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Original Paper

Exogenous Bradykinin Inhibits Tissue Factor Induction and Deep Vein Thrombosis via Activating the eNOS/Phosphoinositide 3-Kinase/Akt Signaling Pathway

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Key Words
Bradykinin • Tissue factor • Deep vein thrombosis • LPS • Monocytes

Abstract
Background/Aims: Bradykinin has been shown to exert a variety of protective effects against vascular injury, and to reduce the levels of several factors involved in the coagulation cascade. A key determinant of thrombin generation is tissue factor (TF). However, whether bradykinin can regulate TF expression remains to be investigated. ***Methods:*** To study the effect of bradykinin on TF expression, we used Lipopolysaccharides (LPS) to induce TF expression in human umbilical vein endothelial cells and monocytes. Transcript levels were determined by RT-PCR, protein abundance by Western blotting. In the *in vivo* study, bradykinin and equal saline were intraperitoneally injected into mice for three days ahead of inferior cava vein ligation that we took to induce thrombus formation, after which bradykinin and saline were injected for another two days. Eventually, the mice were sacrificed and tissues were harvested for tests. ***Results:*** Exogenous bradykinin markedly inhibited TF expression in mRNA and protein level induced by LPS in a dose–dependent manner. Moreover, the NO synthase antagonist L-NAME and PI3K inhibitor LY294002 dramatically abolished the inhibitory effects of bradykinin on tissue factor expression. PI3K/Akt signaling pathway activation induced by bradykinin administration reduced the activity of GSK-3β and MAPK, and reduced NF-κB level in the nucleus, thereby inhibiting TF expression. Consistent with this, intraperitoneal injection of C57/BL6 mice with bradykinin also inhibited the thrombus formation induced by ligation

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of inferior vena cava. ***Conclusion:*** Bradykinin suppressed TF protein expression in human umbilical vein endothelial cells and monocytes *in vitro*; in line with this, it inhibits thrombus formation induced by ligation of inferior vena cava *in vivo*.

Introduction

Accompanied with the changing lifestyle, venous thromboembolism (VTE) is becoming increasingly prevalent and poses a burden on health economy. In spite of this enormous disease burden, little is known about its pathophysiology, which is in marked contrast with arterial thrombosis. VTE is comprised of deep vein thrombosis (DVT) and pulmonary embolism (PE), which can occur in any sections of the venous system, but arises most frequently in the deep veins of the lower extremity. Cases in the upper limbs and 'unusual sites', such as mesenteric veins, constitute just less than 10% [1]. Since 1856, Virchow has postulated that damage of the vessel wall, alterations in the flow, and hypercoagulability of the blood are the three main causes of thrombus formation [2]. A thrombus either arises spontaneously or is caused by clinical conditions including surgery, trauma, or prolonged bed rest. Timely management of DVT is especially important, because embolization of the thrombus to the lung can be fatal.

Tissue factor (TF), the main initiator of the physiological coagulation cascade, is a critical determinant of thrombin generation. It is also essential for embryo development and maintenance of vascular integrity and tissue repair [3-5]. Cellular TF is present in three pools as surface, soluble encrypted and intracellular protein. In contrast, TF circulates in normal plasma, both associated with cell-derived membrane micro vesicles and as a soluble, alternatively spliced form which was referred as circulating or blood-borne TF[6]. The relative importance of circulating versus vessel wall TF in the pathogenesis of thrombosis is controversy [7]. Under normal conditions, TF is highly expressed by cells which are not in contact with the blood, such as smooth muscle cells, mesenchymal and epithelial cells [8]**.** In contrast, in endothelial cells and monocytes, TF is either expressed at a very low level or in an inactive (so-called encrypted) form [9]; as a consequence, there is no appreciable contact of cellular TF with the circulating blood. Nevertheless, TF expression can be significantly increased by various inflammatory stimuli, such as Lipopolysaccharides (LPS), IL-1β, and tumor necrosis factor-α (TNF-α) [9, 10]**.** Tissue factor (TF)–bearing micro vesicles that arise from cells of monocyte/macrophage lineage can fuse with activated endothelial cells and initiate blood coagulation [11]. Hence therapeutic strategies specifically targeting TF and its effectors appear to be an attractive target for the treatment of deep vein thrombosis.

The kallikrein-kinin system (KKS) is a complex multi-enzyme system which is composed of circulating and tissue KKS [12]. As a member of the KKS, bradykinin has been shown to possess a variety of beneficial effects against insults, including the suppression of oxidative stress [13, 14]. Increasing evidences indicate that the circulating KKS plays critical roles in coagulation and fibrinolysis [15, 16]. Previous studies revealed that by binding to the constitutive bradykinin B2 receptor in the intravascular compartment, bradykinin promotes prostacyclin and plasmin formation and tissue plasminogen activator (t-PA) liberation[17]. Consistent with this, William D suggested that bradykinin binding to a-thrombin would substantially reduce the fibrinogen to fibrin conversion and inhibit clot formation [18]. It has also been established that L-Arginine, the principal substrate for nitric oxide synthases, suppressed the induction of TF activity and increased fibrinolysis significantly [19]. Another study revealed that endothelium-derived NO, through its vasodilator and anti-aggregatory properties, can prevent vasospasm and thrombus formation in the circulation [20]. However, whether bradykinin is involved in TF expression regulation and deep vein thrombosis still remains unknown. This study was therefore designed to investigate the effect of bradykinin on TF expression and thrombus formation *in vitro* and *in vivo*.

Material and Methods

Reagents

Lipopolysaccharide (Escherichia Coli 0111:B4) and bradykinin were from Sigma-Aldrich (St. Louis, MO). HOE-140 and N-methyl- L-arginine acetate salt were bought from Sigma-Aldrich. Rabbit monoclonal antibodies against human tissue factor (for western blotting) and mouse tissue factor (for immunohistochemistry) were from Epitomic. Plasma TF activities were measured using an Assay Sense TF Chromogenic Activity Assay kit (Assaypro, St. Charles, MO). Antibodies for western blotting, including rabbit anti -p38(T-P38), anti-phospho-p38 (P-P38), anti-extracellular signal-regulated kinase (T-ERK1/2), anti-phospho-ERK1/2 (P-ERK1/2), anti-JNK (T-JNK), anti-phospho-JNK (P-JNK), anti-Pi3kinase (PI3K), anti-AKT (T-AKT), anti-phospho-AKT T308 (P-AKT), anti -GSK3β (T-GSK3β), and anti-phospho-GSK-3β (P-GSK3β) as well as mouse anti-actin were purchased from Cell Signaling Technology. Antibody against Fibrin II beta was from Accurate Chemical & Scientific Corp (Westbury, NY). All second antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals and reagents were purchased from Sigma-Aldrich unless otherwise specified.

Cell lines and cell culture

The Human Umbilical Vein Endothelial cells (HUVEC) were purchased from ATCC. Cells were grown in RPMI 1640 (Gibco) medium, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 ug/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C. The cells in the logarithmic phase of growth were used in all experiments described below. Monocyte cells THP-1 was also obtained from ATCC and maintained in DMEM with 10% heat-inactivated FBS.

TF activity assay

The TF activity in the plasma was determined by one stage clotting assay according to the manufacturer's instructions.

Western blotting analysis

Cellular protein was extracted with RIPA lysis buffer and the concentrations were measured by using BCA Protein Assay Reagent. Protein samples (20 μg/lane) were separated by 10% SDS-PAGE, and then electrophoretically transferred to PVDF membranes. The membranes were blocked, and then incubated with primary antibodies overnight at 4°C, followed by secondary antibodies against rabbit or mouse IgG conjugated to horseradish peroxidase (1:3000) for 2 hours at room temperature. Finally, after developed with ECL detection reagents, the protein bands of membranes were visualized by exposure to x-ray film. Protein expression was quantified by densitometry and normalized to β-actin expression.

Real-time PCR

Total RNA from cultured cells or frozen samples were extracted with trizol reagent (Invitrogen, Carlsbad, CA), followed by the synthesis of cDNA. Real-time quantitative PCRs were performed using the SYBR-green I Core Kit. The primers were obtained from Qiagen as Table 1 indicates. PCR products were detected in the ABI-PRISM 7700 sequence detection system (Applied Biosystems), and the results were analyzed using the $2^{-\Delta\Delta CT}$ method [21]. The level of expression of mRNA was normalized to β-actin mRNA.

Table 1. The sequence of primers

	Forward	Reverse
TNFα	F 5'-ACGGCATGGATCTCAAAGAC-3'	R 5'-GTGGGTGAGGAGCACGTAGT-3'
IL-1β	F 5′-TCACAGCAGCACATCAACAA-3′	R 5′-TGTCCTCATCCTGGAAGGTC-3′
actin	F 5′-GCAGCTCCTTCGTTGCCGGT-3′	R 5′-ACATGCCGGAGCCGTTGTCG-3'
TF	F 5'-TGACCTCACCGACGAGATTGTGAA-3	R5'TCTGAATTGTTGGCTGTCCGAGGT3

Animals

C57BL/6J mice were purchased from SLAC Laboratory Animal Co, Ltd (Shanghai, China). All procedures were approved by the Animal Research Ethics Board at Huazhong University of Science and Technology and conformed to Directive 2010/63/EU of the European Parliament. Mice were randomly divided into 3 groups: Sham, Model and Bradykinin. Before surgical procedure, the mice of bradykinin group were intraperitoneally injected with bradykinin (10 mg/kg/d) once a day for three days. On the fourth day, after anesthetization by intraperitoneal injection with 60 mg/kg of pentobarbital, the ligation of inferior vena cava (IVC) was executed via a midline laparotomy, dissected at the level of the renal veins, and then ligated below the renal veins as previously described [22]. After ligation, the mice received bradykinin injection for another two days and analgesic therapy was performed using buprenorphine at 0.1 mg/kg body weight for 3 days. The mice in other groups received equal saline as control. Sham animals underwent the same surgical procedures except for IVC ligation. If the dissection was traumatic or the IVC was ruptured during surgery, mice were excluded for further studies. 48 hours later, mice were euthanized by intraperitonial injection with 100 mg/kg of pentobarbital, and the thrombosed IVC were collected, weighed, and measured for length after blood sampling [23]. The samples were snap-frozen or stored for histological analysis.

Statistical analysis

All data were shown as mean ± standard deviation (SD). Comparisons between groups were performed by a one-way analysis of ANOVA with *post hoc* analyses performed using the Student-Newman-Keuls method and the level for significant statistical differences was set at $P < 0.05$.

Results

Bradykinin suppressed LPS-induced tissue factor expression in HUVECs and human monocytes

To determine whether bradykinin regulates TF expression in endothelial cells, Confluent cells were pretreated with bradykinin at various concentrations for 6 hours, after which LPS was used to induce tissue factor expression. HUVECs were harvested 4 hours after LPS treatment. As expected, LPS significantly induced TF expression in protein and mRNA level in a dose dependent manner in HUVECs as shown in Fig. 1A and 1B. TF expression induced by LPS (1.0 μg/ml) was higher than that induced by LPS (0.1 μg/ml) (Fig. 1A and 1B), so LPS (1.0 μg/ml) was used to induce TF expression in the following experiments. As shown in Fig. 1C and 1D, Bradykinin significantly prevented TF expression induced by LPS in protein and mRNA level in a dose dependent manner. In addition, bradykinin (10^{-5} mol/l) obviously inhibited TF expression in protein and mRNA level (Fig. 1C and 1D), and bradykinin (10^{-5} mol/l) was selected to explore the effects of bradykinin on TF expression in the following experiments. Taken together, these data indicated that bradykinin prevented TF expression induced by LPS in HUVECs.

Previous data indicated that monocytes derived TF was also involved in the pathogenesis of deep vein thrombosis [24]. As shown in Fig. 1E and 1F, Bradykinin significantly prevented TF expression induced by LPS in protein and mRNA level in a dose dependent manner in cultured THP-1 cells. Collectively, these data indicated that bradykinin prevented TF expression induced by LPS in monocytes.

The inhibitory effect of bradykinin on tissue factor expression is dependent of PI3K/AKT and MAPK signaling

Previous data indicated that PI3K/Akt signaling pathway negatively regulated TF expression *in vitro* and *in vivo* [25]. We explored whether bradykinin inhibited LPS-induced TF expression via activating PI3K/Akt signaling pathway. As expected, PI3K inhibitor LY294002 abolished the inhibitory effects of bradykinin on tissue factor expression in HUVECs and monocytes as shown in Fig. 2A and 3A. Furthermore, under resting conditions, bradykinin increased Akt phosphorylation in HUVECs (Fig. 2B) and monocytes (Fig. 3B). The PI3K inhibitor LY294002 blocked the activation of Akt by bradykinin, indicating that

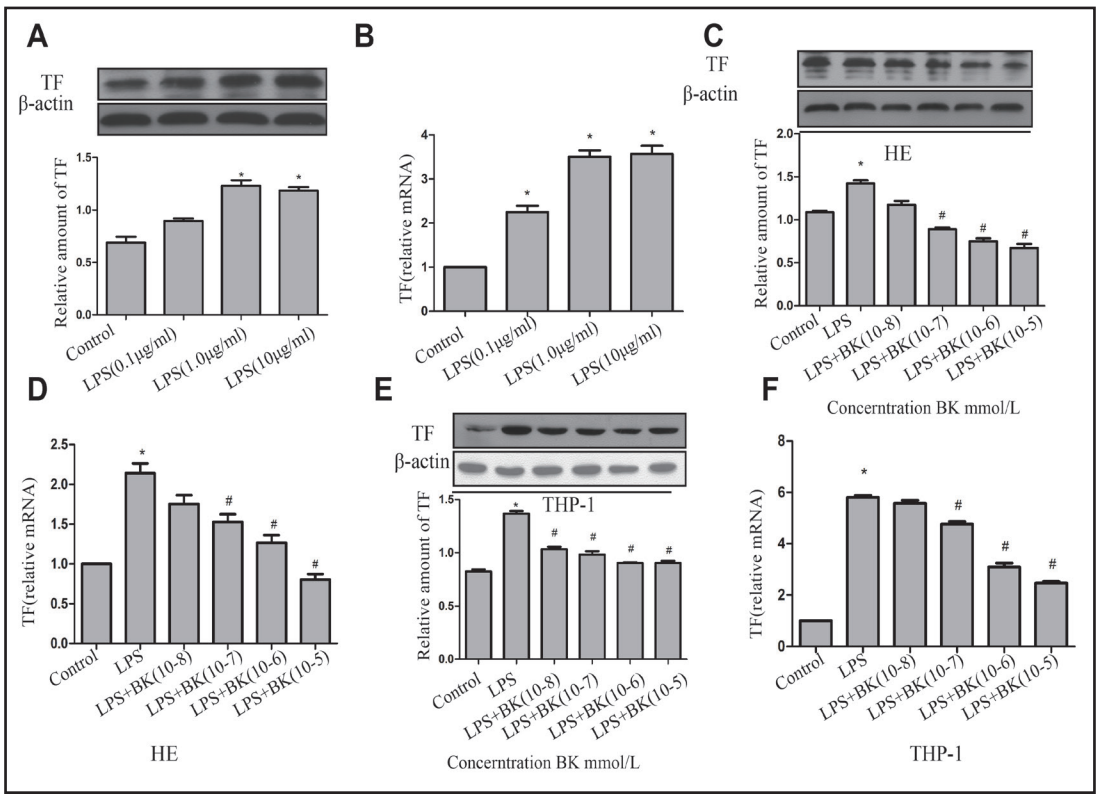

Fig. 1. Bradykinin suppresses LPS-induced TF expression in HUVEC. (A) and (B): HUVEC were treated with LPS at various concentrations for 6 hours, and we observed that LPS induced TF expression peaked at 1μg/mL; (C) and (D): HUVEC were pretreated with bradykinin for 1 hour at various concentrations, followed by stimulation with LPS at 1 μg/mL for 6 hours. The western and real-time PCR revealed that Bradykinin reduced LPS induced TF expression dose dependently. (E) and (F): Effect of bradykinin on LPS induced TF expression in THP-1. Data are shown as means ±SEM of 3 independent experiments. $^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ and $^{\&}P < 0.05$ *vs* LPS.

Akt phosphorylation in bradykinin-treated cells is attributable to bradykinin-mediated PI3K activation. GSK-3β, a crucial molecule for p65 phosphorylation, is one downstream phosphorylation target of Akt [25]. Bradykinin treatment increased the levels of phosphor-GSK3β in HUVECs and monocytes under both resting and LPS-treated conditions (Fig. 2C and 3C), thus inhibited the disassociation of p65 from IκBα and its translocation to nuclear as Fig. 4B indicated. Extracellular signal-regulated kinase (ERK), an important MAPK for TF expression, is the target of the Raf-1 pathway, the latter is regulated by PI3K/Akt signaling [26]. In HUVECs and monocytes, LPS treatment markedly upregulated phosphor-ERK level, and ERK phosphorylation was significantly inhibited by bradykinin (Fig. 2E and 2E). In addition, P38MAPK and JNK phosphorylation was also upregulated by LPS, and moreover, the phosphor-P38MAPK and phosphor-JNK level was also dramatically inhibited by bradykinin in HUVECs and monocytes (Fig. 2D, F and Fig. 3D, F). Interestingly, PI3K inhibitor LY294002 administration efficiently abolished the effects of bradykinin on phosphor-P38MAPK and phosphor-JNK level as well as TF expression as shown in Fig. 2 and 3. Thus, the PI3K/Akt pathway is critically involved in the inhibitory effect of bradykinin on TF expression in HUVECs and monocytes.

Previous study indicated that transcription factors NF-κB activation was involved in tissue factor expression [27]. We examined whether NF-κB participated in the inhibitory effects of bradykinin on tissue factor expression in HUVECs and monocytes. Interestingly, LPS markedly reduced IκBα expression, and increased nuclear NF-κB level in HUVECs and

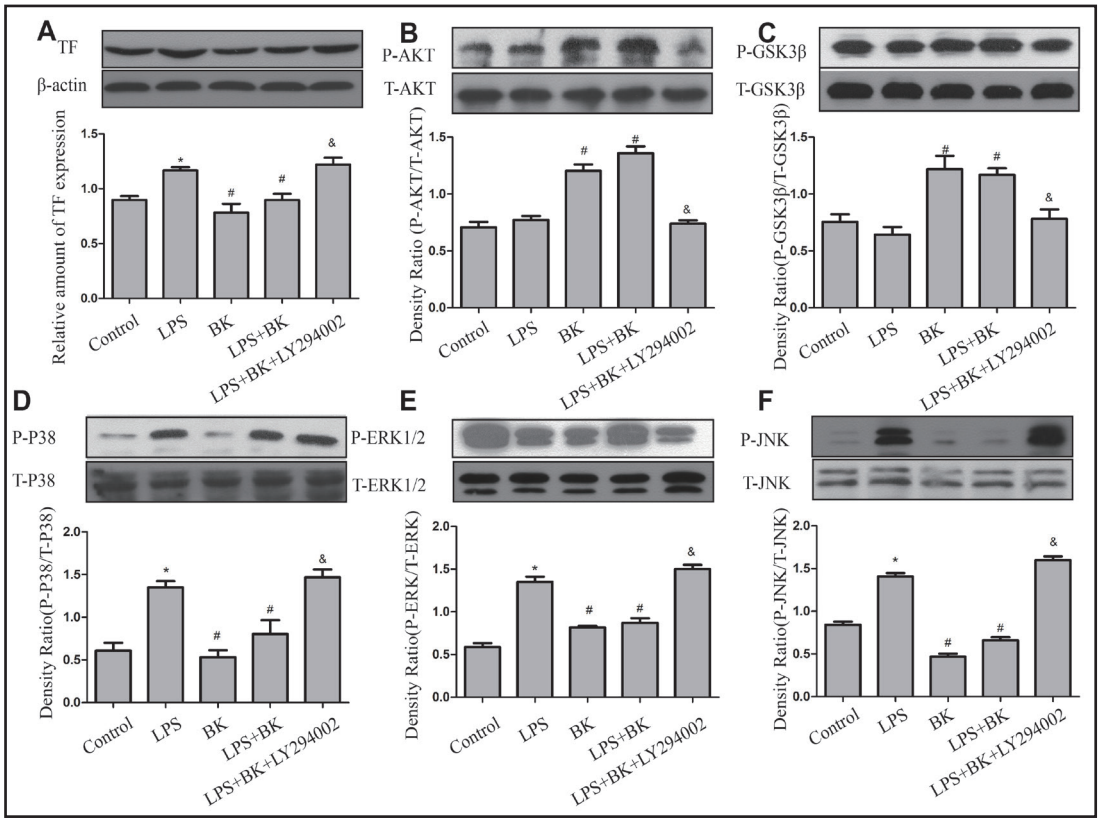

Fig. 2. BK reduced TF expression induced by LPS via activating PI3K/AKT and MAPK signaling pathways in HUVEC. HUVEC were pretreated with bradykinin (1×10^{-6} mol/L) for 1 hour, followed by stimulation with LPS at 1 μg/mL for 2 hours, ahead of which the PI3K inhibitor LY294002 has been added and then the protein was harvested. The expression of signaling molecules was detected by western blot. (A): LPS induced TF expression is attenuated by bradykinin, which is blocked by PI3K inhibitor LY294002; (B): BK and LPS induced AKT phosphorylation under resting condition, and the AKT phosphorylation is blocked by LY294002; (C): LPS reduced the GSK3β phosphorylation and this is reversed by bradykinin preincubation, which is also inhibited by LY294002; (D-F): LPS activated MAPK (P38/JNK/ERK), and bradykinin suppressed this trend, which is interrupted by LY294002. The intensity of each band was quantified by densitometry and expressed as folds of untreated control cells Data are shown as means ±SEM of 3 independent experiments. $^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* LPS; $^{\&}P<0.05$ *vs* LPS+BK.

monocytes, and moreover, bradykinin treatment prevented the decrease in IκBα expression and the increase in nuclear NF-κB level as shown in Fig. 4A, 4B, 4C and 4D. As expected, PI3K inhibitor LY294002 administration significantly and partly abolished the beneficial effects of bradykinin on NF-κB transcriptional activity indirectly characterized by changes in IKBα and nuclear NF-κB level (Fig. 4A, 4B, 4C and 4D) and subsequent TF expression as shown in Fig. 2A and 3A. Collectively, these data indirectly indicated that bradykinin inhibited tissue factor expression via regulating NF-κB-mediated tissue factor transcription in HUVECs and monocytes.

Bradykinin inhibits tissue factor expression by BK B2 receptor–mediated NO release

Bradykinin receptors are mainly divided pharmacologically into two subtypes, B1 and B2. Previous data indicate that most of the known actions of bradykinin (BK) are mediated via B2 receptors [28]. To check whether the protective effect against tissue factor expression is also dependent on the BK B2 receptor, the effect of the BK B2 receptor antagonist HOE-140 (1 μmol/L) on the reduction of TF expression by BK was explored in HUVECs and monocytes. HOE-140 administration totally blocked the beneficial effect of bradykinin on TF expression,

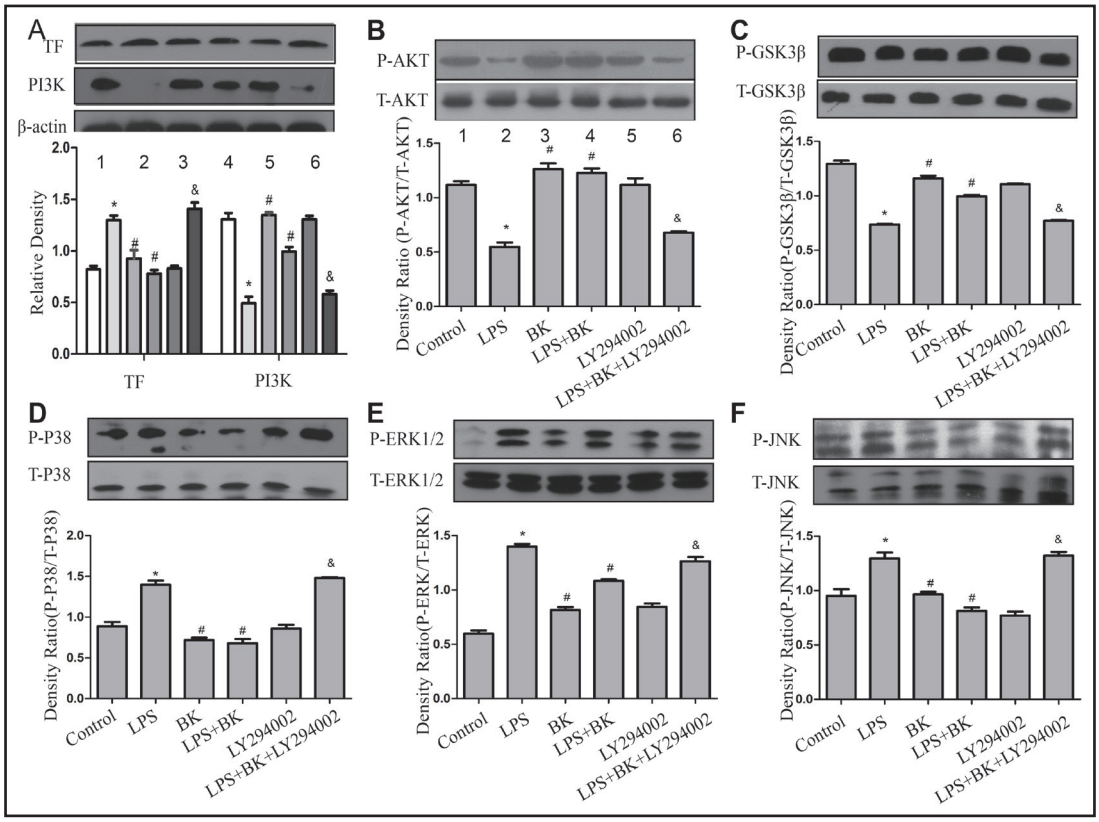

Fig. 3. BK inhibited TF expression induced by LPS via activating PI3K/AKT and MAPK signaling pathways in THP-1 cells. (A): LPS induced TF expression is attenuated by bradykinin, which is blocked by PI3K inhibitor LY294002; (B): BK and LPS induced AKT phosphorylation under resting condition, and the AKT phosphorylation is blocked by LY294002; (C): LPS reduced the GSK3β phosphorylation and this is reversed by bradykinin preincubation, which is also inhibited by LY294002; (D-F): LPS activated MAPK (P38/JNK/ERK) in THP-1, and bradykinin suppressed this trend, which is interrupted by LY294002. The intensity of each band was quantified by densitometry and expressed as folds of untreated control cells Data are shown as means ± SEM of 3 independent experiment $^{*}P<0.05$ *vs* control; $^{\#}P < 0.05$ *vs* LPS; $^{\&}P < 0.05$ *vs* LPS+BK.

and moreover PI3K, phosphor-AKT and phosphor-GSK-3β level were also abolished by HOE-140 treatment (Fig. 5 and 6). Additionally, bradykinin B2 receptor activation leads to the release of NO [28], we also investigated whether NO was involved in the beneficial effects of bradykinin on TF expression. N-methyl- L-arginine acetate salt (L-NAME 10 μmol/L) treatment totally blocked the beneficial effect of bradykinin on TF expression, and moreover PI3K, phosphor-AKT and phosphor-GSK-3β level were also abolished by L-NAME treatment (Fig. 5 and 6). These data suggested that NO was the upstream target of PI3K/AKT signaling pathway, which was involved in the inhibitory effects of bradykinin on TF expression. Collectively, these results indicated that bradykinin inhibited HUVECs and monocytes TF expression induced by LPS via activation of eNOS-NO-PI3K/AKT-ERK signaling pathways.

Bradykinin restrains thrombosis by reducing tissue factor expression induced by inflammation.

TF is also involved in the formation of deep vein thrombosis [29]. Using a mouse model of deep vein thrombosis, we explored whether bradykinin could affect thrombus formation. We injected bradykinin intraperitoneally into C57BL/6J mice for three days before ligation of inferior vena cava, and injection of bradykinin was continued for another 2 days after surgical procedure until sacrifice. Interestingly, Mice treated with vehicle had large thrombi in their

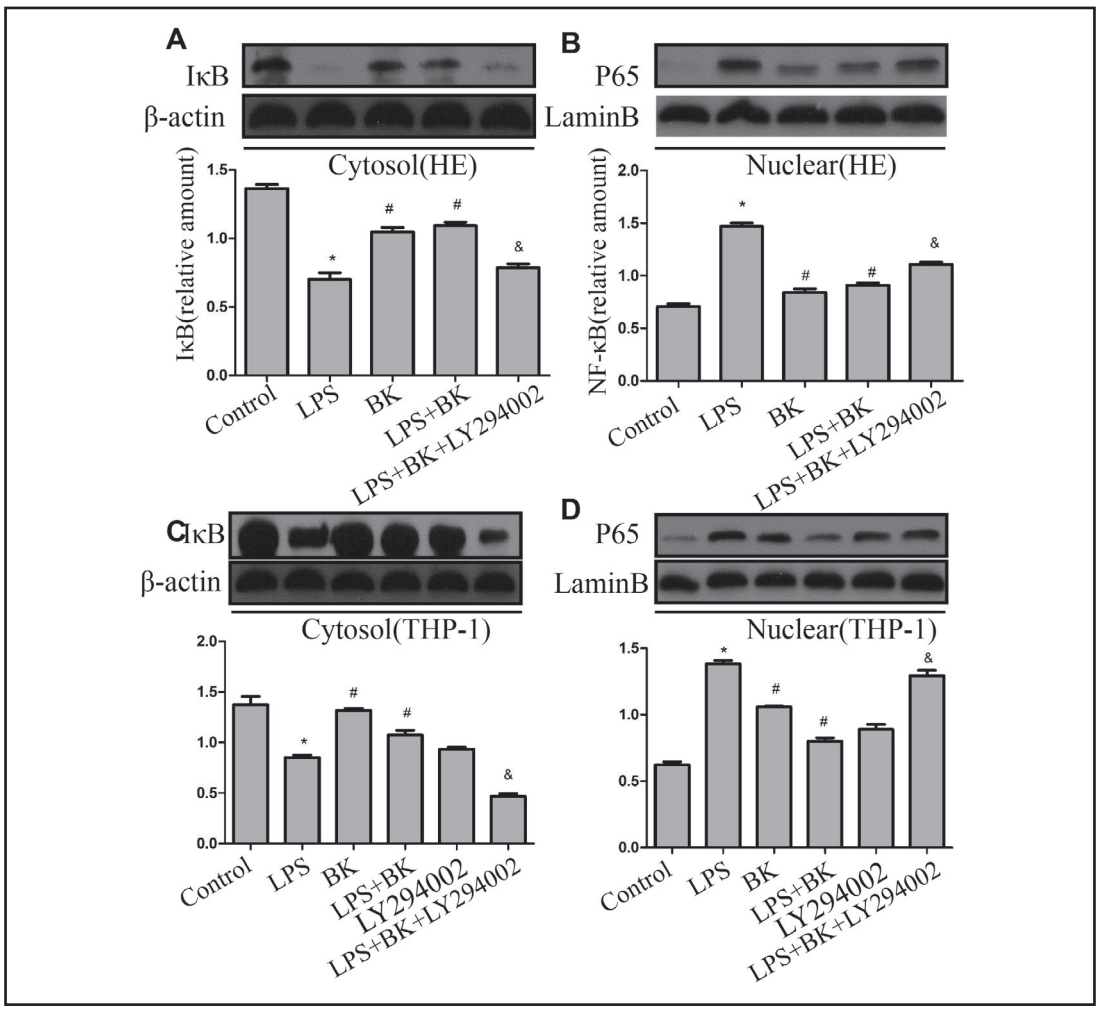

Fig. 4. Role of the NF-κB pathways in bradykinin down-regulated TF expression in endothelial cells and monocytes. Cytosol and Nuclear protein was extracted from HUVEC (A-B) or monocytes (C-D) 2 hours after treatment with LPS (1 μg/mL) or LPS and bradykinin(1×10^{-6} mol/L) with LY294002(10 μmol/L) pretreatment. (A) and (C): LPS reduced IκB expression in cytoplasm, bradykinin prevent LPS induced IκB phosphorylation, and this trend is interrupted by LY294002; (B) and (D): LPS induced NF-κB activation in nuclear is inhibited by bradykinin, and this is blocked by LY294002 .The intensity of each band was quantified by densitometry and expressed as folds of untreated control cells. Data are shown as means ±SEM of 3 independent experiments.$^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* LPS; $^{\&}P < 0.05$ *vs* LPS+BK.

inferior vena cava, whereas mice treated with bradykinin had very small thrombi in their ligated inferior vena cava as shown in Fig. 7A. This beneficial effect was further confirmed by reduced TF activity and TF mRNA (Fig. 7B and C) in thrombi and fibrin expression in liver (Fig. 7D).

The coagulation factor, TF, plays a pivotal role in the formation of deep vein thrombosis. Bradykinin treatment markedly reduced TF expression in thrombi as shown in Fig. 7E. Interestingly, the level of other inflammatory factors (P-selectin, VCAM-1, ICAM-1) in thrombi of mice treated with bradykinin was significantly lower than that treated with vehicle (Fig. 7E). These data indicated that exogenous bradykinin significantly inhibited macrophage derived TF expression and myeloid cells adhesion to the vessel wall via reduced P-selectin, VCAM-1 and ICAM-1 expression. Meanwhile, the P13K/AKT expression in the vascular wall is also elevated (Fig. 7F and 7G), which again prove the involvement of the PI3K/AKT signaling pathway in the regulation of tissue factor expression.

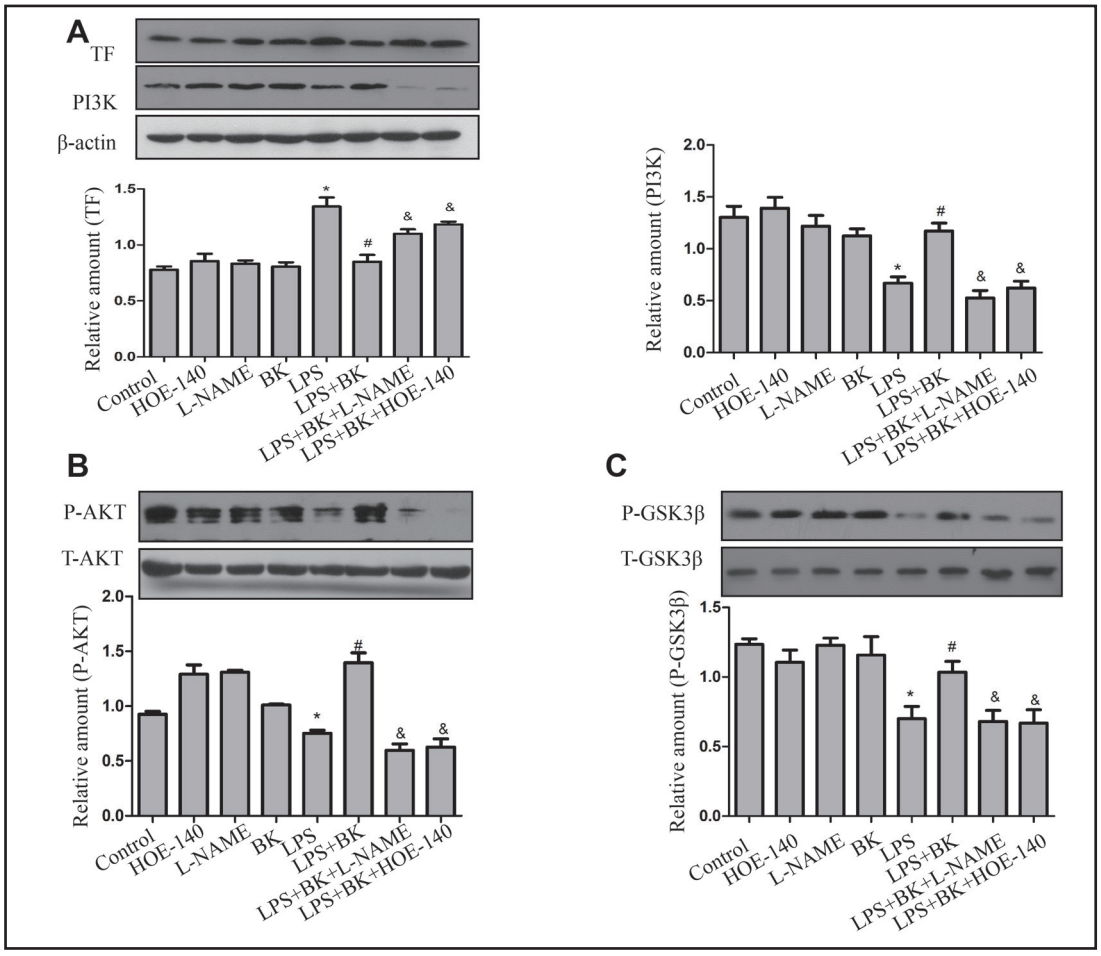

Fig. 5. Bradykinin inhibited tissue factor expression of endothelial cells by BK B2 receptor–mediated NO release. BK was given 30 minutes before the induction of tissue factor expression. The B2R antagonist HOE-140 (1 μmol/l) and eNOS inhibitor N^{ω}-methyl-L-arginine acetate salt (L-NAME) (10 μmol/l) were added 30 minutes before BK treatment, after which BK was added without replacing the medium.6 hours after LPS treatment, cells were harvested for detection. (A): bradykinin suppressed LPS induced TF expression, and this effect is almost completely blocked by HOE-140 and L-NAME, while PI3K exhibit an opposite expression; (B)-(C): bradykinin reversed LPS reduced AKT and GAK3β phosphorylation, which was restrained by HOE-140 and L-NAME. At least 3 separate experiments were performed per treatment. Data are shown as means ± SEM of 3 independent experiments.$^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* LPS; $^{\&}P < 0.05$ *vs* LPS+BK.

Discussion

In the present study, our data demonstrated that bradykinin inhibited TF expression in endothelial cells and in monocytes/macrophages via activation of the eNOS PI3K/Akt signaling pathway. PI3K/Akt signaling pathway reduced the activity of MAPK and GSK-3β, and suppressed NF-κB transcriptional activity, thereby inhibiting TF expression. Importantly, exogenous bradykinin attenuated deep vein thrombosis via reduced TF expression and myeloid cells adhesion to the vessel wall in the mouse model.

Previous studies have demonstrated that the cytokines are intimately associated with TF expression and thrombus formation [30]. Leukocytes can be tethered to P- or E-selectin expressed on activated endothelial cells, and such interactions can promote the transfer of TF to induce thrombosis [9, 26]. Monocytes are the primary source of TF in the stasis induced thrombus formation although endothelial cells also express some TF [31]. LPS treatment could also stimulate TF expression in human umbilical vein endothelial cells and

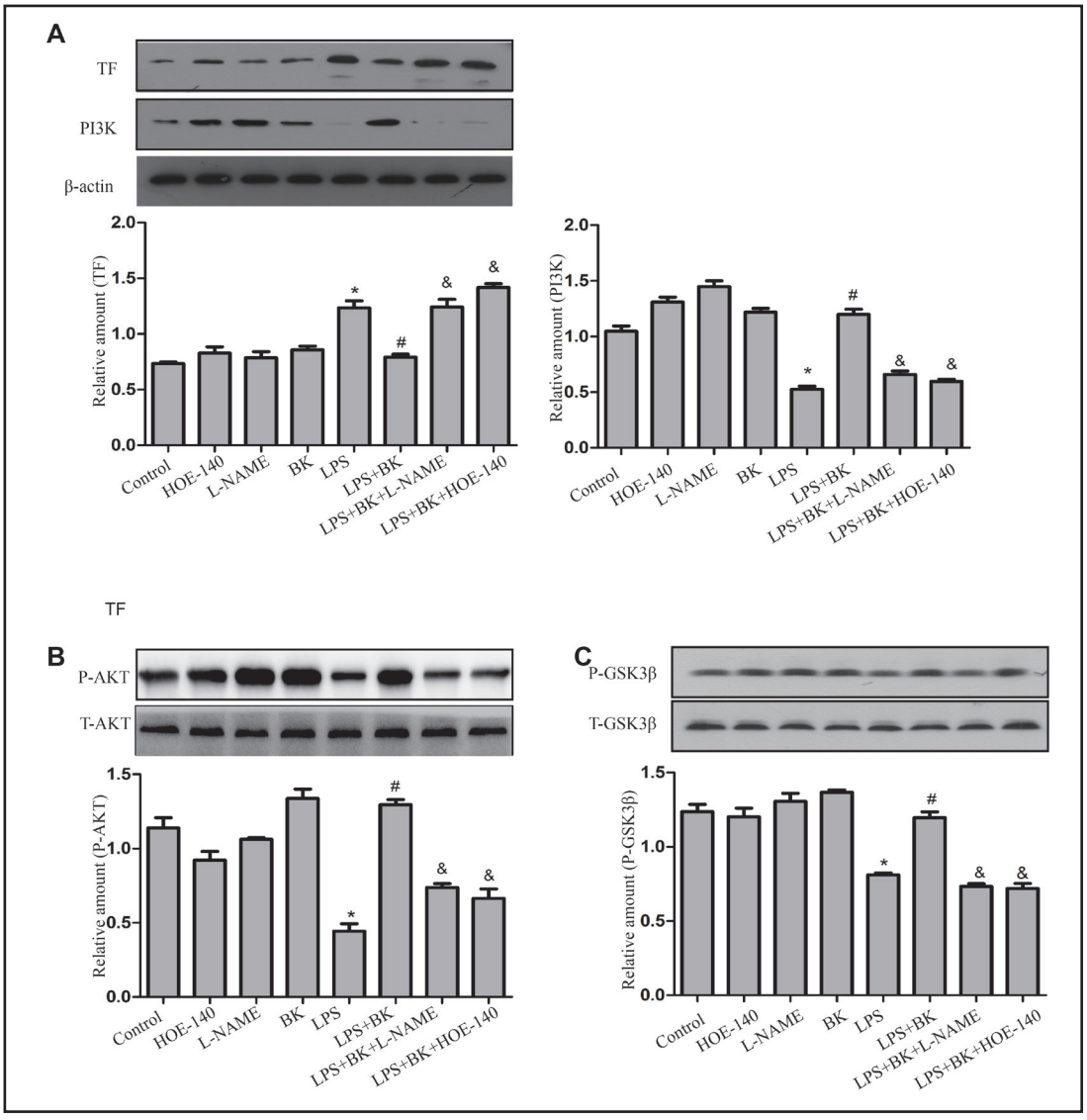

Fig. 6. Bradykinin inhibited tissue factor expression of monocytes by BK B2 receptor–mediated NO release. Data are shown as means ± SEM of 3 independent experiments.*$P < 0.05$ *vs* control; #$P < 0.05$ *vs* LPS; &$P < 0.05$ *vs* LPS+BK.

human peripheral blood monocytes [29]. Kallikrein treatment through kinin B2 receptor activation reduced MAPK and Ikappa-Balpha phosphorylation, NF-κB activation and MCP-1 and VCAM-1 expression [29]. In this study, bradykinin exhibited a consistent inhibitory effect on LPS induced TF protein expression in both monocytes and HUVECs in a dose dependent fashion. Moreover, bradykinin treatment also decreased plasma TF level and reduced deep vein thrombosis in mice treated with ligation of inferior vena cava. Under inflammatory conditions, activated endothelial cells heighten the expression of cell adhesion molecules (CAM), including ICAM-1, VCAM-1, and P-selectin. Leukocytes attach to activated endothelial cells via adhesion molecules which interact with their ligands present on the leukocytes [25, 32]. A number of studies have shown that engagement and cross-linking of the counter receptors for several adhesion molecules, such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin, and P-selectin, will result in transcription of the TF gene and subsequent surface expression of the protein[26, 33]. Previous studies have shown that interference with both P-selectin and E-selectin should have a beneficial effect on decreasing

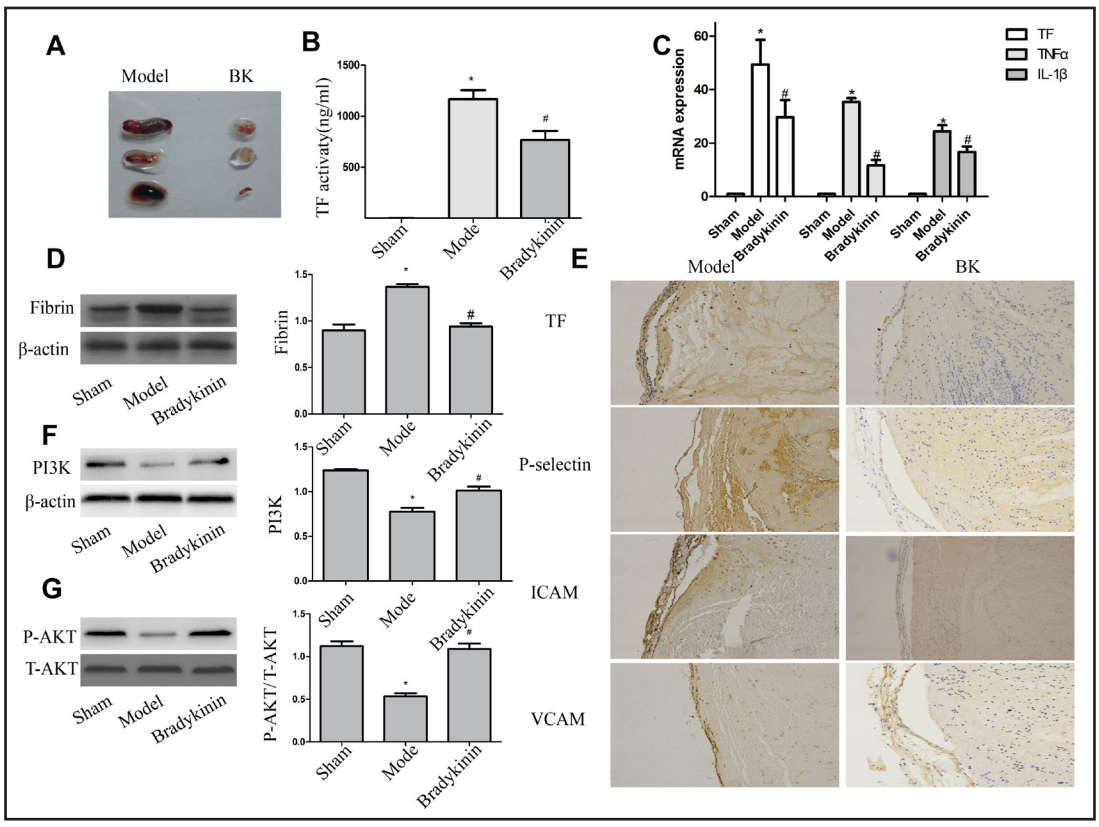

Fig. 7. Bradykinin restrains thrombosis by reducing tissue factor expression induced by inflammation. (A): The size of thrombus in the bradykinin treated team was much smaller than that of the Mode team; (B): The TF activity in the plasma of bradykinin treated team was much lower than that of the Mode team. (C):The relative TF/PI3K/AKT mRNA expression of vascular wall; (D): Ligation of inferior vein cava induced Fibrin expression of liver, while bradykinin attenuated this trend; (E):The inferior vein cava ligation induced expression of TF, VCAM-1, ICAM-1 and P-selectin by endothelial cells was also reduced by bradykinin (100 × magnification); (F-G): The western blot revealed that the bradykinin reduced the inferior vein cava ligation induced tissue factor expression by upregulating PI3K and AKT expression. n = 8, $^{*}P < 0.05$ *vs* Sham; $^{\#}P < 0.05$ *vs* Model.

clot formation during venous thrombosis[34]. Interestingly, in the present study, bradykinin inhibited the LPS-induced expression of pro-inflamatory mediators such as TNF-α, IL-1β, P-selectin and E-selectin in endothelial cells, as well as monocytes activation characterized by inflammatory cytokines expression, which indicated that exogenous bradykinin inhibited the adhesion of leukocytes to the endothelium. It is also speculated that bradykinin inhibited TF expression via reduced adhesion molecules expression, and thus broke the vicious circle in the deep vein thrombosis. Collectively, these data demonstrated that exogenous bradykinin prevented deep vein thrombosis via reduced monocytes and HUVECs TF expression and adhesion of leukocytes to the endothelium.

Furthermore, the angiotensin-converting enzyme breakdown product of bradykinin, Arg-Pro-Pro-Gly-Phe (RPPGF), is a stable metabolite of bradykinin [35, 36], and this peptide has biological activity [37]. Further study indicated that RPPGF inhibited both α- and β-thrombin-induced platelet aggregation and secretion via binding to the active site of thrombin and the extracellular domain of PAR1 to prevent thrombin cleavage after Arg41 [18, 38]. Thus, it is speculated that the inhibitory effects of bradykinin metabolite RPPGF on both α- and β-thrombin-induced platelet aggregation and secretion may be partly involved in the beneficial effects of bradykinin on deep vein thrombosis.

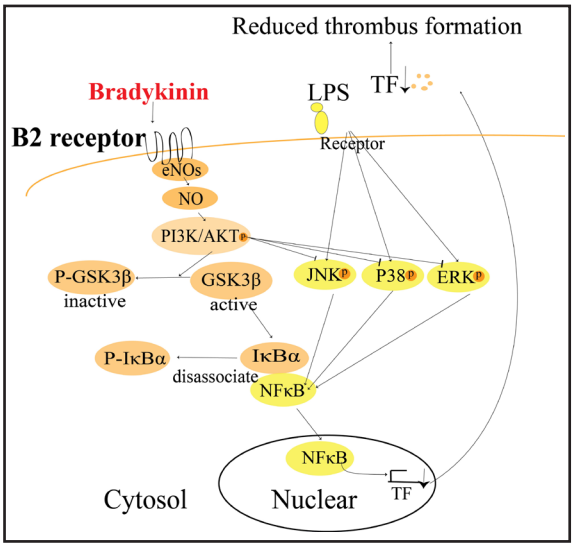

Fig. 8. Proposed model about the role of BK B2 receptor in LPS induced tissue factor expression. AS indicated, LPS promoted MAPK activation and NFκB translocation to nuclear to activate the TF promoter, however, by activating BK B2receptor/eNOS/PI3K/AKT/GSK-3β signaling pathway, the bradykinin inhibited NF-κB translocation. At the same time, MAPK activation was also interrupted thus suppressed tissue factor expression eventually.

In addition, the rapid development of DVT after IVC ligation reflects a combination of stasis-induced vein wall injury and enhanced TF expression in endothelial cells and leukocytes [9]. Furthermore, once venous thrombosis begins, an acute to chronic inflammatory response occurs in the thrombus and vein wall which leads to thrombus amplification. Proinflammatory cytokines such as TNF-α and IL-1β were indeed increased at the occlusion site, which are potent inducer of monocyte-derived TF-bearing micro vesicles. TF-bearing micro vesicles are central in the pathogenesis of DVT in disease states in which monocytes are stimulated to express TF [11]. Whether bradykinin inhibited deep vein thrombosis via reduced TF-bearing micro vesicles level and activity remains to be further elucidated.

The p38MAPK, p44/42MAPK, and c-jun terminal NH2-kinase (JNK) are involved in LPS-induced, TNF-α induced, histamine-induced, and thrombin-induced TF expression [39]. These signal transduction molecules stimulate the TF promoter by activating transcription factors such as nuclear factor (NF)-κB, ultimately resulting in up-regulation of TF mRNA [40]. Unlike MAP kinases, however, the PI3-kinase pathway negatively regulates TF expression [29, 31]. Bradykinin limited myocardial infarction induced by ischemia-reperfusion injury via PI3K/Akt/eNOS signaling pathway in mouse heart [29]. Bradykinin inhibited oxidative stress-induced cardiomyocytes senescence by acting through BK B2 receptor induced NO release [14]. In this study, the PI3K inhibitor LY294002 blocked the activation of Akt by bradykinin, and thus abolished the inhibitory effects of bradykinin on tissue factor expression. HOE-140 administration totally blocked the beneficial effect of bradykinin on TF expression, and moreover PI3K, phosphor-AKT and GSK-3β level were also abolished by HOE-140 treatment. Additionally, N-methyl- L-arginine acetate salt (L-NAME 10 μmol/L) treatment totally blocked the beneficial effect of bradykinin on TF expression, and moreover PI3K, phosphor-AKT and GSK-3β level were also abolished by L-NAME treatment. Exogenous bradykinin inhibited NF-κB translocation to the neucleus, concomitantly with the inhibition of TF. These data indicated that bradykinin inhibited TF expression via activating BK B2 receptor/eNOS/PI3K/AKT/GSK-3β/NF-κB signaling pathway. However, B2R makes a complex with eNOS, and L-NAME may bind eNOS inhibiting BK binding to B2R. Thus whether this point was involved in this study remains unkown, and it warrants further investigation.

In addition, there exists a cross-talk between the MAPK and the PI3K pathway on LPS-induced TF expression [41], our *in vitro* studies indicated that major targets of the PI3K-Akt pathways were the MAPK pathways. Inhibition of the PI3K-Akt pathway was likely to enhance LPS induced activation of the MAPK pathway and the subsequent expression of proinflammatory and procoagulant molecules. The PI3K-Akt pathway also inhibited NF-κB activity [31]. Thus drugs targeting the activation of the PI3K-Akt pathway may limit LPS-induced TF expression and thrombus formation. However, some other studies indicate that

PI3K is required for activation of NF-κB in mouse macrophages and human endothelial cells [42]. The discrepancy is mostly probably because LPS induced activation of the PI3K-Akt pathway is slightly delayed relative to activation of the MAPK pathways [41]. Previous data indicated that the p38MAPK, p44/42MAPK, and c-jun terminal NH2-kinase (JNK) signaling pathways downstream NF-κB activation mediated LPS-induced TF expression [26]. In this study, bradykinin treatment also inhibited p44/42MAPK, p38MAPK and JNK phosphorylation induced by LPS in HUVECs and monocytes, which suggested that attenuated MAPK signaling pathway mediated NF-κB activation was also involved in the inhibitory effects of bradykinin on TF expression, but the detailed roles of this signaling pathway remains to be further elucidated.

In summary, our data demonstrated that bradykinin inhibited TF expression and deep vein thrombosis via activation of BK B2 receptor/eNOS/PI3K/Akt signaling pathways, and subsequently attenuated GSK-3β and MAPK signaling pathways mediated NF-κB transcriptional activity as shown in Fig. 8. Therefore, bradykinin B2 receptor may be a critical therapeutic target of deep vein thrombosis. However, even though different approaches to inhibit TF initially looked promising in animal models, their efficacy in inhibiting TF expression and thrombosis in humans has not yet been examined successfully, and further investigations are necessary.

Abbreviations

TF (tissue factor); LPS (Lipopolysaccharides); KKS (kallikrein-kinin system); NF-κB (nuclear transcription factor kappa B); ICAM-1 (vascular cell adhesion molecule-1); MAPK (mitogen-activated protein kinase); PI3K (phosphatidylinositol 3-kinase); VCAM-1 (vascular cell adhesion molecule-1); IL-1 β (interleukin-1β); TNF-α (tumor necrosis factor-αIL-1β); JNK (c-Jun N-terminal kinase); IL-10 (interleukin10); AKT (protein kinase B, PKB); P38 (p38 mitogen-activated protein kinase); ERK (extracellular regulated protein kinases); IκB (I kappa B kinase).

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Disclosure Statement

The authors have no conflict of interests to declare.

References

1 Ho WK: Deep vein thrombosis--risks and diagnosis. Aust Fam Physician 2010;39:468-474.
2 Dai MX, Zheng XH, Yu J, Yin T, Ma MJ, Zhang L, Liu M, Ma Y, Liu LW, Gao X, Li Y, Song LQ, Wang HC: The impact of intermittent and repetitive cold stress exposure on endoplasmic reticulum stress and instability of atherosclerotic plaques. Cell Physiol Biochem 2014;34:393-404.
3 Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlaenderen I, Demunck H, Kasper M, Breier G, Evrard P, Muller M, Risau W, Edgington T, Collen D: Role of tissue factor in embryonic blood vessel development. Nature 1996;383:73-75.
4 Belting M, Ahamed J, Ruf W: Signaling of the tissue factor coagulation pathway in angiogenesis and cancer. Arterioscler Thromb Vasc Biol 2005;25:1545-1550.
5 Penn MS, Topol EJ: Tissue factor, the emerging link between inflammation, thrombosis, and vascular remodeling. Circ Res 2001;89:1-2.

6 Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y: Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. Nat Med 2003;9:458-462.
7 Steffel J, Luscher TF, Tanner FC: Tissue factor in cardiovascular diseases: molecular mechanisms and clinical implications. Circulation 2006;113:722-731.
8 Xu C, Gui Q, Chen W, Wu L, Sun W, Zhang N, Xu Q, Wang J, Fu X: Small interference RNA targeting tissue factor inhibits human lung adenocarcinoma growth *in vitro* and *in vivo*. J Exp Clin Cancer Res 2011;30:63.
9 Zhou J, May L, Liao P, Gross PL, Weitz JI: Inferior Vena Cava Ligation Rapidly Induces Tissue Factor Expression and Venous Thrombosis in Rats. Arterioscler Thromb Vasc Biol 2009;29:863-869.
10 Zhang WY, Wang H, Huo YQ: Acadesine Inhibits Tissue Factor Induction and Thrombus Formation by Activating the Phosphoinositide 3-Kinase/Akt Signaling Pathway. Circulation 2009;120:S1169-S1169.
11 Lopez JA, Kearon C, Lee AY: Deep venous thrombosis. Hematology Am Soc Hematol Educ Program 2004;10.1182/asheducation-2004.1.439439-456.
12 Xu X, Tu L, Jiang W, Feng W, Zhao CX, Wang DW: Bradykinin prevents the apoptosis of NIT-1 cells induced by TNF-alpha via the PI3K/Akt and MAPK signaling pathways. Int J Mol Med 2012;29:891-898.
13 Oeseburg H, Iusuf D, van der Harst P, van Gilst WH, Henning RH, Roks AJM: Bradykinin Protects Against Oxidative Stress-Induced Endothelial Cell Senescence. Hypertension 2009;53:417-422.
14 Dong RL, Xu XZ, Li G, Feng WJ, Zhao G, Zhao JJ, Wang DW, Tu L: Bradykinin Inhibits Oxidative Stress-Induced Cardiomyocytes Senescence via Regulating Redox State. Plos One 2013;8:e77034.
15 Shariat-Madar Z, Mahdi F, Warnock M, Homeister JW, Srikanth S, Krijanovski Y, Murphey LJ, Jaffa AA, Schmaier AH: Bradykinin B2 receptor knockout mice are protected from thrombosis by increased nitric oxide and prostacyclin. Blood 2006;108:192-199.
16 Ehringer WD EM, Gray RD, Miller FN: Bradykinin antagonizes the effect of alpha-thrombin. Inflammation 1997;21:279-298.
17 Schmaier AH: The kallikrein-kinin and the renin-angiotensin systems have a multilayered interaction. Am J Physiol Regul Integr Comp Physiol 2003;285:R1-13.
18 Hasan AA, Amenta S, Schmaier AH: Bradykinin and its metabolite, Arg-Pro-Pro-Gly-Phe, are selective inhibitors of alpha-thrombin-induced platelet activation. Circulation 1996;94:517-528.
19 Yang Y, Loscalzo J: Regulation of tissue factor expression in human microvascular endothelial cells by nitric oxide. Circulation 2000;101:2144-2148.
20 Tschudi MR, Luscher TF: [Nitric oxide: the endogenous nitrate in the cardiovascular system]. Herz 1996;21 Suppl 1:50-60.
21 Livak KJ1 ST: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-408.
22 Brill A, Fuchs TA, Chauhan AK, Yang JJ, De Meyer SF, Kollnberger M, Wakefield TW, Lammle B, Massberg S, Wagner DD: von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. Blood 2011;117:1400-1407.
23 Westrick RJ, Bodary PF, Xu Z, Shen YC, Broze GJ, Eitzman DT: Deficiency of Tissue Factor Pathway Inhibitor Promotes Atherosclerosis and Thrombosis in Mice. Circulation 2001;103:3044-3046.
24 von Bruhl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, Khandoga A, Tirniceriu A, Coletti R, Kollnberger M, Byrne RA, Laitinen I, Walch A, Brill A, Pfeiler S, Manukyan D, Braun S, Lange P, Riegger J, Ware J, Eckart A, Haidari S, Rudelius M, Schulz C, Echtler K, Brinkmann V, Schwaiger M, Preissner KT, Wagner DD, Mackman N, Engelmann B, Massberg S: Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice *in vivo*. J Exp Med 2012;209:819-835.
25 Zhang WY, Wang JG, Wang H, Tang R, Belcher JD, Viollet B, Geng JG, Zhang CX, Wu CD, Slungaard A, Zhu CH, Huo YQ: Acadesine Inhibits Tissue Factor Induction and Thrombus Formation by Activating the Phosphoinositide 3-Kinase/Akt Signaling Pathway. Arterioscler Thromb Vasc Biol 2010;30:1000-1006.
26 Guha M, Mackman N: The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. J Biol Chem 2002;277:32124-32132.
27 Xia L ZH, Hu L, Xie H, Wang T, Xu Y, Liu J, Zhang X, Yan J.: Both NF-κB and c-Jun/AP-1 involved in anti-β2GPI/β2GPI-induced tissue factor expression in monocytes. Thromb Haemost 2013;109:643-651.
28 Thuringer D, Maulon L, Frelin C: Rapid transactivation of the vascular endothelial growth factor receptor KDR/Flk-1 by the bradykinin B2 receptor contributes to endothelial nitric-oxide synthase activation in cardiac capillary endothelial cells. J Biol Chem 2002;277:2028-2032.

29 Li YD, Ye BQ, Zheng SX, Wang JT, Wang JG, Chen M, Liu JG, Pei XH, Wang LJ, Lin ZX, Gupta K, Mackman N, Slungaard A, Key NS, Geng JG: NF-kappaB transcription factor p50 critically regulates tissue factor in deep vein thrombosis. J Biol Chem 2009;284:4473-4483.
30 Lindmark E, Tenno T, Siegbahn A: Role of platelet P-selectin and CD40 ligand in the induction of monocytic tissue factor expression. Arterioscler Thromb Vasc Biol 2000;20:2322-2328.
31 Muller WA: Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol 2003;24:327-334.
32 Mangano DT, Miao Y, Tudor IC, Dietzel C, McSPI I, IREF: Post-reperfusion myocardial infarction - Long-term survival improvement using adenosine regulation with acadesine. J Am Coll Cardiol(JACC) 2006;48:206-214.
33 Eto M, Kouroedov A, Cosentino F, Luscher TF: Glycogen synthase kinase-3 mediates endothelial cell activation by tumor necrosis factor-alpha. Circulation 2005;112:1316-1322.
34 Muller WA: Leukocyte-endothelial cell interactions in the inflammatory response. Lab Invest 2002;82:521-533.
35 Majima M, Nishiyama K, Iguchi Y, Yao K, Ogino M, Ohno T, Sunahara N, Katoh K, Tatemichi N, Takei Y, Katori M: Determination of bradykinin-(1-5) in inflammatory exudate by a new ELISA as a reliable indicator of bradykinin generation. Inflamm Res 1996;45:416-423.
36 Murphey LJ, Hachey DL, Oates JA, Morrow JD, Brown NJ: Metabolism of bradykinin *in vivo* in humans: Identification of BK1-5 as a stable plasma peptide metabolite. J Pharmacol Exp Ther 2000;294:263-269.
37 Morinelli TA, Webb JG, Jaffa AA, Privitera PJ, Margolius HS: A metabolic fragment of bradykinin, Arg-Pro-Pro-Gly-Phe, protects against the deleterious effects of lipopolysaccharide in rats. J Pharmacol Exp Ther 2001;296:71-76.
38 Hasan AA, Warnock M, Nieman M, Srikanth S, Mahdi F, Krishnan R, Tulinsky A, Schmaier AH: Mechanisms of Arg-Pro-Pro-Gly-Phe inhibition of thrombin. Am J Physiol Heart Circ Physiol 2003;285:H183-193.
39 Myers D, Jr., Farris D, Hawley A, Wrobleski S, Chapman A, Stoolman L, Knibbs R, Strieter R, Wakefield T: Selectins influence thrombosis in a mouse model of experimental deep venous thrombosis. J Surg Res 2002;108:212-221.
40 Schabbauer G, Tencati M, Pedersen B, Pawlinski R, Mackman N: PI3K-Akt pathway suppresses coagulation and inflammation in endotoxemic mice. Arterioscler Thromb Vasc Biol 2004;24:1963-1969.
41 Baker AK, Wang R, Mackman N, Luyendyk JP: Rapamycin enhances LPS induction of tissue factor and tumor necrosis factor-alpha expression in macrophages by reducing IL-10 expression. Mol Immunol 2009;46:2249-2255.
42 Bae MK, Su-Ryun K, Soo-Kyung B: Visfatin enhances ICAM-1 and VCAM-1 expression through ROS-dependent NF-kappa B activation in endothelial cells. Febs J 2009;276:187-188.