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Branched-Chain Amino Acid Catabolism Promotes Thrombosis Risk by Enhancing Tropomodulin-3 Propionylation in Platelets

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BACKGROUND: Branched-chain amino acids (BCAAs), essential nutrients including leucine, isoleucine, and valine, serve as a resource for energy production and the regulator of important nutrient and metabolic signals. Recent studies have suggested that dysfunction of BCAA catabolism is associated with the risk of cardiovascular disease. Platelets play an important role in cardiovascular disease, but the functions of BCAA catabolism in platelets remain unknown.

METHODS: The activity of human platelets from healthy subjects before and after ingestion of BCAAs was measured. Protein phosphatase 2Cm specifically dephosphorylates branched-chain α-keto acid dehydrogenase and thereby activates BCAA catabolism. Protein phosphatase 2Cm–deficient mice were used to elucidate the impacts of BCAA catabolism on platelet activation and thrombus formation.

RESULTS: We found that ingestion of BCAAs significantly promoted human platelet activity (n=5; $P<0.001$) and arterial thrombosis formation in mice (n=9; $P<0.05$). We also found that the valine catabolite α-ketoisovaleric acid and the ultimate oxidation product propionyl-coenzyme A showed the strongest promotion effects on platelet activation, suggesting that the valine/α-ketoisovaleric acid catabolic pathway plays a major role in BCAA-facilitated platelet activation. Protein phosphatase 2Cm deficiency significantly suppresses the activity of platelets in response to agonists (n=5; $P<0.05$). Our results also suggested that BCAA metabolic pathways may be involved in the integrin αIIbβ3–mediated bidirectional signaling pathway that regulates platelet activation. Mass spectrometry identification and immunoblotting revealed that BCAAs enhanced propionylation of tropomodulin-3 at K255 in platelets or Chinese hamster ovary cells expressing integrin αIIbβ3. The tropomodulin-3 K255A mutation abolished propionylation and attenuated the promotion effects of BCAAs on integrin-mediated cell spreading, suggesting that K255 propionylation of tropomodulin-3 is an important mechanism underlying integrin αIIbβ3–mediated BCAA-facilitated platelet activation and thrombosis formation. In addition, the increased levels of BCAAs and the expression of positive regulators of BCAA catabolism in platelets from patients with type 2 diabetes mellitus are significantly correlated with platelet hyperreactivity. Lowering dietary BCAA intake significantly reduced platelet activity in *ob/ob* mice (n=4; $P<0.05$).

CONCLUSIONS: BCAA catabolism is an important regulator of platelet activation and is associated with arterial thrombosis risk. Targeting the BCAA catabolism pathway or lowering dietary BCAA intake may serve as a novel therapeutic strategy for metabolic syndrome–associated thrombophilia.

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Key Words: amino acids, branched-chain ■ blood platelets ■ metabolic syndrome ■ thrombosis ■ TMOD protein, human

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Clinical Perspective

What Is New?

- Ingestion of branched-chain amino acids (BCAAs) significantly enhanced the activity of platelets in response to agonists and increased the risk of the arterial thrombosis.
- The BCAA catabolic pathway–driven propionylation of tropomodulin-3 at K255 is an important mechanism underlying BCAA-facilitated platelet activation.
- Elevated levels of BCAAs and enhanced expression of positive regulators of BCAA catabolism in platelets are probably responsible for the high platelet activity in type 2 diabetes mellitus.

What Are the Clinical Implications?

- BCAAs or BCAA catabolites enhance the risk of arterial thrombosis.
- Restricting BCAA intake or targeting BCAA catabolism may serve as a novel strategy for antithrombosis therapy.

Branched-chain amino acids (BCAAs), members of a unique category of essential amino acids including leucine (Leu), isoleucine (Ile), and valine (Val), can supply energy for long-term exercise, promote muscle protein synthesis, and reduce muscle decomposition.[1] Therefore, oral BCAAs are commonly used for fitness and in professional sports.[2] In addition to sports supplementation, BCAA injections are used for the improvement of severe hepatitis, cirrhosis, and hepatic encephalopathy after hepatobiliary surgery.[3,4]

BCAAs are catabolized mainly in the liver, muscle, fat, kidneys, and brain. Complete catabolism of BCAAs requires a number of enzymatic steps, most of which occur in the mitochondria.[1] Catabolism of Leu, Ile, and Val shares the first 2 catabolic enzymes, branched chain aminotransferase (BCAT) and branched-chain α-keto acid (BCKA) dehydrogenase complex (BCKD). BCAT uses vitamin B_6 as a cofactor to transfer the α-amino group from Leu, Ile, and Val to α-ketoglutarate, which becomes glutamate. Once deaminated, Leu, Ile, and Val form the BCKAs α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV), and α-ketoisovaleric acid (KIV), respectively.[1] These BCKAs can be reaminated into BCAAs. After BCAA deamination, BCKAs can be irreversibly oxidized by BCKD, and BCKD is a rate-limiting enzyme of BCAA catabolism. The activity of BCKD is regulated by BCKD kinase (BCKDK) and protein phosphatase 2Cm (PP2Cm). BCKDK can phosphorylate the E1-α subunit of BCKD on Ser293 to inhibit BCKD activity.[5] In contrast, PP2Cm is a specific phosphatase for the dephosphorylation of BCKD Ser293 that activates BCKD, thereby enhancing BCAA catabolism.[6] Genetic ablation of PP2Cm leads to BCAA catabolic defects, accumulation of BCAAs and BCKAs, and reduced levels of downstream catabolites. BCAA catabolism eventually produces acetoacetyl-coenzyme A (CoA), acetyl-CoA, and propionyl-CoA.[7] The BCAA catabolic pathway widely regulates the metabolism of protein,[8] glucose,[9] and fatty acid[10] and plays essential roles in intestinal development,[11] the immune response,[12] neurotransmission,[13] mitochondrial biogenesis,[14] oxidative stress,[15] etc.

Metabolic syndrome (MS) and type 2 diabetes mellitus (T2DM), which are often characterized by obesity, hyperlipidemia, hyperglycemia, and hypertension, increase the risk of cardiovascular embolic diseases.[16] MS and T2DM can exacerbate atherosclerosis by enhancing inflammatory cell infiltration at plaque sites and can facilitate atherosclerotic thrombosis by interfering with platelet function, the coagulation system, the fibrinolytic system, and vascular endothelial function. Such effects are the major pathological causes of cardiovascular diseases.[17,18] Recently, considerable epidemiological and experimental data have shown that the plasma levels of BCAAs increase gradually during the development of MS, and plasma BCAA levels are positively correlated with cardiovascular disease risk.[19]

Anucleate blood platelets are major participants in atherosclerotic thrombosis and serve as vital target cells for cardiovascular disease therapy.[20] Many studies have shown that platelets in patients with MS or T2DM are often in a state of high activity.[21] This hyperactivity may be caused by increases in oxidized low-density lipoproteins[22] and advanced glycation end products,[23] decreases in insulin levels,[24] and platelet mitochondrial dysfunction.[25] Whether the high plasma BCAA levels in MS relate to high platelet activity and thrombosis facilitation remains unclear.

In this study, we found that ingestion of BCAAs significantly increased the activity of human platelets in response to agonists. BCAA catabolic defects significantly suppressed platelet activation and arterial thrombus formation in PP2Cm knockout mice, indicating that BCAA catabolism is essential for thrombosis. BCAA catabolite–promoted propionylation of tropomodulin-3 (TMOD3) probably plays a key role in this process. The elevated levels of BCAAs and enhanced expression of positive regulators of BCAA catabolism in platelets are correlated with high platelet activity in patients with T2DM. Reducing dietary BCAA intake lowers platelet activation. Our study not only reveals that the risk for arterial thromboembolism is increased with intake of BCAA but also provides a novel strategy for antiplatelet therapy by targeting BCAA catabolism.

METHODS

The data, analytical methods, and study materials will be/have been made available to other researchers for purposes

of reproducing the results or replicating the procedure (available at the authors' laboratories).

Antibodies and Reagents

Detailed descriptions of antibodies and reagents are given in Methods in the Data Supplement.

Mice

PP2Cm knockout mice (*PP2Cm*$^{-/-}$) on the C57BL/6 genetic background were described previously.[6] *Ob/ob* mice[26] or C57BL/6 control mice were purchased from Jackson Laboratories or SLAC Laboratory Animals Company Ltd (Shanghai, China) separately. The Shanghai Jiao Tong University School of Medicine Animal Care and Use Committee approved the animal research.

Oral Administration of BCAAs in Humans and Mice

To observe the effects of BCAA ingestion on human platelet activation, healthy volunteers were instructed to fast overnight and to drink 0.9% NaCl before the ingestion of BCAAs. When the first blood sample (0 hours) was collected, 10 g BCAA mixture (weight ratio, 2:1:1 of Leu:Ile:Val) in 100 mL 0.9% NaCl was ingested immediately, and blood samples were collected at 0.5, 1, 1.5, and 2 hours after ingestion. After blood collection, the platelets were instantly prepared for further study. The study design and possible risks were explained to each subject before written consent was obtained. The study protocol was approved by the Human Research Review Committee of the Shanghai Jiao Tong University School of Medicine.

To investigate the effects of oral BCAAs on mouse platelet activation, mice were fasted overnight followed by oral gavage treatment with 0.9% NaCl or BCAAs.[5] Mouse platelets were prepared 1 hour after administration for further study.

To study the effects of BCAA on *ob/ob* mouse platelet activation, male *ob/ob* and control mice were fed an isocaloric normal-protein diet (20% protein diet, TD91352, Envigo Teklad Diet) or a low-protein diet (6% protein diet, TD90016, Envigo Teklad Diet) for 4 weeks.[26] For the low-protein diet+BCAA group, BCAA in drinking water was started after low-protein diet for 2 weeks and lasted for 2 weeks.[26]

Platelet Preparation, Aggregation, and Spreading; PAC1 Binding; JON/A Binding; and P-Selectin Exposure

Human and mouse washed platelets were prepared and stimulated as described.[27] To explore the function of BCAAs and their metabolites on platelet aggregation, 300 μL 3×10^8/mL platelets were incubated with Leu, Ile, Val, KIC, KMV, KIV, or propionyl-CoA for 30 minutes at 37°C as indicated. Aggregation of platelets was measured in response to collagen, α-thrombin, ADP, and U46619. Analysis of platelet spreading on immobilized fibrinogen was done as described.[27] Platelets were incubated with FITC-conjugated PAC1 antibody (for human platelets), phycoerythrin-conjugated JON/A antibody (for mouse platelets), or phycoerythrin-conjugated anti–P-selectin antibody and stimulated by 0.1 U/mL α-thrombin for 20 minutes at room temperature. PAC1 binding, JON/A binding, and P-selectin exposure were analyzed by flow cytometry (LSR Fortessa/FACS calibur, BD Biosciences).

Measurement of BCAA Levels in Platelets

The levels of BCAA in platelets were measured by gas chromatography–mass spectrometry. Nitrogen quick-frozen platelets were resuspended by 100 μL water, 800 μL chloroform, 700 μL methanol, and 100 μL internal standards (0.08 μg/mL Myristic-d27). Platelets were ultrasonic to fragments and kept on ice for 1 hour. After centrifugation at 14000*g* for 15 minutes, the supernatant was collected and dried in vacuum. The crystallization was redissolved in 30 μL methoxypyridine (20 mg/mL) at 37°C for 1.5 hours. For derivatization, 30 μL N,O-Bis(trimethylsilyl)trifluoroacetamide (containing 1% trimethylsilyl chloride) was added to the sample and incubated at 70°C for 1 hour. After centrifugation, the supernatant was analyzed by gas chromatography–mass spectrometry (5977B GC/MSD, Agilent Technologies Inc). The levels of BCAA relative to Myristic-d27 in platelets were calculated.

Thrombosis and Hemostasis Models

Wild-type (WT) and *PP2Cm*$^{-/-}$ mice were fasted overnight followed by oral gavage treatment with 0.9% NaCl or BCAAs. For arteriole thrombosis, mouse surgery and intravital microscopy of laser-induced cremaster arteriole injury were performed as previously described.[28] For arterial thrombosis, a ferric chloride–induced carotid artery injury murine thrombosis model was processed as described.[27] For experimental deep vein thrombosis, an inferior vena cava stenosis model was performed as described.[29] For hemostasis, a mouse tail bleeding assay was performed as described.[30] The detailed methods are described in Methods in the Data Supplement. The Shanghai Jiao Tong University School of Medicine Animal Care and Use Committee approved the animal research.

Plasmid Constructs and Transfection

Plasmid constructs were made with standard genetic manipulations. Human TMOD3 cDNA was cloned into the CMV4-p-flag vector. Mutation of K255 of TMOD3 to an alanine was based on CMV4-p-flag-TMOD3. The construct plasmid was transfected into HEK293T cells (from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) with Lipofectamine 2000 (Life Technologies Inc, ON, Canada) as suggested by the manufacturer. The cells expressing flag-tagged TMOD3 and flag-tagged TMOD3-K255A fusion proteins were obtained after 48 hours after transfection.

Preparation and Microscopy of αIIbβ3–Chinese Hamster Ovary Cells

αIIbβ3–Chinese hamster ovary (CHO) cells[30] expressing flag-tagged TMOD3 and flag-tagged TMOD3-K255A fusion proteins were obtained 48 hours after transfection. For immunofluorescence staining, αIIbβ3-CHO cells were incubated with 1 mmol/L BCAAs, cultured on coverslips coated with 50 μg/mL fibrinogen for 4 hours, fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100, rinsed with

PBS, and blocked with 3% BSA in PBS. The coverslips were incubated overnight with 100 µL primary antibody solution at 4°C. After incubation with fluorescently labeled secondary antibodies, the coverslips were washed and then mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector). The images were captured with a Zeiss LSM710 confocal fluorescence microscope with a Plan-Apochromat 63×/1.40 oil DIC M27 objective lens. Z-stack digital images were acquired with ZEN imaging software (Carl Zeiss, GmbH).

Immunoprecipitation and Mass Spectrometry

For immunoprecipitation, 500 µL 3×10^8/mL washed human platelets were incubated with BCAAs, BCKAs, and propionyl-CoA for 30 minutes at 37°C individually. Platelets were incubated with anti–propionyl-lysine antibody–beaded agarose (Jingjie PTM BioLab, Hangzhou, China) in the Pierce immunoprecipitation lysis buffer at 4°C overnight. The beads were harvested, and the immunoprecipitation samples or direct cell lysates were analyzed by SDS-PAGE and Western blotting. For identification of propionyl-modified protein, BCAA-treated platelets were lysed and preabsorbed with immunoglobulin G–agarose beads (Roche, Indianapolis, IN), followed by treatment with the anti–propionyl-lysine antibody–beaded agarose at 4°C overnight.

For mass spectrometry, Coomassie blue–stained gel bands containing ≥1 protein species were excised and digested in gel with trypsin. The peptide mixtures were analyzed with Zorbax 300SB-C18 peptide trap columns (Agilent Technologies, Wilmington, DE) and tandem mass spectrometry (Thermo Finnigan Q Exactive, San Jose, CA). Peptides were collected in 300.00 to 1800.00 mass/charge ratio, and the original mgf files were loaded on SEQUEST software (Thermo Fisher Scientific, Waltham, MA) to search the database of IPI Human.3.87. REVERSED for identification of proteins. In-gel digestion, mass spectrometry analysis, and database searching were carried out by the Jingjie PTM BioLab. The presence of propionyl modified proteins was verified by immunoblotting.

mRNA levels of BCAT1; BCAT2; BCKA Dehydrogenase E1, alpha; PP2Cm; and BCKDK in Human Platelets

Total mRNA was extracted from washed platelets. The mRNA expression levels of BCAT1; BCAT2; BCKA dehydrogenase E1, alpha; PP2Cm; and BCKDK in platelets were measured with real-time quantitative polymerase chain reaction (ABI 7500 Real-Time PCR system). Relative mRNA expression levels were normalized to actin expression levels. The sequences of polymerase chain reaction primers were as follows. For BCAT1: forward, 5′-AGTCAAGAAGCCTACCAAAGCC-3′; reverse, 5′-ATGAGCCGTAATTCCCTC CC-3′. BCAT2: forward, 5′-AAGGCGTTCAAAGGCAAAGACCA-3′; reverse, 5′-TGTCGAAACTCGGCAGGCACA-3′. BCKA dehydrogenase E1, alpha: forward, 5′-TGCTGAGCCAA GGCTGGTGG-3′; reverse, 5′-TGGGCGGGCATCTCCTGATA -3′. PP2Cm: forward, 5′-ATAACCGCATTGATGAGCCA-3′; reverse, 5′-TCCGTTTG CCAATCTGTGAG- 3′. BCKDK: forward, 5′-AGACCGTGGT GGAGGAATCGC-3′; reverse, 5′-GGGCA ACCCGAAGCC AAAGC-3′.

Patient Population

Twenty-one patients with T2DM (11 male, 10 female patients) were admitted and treated in the Zhongshan Hospital Affiliated with Fudan University School of Medicine and the No. 9 People's Hospital affiliated with the Shanghai Jiao Tong University School of Medicine. Twenty-one healthy subjects were also included. Antiplatelet therapy, anticoagulants, coronary angiography, and percutaneous coronary intervention were not administered. Peripheral blood was obtained from these subjects. The study protocol was approved by the Zhongshan Hospital Ethics Committee and No. 9 People's Hospital Ethics Committee, and written informed content was obtained from all subjects.

Measurement of ATP Secretion and Thromboxane B2 Production

CHORONO-LUME reagent (Chrono-Log) and a thromboxane B2 ELISA kit (Abcam) were used to measure ATP secretion and thromboxane A2 production in platelets as previously described.[27]

Statistical Analysis

Unless otherwise stated, data were analyzed and evaluated with GraphPad Prism software (GraphPad Inc) with a Student unpaired *t* test, 1-way ANOVA, 2-way ANOVA, or Kruskal-Wallis test as appropriate. Values of $P<0.05$ were considered statistically significant.

RESULTS

Ingestion of BCAAs Enhanced the Activity of Human Platelets

To observe the effects of ingestion of BCAAs on human platelet activation in vivo, healthy volunteers were instructed to fast overnight and to drink 0.9% NaCl before the ingestion of BCAAs. Before (0 hours) and after the ingestion of BCAAs, platelets were instantly prepared as described in the Methods section. The levels of BCAA in the human platelets were first examined by mass spectrometry. The results demonstrated that the levels of Val, Leu, and Ile in platelets reached a peak 1 hour after ingestion (Figure 1A through 1C).

Ingestion of BCAAs significantly promoted agonist-induced human platelet aggregation (Figure 1D and Figure IA through IC in the Data Supplement), and the facilitating effects of the BCAAs reached a peak 1 hour after ingestion (Figure 1D). Moreover, thrombin-induced P-selectin exposure increased in human platelets 1 hour after ingestion of BCAAs (Figure 1E). Ingestion of BCAAs also significantly facilitated ATP secretion and thromboxane B2 production in response to agonists (Figure ID and IE in the Data Supplement). These results demonstrated that ingestion of BCAAs significantly promoted the aggregation and degranulation of human platelets.

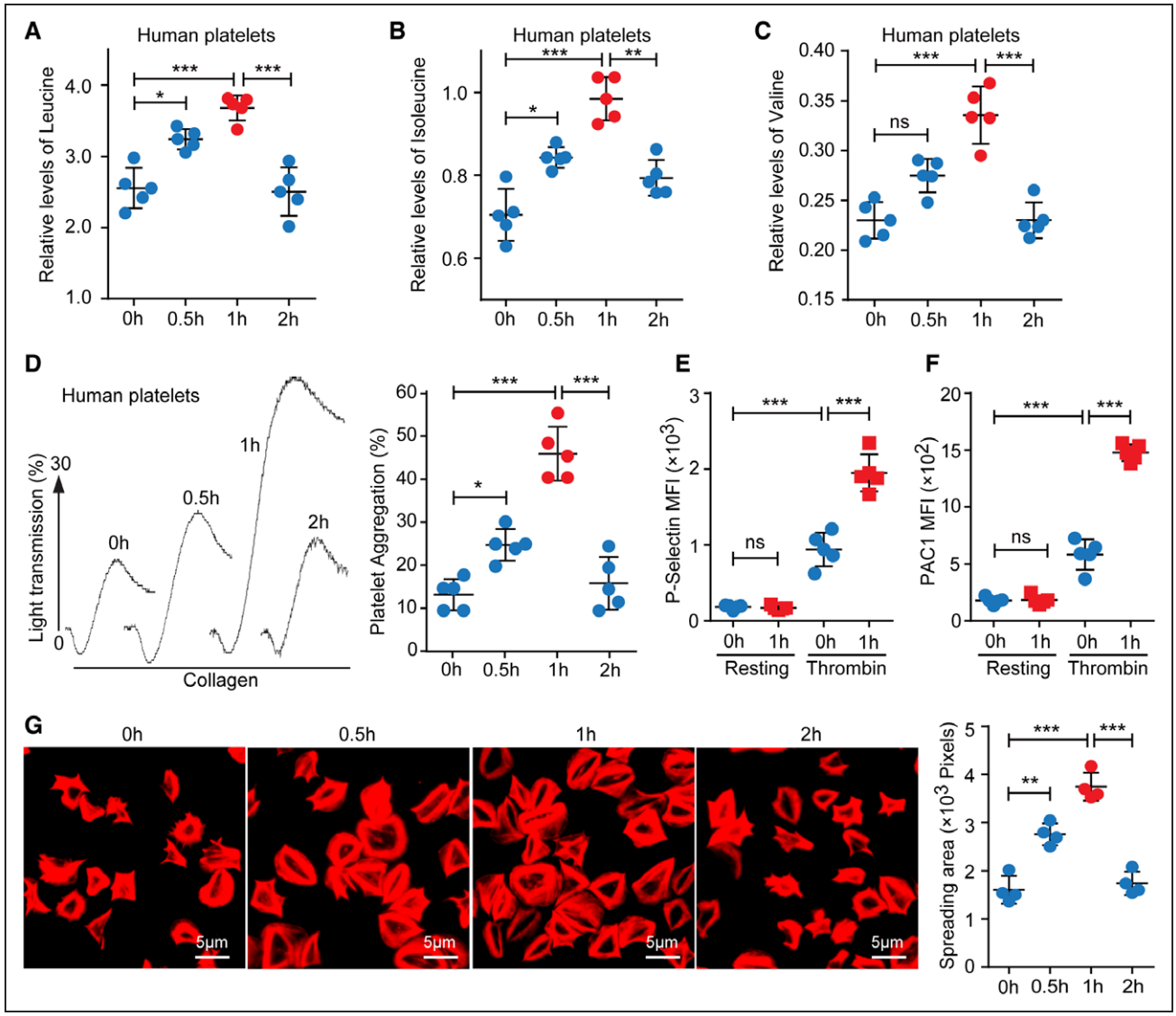

Figure 1. Ingestion of branched-chain amino acid (BCAA) enhanced human platelet activation.
A through **C**, The relative levels of leucine (Leu), isoleucine (Ile), and valine (Val) in human platelets from healthy volunteers before (0 hours) ingestion of BCAAs and after ingestion at different time points (n=5, male=3). **D**, Aggregation of washed human platelets collected from healthy volunteers before and after ingestion of BCAAs in response to 1 μg/mL collagen (n=5, male=3). **E**, P-selectin exposure of washed human platelets stimulated with 0.1 U/mL α-thrombin (n=5, male=3). **F**, Binding of PAC1 to washed human platelets stimulated with 0.1 U/mL α-thrombin (n=5, male=3). **G**, Spreading of human platelets on immobilized fibrinogen. Areas (pixel numbers) of 3 random fields of spreading platelets (n=4, males=3) were quantified. Data were analyzed by 2-way ANOVA with Tukey multiple-comparisons test. Data are presented as mean±SEM. ns indicates nonsignificant ($P>0.05$). *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Integrin αIIbβ3, a major adhesion receptor, mediates bidirectional signaling to regulate platelet activation and plays a critical role in thrombosis and hemostasis.[30] Stimulation of platelets with agonists will induce a conformational change in αIIbβ3 and soluble fibrinogen binding (inside-out signaling), which causes platelet aggregation.[31] The monoclonal antibody PAC1 recognizes an epitope on active human αIIbβ3 and serves as a tool to detect the inside-out signal-driven human αIIbβ3 activation.[32] BCAA ingestion significantly enhanced α-thrombin–induced PAC1 binding to human platelets (Figure 1F), indicating that BCAAs might regulate αIIbβ3-mediated inside-out signaling. Platelets can also adhere to immobilized fibrinogen, which triggers αIIbβ3-mediated outside-in signaling and cell spreading.[33,34] The results in Figure 1G demonstrate that the ingestion of BCAAs enhances human platelet spreading on immobilized fibrinogen, suggesting that BCAAs may also regulate integrin αIIbβ3 outside-in signal-mediated platelet activation.

BCAA Catabolic Defects Suppressed Platelet Activation

BCKD is a rate-limiting enzyme in BCAA catabolism.[1] BCKD is inhibited by phosphorylation and activated by dephosphorylation on the E1-α subunit at Ser293.[5] Phosphatase PP2Cm can dephosphorylate BCKD and

therefore enhance BCAA catabolism. PP2Cm deficiency causes BCAA catabolic defects[5,6] (Figure 2A). To elucidate the roles of BCAA catabolism in platelet activation and thrombosis, *PP2Cm*$^{-/-}$ mice were used in our study (Figure 2B). PP2Cm deficiency had no significant effects on peripheral platelet counts (Figure 2C), indicating that PP2Cm deficiency probably does not affect thrombopoiesis. The aggregation of platelets from *PP2Cm*$^{-/-}$ mice and WT mice was measured in response to agonists. Compared with WT platelets, aggregation of *PP2Cm*$^{-/-}$ platelets in response to agonists was suppressed (Figure 2D and Figure IIA through IIC in the Data Supplement). Moreover, PP2Cm deficiency also decreased platelet α-granule secretion, as determined by detection of P-selectin exposure levels in *PP2Cm*$^{-/-}$ platelets (Figure 2E). PP2Cm deficiency also inhibited thrombin-induced JON/A binding to platelets (Figure 2F), platelet spreading on immobilized fibrinogen (Figure 2G), and ATP secretion (Figure IID in the Data Supplement), suggesting that BCAA metabolic

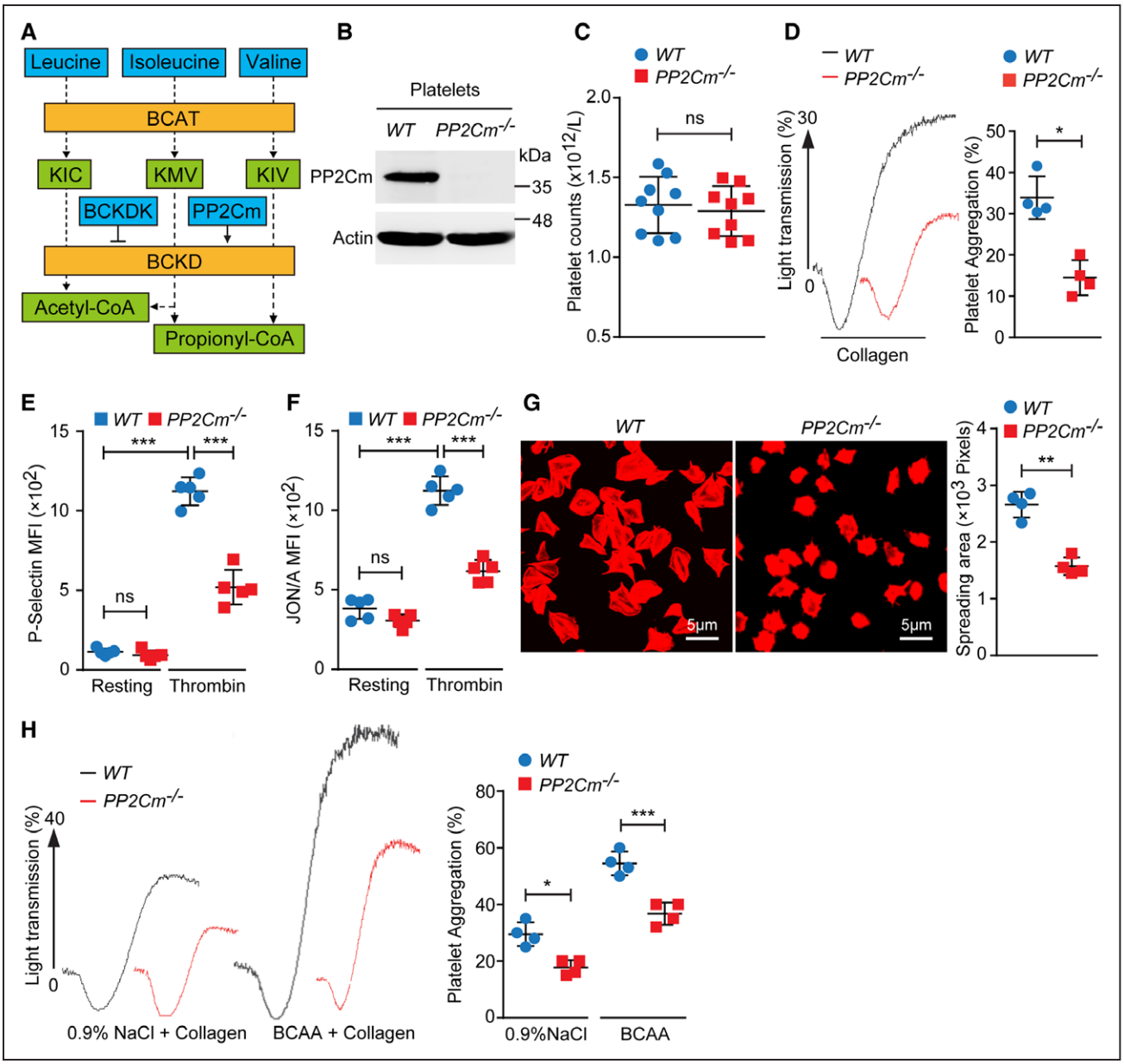

Figure 2. Protein phosphatase 2Cm (PP2Cm) played an important role in branched-chain amino acid (BCAA)–mediated platelet activation.
A, Schematic diagram of the catabolic pathway of BCAAs. **B**, Levels of PP2Cm in wild-type (WT) and *PP2Cm*$^{-/-}$ mouse platelets. **C**, Platelet counts in WT mice and *PP2Cm*$^{-/-}$ mice (n=9). **D**, Aggregation of WT and *PP2Cm*$^{-/-}$ mouse platelets in response to 1.5 μg/mL collagen (n=4). **E**, P-selectin exposure of washed WT and *PP2Cm*$^{-/-}$ mouse platelets stimulated with 0.1 U/mL α-thrombin (n=5). **F**, Binding of JON/A to washed WT and *PP2Cm*$^{-/-}$ mouse platelets stimulated with 0.1 U/mL α-thrombin (n=5). **G**, Spreading of WT and *PP2Cm*$^{-/-}$ platelets on immobilized fibrinogen. Areas (pixel numbers) of 3 random fields of WT and *PP2Cm*$^{-/-}$ platelets were quantified (n=4). **H**, Aggregation of platelets from WT and *PP2Cm*$^{-/-}$ mice with oral gavage treatment of 0.9% NaCl or 1 mg/g (weight) of BCAAs in response to 1.5 μg/mL collagen (n=4). Data were analyzed by 2-way ANOVA with the Tukey multiple-comparisons test. Data are presented as mean±SEM. BCAT indicates branched chain aminotransferase; BCKD, branched-chain α-keto acid dehydrogenase complex; BCKDK, BCKD kinase; CoA, coenzyme A; KIC, α-ketoisocaproic acid; KIV, α-ketoisovaleric acid; KMV, α-keto-β-methylvaleric acid; and ns, nonsignificant (*P*>0.05). **P*<0.05; ***P*<0.01; ****P*<0.001.

pathways may regulate platelet activation by affecting the αIIbβ3-mediated bidirectional signaling pathway. To study the effects of BCAAs on mouse platelets in vivo, WT and *PP2Cm*$^{-/-}$ mice were fasted overnight, followed by oral gavage treatment with 0.9% NaCl or a BCAA suspension. Mouse platelets were obtained at 1 hour after oral gavage treatment. Platelet aggregation, ATP secretion, P-selectin exposure, and JON/A binding were greatly reduced in BCAA-treated *PP2Cm*$^{-/-}$ mice compared with BCAA-treated WT mice (Figure 2H and Figure IIE through IIJ in the Data Supplement).

BCAA Catabolic Defects Suppressed Arterial Thrombosis but Had No Effects on Venous Thrombosis and Tail Bleeding Time

To evaluate the function of BCAA catabolism on thrombus formation, a laser-induced cremaster arteriole injury model was carried out. After oral gavage treatment with 0.9% NaCl or BCAAs, surgery and laser injury thrombosis assays were immediately performed to monitor the living arteriole thrombus formation using DyLight488-conjugated anti-CD42c antibody and intravital microscopy in *PP2Cm*$^{-/-}$ and WT mice. We found that the platelet accumulation increased in the BCAA-treated WT mice compared with 0.9% NaCl-treated WT mice (Figure 3A through 3C), indicating that oral BCAAs could facilitate arteriolar thrombogenesis. Moreover, platelet accumulation was significantly decreased in 0.9% NaCl–treated *PP2Cm*$^{-/-}$ mice compared with 0.9% NaCl–treated WT mice (Figure 3A through 3C). BCAA treatment did not increase platelet accumulation in *PP2Cm*$^{-/-}$ mice (Figure 3A through 3C). Therefore, BCAA catabolism is critical for arteriolar thrombus formation in vivo.

A ferric chloride ($FeCl_3$)–induced carotid artery thrombosis model was also used to evaluate the role of BCAAs in arterial thrombosis. The average occlusion time in the BCAA-treated WT mice significantly decreased compared with that of the 0.9% NaCl–treated WT mice (Figure 3D), indicating that oral BCAAs could facilitate arterial thrombogenesis in vivo. Moreover, the average occlusion time in the BCAA-treated *PP2Cm*$^{-/-}$ mice was longer than that in the BCAA-treated WT mice (Figure 3D). These results demonstrated that BCAA catabolism plays an important role in arterial thrombosis formation in vivo.

In contrast to arterial thrombosis, venous thrombosis is caused by a slow and blocked blood flow, and the main components are red blood cells and fibrin.[29] To further explore the roles of BCAA catabolism in the formation of venous thrombosis, an inferior vena cava ligation model was applied. The results showed that the average thrombus length and weight were not different between the WT and *PP2Cm*$^{-/-}$ mice (Figure 3E and 3F). There was also no significant difference in thrombus length or weight in BCAA-treated WT mice compared with 0.9% NaCl–treated WT mice (Figure 3E and 3F). In addition, the thrombus length and weight showed no difference between the BCAA-treated *PP2Cm*$^{-/-}$ mice and 0.9% NaCl–treated *PP2Cm*$^{-/-}$ mice (Figure 3E and 3F). Therefore, ingestion of BCAA and BCAA catabolism probably did not affect the formation of venous thrombosis.

To elucidate the role of BCAA catabolism on hemostasis, a mouse tail bleeding experiment was performed. The average bleeding times between WT and *PP2Cm*$^{-/-}$ mice were not different (Figure 3G). There was also no difference in tail bleeding time in BCAA-treated WT mice compared with 0.9% NaCl–treated WT mice (Figure 3G). Moreover, the tail bleeding time showed no difference between the BCAA-treated *PP2Cm*$^{-/-}$ mice and 0.9% NaCl–treated *PP2Cm*$^{-/-}$ mice (Figure 3G). Therefore, BCAA catabolism probably had no effect on hemostasis.

BCAA Catabolites Enhanced Agonist-Induced Platelet Activation

It has been reported that BCAA catabolism controls energy homeostasis.[35] To investigate whether BCAA-facilitated platelet activation was the result of energy release during BCAA catabolism, ATP levels in BCAA-treated human platelets and mouse *PP2Cm*$^{-/-}$ platelets were measured. The ATP levels did not differ between BCAA-treated and control human platelets (Figure 4A) or between WT and *PP2Cm*$^{-/-}$ mouse platelets (Figure 4B), indicating that energy release was probably not the major cause of BCAA-facilitated platelet activation.

To further reveal the mechanism underlying BCAA promotion of platelet activation and thrombosis, the individual functions of Val, Ile, Leu, and their catabolites on platelet activation were studied. First, the effects of Leu, Ile, Val, and their respective ketoacid metabolites, KIC, KMV, and KIV, on platelet activation were measured separately. We found that Leu, Ile, and Val could enhance agonist-induced platelet aggregation, P-selectin exposure, PAC1 binding, and ATP secretion individually (Figure 4C through 4E and Figure IIIA through IIID in the Data Supplement). KIC, the ketoacid catabolite of Leu, slightly promoted platelet aggregation, P-selectin exposure, PAC1 binding, and ATP secretion (Figure 4F through 4H and Figure IIIE through IIIH in the Data Supplement). However, the ketoacid metabolite of Val, KIV, greatly enhanced human platelet aggregation, P-selectin exposure, PAC1 binding, and ATP secretion (Figure 4F through 4H and Figure IIIE through IIIH in the Data Supplement). The effects of the Ile ketoacid metabolite, KMV, on platelet activation were between those of KIC and KIV in magnitude (Figure 4F through

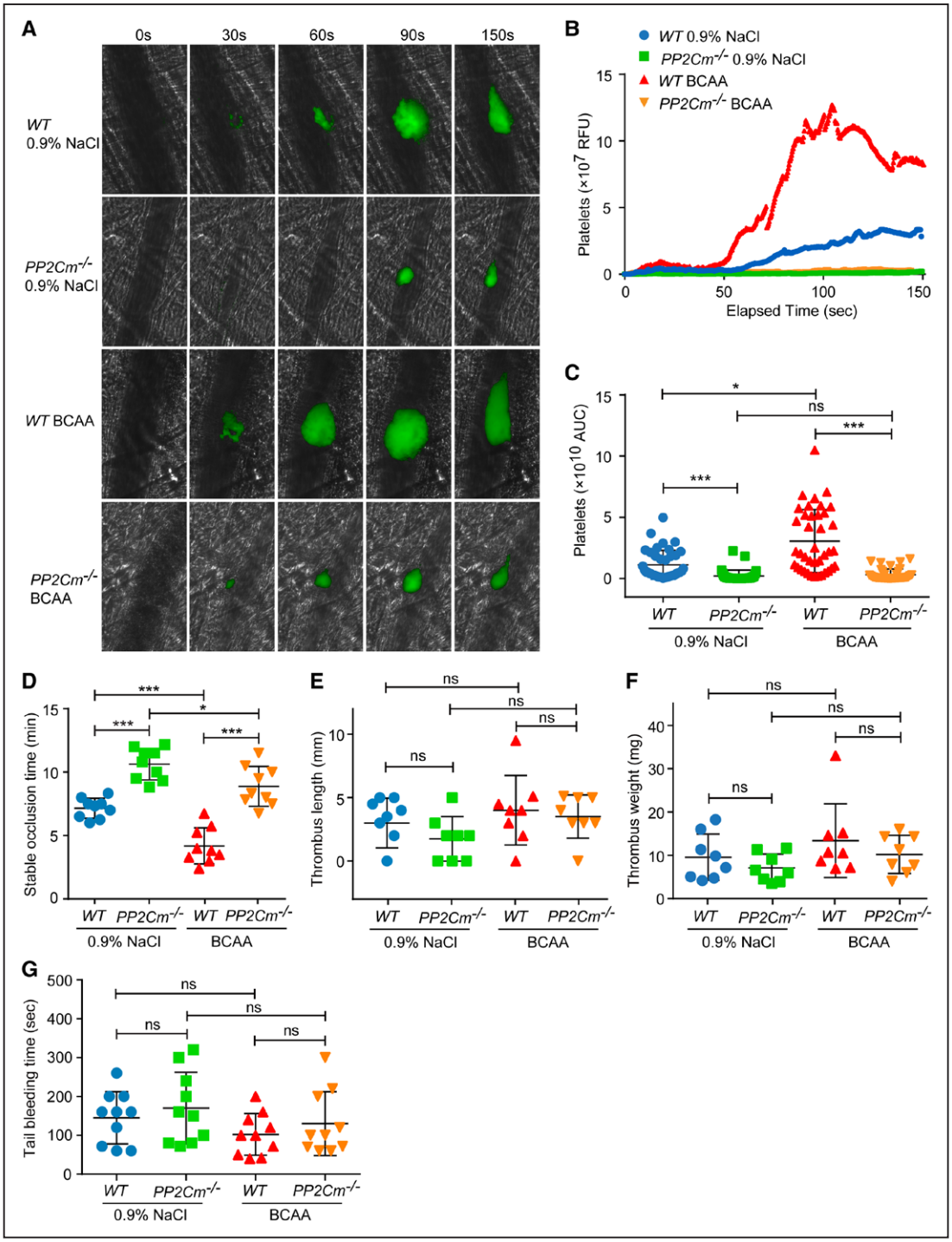

Figure 3. Branched-chain amino acid (BCAA) catabolism facilitated thrombosis formation.
A, Wild-type (WT) and protein phosphatase 2Cm knockout (*PP2Cm*$^{-/-}$) mice were fasted overnight and treated oral gavage with 0.9% NaCl or 1 mg/g (weight) of BCAA mixture. Laser-induced thrombosis of the cremaster arteriole model was then performed. Representative images depicting platelet accumulation at the site of vascular injury at different time points after injury are shown. **B**, Relative fluorescence units (RFUs) over time from at least 30 thrombi of 3 to 4 mice in each group are shown. **C**, Areas under the curve (AUCs) of platelet fluorescence intensity from all thrombi were collected in each group. **D**, The carotid artery was injured with 10% $FeCl_3$. Blood flow were recorded, and the time to full occlusion were measured. The occlusion time was 7.157±0.263 minutes in 0.9% NaCl–treated WT mice, 10.63±0.415 minutes in 0.9% NaCl–treated *PP2Cm*$^{-/-}$ mice, 4.20±0.473 minutes in BCAA-treated WT mice, and 8.879±0.524 minutes in BCAA-treated *PP2Cm*$^{-/-}$ (n=9). **E** and **F**, Inferior vena cava ligation-induced thrombi were measured in WT and *PP2Cm*$^{-/-}$ mice treated with oral gavage treatment of 0.9% NaCl or BCAAs. The thrombus lengths were 3.375±0.603 mm in 0.9% NaCl–treated WT mice, 1.750±0.620 mm in 0.9% NaCl–treated *PP2Cm*$^{-/-}$ mice, 4.013±0.970 mm in BCAA-treated WT mice, and 3.513±0.602 mm in BCAA-treated *PP2Cm*$^{-/-}$ (n=8). The thrombus weights were 9.525±1.887 mg in 0.9% NaCl–treated WT mice, 7.050±1.146 mg in 0.9% NaCl–treated *PP2Cm*$^{-/-}$ mice, 13.360±3.006 mg in BCAA-treated WT mice, and 10.190±1.559 mg in BCAA-treated *PP2Cm*$^{-/-}$ (n=8). **G**, Tail bleeding time of WT and *PP2Cm*$^{-/-}$ mice treated with oral gavage treatment of 0.9% NaCl or BCAAs. The tail bleeding time was 145.2±21.18 seconds in 0.9% NaCl–treated WT mice, 170.2±29.14 seconds in 0.9% NaCl–treated *PP2Cm*$^{-/-}$ mice, 102.4±16.93 seconds in BCAA-treated WT mice, and 130.2±25.94 seconds in BCAA-treated *PP2Cm*$^{-/-}$ (n=10). Data were analyzed by the Kruskal-Wallis test. Data are presented as mean±SEM. ns indicates nonsignificant (P>0.05). *P<0.05; ***P<0.001.

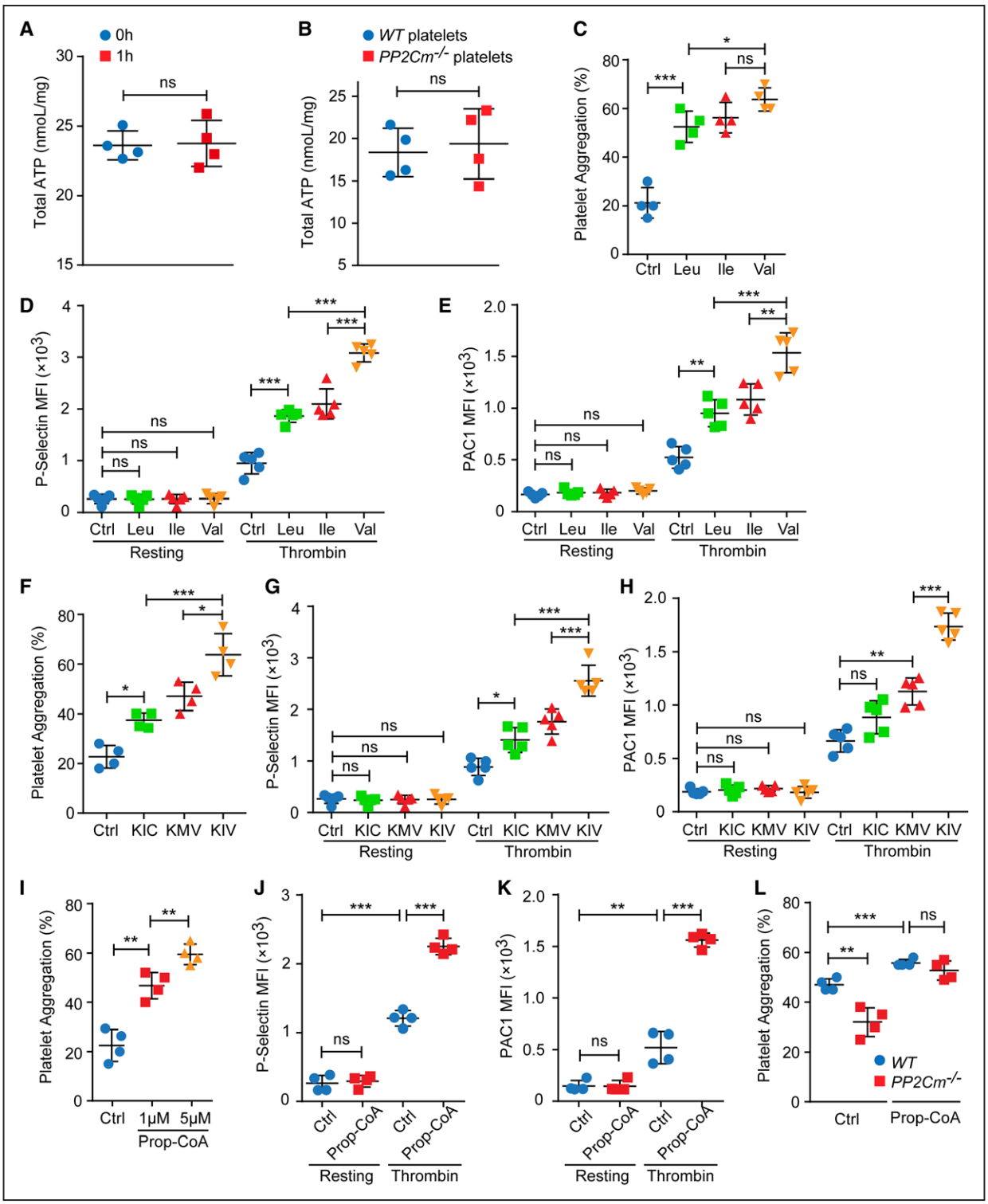

Figure 4. Branched-chain amino acids (BCAAs) and their catabolites enhanced platelet activation.
A, ATP levels in human platelets from healthy volunteers before (0 hours) and after (1 hour) ingestion of BCAAs (n=4). **B**, ATP levels in wild-type (WT) and protein phosphatase 2Cm knockout (*PP2Cm*$^{-/-}$) platelets (n=4). **C**, Aggregation of human platelets in response to 1.5 μg/mL collagen in the presence of 1.25 mmol/L leucine (Leu), 1.25 mmol/L isoleucine (Ile), or 1.25 mmol/L valine (Val; n=4). **D** and **E**, P-selectin exposure and PAC1 binding in human platelets in response to 0.1 U/mL α-thrombin in the presence of 1.25 mmol/L Leu, 1.25 mmol/L Ile, or 1.25 mmol/L Val (n=5). **F**, Aggregation of human platelets in response to 1.5 μg/mL collagen in the presence of 0.15 mmol/L α-ketoisocaproic acid (KIC), 0.15 mmol/L α-keto-β-methylvaleric acid (KMV), or 0.15 mmol/L α-ketoisovaleric acid (KIV; n=4). **G** and **H**, P-selectin exposure and PAC1 binding in human platelets in response to 0.1 U/mL α-thrombin in the presence of 0.15 mmol/L KIC, 0.15 mmol/L KMV, or 0.15 mmol/L KIV (n=5). **I**, Aggregation of human platelets treated with 1 or 5 μmol/L propionyl-CoA (Prop-CoA) in response to 1.5 μg/mL collagen (n=4). **J** and **K**, P-selectin exposure and PAC1 binding in human platelets with or without stimulation of 0.1 U/mL α-thrombin in the presence or absence of 5 μmol/L Prop-CoA (n=4). **L**, Aggregation of WT and *PP2Cm*$^{-/-}$ mouse platelets in response to 1.5 μg/mL collagen in the presence of 5 μmol/L Prop-CoA (n=4). Data were analyzed by 2-way ANOVA with the Tukey multiple-comparisons test. Data are presented as mean±SEM. Ctrl indicates control; and ns, nonsignificant (P>0.05). *P<0.05; **P<0.01; ***P<0.001.

4H and Figure IIIE through IIIH in the Data Supplement). These results demonstrated that Val/KIV and Ile/KMV catabolism plays more important roles in BCAA-mediated platelet activation.

In the BCAA catabolic pathway, Leu catabolism eventually leads to the formation of acetoacetyl-CoA and acetyl-CoA.[36] Val is metabolized into propionyl-CoA. Ile is metabolized into acetyl-CoA and propionyl-CoA (Figure 2A). These acyl-coenzyme A intermediate catabolites of BCAA regulate energy release and a variety of posttranslational modifications.[37] Because propionyl-CoA is the final common metabolite of KIV and KMV oxidation, there is reason to test the effects of propionyl-CoA on platelet activation. Pretreatment with propionyl-CoA dose-dependently enhanced platelet aggregation, P-selectin exposure, PAC1 binding, and ATP secretion (Figure 4I through 4K and Figure IIII through IIIL in the Data Supplement). Moreover, propionyl-CoA promoted *PP2Cm*$^{-/-}$ platelet aggregation to levels comparable to those of WT mouse platelets (Figure 4L). These results indicate that propionyl-CoA is the key mediator in Val/KIV and Ile/KMV metabolic pathway–mediated platelet activation.

BCAA Catabolism Induced TMOD3 Propionylation in Platelets

Propionylation of protein lysine residues (Kpr), which has recently emerged as an important reversible posttranslational modification in mammalian cells, regulates cellular processes and disease progression.[38,39] The results in Figure 5A show that BCAAs dose-dependently increase protein propionylation in human platelets. Preincubation with BCKAs and propionyl-CoA significantly enhanced the levels of protein propionylation in human platelets (Figure 5B). The levels of protein propionylation decreased in *PP2Cm*$^{-/-}$ platelets compared with WT platelets (Figure 5C).

To identify the propionylated proteins in human platelets treated with BCAAs, propionylated protein immunoprecipitation and mass spectrometry were performed (Figure 5D). The mass spectrometry results showed that most of the propionylated proteins were cytoskeletal proteins such as TMOD3, PDM1L1, L-plastin, CAP1, CALR, and PLEK. Among these proteins, only TMOD3 propionylation demonstrated a BCAA dose-dependent pattern in human platelets (Figure 5E and 5F).

It has been reported that TMOD3 plays an essential role in stabilizing the actin cytoskeleton by binding tropomyosins to F-actin and that it is critical in megakaryocyte actin assembly and thus platelet biogenesis.[40] Integrin αIIbβ3 is critical for platelet activation, and αIIbβ3-mediated outside-in signaling is a key regulator for platelet cytoskeletal reorganization.[30,41] αIIbβ3-Expressing CHO cells are a useful model system to study the process of αIIbβ3-mediated platelet activation because of the similar adhesive and dynamic responses when spreading on the immobilized fibrinogen.[30,33] We found that BCAAs significantly improved the spreading of αIIbβ3-CHO cells on immobilized fibrinogen (Figure 5G and 5H) and upregulated the propionylation levels of proteins in both the cytoplasm and nucleus (Figure 5I). TMOD3 propionylation was also enhanced in αIIbβ3-CHO cells treated with BCAAs. These results implied that TMOD3 propionylation was involved in αIIbβ3-mediated platelet activation.

TMOD3 Propionylation on K255 Participated in Integrin αIIbβ3-Mediated Cell Spreading

Mass spectrometry analysis of platelet lysate proteins identified TMOD3 propionylation at K255, a highly conserved site (Figure 6A and 6B). Notably, mutation of K255 to alanine completely abolished the propionylation of TMOD3 in HEK293T cells (Figure 6C and 6D). In the presence of BCAAs, the spreading area of αIIbβ3-CHO cells transfected with the TMOD3-K255A-flag expression construct was significantly diminished compared with that of the αIIbβ3-CHO cells transfected with the TMOD3-flag expression construct (Figure 6E and 6F). In addition, the propionylation level of TMOD3 was greatly reduced in αIIbβ3-CHO cells transfected with the TMOD3-K255A-flag expression construct in the presence of BCAAs (Figure 6G). These results demonstrated that K255 was an important site for TMOD3 propionylation and plays a critical role in cell spreading.

BCAA Catabolism Dysfunction Was Correlated With Platelet Hyperactivation in MS

MS is a key risk factor for cardiovascular diseases.[14,18] Increased levels of plasma BCAAs in patients with MS have recently been reported to be positively correlated with an increased risk of cardiovascular disease.[19] To unveil the potential role of BCAA catabolism in facilitating thrombosis in the context of MS, the activation levels of platelets from patients with T2DM were measured by assessment of platelet activation markers. The levels of P-selectin exposure and PAC1 binding were strikingly increased in platelets from patients with T2DM compared with healthy control subjects (Figure 7A and 7B). In addition, the levels of BCAAs were higher in platelets from patients with T2DM than in those from healthy subjects (Figure 7C through 7E). The expression of BCAA catabolism–positive regulators, especially the BCKD phosphatase PP2Cm, was increased in platelets, indicating that BCAA catabolism is correlated with platelet hyperreactivity and the risk of thrombosis in patients with T2DM (Figure 7F). Leptin-deficient

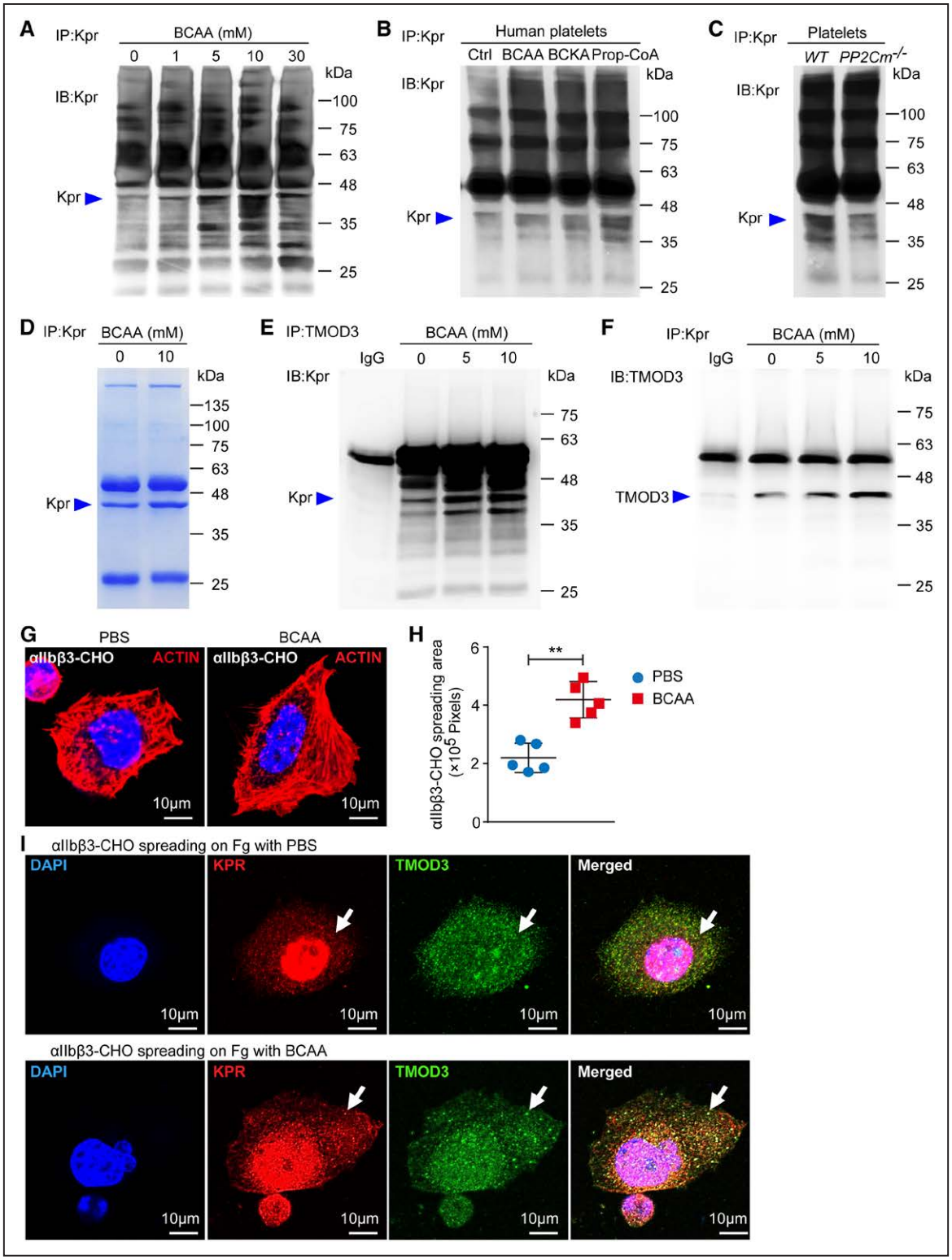

Figure 5. Branched-chain amino acid (BCAA) metabolism enhanced the propionylation of tropomodulin-3 (TMOD3).
A, Propionylation of protein lysine residues (Kpr) levels of protein in human platelets in response to different doses of BCAAs. **B**, Kpr levels of protein in human platelets treated with 5 mmol/L BCAAs (2.5 mmol/L leucine [Leu], 1.25 mmol/L isoleucine [Ile], and 1.25 mmol/L valine [Val]), 0.45 mmol/L branched-chain α-keto acids (BCKAs; 0.15 mmol/L α-ketoisocaproic acid [KIC], 0.15 mmol/L α-keto-β-methylvaleric acid [KMV], and 0.15 mmol/L α-ketoisovaleric acid [KIV]), or 5 μmol/L propionyl-CoA (Prop-CoA). **C**, Kpr levels of protein from wild-type (WT) and protein phosphatase 2Cm knockout (*PP2Cm*$^{-/-}$) mouse platelets treated with 5 mmol/L BCAAs. **D**, Coomassie staining of Kpr proteins from human platelets treated with or without 10 mmol/L BCAAs. **E** and **F**, Kpr levels of TMOD3 were enhanced in human platelets in response to BCAAs in a dose-dependent manner. **G** and **H**, Immunofluorescence images of actin in αIIbβ3-CHO cells spreading on immobilized fibrinogen in the absence or presence of 1 mmol/L BCAAs (0.5 mmol/L Leu, 0.25 mmol/L Ile, and 0.25 mmol/L Val; n=5; Mann-Whitney test). **I**, Immunofluorescence staining of DAPI, Kpr, and TMOD3 in αIIbβ3–Chinese hamster ovary (CHO) cells spreading on immobilized fibrinogen in the absence or presence of 1 mmol/L BCAAs (0.5 mmol/L Leu, 0.25 mmol/L Ile, and 0.25 mmol/L Val). Data are presented as mean±SEM. IB indicates immunoblot; IgG, immunoglobulin G; and IP, immunoprecipitation. ***P*<0.01.

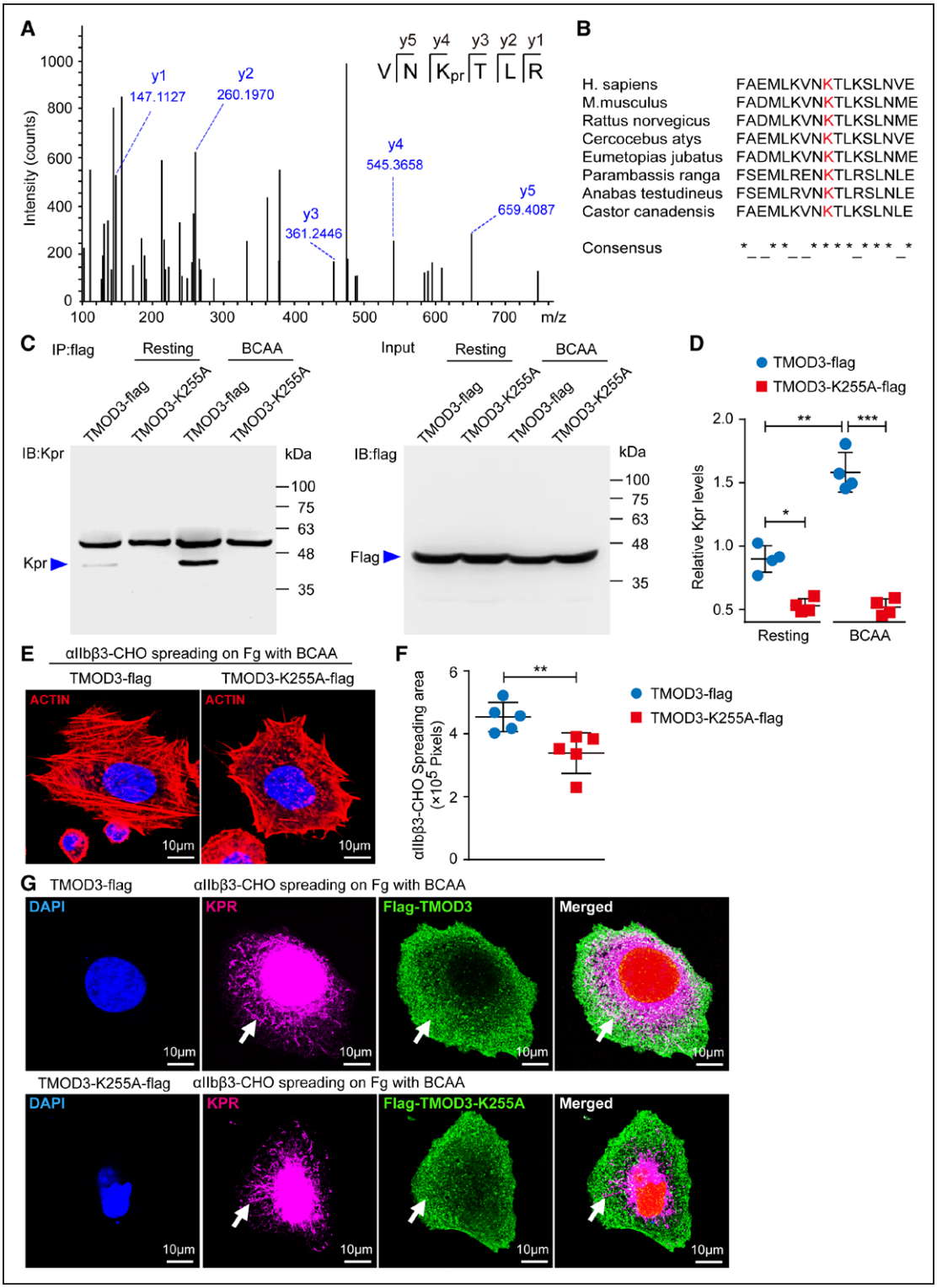

Figure 6. Propionylation of tropomodulin-3 (TMOD3) at K255 regulates αIIbβ3-mediated Chinese hamster ovary (CHO) spreading.
A, Tandem mass spectrometry spectrum of the propionylated peptide (VNKprTLR) from TMOD3 near residue K255. **B**, Sequence alignment analysis of K255 of TMOD3 in *Homo sapiens* (accession identifier [ID]: NP055362.1), *Mus musculus* (accession ID: NP058659.1), *Rattus norvegicus* (accession ID: NP001011997.1), *Cercocebus atys* (accession ID: XP011948499.1), *Eumetopias jubatus* (accession ID: XP027955735.1), *Parambassis ranga* (accession ID: XP028258145.1), *Anabas testudineus* (accession ID: XP026234076.1), and *Castor canadensis* (accession ID: JAV42640.1). *A position that has a single fully conserved residue; the conserved propionylated lysine residue of interest is indicated in red. **C** and **D**, Mutation of K255A disrupted branched-chain amino acid (BCAA)–induced TMOD3 propionylation (n=4). **E** and **F**, Immunofluorescence staining of actin in αIIbβ3-CHO cells transfected with TMOD3-flag or TMOD3-K255A-flag plasmids spreading on immobilized fibrinogen in the presence of 1 mmol/L BCAAs (0.5 mmol/L leucine [Leu], 0.25 mmol/L isoleucine [Ile], and 0.25 mmol/L valine [Val]; n=5; Mann-Whitney test). **G**, Immunofluorescence staining of DAPI, propionylation (Kpr), and flag in spreading αIIbβ3-CHO cells transfected with TMOD3-flag or TMOD3-K255A-flag plasmids in the presence of 1 mmol/L BCAAs (0.5 mmol/L Leu, 0.25 mmol/L Ile, and 0.25 mmol/L Val). Data are presented as mean±SEM. *P<0.05; **P<0.01; ***P<0.001.

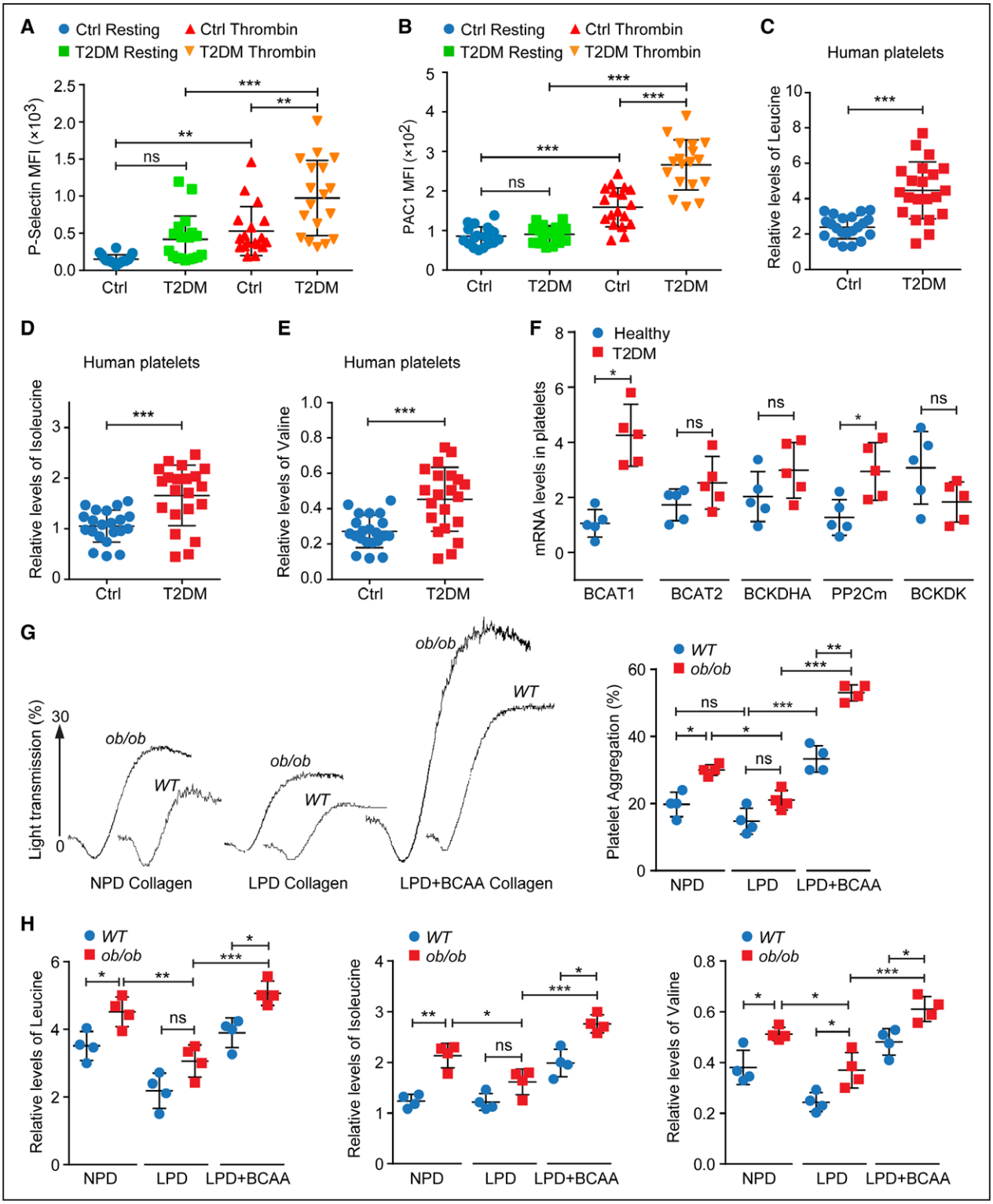

Figure 7. Branched-chain amino acids (BCAAs) are probably the cause of platelet hyperactivity in metabolic syndrome.
A, P-selectin exposure in platelets from healthy volunteers (n=18, male=9) and patients with type 2 diabetes mellitus (T2DM; n=18, male=11) in response to 0.05 U/mL α-thrombin. **B**, PAC1 binding to platelets from healthy volunteers and patients with T2DM in response to 0.05 U/mL α-thrombin. **C** through **E**, Intracellular levels of leucine (Leu), isoleucine (Ile), and valine (Val) in platelets from healthy volunteers (n=21, male=13) and patients with T2DM (n=21, male=12) were measured by gas chromatography–mass spectrometry. **F**, mRNA levels of branched chain aminotransferase (BCAT) 1, BCAT2, branched chain keto acid dehydrogenase E1, alpha (BCKDHA), protein phosphatase 2Cm (PP2Cm), and branched-chain α-keto acid dehydrogenase complex kinase (BCKDK) in healthy people (n=5) and patients with T2DM (n=5, male=3). **G**, Platelet aggregation from *ob/ob* and wild-type (WT; C57BL/6) mice treated with normal-protein diet (NPD), low-protein diet (LPD), or LPD+BCAA diets induced by 1 μg/mL collagen (n=4). **H**, Intracellular levels of Leu, Iso, and Val in platelets from *ob/ob* and WT (C57BL/6) mice treated with NPD, LPD, or LPD+BCAA (3 mg/mL BCAAs in drinking water) diets, respectively (n=4). Data in **A**, **B**, **G**, and **H** were analyzed by 2-way ANOVA with the Tukey multiple-comparisons test. Data in **C** through **E** were analyzed by the Mann-Whitney test. Data are presented as mean±SEM. Ctrl indicates control; and ns, nonsignificant (P>0.05). *P<0.05; **P<0.01; ***P<0.001.

ob/ob mice possess the pathological characteristics of insulin resistance and hyperglycemia, similar to those of human MS and T2DM.[26] BCAA supplementation in drinking water greatly increased the activity of platelets in *ob/ob* mice on the low-protein diet compared with that in WT mice (Figure 7G and 7H). Thus, lowering dietary BCAA intake could reduce platelet activity in *ob/ob* mice. These results imply that increased levels of cellular BCAAs in platelets are important for their hyperactivation in MS.

DISCUSSION

BCAAs are essential nutrients with numerous beneficial effects. BCAAs are commonly used as nutritional supplements to increase muscle protein synthesis and mass during exercise training.[14,42] Clinically, injection of BCAAs is applied for the treatment of acute, subacute, and chronic severe hepatitis, cirrhosis, and hepatic encephalopathy and is used before and after hepatobiliary surgery.[3,4] BCAA α-ketoacid metabolites as the main ingredients can be taken with a low-protein diet to prevent and treat the damage caused by protein metabolic defects in chronic renal insufficiency.[11,42] Despite these benefits of BCAAs, our study unequivocally showed that ingestion of BCAAs facilitates platelet activation and increases thrombosis risk, suggesting that BCAAs should be used with caution for arterial thrombosis risk.

Among the 3 BCAAs, Val and Ile may play a more important role than Leu in the regulation of platelet activation. BCAA catabolism is catalyzed by multiple mitochondrial enzymes and leads to the formation of intermediate metabolites such as BCKAs and acyl-CoAs.[7,10] Although Leu, Ile, and Val individually promoted platelet activation, KIV and KMV (the ketoacid metabolites of Val and Ile, respectively) had more significant effects on enhancing platelet activation than the Leu metabolite, KIC. In addition, propionyl-CoA, a common metabolite of KIV and KMV but not KIC, drastically enhanced platelet activation, further indicating that the Val and Ile metabolic pathways are the major pathways regulating platelet activation. However, our study cannot exclude nonmetabolic functions of Leu in facilitating platelet activation, and more studies are needed to investigate the possible involvement of such functions.

Propionyl-CoA is a high-energy intermediate product of Val and Ile catabolism and a donor molecule for protein propionylation.[7,38,39] Kpr was first identified as a histone modification,[38,43] but little is known about global Kpr substrates and their dynamic functions in cellular physiology. In our study, the propionylation levels of cytoskeletal proteins were first identified to be universally enhanced in platelets treated with BCAAs. Furthermore, increases in propionylation of cytoskeletal proteins were obviously associated with the upregulation of platelet activation.

Cytoskeletal reorganization is the primary physical change associated with platelet shape change and subsequent aggregation.[44] The platelet cytoskeleton is regulated by F-actin and plays critical roles in the platelet activation induced by αIIbβ3,[44] glycoprotein Ib/IX/V,[27] the collagen receptor glycoprotein VI,[45] and G protein–coupled receptors.[46] TMOD3 is the unique isoform of TMOD found in platelets.[40] It caps the pointed end of tropomyosin-coated F-actin filaments to stabilize the cytoskeletal structures.[40] TMOD3 deficiency causes severe macrothrombocytopenia as a result of impaired megakaryocyte cytoplasmic morphogenesis.[40] In the present study, TMOD3 was found to be propionylated in platelets treated with BCAAs, and increased TMOD3 propionylation at K255 played an essential role in enhancing platelet activation, revealing novel functions of protein propionylation and BCAA catabolism.

MS is a major risk factor for cardiovascular diseases with adverse clinical outcomes.[47] T2DM is a typical MS and is characterized by increases in oxidized low-density lipoproteins[22] and advanced glycation end products[23] and decreases in insulin levels in plasma,[24] which are the common causes of MS-facilitated platelet hyperactivity and thrombosis. Frequently, elevated BCAA levels have been reported to mediate the occurrence of cardiovascular diseases.[48] In *ob/ob* mice, BCAA catabolism is impaired in many tissues such as the liver and white adipose tissue.[26] We found that the abundance of BCAAs in platelets was significantly increased in patients with T2DM compared with healthy control subjects, and the expression of BCAA metabolic positive regulators was also increased in the platelets of patients with T2DM, indicating that BCAA catabolism in platelets is correlated with platelet hyperreactivity and the risk of thrombosis.

Overall, the present results reveal a previously unrecognized mechanism linking BCAA catabolism with platelet function and thrombotic risk. These findings also demonstrate that the ingestion of BCAAs may be detrimental for individuals with cardiovascular diseases because of potential thrombosis risk. Moreover, our study indicates that targeting the BCAA catabolism pathway or lowering dietary BCAA intake may serve as a novel therapeutic strategy for MS-associated thrombophilia.

ARTICLE INFORMATION

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Y.X., H.J., H.S., and J.L. designed the experiments, analyzed data, and wrote the paper. Y.X., H.J., L.L., F.C., Y.L., M.Z., J.W., and J.J. performed the experiments. C.F., Y.W., X.L., X.F., L.Z., J.Z., and J.Q. helped with the experiments. The authors thank Kevin Liu from the Shanghai High School International Division for proofreading the article.

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Disclosures

None.

Supplemental Materials

Supplemental Methods
Data Supplement Figures I–III
Data Supplement Movies I–IV

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