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Research Paper

Tea polyphenols affected BP as an endothelium-dependent vasodilator

J. Lv[1#], W. Li[1#], P. Zh[1], L. Li[1], X. Zhou[1], Z. Xu[1, 2]*

[#]J. Lv and W. Li contributed equally to this article

[1] Institute for Fetology, First Hospital of Soochow University, Suzhou, China

[2] Center for Perinatal Biology, Loma Linda University, Loma Linda, California, USA

*Corresponding Author

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ABSTRACT

Tea polyphenols are major active components of tea with important cardiovascular protective effects as suggested. This study used telemetrically recording to test the effects of tea polyphenols on blood pressure in freely moving rats *in vivo*, and vascular and cellular functions in isolated human umbilical veins as well as cells *in vitro*. Tea polyphenols (20, 50, and 100mg/kg) decreased blood pressure significantly without affecting heart rate and body temperature in the conscious rats. Vascular tone was reduced by tea polyphenols in umbilical veins, which could be suppressed by pre-treatment with L-NG-Nitroarginine Methyl Ester in organ bath. Removing vessel endothelium also significantly inhibited tea polyphenols-mediated vasodilatation. In the cultured human umbilical vein endothelial cells, angiotensin II-decreased nitric oxide levels were reversed by tea polyphenols. In addition, angiotensin II-increased endothelin release in the cultured endothelial cells was significantly inhibited by application of tea polyphenols. The results demonstrated the influence of tea polyphenols on blood pressure *in vivo* in the association of its vasorelaxation *in vitro*, which may be linked to the signaling pathways in the vascular cells. Since the present study was the first to test the effects of tea polyphenols or tea ingredients in the blood vessels of human umbilical cords, the data achieved should be important to feto-maternal medicine and clinical research.

Key words: Tea polyphenols; Blood pressure; Vasorelaxation.

INTRODUCTION

Epidemiological studies have shown that tea ingestion has been associated with a reduced risk in the development of cardiovascular diseases, and tea was shown its

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ability in the decrease in blood pressure (Chong et al., 2010; Dohadwala et al., 2009; Habauzit et al., 2012). Tea polyphenols (TP) are major active components of tea, and suggested to be responsible for the cardiovascular protective action (Khurana et al., 2013; Li et al., 2014). However, there has been very limited data or information on the effects of tea or its ingredients on isolated blood vessels and vascular cells in human. The present study used umbilical cord blood vessels from healthy human to test direct effects of TP on the isolated umbilical veins *in vitro*. In addition, the cell line of human umbilical vein endothelial cells (HUVEC) was used in the determination of the effects of TP at cellular levels.

The mechanism of the beneficial effects of TP on cardiovascular systems may be due to their antioxidant activity (Hatia et al., 2014; Newsome et al., 2014). However, many polyphenols are metabolized in the body, and these metabolites have much lower antioxidant capacity than their parent compounds, suggesting that antioxidant activity may not be the only mechanism of actions of tea (Sonja et al., 2010). Tea ingestion may improve endothelium-dependent vasorelaxation, and may be linked to nitric oxide (NO)-dependent vasorelaxation in healthy individuals under various physiological conditions (Tangney et al., 2013; Minatti et al., 2012). The ability of TP to activate endothelial nitric oxide synthase (eNOS) is likely the underlying mechanism for the improvement of endothelial functions and influence of blood pressure (Jiménez et al., 2012; Gómez-Guzmán et al., 2012). Accumulated evidence suggests that the mechanisms of actions of TP may go beyond their antioxidant activity and the attenuation of oxidative stress. Therefore, there is a need for more investigations on intracellular and molecular pathways underlying TP-induced cardiovascular protection. Thus, the present study focused on the possible roles of TP in vascular endothelial functions as well as in human umbilical vein endothelial cells.

Angiotensin II (Ang II) is well known for its effects on hypertension and vascular tension (Yu et al., 2005). Nitric oxide (NO) and endothelium (ET) also play important roles in regulation of vascular tension (Quaschning et al., 2003; Yukihito et al., 2004). Previous studies demonstrated that Ang II could change vascular endothelial functions (Alia et al., 2011). Notably, Ang II was shown its significant effects on the reduced levels of nitric oxide (NO) in coronary vessels, resistant arteries, and vascular smooth muscle cells (Dikalov et al., 2008; Dikalova et al., 2005; Dikalova et al., 2010), and stimulation of ET release in endothelial cells (Wenzel et al., 2011). Thus, in the present study, we used human umbilical vein cell line (HUVC) as well as vascular stimulator Ang II in the determination of possible effects and mechanisms for TP-induced changes in the human vascular cells. Together, the information gained is not only important for further understanding the vascular effects and TP as well as its mechanisms, but also valuable to clinical practice regarding regulations of vascular functions. In particular, since the present study was the first to test the effects of TP or tea on umbilical blood vessels, and this type of work-activity of tea polyphenols is reported first time here, the data achieved should be important to feto-maternal medicine and clinical research.

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MATERIALS AND METHODS

Animals and treatment: Male Sprague Dawley rats (250g) were from the Center for Experimental Animals of Soochow University, and randomly divided into four groups (n=8). Three groups of animals were given 20mg/kg, 50mg/kg, or 100mg/kg TP (Xianlin Pharmaceutical Ltd. Wuhan, China) subcutaneously, and one group was given same volume 0.9% saline as the control. All animals were treated humanely and followed the Guild-lines for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All approaches in this study were approved by the Institute Ethics Board of First Hospital of Soochow University with number 20120205 on February 5, 2012.

Telemetrically recording in vivo: Implantable transmitters (AD Instrument, Dunedin, New Zealand) was used in conscious and freely moving rats for measuring mean arterial pressure (MAP) and heart rate (HR). Rats were anesthetized with a mixture of ketamine (1.1mg/kg) and xylazine (15mg/kg). The abdominal aorta of the rat was accessed with a ventral midline incision. The abdominal aorta was isolated with fine-tipped vessel dilation forceps. Two occlusion sutures were placed beneath the artery. The elevated artery was punctured with a catheter introduced, and the telemetry catheter was inserted into the vessel. The catheter tip was advanced so that 3 mm of the thin-walled tip section could reside in the abdominal aorta. The sutures were tied and secured with tissue adhesive. The body of the transmitter was slipped into the pocket and secured with tissue adhesive. The ventral incision was then closed. All rats were allowed 5 days of recovery after transmitter implantation before experiments started. This time interval is necessary for rats to regain their circadian blood pressure and heart rate rhythm. Thereafter, MAP and HR were recorded and stored in the Dataquest ART data acquisition system (AD Instruments, Dunedin, New Zealand). Signals were acquired continuously and expressed as beat-to-beat values. MAP and HR were derived from the electrocardiogram waveforms and were continuously recorded for 12 hours following the TP treatment. Core body temperature was monitored and recorded continuously by using thermocouple temperature probe (AD Instruments, New Zealand) throughout all experiments.

Vascular tension in human umbilical vein: Human umbilical cord was collected from pregnant women immediately after delivery at term (median gestation: 38-40 weeks) at the Suzhou Municipal Hospital, and kept in 4℃ Krebs–Henseleit buffer (NaCl 119, KCl 4.7, $NaHCO_3$ 25, KH2PO$_4$ 1.2, CaCl2 2.5, $MgSO_4$ 1.0, D-glucose 11), bubbled with 95% O_2 and 5% CO_2. All umbilical cords were collected following spontaneous delivery without complications. For those subjects, there was no evidence of hypertensive disease, gestational diabetes mellitus, and other diseases. The mean body mass index was 23 kg/m^2. All procedures were approved by the Institute Ethic Committee.

Human umbilical veins (HUV) were isolated and cut into rings with 5mm length approximately. Half rings were denuded of endothelium by wiping the luminal surface with a piece of wet paper towel held in fine forceps. The failure of acetylcholine (1uM) to relax a contraction induced by PGF2 (prostaglandin F) (0.1uM)

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demonstrated the effectiveness of removal of endothelium. After the rings were mounted on the bath, a passive tension of 2g was applied to each ring and isometric tension was measured with a force transducer. During the equilibration period, Krebs'solution was maintained at 37℃ and pH 7.4 with constant bubbling of 95% O_2/5%CO_2. The bath solution was replaced every 15min. After 120min of equilibration period, each preparation was contracted with 60mM KCl, and then washed. After third KCl challenge, we added TP or NG-nitro L-arginine methyl ester (L-NAME, Sigma, USA) or saline as a control. Strips with a KCl response of<1g were excluded. Optimal passive tension was adjusted throughout the equilibration period. Experiments were performed in parallel of rings from the same tissue.

Pharmacological experiments: Cell line of human umbilical vein endothelial cells (HUVEC) was provided by the American Type Culture Collection. Cells were cultured in DMEM (Hyclone, UT, USA) with 15% FBS (Hyclone, UT, USA), 100 U/ml penicillin (Hyclone, UT, USA), and 10 mg/ml streptomycin (Hyclone, UT, USA) at 37°C, 5% CO_2. Cells were inoculated into six-well culture dishes in the density of 4×10^5 following process of resuscitation and passage, and then were treated with Ang II(10uM)(Sigma, St. Louis, MO, USA) or Ang II(10uM)+TP (10mg/kg, 50mg/kg，100mg/kg, 200mg/kg) or same volume cell culture medium as control for 4-72 hours. They were exposed to serum-free culture medium in the presence of 0.1% bovine serum albumin for 6 hours prior to treatments.

Determination of expression of eNOS and ET: Following the treatments, cells were washed twice with PBS, then lysed in extraction buffer (composition in mmol/liter: Tris/HCl 20 (pH 7.5), NaCl 150 Na3VO4 1, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01, a tablet of protease inhibitor and 1% Triton X-100). Total proteins (20μg) were separated on 10% SDS-polyacrylamide gels at 110 V for 2 hours. Separated proteins were transferred electrophoretically onto polyvinylidine difluoride membranes at 200mA for 120 min. Membranes were blocked with blocking buffer containing 5% bovine serum albumin, Tris-buffered saline solution and 0.1 % tween 20 (TBS-T) for 1 hour. For detection of eNOS and ET, membranes were incubated with the primary antibody (ET or eNOS; 1:1,000; Cell Signaling Technology, Beverly, MA) overnight at 4℃. After washing, membranes were incubated with the secondary antibody (anti-rabbit IgG, 1:5,000 for ET, and anti-mouse IgG; 1:20,000 for eNOS; Cell Signaling Technology, Beverly, MA) at room temperature for 60 min. Pre-stained markers (Invitrogen, Life Technologies, Carlsbad, CA) were used for molecular mass determinations. The immunoreactive bands were detected by enhanced chemiluminescence.

Determination of NO and ET levels in cell culture: NO and ET released from cells in the culture medium were measured using radioimmunoassay (RIA) by Beijing Sino-UK institute of Biological Technology uses radioimmunoassay (Ding et al., 2010). The samples and data were handled in a blinded manner.

Statistical analysis: Data were expressed as means±SEM. Statistical evaluation was performed by two-way ANOVA. Values of $P<0.05$ were considered statistically significant.

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RESULTS

Blood pressure and heart rate: The rats showed a significant decrease of MAP compared with the control at 3 hours after receiving both TP 20mg/kg and 50mg/kg, which lasted for 0.5 hours before returning to the baseline level. There was significant drop in blood pressure at 3 and 3.5 hours after administration of TP 20mg/kg (TP: 102±3mmHg vs control: 111±4 mmHg; TP: 101±4mmHg vs control: 109±3mmHg) or 50mg/kg (TP: 100±3mmHg vs control: 111±4mmHg; TP: 100±4 mmHg vs control: 109±3mmHg). The rats receiving TP 100mg/kg showed significantly enhanced MAP that reached the peak at 2 hours (TP: 125±3mmHg vs control: 109±3mmHg). At 3 hours the MAP was (TP: 121±5mmHg vs control: 111±4mmHg), then significantly falling at 3.5 hours (TP: 100±5mmHg vs control: 109±3mmHg) and at 4 hours (TP: 100±5mmHg vs control: 108±2mmHg) (**Table-1**).

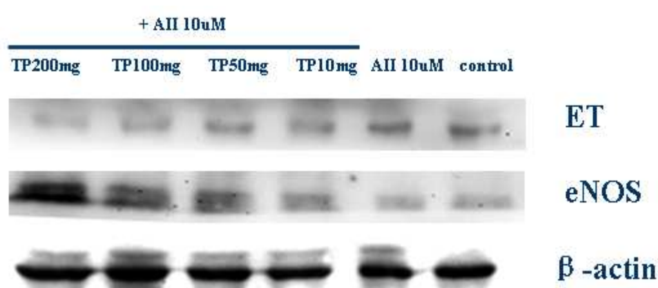

Figure-1: Dose dependent increase of TP increased the expression of eNOS protein in HUVECs, with concomitant reduction in ET expression.

Table-1: The effect of TP on MAP in the rats (n=8, each group).

Treatment	Mean arterial pressure (mmHg)DO					
	0 h	2 h	3 h	3.5 h	4 h	6 h
0.9% saline	110±2	109±3	111±4	109±3	108±2	108±3
Tea polyphonel 20mg/kg	110±2	110±2	102±3*	101±4*	110±4	110±4
Tea polyphonel 50mg/kg	111±4	110±2	100±3*	100±4*	110±6	109±5
Tea polyphonel 100mg/kg	110±5	125±3*	121±5*	100±5*	100±5*	111±6

- *P<0.05, compared to 0.9% saline as control

TP administrated via implanted catheters did not significantly affect body temperature in rats; core body temperature remained around 38°C (**Table-2**). In addition, rat HR remained around 300-320t/m, regardless of concentrations of TP, indicating that TP did not affect HR.

Table-2: The effect of TP on core body temperature in the rats (n=8, each group).

Treatment	Core body temperature (℃)					
	0h	2h	3h	3.5h	4 h	6h
0.9%Saline	38.4±0.3	38.2±0.4	38.1±0.6	38.5±0.4	38.4±0.6	38.2±0.7
Tea polyphonel 20mg/kg	38.4±0.3	38.2±0.4	38.1±0.8	38.4±0.3	38.3±0.4	38.2±0.3
Tea polyphonel 50mg/kg	38.3±0.2	38.5±0.6	38.4±0.3	38.2±0.4	38.4±0.4	38.3±0.6
Tea polyphonel 100mg/kg	38.2±0.4	38.4±0.6	38.5±0.7	38.3±0.3	38.6±0.4	38.1±0.8

- No significant difference was observed

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Vascular responses to TP: Following KCl-increased vessel tension, the vascular rings obtained from HUV showed a significantly reduced vessel tension to TP, the maximum tension reduced by TP is 57±4 (%) vs control 95±8(%). The treatment with L-NAME significantly reduced TP-mediated vasodilatation in HUV, the maximum tension induced by L-NAME is 78±6 (%) vs TP 57±4(%), where removing the endothelium from the vessel rings completely inhibited TP-mediated vasodilatation in HUV, TP+without endothelium 94±7(%) vs TP 95±8(%) (**Table-3**).

Table-3: TP affect the human umbilical vein tension.

Treatment	Tension/KCl/%((Log(TP)/g/l))					
	-4	-3	-2	-1	0	1
Control	99±8	98±9	97±5	97±6	96±7	95±8
TP	99±4	86±5*	82±6*	76±5*	60±4*	57±4*
TP+L-NAME	99±7	97±7#	96±9#	93±8#	79±6#	78±6#
TP + without endothelium	99±6	98±5★	99±11★	97±8★	96±8★	94±7★

- *P<0.05, TP vs control; ★ P <0.05, TP vs TP without endothelium; # P <0.05, TP vs TP + L-NAME

ET and NO levels in HUVECs culture: Ang II (10uM) was used to treat HUVECs for 24 hours or 48hours, ET (Endothelin) levels in HUVECs culture were significantly increased. At 24h (Ang II 15.3±1.3pg/ml vs control 11.4±1.2pg/ml); at 48h (Ang II 15.9±0.6pg/ml vs control 13.1±0.7pg/ml). Interestingly, TP attenuated Ang II-induced ET increase. As shown in Table-4, HUVECs treated with TP for 24h, ET concentrations were (10mg/L 12.7±1.2pg/ml, 50mg/L 10.0±0.6pg/ml, 100mg/L 10.7±0.7pg/ml, 200mg/L 9.4±0.6pg/ml), at 48h the ET levels were (10mg/L 9.7±0.7pg/ml, 50mg/L 9.8±0.6pg/ml, 100mg/L 9.5±0.4pg/ml, 200mg/L 5.2±0.3 pg/ml), indicating a dose-dependent pattern.

Table-4: The effect of TP on endothelin levels of in HUVECs culture.

Treatment	ET level of HUVEC culture (pg/ml)				
	4h	12h	24h	48h	72h
control	13±1.1	16±0.9	11.4±1.2	13.1±0.7	12±1.4
A II 10uM	13.7±1.2	9.6±0.8*	15.3±1.3*	15.9±0.6*	12.2±1.1
A II+TP 10mg/L	14.5±0.8	10.9±0.9	12.7±1.2#	9.7±0.7#	12.4±1.3
A II+TP 50mg/L	14.5±0.9	9.0±0.7	10.0±0.6#	9.8±0.6#	12.12±0.7
A II+TP 100mg/l	14.0±0.8	9.9±0.8	10.3±0.7#	9.5±0.4#	11.6±0.5
A II+TP 200mg/L	12.5±1.1	10.8±0.6	9.4±0.6#	5.2±0.3#	12.7±0.6

- AII: Angiotensin II. *P<0.05, compared to the control, #P <0.05, compared to AII group.

The levels of NO in HUVECs culture were significantly elevated corresponding to TP at 24 or 48 hours. As shown in **Table-5**, TP (10, 50, 100, and 200mg/L) significantly inhibited Ang II-reduced NO release, at 24h (Ang II 11.74±1.2umol/L, TP 10mg/L 16.03±1.1 umol/L, 50mg/L 18.78±1.4umol/L, 100mg/L 21.02±1.7umol/L, 200mg/L 25.38±2.1umol/L), at 48h (Ang II 9.9±0.9umol/L, TP 10mg/L 16.1±1.3umol/L, 50mg/L 16.8±1.6umol/L, 100mg/L 16.5±1.4umol/L, 200mg/L 15.2±1.2umol/L). Similarly these data showed dose-dependent TP-increased NO secretion.

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Table-5: Nitric oxide (NO) levels in HUVECs culture affected by TP.

Treatment	NO level of HUVECs culture (umol/L)				
	4h	12h	24h	48h	72h
Control	14.7±0.8	16.7±1.2	14.48±0.6	13.1±0.7	14±0.7
AII 10uM	15.2±0.6	15.6±1.3	11.74±1.2*	9.9±0.9*	14.2±0.8
AII+TP 10mg/L	15.6±0.7	15.5±1.4	16.03±1.1#	16.1±1.3#	14.4±0.6
AII+TP 50mg/L	15.3±0.6	15.4±1.1	18.76±1.4#	16.8±1.6#	14.12±1.2
AII+TP100mg/L	15.2±1.1	14.9±1.5	21.02±1.7#	16.5±1.4#	13.6±1.5
AII+TP200mg/L	15.1±1.2	14.9±1.6	25.38±2.1#	15.2±1.2#	14.7±1.9

- AII: Angiotensin II. *$p<0.05$, compared to the control; #$p<0.05$, compared to the AII group.

The expression of eNOS and ET protein in HUVECs: TP increased the expression of eNOS protein in HUVECs. On the contrary, TP reduced ET expression in HUVECs. The eNOS standardized values (vs β-action) following TP treatment at 48h were (10mg/L 0.096, 50mg/L 0.116, 100mg/L 0.135, and 200mg/L 0.4); the ET standardized value (vs β-action) following TP treatment at 48h were (10mg/L 0.07, 50mg/L 0.054, 100mg/L 0.036, and 200mg/L 0.011) (**Table-6**).

Table-6: The expression of eNOS and endothelin (ET) protein in HUVECs.

Protein ratio	Grey value of relative protein expression					
	Control	AII 10uM	AII 10uM+ TP 10mg/L	AII 10uM+ TP 50mg/L	AII 10uM+ TP 100mg/L	AII 10uM+ TP200mg/L
ET *vs* β-action	0.09	0.126	0.07	0.054	0.036	0.011
eNOS *vs* β-action	0.056	0.059	0.096	0.116	0.135	0.4

- AII: Angiotensin II.

DISCCUSION

Previous studies demonstrated cardioprotective potentials of tea (Li et al., 2014; Hintzpeter et al., 2014). However, underlying cellular mechanisms of tea-affected blood pressure remained unclear. The present study used telemetrically recording in determination of effects of TP on blood pressure *in vivo*. Telemetrically recording provides advantages in measurement of biological activity. For example, it can measure blood pressure and HR continuously without stress on animals, which is important for evaluating blood pressure. In addition, our system could measure core body temperature simultaneously. Our data showed that TP at relatively low or moderate doses (20, 50mg/kg) significantly lowered MAP at 3 hours after the administration, and the reduced MAP lasted for half of hour, indicating that the TP-reduced MAP took a relatively long period before showing the significant effect. For the high dose of TP (100mg/kg), it caused an increase of MAP for a short time first, and followed by a fall of MAP at 3.5 hours after administration of the chemical. Possible causes for the increased MAP by high dose of TP include: tea or its ingredients at high concentrations may affect central neural activities (Schlue et al., 1977) that might influence cardiovascular functions indirectly, which is worth future investigation. Notably, all doses of TP in the present study did not produce significant

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changes in heart rate and body temperature, suggesting that the rats were not stressed and the TP's effect on blood pressure could be specific. This is consistent with other work that demonstrated that green tea extracts reduced blood pressure in hypertensive patients (Hiroko et al., 2004; Pawel et al., 2012).

In the determination of possible mechanisms, we did experiments on isolated vessel and cells *in vitro*. Since TP decreased MAP *in vivo*, we tested if TP can affect vascular tension in isolated human blood vessels. Umbilical cord vein in human has been often used in pharmacological testing for various drugs (Pawel et al., 2012; Tezuka et al., 1993). In the present study, application of TP could significantly suppress KCl-increased vessel tension in the HUV. The pre-treatment with L-NAME significantly reduced TP-mediated vasodilatation. In addition, removing endothelium from the vessel inhibited TP-mediated vasodilatation in the HUV. To the best of our knowledge, this was the first to show the effects of TP or tea on healthy human blood vessels, and demonstrated the inhibitory effect against vascular tension by TP *in vitro,* which may contribute to the observed TP-reduced blood pressure *in vivo*.

L-NAME has been shown an inhibition of NO pathway (Farhad et al., 2004), and NO is rich in vascular endothelium (Palmer et al; 1988). Previous studies showed that TP enhanced production of vasodilating factors (such as nitric oxide, endothelium-derived hyper-polarizing factor, and prostacyclin) and inhibited synthesis of vasoconstrictor endothelin-1 in the endothelial cells (Stoclet et al., 2004). In the present study, L-NAME significantly reduced TP-suppressed vascular tension, while removing the vessel endothelium also showed a completely inhibition for TP-reduced vascular tension, indicating that NO pathways in the vessel played roles in TP influenced vascular tone. However, not only NO, but also other vascular relaxation factors in the endothelial tissue might contribute to the effects of TP in the HUV. This deserves further investigation.

Following testing of vessel tissues in HUV, we performed the experiments on the human umbilical vein endothelial cells (HUVECs). In the cellular model, Ang II, an important vascular regulator (Quy et al., 2002), was used. Ang II significantly increased ET levels in HUVECs culture at 24 and 48 hours following the treatment. This effect could be inhibited by adding TP. It is known that ET can increase vascular tension (Adviye et al., 2011). In the present study, Ang II decreased NO levels in HUVECs culture, which was inhibited by TP. The results suggest that the actions of TP on the vascular endothelium or cells may be linked to both ET and NO signaling pathways. Our subsequent experiments revealed that TP at various concentrations significantly suppressed AngII-increased ET expression and up-regulate eNOS expression in HUVECs. Together, those *in vitro* data showed: TP-inhibited vascular tension was dependent on the presence of an intact and functional endothelium; this endothelium-dependent relaxation was largely inhibited by L-NAME, a competitive inhibitor of NOS; TP in HUVECs could affect the release of NO as well as ET in the cells. These findings indicate that TP is an interesting endothelium-dependent vasodilator in vascular systems related to NO and ET signaling pathways.

Previous studies showed TP increased release of NO appeared to be associated

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with the intracellular Ca^{2+} in the endothelium, which is required for activation of endothelial NOS (Yung et al., 2008). Because the activity of endothelial NO synthesis is calcium dependent, an increase in intracellular free calcium will activate NO enzyme, with a resultant increase of NO release (Félétou et al., 2011). Notably, essential hypertension is associated with endothelial dysfunction, which could be caused by production of oxygen-free radicals that destroy NO and impair its beneficial effects on the vessel wall (Taddei et al., 2000). Improved endothelial functions should be an appropriated anti-hypertension approach (Rocha et al., 2010; Asgary et al., 2013; van- den -Elsen et al., 2014). The new information gained support the idea that TP, as the major ingredients of tea, may have special abilities to improve endothelial functions by reducing ET release and increasing NO formation, and this natural product may have important potentials against hypertension.

CONCLUSION

This study demonstrated that TP affected blood pressure in un-stressed and freely moving rats *in vivo*; the underlying mechanisms may be linked to TP's influence on vascular endothelial functions in both tissue and cells in human vascular samples.

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Conflict of interest: None

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