressure values are reached between 170 and 190 mmHg after 4

weeks induction [18]. N-nitro-L-arginine-methylester (L-NAME) is a nonspecific inhibitor of all three NO synthase (NOS) isoforms (neuronal - nNOS; inducible - iNOS; endothelial- eNOS) and causes an increase of blood pressure in a dose dependent manner when administered orally to the experimental animals [19]. In this study we used the dose of 1.75 mg/25g BW/day diluted in drinking water, after adjustment of standard dose of 40 mg/Kg [17,18,19]. The adjusted dose was derived from calculating the rat dose by 0.14 as conversion factor from rat to mouse [20]. Blood pressures rised to increase as confirmed by both the SBPs and MAPs after two weeks of L-NAME induction (Table 1), and after two weeks of treatment (Table 1). This finding coupled with the level of plasma renin, since it was still high in L-NAME group, but decreases in captopril and valsartan group after the next two weeks of treatment (Table 1). This was also confirmed by the positive correlations between plasma renin level with systolic blood

pressure/SBP ( $r = 0.700$ ;  $p = 0.003$ ) and MAP ( $r = 0.669$ ;  $p = 0.005$ ). Posttest analysis of ACE inhibitor (captopril) and angiotensin II type-1 receptor blocker (valsartan) effect within two weeks treatment shows that both were effective in lowering blood pressure ( $p > 0.05$ ), confirming previous studies [9, 10, 11]. Our results in this study proof

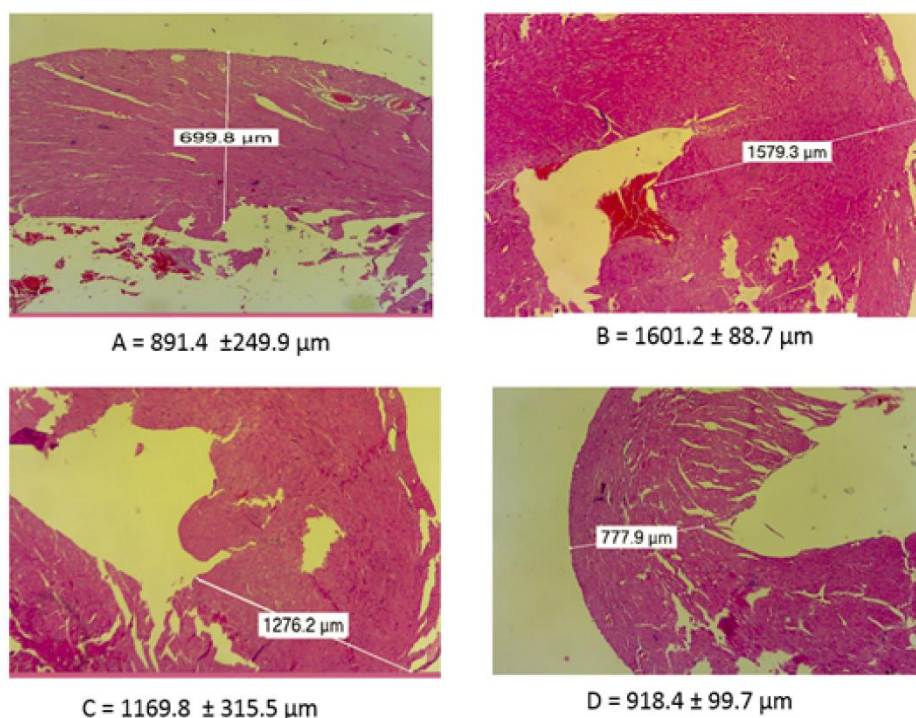


Figure 1. Samples of individual thickness of ventricular wall representing group. Were measured under light microscope in 40x magnification (H&E staining). A=Aquadest, B= L-NAME; C= captopril; D = valsartan. Valsartan could significantly reduce the ventricular wall thickness to normal level (D vs A)

that oral route of L-NAME in adjusted dose within two weeks has successfully inhibited NOS, increased plasma renin level and made the mice model to be hypertensive. Renin, the well-known enzyme that activates angiotensinogen to angiotensin 1, firstly initiates cascade in the RAAS, resulting the increase of blood pressure [26]. Captopril and valsartan have similar effects in enhancing nitric oxide production, reducing plasma renin level and lowering blood pressure, either through bradykinin or AT<sub>2</sub> receptor pathway.

In H&E histological slides examinations we found that ventricular wall thickness of -L-NAME group ( $1601.2 \pm 88.7 \mu\text{m}$ ) is significantly highest compared to aquadest ( $891.4 \pm 249.9 \mu\text{m}$ ) and valsartan group ( $918.4 \pm 99.7 \mu\text{m}$ ) ( $p < 0.05$ ). The ventricular wall thickness of captopril group ( $1169.8 \pm 315.5 \mu\text{m}$ ) is seemed to be lower than L-NAME group, but the difference is not significant ( $p > 0.05$ ). Microscopic sample slides representing one group each, are presented on Figure 1. In this study we examined the histological specimens of hearts using hematoxylin and eosin (H&E) staining and measured the left ventricular wall thickness in order to reflect the LVH. The result

showed that induction of L-NAME within 14 days, then continued with the next 14 days without antihypertensive treatment (totally four weeks induction with L-NAME), resulted in a significant thickening of ventricular wall compared with normal ( $p < 0.05$ ). Furthermore, both of captopril and valsartan were significantly able to reduce the wall thickness until nearly normal (Table 1).

Our results showed that there was also significant correlation between wall thickness with SBP and MAP but not with the level of plasma renin and plasma sST<sub>2</sub>. Although Captopril and valsartan showed similar efficacy in lowering blood pressure, valsartan seemed to be more effective in reducing ventricular wall thickness (Table 1). The low significance may due to the small number of samples or measurements of wall thickness of HE staining slides. At hematoxylin and eosin (H&E) staining for any histopathological examination, ideally dystrophic striated muscle pathology can be easily assessed on sections stained with H&E. In proper staining, hematoxylin will stain eosinophilic structures (e.g. muscle sarcoplasm) pink and eosin will stain basophilic structures (e.g. nuclei) dark purple, but it is time-consuming and need ex-

peritise [29]. Since the LVH is initiated by fibrosis process, so that in order to detect early process of LVH and to measure the degree of fibrosis we suggest to use a specific staining such as Masson's trichrome staining [30], Picrosinus red staining which is automatically scanned or immunohistochemistry staining, as what Hadi *et al.* had done [30].

Interestingly, in this study the levels of plasma sST2 post treatment did not correlate, either with systolic blood pressure, MAP, plasma renin level or ventricular wall thickness. In contrast, many studies and literatures mentioned that plasma sST2 is a specific cardiac biomarker which has high prognostic value with respect to almost all-cause and cardiovascular death including patients with myocardial infarction (MI), acute heart failure (AHF) and chronic heart failure [15, 27, 28]. Soluble ST2 is also markedly induced in mechanically overloaded cardiac myocytes and serves as cardiac stress biomarker which associated with LVH [14, 15, 16]. Meta-analysis study performed by Aimo *et al.* (2017) mentioned that plasma sST2 has prognostic value with respect to all-cause and cardiovascular death [31], and associated with cardiac concentric hypertrophy in patients with metabolic syndrome [32]. Circulating sST2 levels also correlate with a worse phenotype of disease including adverse remodeling and fibrosis, cardiac dysfunction, impaired hemodynamics and higher risk of progression [33, 34]. Therefore, it was supposed that plasma level of sST2 would increase in the early stage of LHV. In this study, induction of with L-NAME within two weeks, did not induce significant increase of plasma sST2 of mice models. There was no difference of plasma level of sST2s among the four study groups. This finding suggests that the level of plasma sST2 is not always mirroring or correlating with improving or worsening of clinical condition, including the ventricular wall fibrosis and thickness. It is relevant with the study reported by Yang *et al.*, that in sepsis patients sST2 showed moderate correlation with MAP but no correlation with LV ejection fraction [35]. The serum concentration of sST2 in metabolic syndrome patients was not linked to age, gender, or history of hypertension or diabetes [33]. In the Dallas Heart Study (DHS) sST2 concentration were not significantly associated with most traditional cardiovascular risk factors or sub-clinical cardiovascular disease [36]. Ojji *et al.* [14]

mentioned that blood pressure in hypertension patients did not influence sST2 concentration. Willems *et al.* [37] also reported that sST2 levels have no predictive value for future cardiovascular events patients with significant carotid artery stenosis. Malek *et al.* [38] also mentioned that concentration of cardiac biomarkers including sST2 did not correlate with echocardiography parameters. Study which was done by Gao *et al.* [39] showed that serum IL-33 and sST2 were overexpressed in acute-on-chronic hepatitis B liver failure (ACHBLF) and sST2 might potentially serve as a prognostic marker for it. We suggest that soluble ST2 may not be specific for cardiac stress biomarker which associated with LVH and useful as predictive tool of future cardiac events anymore.

### Conclusion

Captopril and valsartan had similar effect in lowering plasma renin level, blood pressure and LVH regression in Hypertension mice models, while sST2 seems to be not involved in this model of LVH regression obtained after blocking renin - angiotensin system.

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