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ORIGINAL ARTICLE

Puerarin Protects Against LPS-Induced Vascular Endothelial Cell Hyperpermeability *via* Preventing Downregulation of Endothelial Cadherin

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Abstract— In the present study, we aimed to investigate the effects of puerarin on the hyperpermeability of vascular endothelial cells induced by lipopolysaccharide (LPS) and its underlying mechanisms. Human umbilical vein endothelial cells (HUVECs) were pre-incubated with puerarin (25, 50, and 100 μM) for 1 h, and then exposed to LPS (1 μg/mL). The monolayer permeability of endothelial cells was assessed by measuring the paracellular flux of FITC-dextran 40,000 (FD40). The expression of vascular endothelial cadherin (VE-cadherin) in HUVECs was examined by Western blotting analysis. A total of 18 mice were randomly assigned into three groups as follows: control group, LPS group, and puerarin group. The pulmonary W/D ratio (wet-to-dry weight ratios) was calculated, and the lung morphology was examined. The levels of TNF-α and IL-1β in cell supernatant and mouse serum were determined by ELISA. Compared with the control group, LPS obviously increased the flux of FD40 and the monolayer permeability, raised the levels of TNF-α and IL-1β in cell supernatant, and reduced the VE-cadherin expression in HUVECs. However, puerarin (25, 50, and 100 μM) was able to relieve such LPS-induced increase in flux of FD40 and then reduce the hyperpermeability. Puerarin decreased the levels of TNF-α and IL-1β in cell supernatant and increased the VE-cadherin expression in HUVECs ($P < 0.05$). Moreover, LPS obviously increased the levels of TNF-α and IL-1β in mouse serum and elevated the pulmonary W/D ratios, resulting in lung injury. However, all of above-mentioned LPS-induced changes were improved by puerarin pre-treatment. Puerarin could alleviate LPS-induced hyperpermeability in endothelial cells *via* preventing downregulation of endothelial cadherin.

KEY WORDS: puerarin; lipopolysaccharide; permeability; inflammatory factor; VE-cadherin.

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INTRODUCTION

Sepsis is the main disease diagnosed in Intensive Care Unit globally, and its incidence is increasing year by year. Although anti-infective treatments continue to progress and there is strong support from modern intensive care, the mortality rate of sepsis remains high [1]. Increased vascular permeability is a hallmark of sepsis, the initial manifestation of its occurrence, and the pathological basis leading to other subsequent changes [2]. On the one hand, capillary leakage based on the increased endothelial cell permeability causes intercellular edema and increased intercellular distance, resulting in hypoxia of tissues and cells [3]. On the other hand, inflammatory cytokines are concentrated in local tissues, directly causing tissue and organ damage or even multiple organ dysfunction syndrome (MODS) [4]. Endothelial cell junctions, which are closely related to vascular permeability, consist of tight junctions and adhesion junctions [5]. As a characteristic calcium-dependent transmembrane adhesion protein of endothelial cells, the cadherin is a major component of intercellular adhesion junctions [6]. Previous study has shown that the increase of pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β), can disrupt the expression of vascular endothelial cadherin (VE-cadherin) [7].

As a structural component of the outer membrane of Gram-negative bacteria, lipopolysaccharide (LPS) is also known as endotoxin. Exposure to LPS can result in systemic inflammatory responses in the development of sepsis, such as vasomotor dysfunction, enhanced permeability of vascular endothelial cells, and activation of coagulation system [8–10]. Animal experiments have shown that LPS can trigger leakage of fluid in the blood vessels [11]. In addition, LPS can also significantly increase the permeability of vascular endothelial cells cultured *in vitro* [12]. Moreover, the permeability of vascular endothelial cells can be directly or indirectly elevated by LPS *via* upregulating various inflammatory factors, such as TNF-α, IL-1, and IL-8 [12, 13].

Puerarin is an isoflavone derivative isolated from Pueraria radix [14]. Many studies have shown that puerarin can improve endothelial function, and it has a wide range of anti-inflammatory activities. For example, puerarin can also protect against endothelium diastolic dysfunction induced by glycosylated bovine serum albumin and lysophosphatidylcholine [15, 16]. It has been shown that puerarin can decrease the plasma level of C-reactive protein in patients with unstable angina [17] and reduce the expressions of IL-1 and TNF-α at the mRNA level in the myocardial cells induced by LPS [18]. However, it remains unclear whether puerarin protects against LPS-induced hyperpermeability of vascular endothelial cells. In the present study, we aimed to investigate the protective effects of puerarin on the permeability induced by LPS and explore the mechanisms underlying such protective effects of puerarin on LPS-induced hyperpermeability in human umbilical vein endothelial cells (HUVECs). Moreover, we also assessed whether puerarin prevented the lung injury *via* mitigating endothelial injury induced by LPS.

MATERIALS AND METHODS

Reagents

Puerarin and LPS (*Escherichia coli* 055:B5) were purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). The puerarin and LPS powder were dissolved in deionized water, filtered, sterilized, and then stored at −20 °C. The stock solutions of puerarin and LPS were diluted to desired concentrations with DMEM before use. Rabbit anti-human VE-cadherin antibody was obtained from Abcam (Cambridge, UK), and mouse anti-human GAPDH antibody was provided by Santa Cruz Biotechnology Inc. (Dallas, TX, USA). The kits for enzyme-linked immunosorbent assay (ELISA) of TNF-α and IL-1β were supplied by ABclonal Biotechnology Co., Ltd. (Boston, MA, USA).

Cell Culture and Treatment

HUVECs were obtained from the Experimental Center, Xiangya School of Medicine, Central South University. Briefly, cells were maintained in DMEM supplemented with various growth factors, 10% fetal bovine serum (FBS), and 0.1% penicillin and streptomycin. Sub-confluent HUVECs were pretreated with puerarin at desired concentrations (0, 25, 50, and 100 μM) for 1 h, followed by challenge with LPS (1 μg/mL) for 12 h.

Measurement of Endothelial Permeability

Endothelial monolayer permeability was determined by a 40,000-kD fluorescein isothiocyanate (FITC)-dextran kit based on a transwell model (Millipore, USA). Briefly, HUVECs (2×10^5/well) were seeded into a transwell culture plate. The inserts were placed into 24-well plates containing 500 μL DMEM, and the cells were allowed to grow to full confluence until a monolayer formed. After the indicated treatments, 200 μL FITC-dextran (2 mg/mL) was added into the insert and allowed to pass through the cell monolayer for 2 h. Subsequently, 50 μL culture medium from the low chamber was harvested and used to determine the fluorescence intensity on a fluorescent microplate reader at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

Measurement of TNF-α and IL-1β Levels by ELISA

The ELISA kits were used to determine the levels of TNF-α and IL-1β in cell culture supernatant and mouse serum according to manufacturer's instructions. The microplate was pre-coated with a polyclonal antibody specific for TNF-α or IL-1β. TNF-α or IL-1β in standards and samples was sandwiched by the immobilized antibody and the biotinylated polyclonal antibody specific for TNF-α or IL-1β, respectively, which was recognized by a streptavidin-peroxidase conjugate. All unbound materials were then removed, followed by addition of a peroxidase enzyme substrate. The color development was terminated, and the color intensity was determined at a wavelength 450 nm based on the standard curve. The contents of TNF-α and IL-1β were expressed in pg/mL.

Determination of VE-Cadherin Expression by Western Blotting Analysis

Total cellular protein was isolated using the RIPA buffer (Beyotime, China). Equal amounts of proteins (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electro-transferred onto polyvinylidene difluoride (PVDF) membranes. Blots were blocked with 5% albumin bovine serum (BSA) in Tris-buffered saline containing Tween-20 (TBST) and then incubated with primary antibodies against VE-cadherin (1:1000) and anti-GAPDH (1:800) at 4 °C overnight. Subsequently, the blots were then incubated corresponding secondary antibodies (goat anti-rabbit IgG/HRP, 1:4000 or goat anti-mouse IgG/HRP, 1:8000) at room temperature (RT) for 2 h. Immunoreactive bands were visualized by enhanced chemiluminescence reagents (EMD Millipore, Billerica, MA, USA). The expressions at the protein level were quantitatively analyzed by Gel-Pro v. 4.0 analyzer (Media Cybernetics, Inc., Rockville, MD, USA).

Animals and Treatment

The animal-related experiments were carried out in accordance with internationally recognized guidelines on animal welfare and conducted according to the guidelines of the Chinese Council on Animal Care, and the experimental protocol was approved by Medical Ethic Committee of Xiangnan University. A total of 18 mice (weighing 18–20 g, 7–8 weeks of age) were purchased from Hunan SJA Laboratory Animal Co., Ltd. in Changsha, China. The animals were housed in a temperature-controlled facility (20–25 °C) with a relative humidity of 45–65% and a 12-h light/dark cycle. After 1 week of adaptation to laboratory conditions, the mice were randomly assigned into the following three groups: (1) control group ($n = 5$); (2) LPS group ($n = 6$); and (3) puerarin group ($n = 7$). Mice in the LPS group were intraperitoneally injected with saline for 3 days, followed by intraperitoneal injection of LPS (5 mg/kg) on the fourth day. Mice in the puerarin group were intraperitoneally injected with puerarin (50 mg/kg) for 4 days, followed by intraperitoneal injection of LPS on the fourth day. Mice in the control group were intraperitoneally administered with equal volume of saline for 4 days. The serum and lung tissues were collected on the fifth day.

Observation of Histological Changes in the Lung

The left lung tissues were dissected and fixed with 4% formaldehyde overnight. The specimens were dehydrated, paraffin embedded, and then cut into 4-μm sections, followed by hematoxylin and eosin staining. The histological changes in the lungs were examined under a photograph microscope (Leica Microscope Ltd., Wetzlar, Germany).

Calculation of Pulmonary Wet-to-Dry Weight Ratios

The right lung tissues were excised. The surface blood and water on the lung were absorbed by filter paper. The wet weight (W) was immediately measured, and then the tissue was placed in an oven at 70 °C for 72 h until a constant weight was obtained [dry weight (D)]. The ratio of

the wet weight over the dry weight of the same lung (W/D) was determined to assess pulmonary edema.

Statistical Analysis

Experimental data were expressed as means ± standard of mean (*SD*) and analyzed by GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). One-way ANOVA was employed to assess differences among different groups, followed by the Newman-Keuls multiple comparison test. A $P < 0.05$ was considered as statistically significant.

RESULTS

Puerarin Prevents the Increased Permeability of HUVEC Monolayer Induced by LPS

Endothelial monolayer permeability was determined by a transwell assay. HUVECs were challenged with LPS (1 μg/mL) for 12 h, and endothelial permeability was determined based on FD40 fluorescence intensity from the lower chamber. Transwell assay revealed that LPS significantly increased the endothelial permeability (Fig. 1).

Interestingly, when HUVECs were pretreated with puerarin for 1 h and then challenged by LPS for another 12 h, puerarin decreased the hyperpermeability induced by LPS in a dose-dependent manner (Fig. 1).

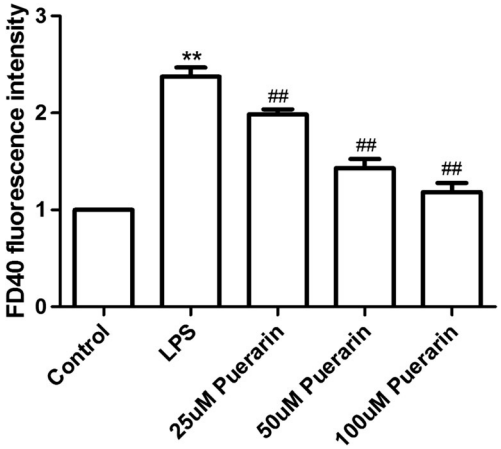

Fig. 1. Puerarin reduces the LPS-induced hyperpermeability of HUVEC monolayer. HUVEC monolayer in the upper chamber of transwells was treated with vehicle, LPS (1 μg/mL), or different concentrations of puerarin (25, 50, and 100 μM) and LPS (1 μg/mL), and then the amount of FITC-dextran diffused from the upper chamber to the bottom chamber was analyzed. $^{**}p < 0.01$ *versus* control. $^{\#\#}p < 0.01$ *versus* LPS group.

Puerarin Reverses the Downregulation of VE-Cadherin Induced by LPS

Adhesion junction proteins have been shown to be involved in maintaining the integrity of endothelial barrier. To further explore the action underlying puerarin on paracellular permeability, we evaluated the effect of puerarin on the expressions of adhesion junction proteins in LPS-treated HUVECs by Western blotting analysis. LPS challenge for 12 h significantly decreased the expression of VE-cadherin at the protein level. When HUVECs were pretreated with puerarin before LPS challenge, the decreased VE-cadherin expression in LPS-stimulated HUVECs was largely restored by puerarin in a concentration-dependent manner (Fig. 2).

Puerarin Reduces the Upregulation of Inflammatory Cytokines TNF-α and IL-1β Induced by LPS

As important inflammatory cytokines, TNF-α and IL-1β can increase endothelial permeability by disrupting the expression of VE-cadherin. In the present study, we determined the levels of TNF-α and IL-1β in the cell culture supernatant of HUVECs after LPS challenge. Compared with the control group, the levels of TNF-α and IL-1β were significantly increased in the cell culture supernatant upon LPS challenge. However, puerarin pre-treatment inhibited the LPS-triggered upregulation of TNF-α and IL-1β in the cell culture supernatant in a dose-dependent manner (Fig. 3). In the mouse serum, the levels of TNF-α

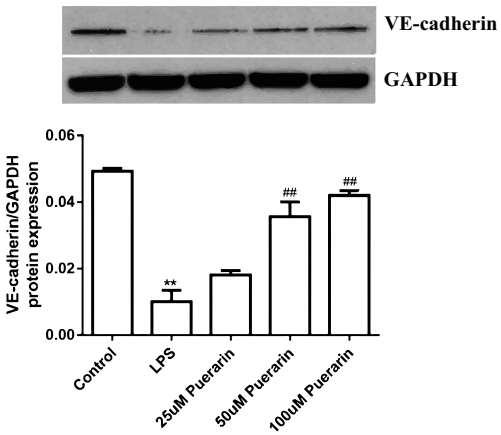

Fig. 2. Puerarin reverses the downregulation of VE-cadherin induced by LPS. LPS (1 μg/mL) for 12 h after pre-treatment with puerarin (0, 25, 50, and 100 μM) for 1 h. Western blotting analysis of VE-cadherin was performed. $^{**}p < 0.01$ *versus* control. $^{\#\#}p < 0.01$ *versus* LPS group.

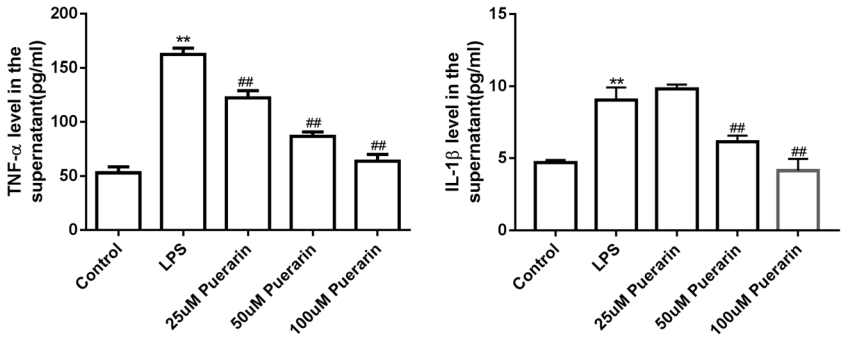

Fig. 3. Puerarin reduces the upregulation of inflammatory cytokines TNF-α and IL-1β in the cell culture supernatant of HUVECs after LPS challenge. Cell culture supernatant of HUVECs was collected after puerarin pre-treatment (0, 25, 50, and 100 μM) for 1 h and subsequent LPS treatment (1 μg/mL) for 12 h, and the concentrations of TNF-α and IL-1β were measured by the ELISA. $^{**}p < 0.01$ *versus* control. $^{\#\#}p < 0.01$ *versus* LPS group.

and IL-1β were also increased after LPS treatment at 24 h. In addition, the contents of TNF-α and IL-1β in the puerarin group were decreased compared with the LPS group (Fig. 4).

Puerarin Reduces the Augment of Pulmonary W/D Ratio in LPS-Challenged Mice

The W/D ratio in the LPS group was significantly higher than that of the control group. However, the W/D ratio in the puerarin group was significantly lower than that of the LPS group (Fig. 5).

Puerarin Improves Pulmonary Histopathological Changes in LPS-Challenged Mice

The alveolar structure in the lungs of mice in the control group was intact, showing normal alveolar interval, the lung interstitium was not edematous, and there was no infiltration of inflammatory cells in the alveolar cavity and the lung interstitium. The alveolar structure in the lungs of mice in the LPS group was severely damaged, and the alveolae and interstitium were significantly edematous. The alveolar interval was widened, and a large number of inflammatory cells infiltrated into the alveolar cavity and the lung interstitium. These manifestations were evidently improved by puerarin treatment (Fig. 6).

DISCUSSION

Our findings demonstrated that puerarin significantly attenuated LPS-induced hyperpermeability. Moreover, puerarin decreased the LPS-induced upregulation of TNF-α and IL-1β in the cell culture supernatant and mouse serum. Furthermore, puerarin simultaneously prevented the downregulation of VE-cadherin in LPS-challenged HUVECs. In addition, puerarin alleviated pulmonary W/D ratio and pulmonary histopathological damages induced by LPS. Therefore, we speculated that puerarin exerted its protective effect on LPS-induced hyperpermeability in

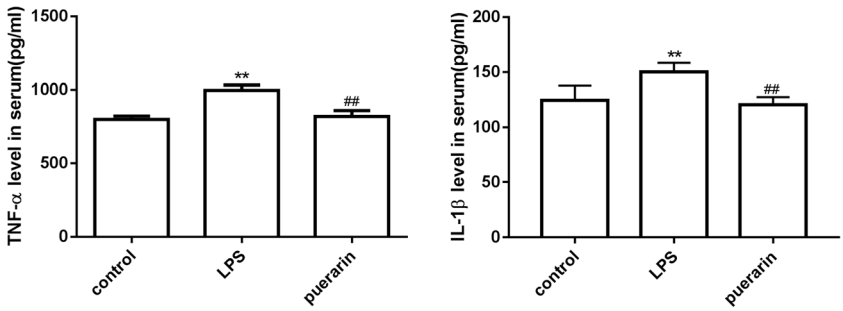

Fig. 4. Puerarin reduces the upregulation of inflammatory cytokines TNF-α and IL-1β in the mouse serum after LPS treatment. Mice were intraperitoneally injected with puerarin (50 mg/kg) for 4 days and then treated with LPS (5 mg/kg). The mouse serum was collected after application of LPS for 24 h, and the concentrations of TNF-α and IL-1β were measured by the ELISA. $^{**}p < 0.01$ *versus* control. $^{\#\#}p < 0.01$ *versus* LPS group.

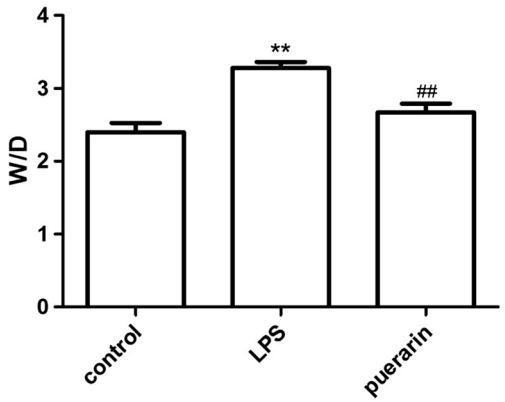

Fig. 5. Puerarin reduces the augment of pulmonary W/D ratio in LPS-challenged mice. Mice were intraperitoneally injected with puerarin (50 mg/kg) for 4 days and then treated with LPS (5 mg/kg). The mouse right lungs were collected after application of LPS for 24 h, and the ratio of the wet weight over the dry weight of the same lung (W/D) was determined. $^{**}p < 0.01$ *versus* control. $^{\#\#}p < 0.01$ *versus* LPS group.

HUVECs by inhibiting TNF-α and IL-1β and subsequently preventing downregulation of endothelial cadherin.

As an important structure, the endothelial barrier plays an essential role in maintaining the integrity of the blood-fluid compartment [19]. Intercellular junctional complexes composed of tight junctions and adherent junctions seal the paracellular pathway between adjacent endothelial cells [20]. Pro-inflammatory cytokines induce downregulation of relevant junction proteins, resulting in a loss of cell-cell association and inhibition of cell contact as well as uncontrolled growth and degradation of the basement [4]. VE-cadherin is one of the main adherent junctions, which is a special cadherin that appears in all endothelial cells found throughout all types of vessels [5]. LPS has been reported to elicit endothelial dysfunction *via* pleiotropic mechanisms, including apoptosis, upregulation of intercellular adhesion molecules, and barrier disruption [21]. Our experiments showed that LPS appeared to result in endothelial hyperpermeability *in vitro*, including TNF-α and IL-1β signaling pathways. This was also related to a subsequent downregulation of VE-cadherin from the endothelial junctions.

Although there are a lot of reports about the protective effects of puerarin on endothelial dysfunction [15, 16], little information is available regarding the pharmacological role of puerarin against endothelial hyperpermeability induced by LPS. In our present study, the findings validated the protective effects of puerarin on LPS-induced endothelial hyperpermeability. Our data demonstrated that LPS increased the levels of TNF-α and IL-1β and reduced the expression of VE-cadherin in HUVECs, while such alterations were restored by puerarin treatment. These results prompted that puerarin prevented LPS-induced hyperpermeability *via* decreasing the contents of TNF-α and IL-1β, subsequently avoiding downregulation of endothelial cadherin.

To investigate whether the protective effect of puerarin on vascular endothelial permeability could reduce organ damage, we further assessed the role of puerarin in LPS-induced lung injury in mice. LPS challenge increased the levels of TNF-α and IL-1β in the serum and enhanced the W/D ratio. At the morphological level, LPS triggered pulmonary edema, the alveolar space widening, and infiltration of inflammatory cells. However, above LPS-induced changes were improved by puerarin treatment. These findings indicated that puerarin attenuated the lung injury by reducing vascular endothelial hyperpermeability induced by LPS.

Collectively, these observations suggested that puerarin could be potentially used in the amelioration of LPS-mediated endothelial barrier dysfunction. This beneficiary effect of puerarin was correlated with inhibition of TNF-α and IL-1β levels as well as preventing downregulation of VE-cadherin. However, it remains largely unclear whether such protective effect of puerarin can be applied to other

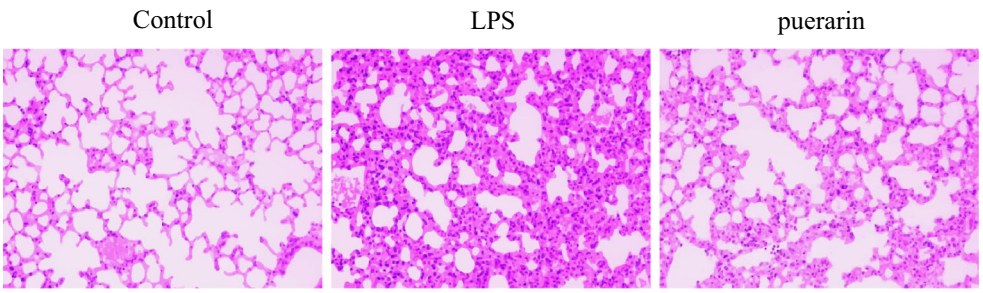

Fig. 6. Puerarin decreases pulmonary histopathological damages in LPS-challenged mice (× 200). Mice were intraperitoneally injected with puerarin (50 mg/kg) for 4 days and then treated with LPS (5 mg/kg). The mouse left lungs were collected after application of LPS for 24 h. The tissues were stained by hematoxylin and eosin, and the histological changes were examined under a photograph microscope.

stimulations. Future investigations would provide us a complete mechanism underlying the protective effects of this drug.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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