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Pharmacology

Age-dependent increase in activity of eukaryotic elongation factor 2 kinase in mesenteric arteries from spontaneously hypertensive rats

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ABSTRACT. Eukaryotic elongation factor 2 (eEF2) kinase (eEF2K) negatively regulates protein translation through the phosphorylation of its specific substrate, eEF2. We previously found that expression of eEF2K was increased in arteries from 13–15-week-old spontaneously hypertensive rats (SHR) as well as in left ventricles of cardiac hypertrophy models. Furthermore, we demonstrated that eEF2K mediates the development of essential hypertension and pulmonary arterial hypertension in animal models. Protein expression changes with age during development of hypertension in SHR. In the present study, we examined whether activity and expression of eEF2K change in isolated mesenteric arteries dependent on the age. After superior mesenteric arteries were isolated from 4–10-week-old Wistar Kyoto rats (WKY) and SHR, Western blotting was performed. The phosphorylation of eEF2K at Ser500, an activating phosphorylation site, was increased in the arteries from 10-week-old SHR, whereas the phosphorylation of eEF2K at Ser366, an inactivating phosphorylation site, was increased in the arteries from 4–5-week-old SHR compared with WKY. The expression of eEF2K was increased in the arteries from 10-week-old SHR compared with WKY. The phosphorylation of eEF2 at Thr56 was decreased in the arteries from 4–5-week-old SHR, whereas it was increased in the arteries from 10-week-old SHR compared with WKY. We for the first time revealed that eEF2K activity is lower in prehypertensive stage but higher in hypertensive stage in SHR, suggesting that an inhibition of eEF2K activity may be a potential therapeutic strategy for the treatment of essential hypertension.

KEY WORDS: age, artery, eukaryotic elongation factor 2 kinase, hypertension, spontaneously hypertensive rats

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Eukaryotic elongation factor 2 (eEF2) kinase (eEF2K) is a calmodulin (CaM)-dependent protein kinase that negatively regulates protein translation. eEF2K has a catalytic domain at the N-terminus and phosphorylates eEF2, a specific substrate, when activated by a Ca^{2+}/CaM complex. As a result, the eEF2 activity is suppressed, and the elongation reaction of peptide chain is suppressed by inhibiting the movement of ribosome from the P to A position. Expression of eEF2K has been reported to be increased in tumor cells such as glioblastoma [11], breast cancer cells [12], and pancreatic cancer cells [2]. We previously demonstrated that expression of eEF2K was increased in aorta and mesenteric arteries from 13–15-week-old spontaneously hypertensive rats (SHR) [15] as well as in left ventricular tissues of isoproterenol- and transverse aortic constriction-induced cardiac hypertrophy models [9]. Furthermore, we have previously demonstrated that eEF2K mediates the development of essential hypertension via reactive oxygen species (ROS)-dependent vascular inflammation [14]. In addition, we have previously demonstrated that eEF2K mediates pulmonary arterial wall remodeling via NADPH oxidase (NOX)-1/ROS/matrix metalloproteinase-2 pathway [8]. These reports suggest that eEF2K is at least partly involved in the pathogenesis of several cardiovascular diseases including hypertension.

Hypertension is medically defined as a sustained increase in blood pressure (BP) of ≥130/80 mmHg. Hypertension is positively correlated with the risk of stroke, myocardial infarction, heart failure, and kidney disease as well as mortality [4]. Approximately 95% of adult hypertension is essential hypertension, the cause of which is unknown. It has been reported that the protein expression was changed with age in SHR, a rat model of essential hypertension. It has been reported that protein expression of voltage-dependent Ca^{2+} channel (CaV) 1.2α2δ1 was increased in cerebral arterial smooth muscle cells derived from 12-week-old

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SHR compared with 6-week-old SHR, which led to the enhanced vasocontraction via an increased CaV1.2 current [3]. It has also been reported that CaV expression was decreased in the isolated aorta derived from 40-week-old SHR compared with 8-week-old SHR [5]. Furthermore, it has been reported that NOX-4 expression was increased in the isolated aorta from 8- and 12-week-old SHR but not from 6-week-old SHR, while endothelial nitric oxide synthase expression was decreased in the isolated aorta from 12-week-old SHR [1]. Although it is logical to hypothesize that eEF2K expression and its activity differ dependent on the age of SHR during the development of hypertension, it has not been explored. In the present study, we aimed to test the hypothesis. As a result, we showed for the first time that activity and expression of eEF2K are changed dependent on the age of SHR.

MATERIALS AND METHODS

Animals

Four- to 10-week-old male Wistar Kyoto rats (WKY) and SHR (Hoshino Laboratory Animal Inc., Ibaraki, Japan) were used. Animal care and treatment were conducted in conformity with the institutional guidelines of the Kitasato University. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Kitasato University. We confirmed that BP in SHR is increased with aging compared with WKY (Table 1).

Antibodies

Antibody sources were as follows: anti-phospho-eEF2K (p-eEF2K) Ser500 (1:200 dilution) (No. EP4451) (ECM Biosciences, Versailles, KY, USA); anti-p-eEF2K Ser366 (1:500 dilution) (No. A0071) (Assay Biotech, Sunnyvale, CA, USA); anti-total-eEF2K (t-eEF2K) (1:1,000 dilution) (No. sc-39071) (Santa Cruz Biotechnology, Dallas, TX, USA); anti-phospho-eEF2 (p-eEF2) Thr56 (1:500 dilution) (No. 905-775-100) (Assay designs, Ann Arbor, MI, USA); anti-total-eEF2 (t-eEF2) (1:500 dilution) (No. A301-688A-T) (Bethyl Laboratories, Montgomery, TX, USA); and anti-total-actin (t-actin) (1:1,000 dilution) (No. MAB1501) (Sigma-Aldrich, St. Louis, MO, USA).

Western blotting

Western blotting was performed as described previously [10]. SHR and WKY were deeply anesthetized with urethane (1.5 g/kg, i.p.) and euthanized by exsanguination. The main branch of superior mesenteric arteries was isolated. After removal of fat and adventitia, the mesenteric arteries were mashed with a lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, and 1 mg/ml leupeptin; Cell signaling Technology, Danvers, MA, USA, and 0.1% protease inhibitor mixture; Nacalai Tesque, Kyoto, Japan) by the Cell destroyer (Bio Medical Science Inc., Tokyo, Japan). Protein concentration was determined by using a bicinchoninic acid method (Pierce, Rockford, IL, USA). After equal amounts of proteins (10 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) at 80–120 V for 1.5–2 hr, they were transferred to a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, USA) at 400 mA for 1.5 hr. The membranes were blocked with 3% bovine serum albumin (for phosphorylated proteins) or 0.5% skim milk (for total proteins), and then incubated overnight at 4°C with primary antibody. The signals were detected by using a horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution in Tris-buffered saline with Tween 20, 45 min at room temperature) and the EZ-ECL system (Biological Industries, Kibbutz, Beit-Haemek, Israel). Equal loading of protein was confirmed by measuring t-actin expression. The results were analyzed using a CS analyzer 3.0 software (ATTO, Tokyo, Japan).

Statistics

Data were shown as means ± standard error of the mean. Statistical evaluations were done with unpaired student's *t*-test. Results were considered significant when *P* was less than 0.05.

RESULTS

Phosphorylation of eEF2K at Ser500 in isolated mesenteric arteries from WKY and SHR

We first compared phosphorylation of eEF2K at Ser500, an activating phosphorylation site, in isolated mesenteric arteries from

Table 1. Systolic blood pressure (SBP) in 4–10-week-old Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR)

	SBP (mmHg)	
	4–5-week-old (n=5)	10-week-old (n=4)
WKY	90.5 ± 9.0	116.8 ± 2.6$^{\$}$
SHR	98.3 ± 6.8	179.9 ± 2.3$^{**, \#\#}$

SBP was measured by a tail cuff method. The results were shown as mean ± standard error of the mean [n=5 (4–5-week-old), n=4 (10-week-old)]. $^{\$}P<0.05$ vs. 4–5-week-old WKY, $^{**}P<0.01$ vs. 4–5-week-old SHR, $^{\#\#}P<0.01$ vs. 10-week-old WKY.

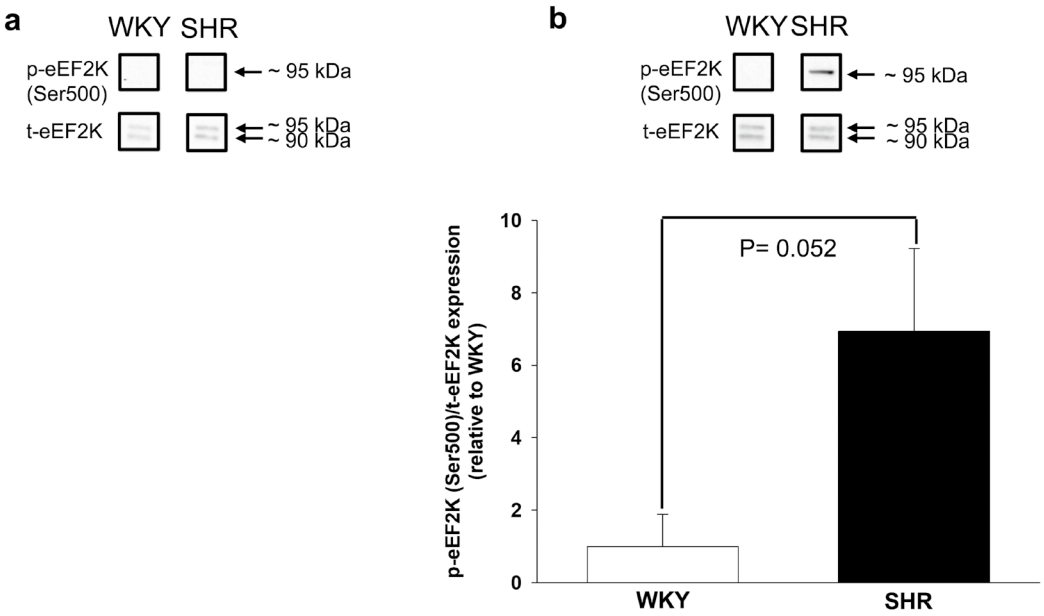

Fig. 1. Phosphorylation of eukaryotic elongation factor 2 kinase (eEF2K) at Ser500 in isolated mesenteric arteries from 4–10-week-old Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Phosphorylation of eEF2K at Ser500 was determined by Western blotting. Phosphorylation of eEF2K at Ser500 was normalized to total-eEF2K (t-eEF2K). The results were shown as fold increase relative to WKY [a (4–5-week-old): n=4, b (10-week-old): n=4]. Please note that representative t-eEF2K blots in Fig. 1a, Fig. 2a, and Fig. 3a were from the same origins.

WKY and SHR. The phosphorylation of eEF2K at Ser500 was not detectable in isolated mesenteric arteries from 4–5-week-old WKY and SHR (Fig. 1a, n=4). The phosphorylation of eEF2K at Ser500 was increased in mesenteric arteries from 10-week-old SHR compared with WKY at the same age (Fig. 1b, n=4).

Phosphorylation of eEF2K at Ser366 in isolated mesenteric arteries from WKY and SHR

We next compared phosphorylation of eEF2K at Ser366, an inactivating phosphorylation site, in isolated mesenteric arteries from WKY and SHR. The phosphorylation of eEF2K at Ser366 was increased in mesenteric arteries from 4–5-week-old SHR compared with WKY at the same age (Fig. 2a, n=5), while it did not change in the arteries from 10-week-old SHR compared with WKY at the same ages (Fig. 2b, n=4).

Expression of eEF2K in isolated mesenteric arteries from WKY and SHR

We further compared expression of eEF2K protein in isolated mesenteric arteries from WKY and SHR. The expression of eEF2K was significantly increased in mesenteric arteries from 10-week-old SHR compared with WKY at the same age (Fig. 3b, n=4, $P<0.05$), while it did not change in the arteries from 4–5-week-old SHR compared with WKY at the same ages (Fig. 3a, n=5).

Phosphorylation of eEF2 at Thr56 in isolated mesenteric arteries from WKY and SHR

We finally compared phosphorylation of eEF2 at Thr56, an inactivating phosphorylation site, in isolated mesenteric arteries from WKY and SHR. The phosphorylation of eEF2 at Thr56 was decreased in mesenteric arteries from 4–5-week-old SHR compared with WKY at the same age (Fig. 4a, n=4). It was significantly increased in mesenteric arteries from 10-week-old SHR compared with WKY at the same age (Fig. 4b, n=4, $P<0.05$).

DISCUSSION

In present study, we examined whether activity and expression of eEF2K are changed in isolated mesenteric arteries dependent on the age of SHR during the development of hypertension. The major findings are as follows (summarized in Table 2): 1) The phosphorylation of eEF2K at Ser500, an activating phosphorylation site, was increased in the arteries from 10-week-old SHR compared with WKY (Fig. 1). 2) The phosphorylation of eEF2K at Ser366, an inactivating phosphorylation site, was increased in the arteries from 4–5-week-old SHR compared with WKY (Fig. 2). 3) The expression of eEF2K was significantly increased in the arteries from 10-week-old SHR compared with WKY (Fig. 3). 4) The phosphorylation of eEF2 at Thr56, a substrate of eEF2K, was decreased in the arteries from 4–5-week-old SHR, while it was significantly increased in 10-week-old SHR compared with WKY (Fig. 4). In summary, it has been for the first time revealed that eEF2K activity is lower in 4–5-week-old SHR and higher in 10-week-old SHR.

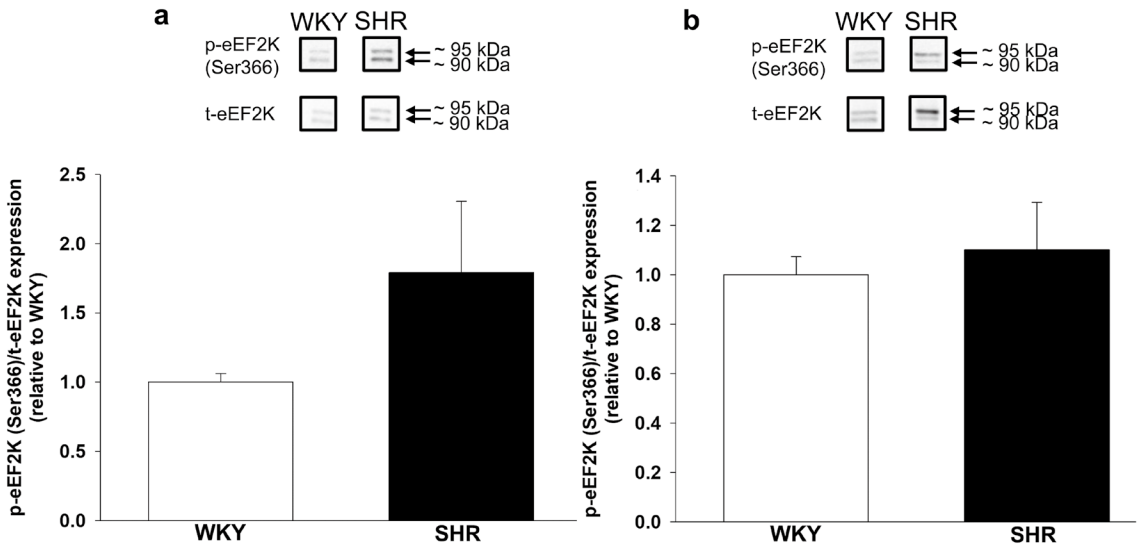

Fig. 2. Phosphorylation of eukaryotic elongation factor 2 kinase (eEF2K) at Ser366 in isolated mesenteric arteries from 4–10-week-old Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Phosphorylation of eEF2K at Ser366 was determined by Western blotting. Phosphorylation of eEF2K at Ser366 was normalized to total-eEF2K (t-eEF2K). The results were shown as fold increase relative to WKY [a (4–5-week-old): n=5, b (10-week-old): n=4].

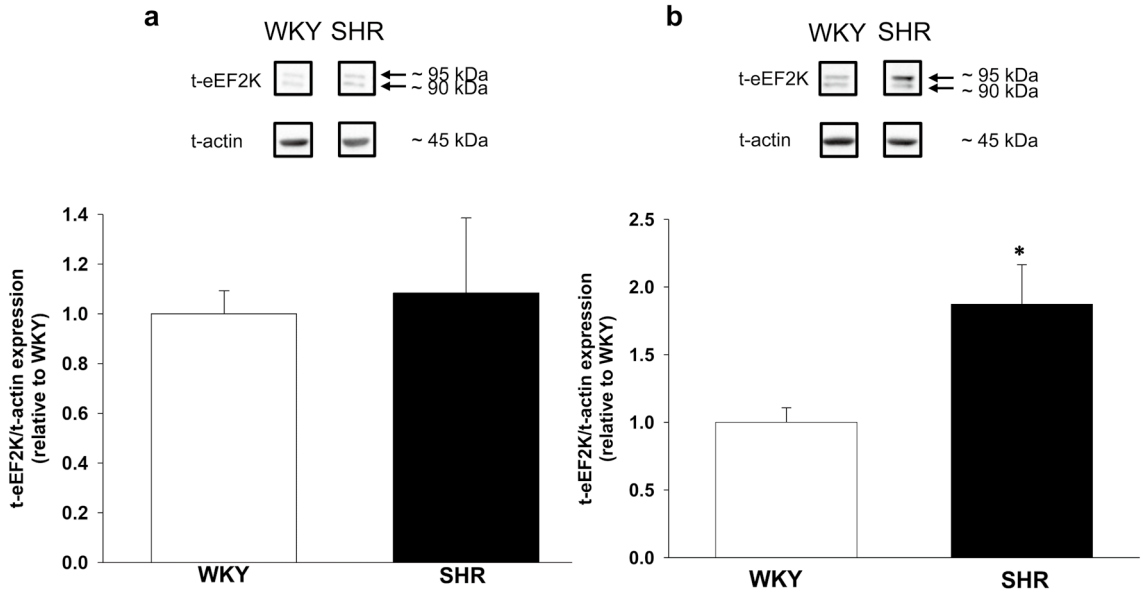

Fig. 3. Expression of eukaryotic elongation factor 2 kinase (eEF2K) protein in isolated mesenteric arteries from 4–10-week-old Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Expression of eEF2K protein was determined by Western blotting. Expression of eEF2K was normalized to total actin (t-actin). The results were shown as fold increase relative to WKY [a (4–5-week-old): n=5, b (10-week-old): n=4]. **P*<0.05 *vs.* WKY.

SHR, a rat model of essential hypertension, exhibits age-dependent hypertension without any treatment. ROS production in the vascular system is mainly mediated by NOX. It has been reported that expression of NOX-1 and 4 and ROS production were increased in isolated mesenteric arteries from hypertensive 12-week-old SHR, while they did not change in prehypertensive 6-week-old SHR [1]. Increased ROS production is involved in the pathological mechanisms of hypertension, including vascular endothelial dysfunction, increased vascular contractility, vascular smooth muscle cell proliferation and migration, and vascular inflammation [13]. It is then suggested that the increased ROS production may at least partly be involved in the age-dependent increase of BP in SHR. We have previously revealed that eEF2K mediates the development of hypertension partly via promoting ROS-dependent vascular inflammation [14]. In addition, we have revealed that eEF2K mediates the development of pulmonary

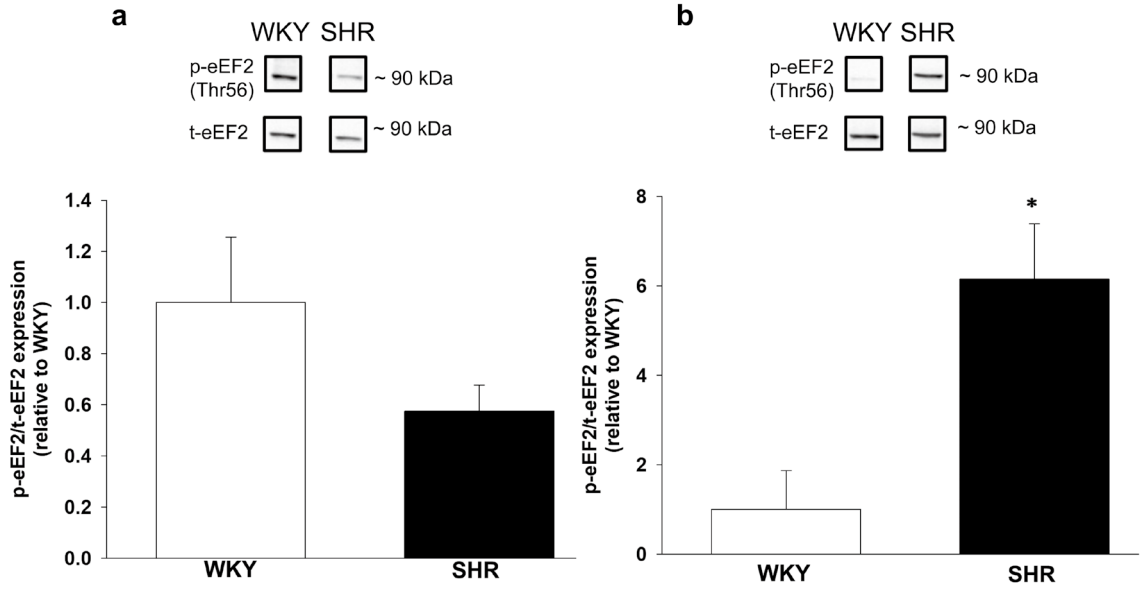

Fig. 4. Phosphorylation of eukaryotic elongation factor 2 (eEF2) at Thr56 in mesenteric arteries from 4–10-week-old Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Phosphorylation of eEF2 at Thr56 was determined by Western blotting. Phosphorylation of eEF2 at Thr56 was normalized to total-eEF2 (t-eEF2). The results were shown as fold increase relative to WKY [a (4–5-week-old): n=4, b (10-week-old): n=4]. **P*<0.05 *vs.* WKY.

Table 2. Summary of the phosphorylation and protein expression of eukaryotic elongation factor 2 (eEF2) and eEF2 kinase (eEF2K) in isolated mesenteric arteries from 4–10-week-old spontaneously hypertensive rats (SHR)

	SHR	
	4–5-week-old	10-week-old
p-eEF2K (Ser500)/t-eEF2K	N.D.	↑
p-eEF2K (Ser366)/t-eEF2K	↑	N.C.
t-eEF2K/t-actin	N.C.	↑↑
p-eEF2 (Thr56)/t-eEF2	↓	↑↑

Two upper-arrows represent a significant increase and one upper- or lower-arrow represents a slight change. N.D.: not detectable, N.C.: no change, p-eEF2K: phospho-eEF2K, t-eEF2K: total-eEF2K, t-actin: total-actin, p-eEF2: phospho-eEF2, t-eEF2: total-eEF2.

arterial hypertension partly via promoting pulmonary arterial wall remodeling through NOX-1/ROS/matrix metalloproteinase-2 pathway [8]. Furthermore, it has been reported that eEF2K is involved in the development of Alzheimer's disease [7] and Parkinson's disease [6] via ROS production. In present study, we have revealed that eEF2K activity is lower in 4–5-week-old prehypertensive SHR but higher in hypertensive 10-week-old SHR. The results indicate that the increased ROS production due to age-dependent increase in eEF2K activity may partly contribute to development of hypertension in SHR.

In conclusion, we have for the first time revealed that eEF2K activity is lower in prehypertensive stage but higher in hypertensive stage in SHR. Therefore, it is suggested that an inhibition of eEF2K activity may be a potential therapeutic strategy for the treatment of essential hypertension.

CONFLICT OF INTEREST. The authors have nothing to disclose.

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