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Xanthohumol isolated from *Humulus lupulus* prevents thrombosis without increased bleeding risk by inhibiting platelet activation and mtDNA release

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Abstract

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Conflict of interest statement

No conflicts of interest to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2017.02.018.

Aim—As the global population has reached 7 billion and the baby boom generation reaches old age, thrombosis has become the major contributor to the global disease burden. It has been reported that, in moderate doses, beer may protect against thrombosis. Xanthohumol (XN), an antioxidant, is found at high concentrations in hop cones (*Humulus lupulus* L.) and is a common ingredient of beer. Here, the aim of the present work was to investigate the effects of XN on antithrombotic and antiplatelet activities, and study its mechanism.

Approach and Results—Using ferric chloride-induced carotid artery injury, inferior vena cava ligation model, and platelet function tests, we demonstrated that XN uniquely prevents both venous and arterial thrombosis by inhibiting platelet activation. Interestingly, in tail bleeding time studies, XN did not increase bleeding risk, which is recognized as a major limitation of current antithrombotic therapies. We also demonstrated that XN induces Sirt1 expression and thereby decreases reactive oxygen species (ROS) overload, prevents mitochondrial dysfunction, and reduces activated platelet-induced mitochondrial hyperpolarization, respiratory disorders, and associated membrane damage at low concentrations. In mitochondrial function assays designed to detect amounts of extracellular mitochondrial DNA (mtDNA), we found that XN prevents mtDNA release, which induces platelet activation in a DC-SIGN-dependent manner.

Conclusions—XN exemplifies a promising new class of antiplatelet agents that are highly effective at inhibiting platelet activation by decreasing ROS accumulation and platelet mtDNA release without incurring a bleeding risk. This study has also provided novel insights into mechanisms of thrombotic diseases with possible therapeutic implications.

Keywords

Xanthohumol; Beer; Mitochondrial DNA; Thrombosis; Bleeding risk

1. Introduction

Thrombosis-related diseases, such as myocardial infarction, stroke, and venous thromboembolism, account for substantial morbidity and mortality in the United States and developed countries [1,2]. Furthermore, thrombosis is the leading cause of morbidity and mortality in patients with diabetes mellitus, with a reported 65% of diabetic patients eventually dying from thrombotic diseases [3]. Cancer-associated thrombosis is also an important cause of morbidity and mortality in cancer patients [4]. As more people reach old age and the global population pushes past 7 billion, the number of deaths from cardiovascular diseases is on the rise, as aging is associated with an increased incidence of thromboembolism. So far, thrombotic disorders have become the major contributor to the global disease burden. Platelets have an essential role both in the pathogenesis of thrombosis and in maintaining normal hemostasis, making platelets a potential target for the development of antithrombotic therapy [5]. Antiplatelet therapy has been successful in reducing the mortality and morbidity of cardiovascular disease. However, current antiplatelet agents approved by the US FDA have serious side effects, including bleeding episodes, gastrointestinal toxicity, neutropenia, thrombocytopenia and incidence of resistance. As stated previously, a recognized limitation of current antiplatelet therapies is their inability to separate thrombotic events from bleeding occurrences. In addition, previous work has shown that 10–40% of patients are biochemically resistant to aspirin, which is commonly used in

clinical practice to prevent and treat thrombosis. Therefore, the development of novel antithrombotic drugs or antiplatelet drugs with minimal side effects is urgently needed [6].

Xanthohumol is the major prenylated flavonoid that presents in the female inflorescences of the *Humulus lupulus* (hop plant), and the most important dietary source of XN is beer [7]. The FDA estimates that a daily average consumption of beer (225 mL) will provide an intake of 0.14 mg of prenylflavonoids (including XN). XN is detected (6.13 ± 5.4 ng/mL) in plasma up to 4 h after consumption of beer [8,9]. Moreover, in studies by Danish researchers [10], moderate beer drinking was associated with a lower risk of blood clots. Interestingly, previous work by Pomp and colleagues [11] also demonstrated that beer in moderate doses may protect against thrombosis, but consumption of more than 14 standard drinks per week increases the risk of the same condition, in particular pulmonary embolism, in both men and women. These consistent findings suggest that a constituent in beer can affect thrombosis [11]. However, whether XN is associated with antithrombotic activity is not known.

It has been reported that XN exerts a dual effect on ROS production in a dose-dependent manner and exhibits potent antioxidant activity by inducing Sirt1 expression at low concentrations and promoting ROS formation by disrupting mitochondrial functions at high concentrations [9]. Numerous studies have reported that XN protects cells from oxidative DNA damage and oxidative stress in a dose-dependent manner at concentrations up to 14 μ M. However, once the XN concentration exceeds a threshold (14 μ M), XN triggers the opposite effect, leading to oxidative DNA damage and genotoxicity [12,13]. Recently, numerous studies have showed that ROS and extracellular DNA were associated with platelet activation and thrombosis [14–16]. However, it is unknown whether XN reduces ROS production of activated platelets and inhibits platelet mtDNA damage. Moreover, the effect of mtDNA on platelet activation is also unknown. We hypothesized that XN prevents platelet activation and platelet-activated blood coagulation by inhibiting platelet ROS overload and mtDNA damage.

Initially, we evaluated the antithrombotic and antiplatelet effect of XN, and found that XN prevents thrombosis without increased bleeding risk by inhibiting platelet activation. Furthermore, we evaluated the potential role of XN in platelet ROS generation and mtDNA release. Our results suggested that XN reduces activated platelet-induced ROS accumulation by increasing Sirt1 expression, and thereby inhibits mitochondrial dysfunction (mitochondrial hyperpolarization, respiratory disorders, associated membrane damage) and mtDNA release at low concentrations. In this study, we have demonstrated that mtDNA induces platelet activation in a dose-dependent manner *in vivo* and *in vitro*. To our knowledge, this study is the first to reveal that mtDNA released from activated platelets can act as a cellular damage factor and subsequently induce platelet activation may via a DC-SIGN-dependent pathway. Our work presented here is consistent with other recent findings and suggests a possible mechanism for XN's role in reduced thrombosis, related to prevention of ROS overload, mitochondrial dysfunction, reduction in the release of free mtDNA, and inhibition of platelet activation.

2. Materials and methods

2.1. Extraction and isolation of xanthohumol

Xanthohumol was extracted and isolated as described in previous reports [13,17]. *Humulus lupulus* cones were purchased from BioPlanta s.a.s., Irsina (MT), Italy (lot no. LTH 1002). The plant material (500g) was extracted with MeOH (2.0 L $\times$ 3) for 21 days, and removal of the marc by gravity filtration. The solvent was removed under reduced pressure to provide a crude extract. This fraction was first fractionated using a petroleum ether-EtOAc-MeOH gradient by vacuum-liquid chromatography over silica gel. XN was identified by comparison of the MS and NMR data with previous literature [13,17].

2.2. Animal models and diets

All animals were treated according to the experimental protocols approved by the Bioethics Committee of West China Center of Medical Sciences. C57/BL6 mice rats and Sprague-Dawley (SD) were obtained from the West China Hospital Experiment Animal Center (Chengdu, China). The rats were maintained in an animal house with a 12/12-h of light/dark cycle, a constant temperature of 25 °C, and food and water were provided ad libitum. All animals in the study were aged 9–12 weeks and included 9–10 mice or rats per group at the time of study. Animals were randomly allocated to each group. XN was administrated orally to animals at 10 mg/kg twice daily for 7 d, and aspirin was administrated at 15 mg/kg twice daily for 7 d. Ferric chloride-induced venous thrombosis and carotid arterial thrombosis experiments were conducted according to the previous reports [18]. Briefly, a piece of filter paper (10 mm $\times$ 10 mm) pre-saturated with 20 μ L of 2 mM FeCl₃ solution or (2 mm $\times$ 10 mm) pre-saturated with 3 μ L of 1 mM FeCl₃ solution was placed on the venous or arterial cava. Blood flow was monitored until stable occlusion occurred, and stable occlusion was defined that as the time at which blood flow remained absent for more than 10 min. Finally, length and weight were measured. Partial inferior vena cava ligation to the venous thrombosis was conducted as described previously [19]. First, rats were anesthetized by chloral hydrate and a midline laparotomy was performed. During this procedure, sterile saline was applied to prevent drying. Then, the inferior vena cava (IVC) was ligated by a 7.0 polypropylene suture below the renal veins for obtaining complete blood stasis. After surgery, skin and peritoneum were closed, and then the animal was allowed to recover. After 48 h, the IVC was harvested for analysis. Another model of arterial thrombosis induced by simultaneous treatments of ligation and cuff placement in the mouse carotid artery was performed according to the previously described methods [20]. Pulmonary thromboembolism was conducted as described previously [19]. The number of paralyzed or dead mice was recorded, and the percentage of protection was calculated. The bleeding time was measured as previous reports [18,21].

2.3. Chronic-type gastric ulcer

C57/BL6 mice (20 $\pm$ 3 g) were administrated orally XN 10 mg/kg twice a day for 60 days, and aspirin was administrated 15 mg/kg twice a day for 60 days. And the mice were sacrificed on the 61st day. The stomach was evaluated by an observer unaware of treatments, and any macroscopically visible ulcers were measured by calipers. Samples of macroscopically damaged and normal tissue were excised, fixed in neutral buffered

formalin, and subsequently, processed by routine techniques for subsequent histological evaluation [22].

2.4. Blood clot dissolution assay in vitro

The dissolution of blood clot assay was measured using previously described methods with a minor modification. Briefly, the venous blood of rats was gathered into a dry Petri dish, and then clotted at room temperature. The blood clot was cut into 100 ± 20 mg weight slices after clotting. Subsequently, clot slices were put into a 12-well plate with one slice per well, then incubated with or without XN (XN: $0.5 \mu\text{M}$, 6 h, 37°C). The blood clot dissolution rate was expressed as Dissolution rate (%) = $[(W1-W2)/W1]*100$; where W1 indicates clot weight, and W2 represents the residual clot weight [23].

2.5. Hematological parameters

The whole blood was collected by cardiac puncture, and treated with anticoagulants. Platelet-poor plasma (PPP) was obtained by centrifugation at $1000g$ for 10 min. Platelet-rich plasma (PRP) was prepared by centrifugation at $100g$ for 10 min. Blood coagulation factor was measured with a kaolin-activated one-stage partial thromboplastin time method using mice factor plasma (George King Biomedical). Thrombelastogram was measured by thrombelastography (Haemoscope, USA). The viscosity of those samples was evaluated by an automated hematology analyzer. Mean platelet volume (MPV), platelet distribution width (PDW), platelet counts (PLT), immature platelet fraction (IPF) and plateletcrit (PCT) of blood samples were measured by an automatic blood cell analyzer (SYSMEX SF-3000, Japan). The platelet aggregation assay was performed by a Lumi-Aggregometer (Chrono-log) at 37°C . Aggregation was initiated by the addition of ADP solution (final concentration, $20 \mu\text{M}$), arachidonic acid (AA: $900 \mu\text{M}$) and thrombin (0.1 U/mL) *in vitro* (pre-incubated with $0.5 \mu\text{M}$ XN, $40 \text{ ng}/\mu\text{L}$ mtDNA or control buffer for 6 h at 37°C) and *in vivo* (pre-treated with 20 mg/kg/d XN, $50 \mu\text{g/kg/d}$ mtDNA or control buffer for 7 d), and recorded for 5 min. In addition, platelets were pre-incubated with neutralizing antibodies against DC-SIGN (120507; R & D Systems, USA, $25 \mu\text{g/mL}$) for 0.5 h at 37°C in selected experiments [16,19,24–26].

2.6. Platelet preparation

Washed platelets were prepared with differential centrifugation by a method as described previously. In brief, venous blood samples were collected by the standard venous puncture in citrate from rats. Then, whole blood was centrifuged at $160g$ for 10 min for obtain PRP in the presence of PGI2 (1 mM). Platelets were centrifuged $1000g$ for 5 min to obtain platelet pellet and then suspended in a washing buffer containing Tyrode's buffer (2 mM EDTA, 10% of acid-citrate-dextrose solution and 1 mM PGI2), wash twice with buffer. Subsequently, the samples were resuspended in modified Tyrode buffer. The purity of platelet was confirmed with CD41a expression by flow cytometric measurement [16,19,27].

2.7. Platelet adhesion under flow conditions assay

Washed platelets were pre-incubated in the absence or presence of XN ($0.5 \mu\text{M}$) at 37°C for 6 h. Then, platelets were incubated with phalloidin (200 nM) (Sigma-Aldrich) at 37°C for

30 min. Subsequently, a collagen-coated coverslip was mounted on a custom-made flow chamber (Chamlide CF; Live Cell Instruments). The platelets were perfused over a matrix of collagen with a syringe pump. Non-adherent platelets were washed by PBS in the chamber. Adherent platelets were fixed by 4% paraformaldehyde for 15 min and washed with PBS. The platelets were live-monitored by fluorescence microscope (Nikon TE-2000S; Nikon, USA) [27,28].

2.8. P-selectin and integrin α IIb β 3 assay

After treatment (*in vitro*: XN: 0.5 μ M, 6 h, 37 °C; mtDNA: 40 ng/ μ L, 6 h, 37 °C. *in vivo*: XN: 10 mg/kg twice a day for 7 days; mtDNA: 25 μ g/kg twice a day for 7 days), platelets were incubated with PAC-1-FITC (Sigma, USA) or PE-CD62P (Sigma, USA) for 30 min in the dark, and then reaction was stopped by cold PBS. The integrin- α IIb β 3 and P-selectin expression on the platelets were measured at 530 nm (FL1) and 585 nm (FL2) by flow cytometer (Becton-Dickinson, San Jose, USA). In selected experiments, platelets were pre-incubated with DNase I (Genentech, USA, 20 μ g/mL) or neutralizing antibodies against DC-SIGN (120507; R & D Systems, USA, 25 μ g/mL) for 0.5 h at 37 °C [16,29].

2.9. Lipid Peroxidation, ROS, Ca^{2+} and ATP assay

Platelets were incubated with XN (0.5 μ M) for 1 h, 3 h or 6 h at 37 °C. Subsequently, platelets were loaded with MitoSOX™ Red Mitochondrial Superoxide Indicator (Thermo Fisher Scientific, USA), BODIPY® 581/591 C11 (Lipid Peroxidation Sensor, Thermo Fisher Scientific, USA), Fluo-3-AM (Intracellular calcium indicator, Sigma, USA) or CM-H₂DCFDA (General Oxidative Stress Indicator, Thermo Fisher Scientific, USA) at room temperature in the dark, and then measured by a fluorescence microplate reader (Aynergy Mx, BioTek, USA). ATP levels were determined using ATP Assay Kit (FLAA-1KT, Sigma, USA) [30–33].

2.10. Mitochondrial membrane potential assay

Washed platelets were incubated with XN (0.5 μ M) for 6 h at 37 °C. Subsequently, platelets were loaded with JC-1 solution (10 μ g/mL) at 37 °C for 15 min. Subsequently, the ψ of labeled platelets was measured by a confocal microscope (Nikon ECLIPSE Ti, Japan) [31,32].

2.11. Mitochondrial routine respiration assay

After stabilization of platelet routine respiration in MiR05 medium, 0.5 μ M XN, either vehicle or 12.5 nM rotenone was added. Then oligomycin (1 μ g/mL), an ATP-synthase inhibitor, was added to assess LEAK respiration. Using FCCP titration reached maximal uncoupled respiratory electron transport system capacity that was supported by endogenous substrates (ETS). Sequentially, respiration was blocked by rotenone (2 μ M), antimycin (1 μ g/mL) and sodium azide (10 mM) to assess residual oxygen consumption [31].

2.12. Transmission electron microscopy imaging of platelets and mitochondria assay

Washed platelets were incubated with or without XN (0.5 μ M, 6 h) and activated or not by agonists. They were then fixed by 2.5% glutaraldehyde for 24 h, and processed for standard

dehydration. In brief, samples were washed (3×10 min) with Tyrode's buffer and fixed with osmium tetroxide for 90 min. Subsequently, samples were washed again (3×10 min) with Tyrode's buffer and processed for standard alcohol dehydration. Then, samples were dipped in 100% EtOH and air-dried overnight. Finally, samples were coated with palladium and then observed with a JEOL 6360LV transmission electron microscope (Tokyo, Japan) [34–36].

2.13. mtDNA preparation

The mtDNA was isolated from washed platelets using a commercial kit (Mitochondria DNA Isolation Kit, Biovision). The purity of the mtDNA preparations was demonstrated by Western blotting to confirm the absence of non-mitochondrial proteins. The concentration of mtDNA was determined by qPCR assay with a PRISM 7300 sequence detection system (Applied Biosystems) [34]. Consistent approaches were used in all below studies.

2.14. mtDNA quantification in vitro

Washed platelets were treated with $0.5 \mu\text{M}$ XN for 6 h at 37°C . After centrifugation at $700g$ for 10 min to remove the pellet (platelet), extracellular mtDNA was isolated from the supernatant using a Mitochondrial DNA Isolation Kit (Catalog K280-50, Biovision, USA). The purity of the mtDNA was demonstrated as described above. The concentration of mtDNA was determined by a spectrophotometer (Nanodrop2000, Thermo) or qPCR assay using a PRISM 7300 sequence detection system (Applied Biosystems) [34,37–39].

2.15. mtDNA quantification in vivo

After seven days treatment with 20 mg/kg/d XN, the rats were sacrificed and blood was collected. Then mtDNA was extracted by Mitochondrial DNA Isolation Kit (Catalog K280-50, Biovision, USA) for analysis. The purity of the mtDNA preparations and concentration of mtDNA were determined as described above. Rat NADH dehydrogenase 1 gene (mtDNA) [forward CGCCTGACCAATAGCCATAA, reverse ATTCGACGTTAAAGCCTGAGA] was the primer sequence. All samples were measured with standards at the same time [34,37–39].

2.16. Western blot analysis

The platelets were harvested by scraping them out with lysis buffer after treatment with XN. Subsequently, the protein content was determined by a bicinchoninic acid protein assay kit. Forty mg of protein from platelets lysates were separated on a SDS-PAGE gel and then electrotransferred onto nitrocellulose membranes. Afterwards, membranes were incubated in a blocking solution of 5% non-fat powdered milk in Tris-buffered saline-Tween. Antisera against Sirt1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein bands were visualized with Immun-Star[®] Western C[®] Chemiluminescent Kit (Bio-Rad, California, USA) Western blotting detection systems. Finally, results were analyzed by Quantity One Software (Bio-Rad, California, USA) [9].

2.17. DC-SIGN expression assay

Washed platelets were incubated with or without mtDNA (mtDNA: 40 ng/μL, 6 h). Immunoprecipitation of protein assemblies was performed with incubation of these pools by purified and identified monoclonal antibodies against DC-SIGN (MR-1, Santa Cruz Biotech, USA). Subsequently, the capture of the immune complexes on Protein G superparamagnetic Microbeads (Milenyi Biotec, Germany) was performed as previously described. Immunoprecipitates were subjected to Western blot by polyclonal antibodies against DC-SIGN and then immunoprecipitated with Streptavidin-agarose (Sigma Aldrich, USA). Subsequently, the immunoprecipitated material was analyzed by SDS-PAGE and Western blot with antibodies specific for DC-SIGN [40].

2.18. Statistical analysis

The data are expressed as mean ± SD. Multiple comparisons were made by appropriate analysis of variance, and individual group samples were compared by Dennett's test. *P* values were two tailed and *P* values < 0.05 were considered to indicate significance.

3. Results

3.1. Xanthohumol (XN), a prenylated chalcone isolated from the hop plant

Xanthohumol was extracted and isolated from *Humulus lupulus* cones and identified by comparison of the MS and NMR data with previous reports (Fig. 1A and B) [13,17]. The purity of XN samples was 99.08% as detected by HPLC (0.2% formic acid in water: methanol). ¹H NMR (400 MHz, DMSO) δ 14.65 (s, 1 H), 7.77 (d, *J* = 15.5 Hz, 1 H), 7.68 (d, *J* = 15.5 Hz, 1H), 7.58 (d, *J* = 8.6 Hz, 2H), 6.85 (d, *J* = 8.6 Hz, 2H), 6.09 (s, 1 H), 5.14 (t, 1H), 3.87 (s, 3H), 3.14 (d, *J* = 7.0 Hz, 2H), 1.70 (s, 3H), 1.61 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 191.64 (s), 164.60 (s), 162.41 (s), 160.50 (s), 159.91 (s), 142.50 (s), 130.48 (s), 129.88 (s), 126.03 (s), 123.76 (s), 123.01 (s), 115.96 (s), 107.31 (s), 104.55 (s), 90.94 (s), 55.73 (s), 25.46 (s), 21.03 (s), 17.65 (s). HRMS (TOF MS ES⁺) for C₂₁H₂₂O₅ (M+Na⁺) calcd 377.1365, found 377.1382.

3.2. XN inhibits the formation of carotid arterial thrombosis and inferior vena cava thrombosis in animals

Initially, we evaluated the effect of XN on thrombus (XN: 10 mg/kg twice a day for 7 days). And our data showed that XN decreased the weight (Fig. 2A, D, G) and length (Figs. 2B, 2E, 2H) of FeCl₃-induced carotid arterial thrombosis, FeCl₃-induced inferior vena cava thrombus, and partial inferior vena cava ligation-induced venous thrombosis in rats [18–21]. Also, the average time to occlusive thrombosis in FeCl₃-induced carotid arterial thrombosis and inferior vena cava thrombosis was longer in XN-treated versus untreated animals (Fig. 2C and F). In addition, the oral administration of XN suppressed the average T/L ratio (thrombus area/lumen area) compared with control group in the carotid artery with ligation-cuff treatment. (Supplementary Fig. 1 A). Thus, XN treatment significantly reduced the incidence of pulmonary embolism (Fig. 2I) and also significantly improved blood viscosity in rats (Supplementary Table 1).

A well-known major limitation of current antithrombotic therapies is their inability to separate thrombotic events from bleeding occurrences [6]. Interestingly, the XN treatment did not significantly prolong bleeding time in rats (Fig. 2J). In addition, the mice treated with XN did not show any spontaneous bleeding and had decreased incidence of gastric ulcer than those treated with aspirin, the most commonly used oral antithrombotic drug (Fig. 2K). In total, our results suggest that XN prevents both venous and arterial thrombosis without a significant risk of bleeding.

3.3. XN has insignificant effects on coagulation factor and thrombolysis system

To further study the effect of XN on the coagulation system, we also examined the effect of XN on coagulation factor, blood clot, thromboelastogram, and platelet properties [19,24,26,41]. We found that XN treatment did not significantly affect prothrombin time (PT), activated partial thrombin time (APTT), thrombin time (TT), or fibrinogen (FIB) (Fig. 3A). XN treatment also appeared to have no significant influence on coagulation factors II, V, VII–XII, and blood clotting (Fig. 3B and C). Analysis of a thromboelastogram (TEG) also demonstrated that XN treatment significantly improved the indexes for viscoelastic properties of platelet activation, developing clot, and thrombogenesis (Fig. 3D). Our study also showed that XN has insignificant effects on platelet properties, including platelet count, platelet distribution width, thrombocytocrit, platelet ratio, mean platelet volume, and immature platelet fraction in mice (Supplementary Table 2). These results indicate that XN prevents venous and arterial thrombosis likely by inhibiting platelet activation.

3.4. XN inhibits platelet activation in vivo and in vitro

It is well known that thrombosis is mediated by activation of platelets includes aggregation and adhesion [42]. Thus, to further study the effect of XN on platelet activation, we examined the XN's effect on aggregation of platelets induced by ADP, AA, and thrombin *in vitro* (Fig. 4A, XN: 0.5 μ M, 6 h, 37 $^{\circ}$ C) and *in vivo* (Fig. 4B, XN: 20 mg/kg/d, 7 d) [27,41,42]. Our studies revealed that XN significantly suppressed platelet aggregation in all assays. We also observed decreases in α IIb β 3 expression (Fig. 4C and D), P-selectin (Fig. 4E and F), and cytosolic calcium (Fig. 4G and H) that three key indicators of platelet activation *in vitro* and *in vivo*. Furthermore, we also examined the effects of XN on platelet adhesion on collagen-coated surfaces. The data indicated that XN effectively inhibited these processes (Fig. 4I and J).

3.5. XN prevents intracellular ROS accumulation of activated platelets by increasing Sirt1 expression

The role that ROS plays in platelet activation has been studied, and early reports primarily showed that ROS overload induces platelet activation [14,43]. In this study, we examined the effect of XN on platelet ROS accumulation. We found that XN inhibited ROS accumulation of activated platelets at low concentrations time-dependently (Fig. 5A–C). Mitochondria are believed to be the major source of ROS [44], accordingly, we examined the effect of XN on platelet mitochondrial ROS accumulation. At a low concentration (0.5 μ M), XN inhibited mitochondrial ROS accumulation in platelets activated by various inducers (Fig. 5D). Reports have indicated that ROS accumulation enhances lipid peroxidation leading to damage of the bio-membrane system [34–36,45]. We found that XN prevented such

membrane damage as shown by transmission electronic microscopy imaging (Fig. 5E). A likely explanation is that XN inhibited ROS overload in activated platelets and thereby also reduced ROS-induced platelet membrane damage. Activated platelets treated with XN also showed lowered lipid peroxidation levels as measured by Lipid Peroxidation Sensor, BODIPY® 581/591 C11 (Fig. 5F). A prior study reported that XN exhibits potent antioxidant activity at low concentrations through induction of Sirt1 expression [9]. The results we obtained are consistent with these recent findings and suggest that XN inhibits activated platelet-induced ROS accumulation by inducing Sirt1 expression at low concentrations in a time-dependent manner (Fig. 5G and H). Furthermore, to clarify the role played by Sirt1 expression, we evaluated the effects of EX-527, a Sirt1 inhibitor, on the effect of XN on platelet activation and ROS accumulation [46]. In the presence of EX-527 + XN, we saw much lower levels of inhibition of ROS accumulation and platelet aggregation (Fig. 5I and J) than with XN alone. In future studies, we intend to use knockout approaches and other pharmacological to confirm that XN inhibits platelet ROS accumulation by increasing Sirt1 expression at low concentrations.

3.6. XN suppresses activated platelet-induced mtDNA release and mitochondrial dysfunction by inhibiting ROS generation

Many studies had reported mitochondrial dysfunction is associated with platelet activation [34,40,47,48]. For example, Brownlee and colleagues demonstrated that mitochondrial hyperpolarization induces ROS generation and platelet activation [47]. Cardenes *et al.* found that mitochondrial dysfunction may lead to platelet activation *in vitro* and *in vivo* [48]. In this study, we determined the hyperpolarization level of the mitochondrial (Fig. 6A), mitochondrial routine respiration (Fig. 6B), and intracellular ATP level (Fig. 6C), and found that these three key indexes for evaluating mitochondrial function were lower when XN was added to ADP-activated platelets. We further determined the amounts of extracellular mtDNA detected from normal platelets or activated platelets *in vitro* (Fig. 6D–F) and *in vivo* (Fig. 6G) in the absence and presence of XN with the qPCR assay [34,38–40]. Our results demonstrated that XN inhibited platelet mtDNA release at low concentrations. Furthermore, we found that the inhibitory effect of XN on mtDNA release was less when EX-527, a Sirt1 inhibitor, was also added along with XN (Fig. 6J). Next, we evaluated the effects of ROS on mtDNA release and identified a hydrogen peroxide-induced increase in free mtDNA from activated platelets (Fig. 6H). Meanwhile, addition of the ROS scavenger N-acetylcysteine (NAC) decreased the amount of mtDNA release (Fig. 6I). At low concentrations, XN had no effect on apoptosis of platelets. Therefore, it was concluded that the change of extracellular mtDNA level may not be caused by the variation of platelets apoptotic bodies [9].

3.7. mtDNA induced platelet activation may occur via a DC-SIGN-dependent pathway

We performed additional related experiments to study the effect of mtDNA on platelet activation. First, we demonstrated that mtDNA caused dose-dependent increased levels of platelet aggregation (Fig. 7A) and α IIB β 3 expression (Fig. 7B) *in vitro* (6 h, 37 °C). We then used a mouse model to confirm that mtDNA can also increase levels of platelet aggregation (Fig. 7C) and α IIB β 3 expression (Fig. 7D) *in vivo* (mtDNA: 50 μ g/kg/d, 7 d). To confirm the importance of mtDNA on platelet activation, we added a DNase to determine whether DNA elimination affected platelet activation. Indeed, platelet aggregation and α IIB β 3 expression

were lowered in the presence of the DNase and no further reduction was seen when XN was also added (Fig. 7E and F). Thus, addition of the DNase also prevented XN-related inhibitory effects.

Next, we assessed the downstream signaling events of mtDNA-induced platelet activation. DC-SIGN is a surface molecule that acts as a critical receptor in further platelet activation and is expressed in platelets [26,40]. We found that more DC-SIGN was expressed in the presence of mtDNA *in vitro* and *in vivo* (Fig. 8A and B). Furthermore, XN reduced platelet DC-SIGN expression in FeCl₃-induced carotid arterial thrombosis and partial inferior vena cava ligation induced venous thrombosis models (Fig. 8C). In addition, we found that the neutralizing antibody against DC-SIGN abolished the potentiating effects of mtDNA on platelet activation, further confirming the critical roles of DC-SIGN in mtDNA-mediated platelet activation (Fig. 8D and E) [26,40]. In the future studies, we intend to use knockout approaches to confirm that mtDNA induced platelet activation in a DC-SIGN-dependent manner.

3.8. Long-term medication with XN is quite safe for rats

To predict the chronic toxicity of XN, we examined its effect on platelet and basic metabolic properties, complete blood count, total protein, and liver function. In our mouse studies, XN did not have deleterious effects on platelet or basic metabolic properties, complete blood count, total protein, and liver function compared with the control group (Supplementary Tables 2 and 3). Therefore, our observations indicate that, most likely, long-term medication with XN would not cause significant negative effects [21–23].

4. Discussion

Thrombosis is a primary cause of mortality and morbidity in the developed world. Each year, 1.1 million Americans experience acute myocardial infarction. Another million experience a thrombotic cerebrovascular accident, and DVT (deep vein thrombosis) is a major problem for surgical and other hospitalized patients [1,2,5,49,50]. However, current antithrombotic agents have serious side effects, mainly bleeding episodes [6]. Therefore, development of a novel, more effective antithrombotic drug with minimal side effects is urgently needed. XN is an antioxidant and a common ingredient of beer. It has been reported that moderate beer consumption may protect against venous thrombosis [10,11]. However, it is not known whether XN is associated with the antithrombotic activity.

The results presented herein are consistent with other findings and suggest a possible mechanism for XN's role in preventing thrombosis without increased bleeding risk, related to inhibition of platelet activation, reduction of ROS production, prevention of mitochondrial dysfunction, and reduction in free mtDNA release. By using models of carotid artery and inferior vena cava thrombosis, we demonstrated first that thrombus formation was strongly impaired in XN-treated rats. We also showed that XN-treated mice were more resistant to pulmonary thromboembolism than control mice. Furthermore, XN treatment significantly improved blood viscosity in rats. These results suggest that XN may have great potential as an anti-thrombotic drug and are consistent with published reports stating that beer at moderate levels may protect against thrombosis and XN reduce infarct volume in rats with

cerebral ischemia [10,11,51]. A well-known major limitation of current antithrombotic therapies is their inability to separate bleeding occurrences from thrombotic events as well as various serious side effects [6]. Intriguingly, XN-treated mice did not show any spontaneous bleeding or gastrointestinal toxicity. Therefore, long-term medication with XN would likely not lead to significant negative effects.

Platelets have essential roles in the maintenance of normal hemostasis and the thrombosis pathogenesis [6]. Our results demonstrated that the platelet prothrombinase activity was markedly impaired in the presence of XN. This finding is consistent with the observed decrease in P-selectin, α Ib β 3 expression, and cytosolic calcium of XN-treated platelets. Our findings also suggest that the platelet adhesive function was reduced after administration of XN. However, XN treatment appeared to have no significant influence on clot weight, coagulation factors, or platelet properties. Our observations indicate that further investigation of XN could lead to a novel antiplatelet therapy, which is able to separate antithrombotic activity from bleeding occurrences. Increased understanding of XN's antiplatelet activity should lead to the development of new antithrombotic drugs that are potentially more effective without causing bleeding. Such new therapies targeting the underlying mechanism for platelet dysfunction are urgently warranted.

It has been reported that ROS promotes platelet hyper-activation and thrombosis and that XN exhibits potent antioxidant activity at low concentrations by inducing Sirt1 expression [9,14–16]. We evaluated the effect of XN on ROS generation in platelets and demonstrated that XN can prevent ROS accumulation of activated platelets by increasing Sirt1 expression. In agreement with this conclusion, EX-527, a Sirt1 inhibitor decreased the inhibitory effects of XN on ROS accumulation and platelet activation. In our subsequent research, we intend to study the downstream signaling events affected by XN's inhibitory effects on platelet activation and ROS overload.

It is well known that mitochondria play a key role in platelet activation and thrombosis [34,43,47,48]. Cardenes and colleagues demonstrated that mitochondrial dysfunction causes platelet activation *in vitro* and *in vivo* [48]. In addition, Boudreau et al. reported that activated platelets release mtDNA [34]. We determined intracellular ATP level, the hyperpolarization level of the mitochondrial membrane potential, and mitochondrial routine respiration, and found that these three key indexes for evaluating mitochondrial function improved with XN addition to activated platelets. We further assessed the amounts of extracellular mtDNA detected from normal platelets or activated platelets *in vitro* and *in vivo* in the absence and presence of XN with a qPCR assay. In this study, we also observed that XN inhibits mtDNA release from activated platelets. We evaluated the effects of ROS on mtDNA release to assess the relationship of ROS and mtDNA release. Also, we identified a hydrogen peroxide-induced increase in free mtDNA from activated platelets, while NAC (an ROS scavenger) decreased the inhibition of XN on mtDNA release. Furthermore, we found that EX-527 (a Sirt1 inhibitor) also decreased the inhibition of XN on platelet mtDNA release. Taken together, our results indicated that XN inhibits mtDNA release by decreasing ROS accumulation.

Activated platelets can release mtDNA [34], however, it is unknown whether mtDNA induces platelet activation. Additional related experiments were performed to study the relationship between mtDNA and platelet activation. First, we examined the effect of mtDNA on platelet pro-coagulant activity and demonstrated that mtDNA-treated platelets can support efficient platelet activation *in vitro* and *in vivo*. We also demonstrated that DNase decreases the inhibitory effects of XN on the platelet aggregation and $\alpha\text{IIb}\beta 3$ expression of activated platelets. Through additional related experiments, we furthermore found that mtDNA-induced platelet activation may occur via a DC-SIGN-dependent pathway. Previous studies have shown that DC-SIGN is associated with platelet activation. However, DC-SIGN was not linked previously to mtDNA and platelet activation [22,40]. To the best of our knowledge, we are the first to report that released mtDNA induces platelet activation via a DC-SIGN-dependent pathway and, thus, is a potential target for the development of antithrombotic therapy. Next, we considered to confirm that mtDNA induces platelet activation in a DC-SIGN-dependent manner with knockout approaches.

In this study, we have demonstrated that XN prevents both venous and arterial thrombosis without bleeding risk by inhibiting platelet activation, mtDNA release, and ROS overload. Specifically, XN induces Sirt1 expression and thereby decreases ROS overload, prevents mitochondrial dysfunction, and reduces activated platelet-induced associated membrane damage and mitochondrial hyperpolarization. In mitochondrial function assays designed to detect amounts of extracellular mtDNA, we found that XN prevents mtDNA release, which acts as a cellular damage factor and subsequently may induce platelet activation in a DC-SIGN-dependent manner. This study has provided novel insights into mechanisms of thrombotic diseases with possible therapeutic implications. We plan to continue with future studies to establish mechanistic details and structural pharmacophores to further develop XN as a potential new agent combining antiplatelet efficacy with minimal side effects. In the future, we will use other pharmacological and knockout approaches to assure and confirm XN reduces ROS accumulation by increasing Sirt1 expression. In this study, we also demonstrate that platelets release mtDNA as time goes by *in vitro*, and it was reported that mtDNA induce the inflammation and transfusing blood that after longer durations of refrigerated storage may induce SIRS (systemic inflammatory response syndrome) [38,52,53]. Next, we will evaluate the potential role of mtDNA in mechanisms of transfusion-induced immunomodulation. And research how to decrease the level of mtDNA during blood bank cold storage. In addition, it will be very interesting to research a new brewing method that increases the content of XN in beer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Xanthohumol (XN), a prenylated chalcone isolated from the hop plant. **(A)** The molecular structure and ¹H NMR spectrum of xanthohumol (2',4',4-trihydroxy-6'-methoxy-3'-prenylchalcone). **(B)** MS spectrum of xanthohumol.

Fig. 2.

XN inhibits formation of carotid arterial thrombosis and inferior vena cava thrombosis in animals. XN decreases weight (**A**, **D**) and length (**B**, **E**) of FeCl₃-induced carotid arterial thrombosis and FeCl₃-induced inferior vena cava thrombus in rats (pre-treated with 20 mg/kg/d XN for 7 d). XN decreases weight (**G**) and length (**H**) of partial inferior vena cava ligation-induced venous thrombosis in rats. (**C**, **F**) The average time to occlusive thrombosis in FeCl₃-induced inferior vena cava and carotid arterial injury was longer in XN treated (XN: 20 mg/kg/d, 7 d) rats compared with untreated rats. (**I**) XN reduces mortality from pulmonary thromboembolism in mice (XN: 20 mg/kg/d, 7 d). (**J**) XN does not significantly prolong bleeding times in C57/BL6 mice (XN: 20 mg/kg/d, 7 d). (**K**) XN decreases the incidence of gastric ulcer in normal rats compared with the use of aspirin (XN: 20 mg/kg/d, 60 d. Aspirin: 30 mg/kg/d, 60 d). Data are expressed as mean $\pm$ SD. $n = 9-10$. * $P < 0.05$ vs Control.

Fig. 3.

XN has insignificant effects on coagulation factor and thrombolysis system. Effects of XN treatment in rats (XN: 20 mg/kg/d, 7 d) on PT, APTT, TT, FIB (**A**), coagulation factors (**B**), and clot weight reduction (**C**). (**D**) Thromboelastogram of whole blood from rats treated with or without XN. Data are expressed as mean $\pm$ SD. $n = 9-10$. * $P < 0.05$ vs Control.

Fig. 4. XN inhibits platelet activation *in vivo* and *in vitro*. (**A, B**) Influence of XN on aggregation of platelets induced by ADP, AA, and thrombin *in vitro* (XN: 0.5 μ M, 6 h; aspirin: 0.1 mM, 6 h) and *in vivo* (XN: 20 mg/kg/d, 7 d; aspirin: 30 mg/kg/d, 7 d). (**C, D**) α IIb β 3 expression in ADP-, AA-, and thrombin-activated platelets with XN *in vitro* and *in vivo*. (**E, F**) P-selectin expression in ADP-, AA-, and thrombin-activated platelets with treatment *in vitro* and *in vivo*. (**G, H**) Influence of XN on the elevation of cytosolic calcium levels in ADP-, AA-, and thrombin-activated platelets *in vitro* and *in vivo*. (**I, J**) Effect of XN on platelet adhesion to collagen-coated surfaces (phalloidin-labeled platelets). Data are expressed as mean $\pm$ SD. $n = 9-10$. * $P < 0.05$ vs Control.

Fig. 5.

XN prevents ROS accumulation of activated platelets by increasing sirtuin 1 expression. (A, B) Dose and time effects of XN on platelet ROS production. (C, D) Low concentrations of XN significantly decrease ROS levels of ADP-, AA-, and thrombin-activated platelets *in vitro* (XN: 0.5 μM , 6 h). (E) Transmission electronic microscopy imaging of platelets and associated membrane damage (black arrows) treated with XN or without. (F) XN inhibits ADP-, AA- and thrombin-activated platelets lipid oxidation (C11-BODIPY 581/591, lipid peroxidation sensor) (XN: 0.5 μM , 6 h). (G, H) XN at low concentrations increases Sirt 1 expression in a time-dependent manner. (I) EX-527, a Sirt 1 inhibitor, decreases inhibition of XN on ROS accumulation of activated platelets. (J) EX-527 decreases inhibition of XN on ADP-, AA- and thrombin-induced platelets aggregation. Data are expressed as mean $\pm$ SD. $n = 8$. * $P < 0.05$ vs Control, # $P < 0.05$ vs Agonist or XN.

Fig. 6.

XN suppresses activated platelet-induced mtDNA release and mitochondrial dysfunction via inhibition of ROS generation. (A) mitochondrial hyperpolarization (JC-1 fluorescence ratio, FL2/FL1) Ψ , (B) mitochondrial routine respiration, (C) ATP, values in activated platelets treated with XN (XN: 0.5 μ M, 6 h). (D, E) The effects of XN on mtDNA release at different concentrations and times. (F) XN inhibits mtDNA escape from activated platelets *in vitro* (XN: 0.5 μ M, 6 h). (G) XN decreases the amounts of plasma mtDNA in FeCl₃-induced carotid arterial thrombosis and partial inferior vena cava ligation-induced venous thrombosis (XN: 20 mg/kg/d, 7 d). (H) ROS (hydrogen peroxide, 6 h) induces mtDNA escape from platelets. (I) NAC reduces mtDNA release from platelets (NAC: 10 mM, 6 h). (J) EX-527 decreases inhibition of XN on platelet mtDNA release *in vitro*. Data are expressed as mean $\pm$ SD. $n = 8$. * $P < 0.05$ vs Control, # $P < 0.05$ vs ADP or Agonist.

Fig. 7.

mtDNA induced platelet activation *in vivo* and *in vitro*. (A, C) mtDNA affects aggregation of platelets *in vitro* (mtDNA: 40 ng/ μ L, 6 h) and *in vivo* (mtDNA: 50 μ g/kg/d, 7 d). (B, D) mtDNA influences α IIb β 3 level *in vivo* and *in vitro*. (E, F) DNase decreases inhibition of XN on platelet aggregation and α IIb β 3 level induced by ADP *in vitro* (Pre-incubated for 0.5 h at 20 μ g/mL DNase I). Data are expressed as mean $\pm$ SD. $n = 8$. * $P < 0.05$ vs control, # $P < 0.05$ vs ADP.

Fig. 8. mtDNA induces platelet activation may through a DC-SIGN dependent pathway. (**A** and **B**) mtDNA increases DC-SIGN expression *in vitro* (mtDNA: 40 ng/μL, 6 h) and *in vivo* (mtDNA: 50 μg/kg/d, 7 d). **C**, XN reduces platelet DC-SIGN expression in FeCl₃-induced carotid arterial thrombosis and partial inferior vena cava ligation induced venous thrombosis. **D** and **E**, Anti-DC-SIGN (Pre-incubated for 0.5 h at 25 μg/mL) agent decreases platelet aggregation and αIIbβ3 level induced by mtDNA *in vitro*. Data are expressed as mean ± SD. *n* = 8. **P* < 0.05 vs Control, #*P* < 0.05 vs mtDNA.