A Novel Antithrombotic Mechanism Mediated by the Receptors of the Kallikrein/Kinin and Renin–Angiotensin Systems

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The contact activation (CAS) and kallikrein/kinin (KKS) systems regulate thrombosis risk in two ways. First, the CAS influences contact activation-induced factor XI activation and thrombin formation through the hemostatic cascade. Second, prekallikrein (PK) and bradykinin of the KKS regulate expression of three vessel wall G-protein-coupled receptors, the bradykinin B2 receptor (B2R), angiotensin receptor 2, and Mas to influence prostacyclin formation. The degree of intravascular prostacyclin formation inversely regulates intravascular thrombosis risk. A 1.5- to 2-fold increase in prostacyclin, as seen in PK deficiency, increases vessel wall Sirt1 and KLF4 to downregulate vessel wall tissue factor which alone is sufficient to lengthen induced thrombosis times. A twofold to threefold increase in prostacyclin, as seen the B2R-deficient mouse, delays thrombosis and produces a selective platelet function defect of reduced GPVI activation and platelet spreading. Regulation of CAS and KKS protein expression has a profound influence on thrombosis-generating mechanisms in the intravascular compartment.

Keywords: prekallikrein, bradykinin B2 receptor, Mas receptor, angiotensin receptor 2, factor XII, high molecular weight kininogen, prostacyclin, tissue factor and coagulation

The contact activation system (CAS) is known to initiate thrombus formation in surface-activated blood coagulation of plasma by factor XII (FXII) autoactivation with subsequent activation of plasma prekallikrein (PK), amplification of FXII and PK activation by each other, and subsequent activation of factor XI initiating a cascade of proteolytic reactions leading to thrombin formation (Figure 1). Plasma high-molecular weight kininogen (HK) accelerates these reactions. In a rabbit model of extracorporeal membrane oxygenation (ECMO), a novel Fab 3F7 directed to FXIIa and betaFXIIa reduced fibrin formation on the membrane oxygenator to the same extent as heparin (1). These data indicate that activations of the CAS leading to thrombin formation are blocked by inhibitors to FXIIa. Also in mice, CAS inhibition results in improved survival of F12−/− mice in the surface-activated collagen-, epinephrine-, or long-chain polyP-induced pulmonary embolism models when compared to wild type (WT) (2–4). In humans, FXIIa inhibition is useful to prevent in vivo contact activation from sepsis from any cause, adult respiratory distress syndrome, and when human blood comes in contact with artificial surfaces of medical devices such as ECMO, cardiopulmonary bypass, left-ventricular assist devices, and indwelling intravenous catheters (5). HK-deficient mice (Kgn1−/−) are also protected from thrombosis, but the mechanism for thrombosis delay is less well characterized (6). They appear to be like F12−/− mice, since Kgn1−/− mice also have improved survival after long-chain polyP infusions (4).
However, other gene-deleted animals of the plasma CAS and kallikrein/kinin system (KKS) do not behave like F12−/− mice on contact activation-induced thrombosis assay. Plasma PK (Klkb1−/−) and bradykinin B2 receptor knockout (Bdkrb2−/−) mice do not have increased survival in the collagen-, epinephrine-, or long-chain polyp-induced pulmonary embolism models (5, 7). Klkb1−/− mice have reduced contact activation as indicated by delayed contact activation thrombin generation times, long aPTT, and reduced lung edema after collagen–epinephrine insult (7). However, this reduced contact activation is not enough to provide thrombosis protection. Another mechanism is involved in its thrombosis protection. This assessment is the basis of the present review. Deficiencies of proteins of the plasma KKS (PK, bradykinin B2 receptor) allow for a novel mechanism for thrombosis protection through regulation of vessel wall tissue factor (TF) (Figure 1). Characterization of this mechanism is the basis of this report. In this pathway, regulation of vessel wall TF is mediated by vascular G-protein-coupled receptors (GPCRs) of the KKS and renin–angiotensin system (RAS) and their influence on prostacyclin.

**CHARACTERIZATION OF Klkb1−/− MICE**

Detailed mechanistic investigations on PK-deficient mice reveal a previously unappreciated thrombosis protection pathway that modulates in vivo thrombosis risk through regulation of vessel wall TF expression. The mechanism for this pathway is not obvious but was discovered by following the data from research observations. When we realized that Klkb1−/− mice have expected reduced contact activation, but do not have protection on contact activation-induced thrombosis assays, other mechanisms were sought. We observed that Klkb1−/− mice have reduced bradykinin (BK) to about 50% of the level of normal and higher than Kgn1−/− mice that have none (6, 7). We had expected that the bradykinin B2 receptor (B2R) that is constitutively expressed would be increased in response to low BK. To our surprise, the B2R was decreased. The B2R is known to make heterodimeric complexes with the angiotensin receptor 2 (AT2R) and the Mas receptor (Mas) (8, 9). Furthermore, the B2R, AT2R, and Mas, when stimulated, all produce prostacyclin and NO. Finally, we knew from previous studies that in the absence of the B2R, AT2R and Mas become overexpressed (see below) (10, 11).

Investigations next determined if the AT2R and/or Mas receptor levels are increased. We observe increased Mas receptor mRNA and protein with reduced AT2R and angiotensin-converting enzyme (ACE) mRNA and normal angiotensin-(1–7) [Ang-(1–7)] levels (Figure 2). We next determined that a Mas receptor antagonist, A-779, corrects the prolonged thrombosis time in Klkb1−/− mice (7). Studies subsequently document that plasma prostacyclin, as measured by its stable plasma breakdown product 6-keto-PGF1α, is elevated, and the Mas antagonist A-779 corrects it to normal and nimesulide, a cyclooxygenase 2 inhibitor, shortens Klkb1−/− mice thrombosis times to normal (8). Even though Klkb1−/− mice have elevated prostacyclin, thrombosis delay is not due to decreased platelet function. Klkb1−/− mice have normal bleeding times and platelet function as determined by thrombin-, CRP-, or ADP-induced platelet activation. Klkb1−/− mice also have normal platelet spreading on a collagen or fibrinogen matrix. Therefore, other mechanisms were sought.

Klkb1−/− mice have reduced platelet adherence on the cremasteric mouse laser injury thrombosis model. Since they have normal platelet function, we asked if there were reduced vessel wall TF (8). Aortic and carotid artery TF mRNA, protein, activity, and expression were reduced. Furthermore, plasma of Klkb1−/− mice has normal TF-induced thrombin generation times when 5 pM rTF is used. However, when 0.5 pM TF is employed, plasma from Klkb1−/− mice has reduced TF-induced thrombin generation. Recent studies by Barbieri and coworkers show that COX2−/− mice have increased vessel wall TF mediated by reduced prostacyclin, allowing for reduction of the vasculoprotective transcription factor Sirt1 (12). We determined if Klkb1−/− mice have increased sirtuin 1 (silent mating type
Figure 2: Model for vessel wall tissue factor regulation in prekallikrein deficiency. In the absence of plasma prekallikrein (PK), there is reduced bradykinin (BK) and the bradykinin B2 receptor (B2R). This result is also associated with reduced angiotensin-converting enzyme activity (ACE) and the angiotensin receptor 2 (AT2R) with elevation of the G-protein-coupled receptor Mas with normal angiotensin-(1–7) [Ang-(1–7), its natural ligand]. Increased Mas receptor alone is associated with a 1.5- to 2-fold elevation of prostacyclin (PGI2) with increased vessel wall Sirt1 and KLF4 and reduced vessel wall tissue factor (TF). There is no platelet function defect in prekallikrein-deficient platelets. The delayed thrombosis in PK-deficient mice is due to reduced vessel wall TF alone.

Characterization of Bdkrb2−/− Mice

We next asked if the thromboprotection mechanism seen in Klkb1−/− mice is observed in any other animals? Previous investigations in our laboratory show that Bdkrb2−/− mice also have delayed times to arterial thrombosis (10, 11). Our investigations reveal that in the absence of the B2R, both the AT2R and Mas receptors are increased as determined by mRNA and protein in kidney and aorta of these animals. Unlike Klkb1−/− mice, Bdkrb2−/− mice have elevated plasma BK, angiotensin II (AngII), and Ang-(1–7) (10, 11) (Figure 3). Also, plasma PK and plasminogen activator inhibitor-1 are increased, and factor XI is decreased (10). Antagonists to the Mas receptor (A-779) or AT2R (PD123319) are able to correct the delay in thrombosis occlusion to normal and both antagonists together are not additive (11). Furthermore, unlike Klkb1−/− mice, Bdkrb2−/− mice have long bleeding times (10, 11). Their plasma 6-keto-PGF1α levels (259 ± 42 pg/ml) are significantly higher than Klkb1−/− mice (129 ± 12 pg/ml) (p < 0.003) and normals (75 ± 10 pg/ml) (7), (11). Twofold higher 6-keto-PGF1α levels produce the long bleeding times and the selective platelet function defect in Bdkrb2−/− mice (11). Bdkrb2−/− mice have a selective GPVI activation defect to collagen-rich peptide and convulxin. They manifest reduced murine activated integrin expression using the JON/A antibody and P-selectin expression. They also have a spreading defect on collagen, fibrinogen, and GFOGER, a β1 integrin adhesive peptide (11).

On the other hand, Bdkrb2−/− mice have normal thrombin- and ADP-induced platelet activation and fibrinogen binding, respectively. Finally, the cyclooxygenase inhibitor, nimesulide, shortens the bleeding time to normal and corrects the time to thrombosis in these animals. However, the platelet inhibition mechanism in Bdkrb2−/− mice is not complete story for their delayed thrombosis times. Bdkrb2−/− mice also have elevated Sirt1 and KLF4 mRNA in their vessel wall. We suspect that these animals too have reduced TF in their vessel walls. In support of that assessment, a recent investigation shows that BK reduces vessel wall TF via cell activation and this translates into reduced thrombosis risk (13). A summary of this mechanism for thrombosis delay in Bdkrb2−/− mice is shown in Figure 3.
Figure 3 | Mechanisms for thrombosis delay in the bradykinin B2 receptor-deleted mice. In the absence of the bradykinin B2 receptor (B2R), there is increased plasma bradykinin (BK), its ACE breakdown product, BK-(1–5) (RPPGF). There is also increased ACE, angiotensin II (AngII), and angiotensin-(1–7) [Ang-(1–7)]. Elevated AngII and Ang-(1–7) bind to increased angiotensin receptor 2 (AT2R) and Mas to increase prostacyclin (PGI2) levels twofold to threefold above normal. This elevation in plasma PGI2 results in increased vessel wall Sirt1 and KLF4 with reduced TF and a selective platelet function defect. The selective platelet function defect is reduced GPVI activation with normal thrombin- and ADP-induced platelet activation and reduced spreading on collagen and adhesive glycoproteins through β1 integrin binding. The delayed thrombosis in B2R-deficient mice is reduced vessel wall tissue factor a selective platelet function defect of GPVI activation and spreading.

Figure 4 | The prostacyclin axis-induced thrombosis protection. PK and B2R deficiency produces thrombosis protection through the prostacyclin axis. In the absence of PK or the B2R, there is increased prostacyclin production due to overexpression of the AT2R and/or Mas receptors to compensate for reduced or absent B2R. Prostacyclin induces a graded increase in thrombosis protection. First, at levels up to twofold increased, it effects the vessel wall reducing TF production. Second, at levels up to twofold to threefold increased, it downregulates vessel wall TF and induces a selective platelet function defect of reduced GPVI activation and spreading on collagen- and integrin-binding adhesive glycoproteins. Finally, at levels greater that threefold, prostacyclin produces the overall platelet anesthesia generally recognized with it.
Finally, higher concentrations of prostacyclin provide a general protective platelet GPVI activation and spreading defect (Figure 4). Second, higher elevations (twofold to threefold increases) result in a selective platelet GPV1 activation and spreading defect (Figure 4). These higher levels of prostacyclin lengthen bleeding times. Finally, higher concentrations of prostacyclin provide a general platelet anesthesia and give increased risk to bleed. Modulating vessel wall TF only through these three GPCRs may provide a novel approach to reduce thrombosis risk without enhanced risk to bleed.

AUTHOR CONTRIBUTIONS

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