Platelet Kainate Receptor Signaling Promotes Thrombosis by Stimulating Cyclooxygenase Activation

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Rationale: Glutamate is a major signaling molecule that binds to glutamate receptors including the ionotropic glutamate receptors; kainate (KA) receptor (KAR), the N-methyl-D-aspartate receptor, and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor. Each is well characterized in the central nervous system, but glutamate has important signaling roles in peripheral tissues as well, including a role in regulating platelet function.

Objective: Our previous work has demonstrated that glutamate is released by platelets in high concentrations within a developing thrombus and increases platelet activation and thrombosis. We now show that platelets express a functional KAR that drives increased agonist induced platelet activation.

Methods and Results: KAR induced increase in platelet activation is in part the result of activation of platelet cyclooxygenase in a mitogen-activated protein kinase–dependent manner. Platelets derived from KAR subunit knockout mice (GluR6−/−) are resistant to KA effects and have a prolonged time to thrombosis in vivo. Importantly, we have also identified polymorphisms in KAR subunits that are associated with phenotypic changes in platelet function in a large group of whites and blacks.

Conclusions: Our data demonstrate that glutamate regulation of platelet activation is in part cyclooxygenase-dependent and suggest that the KAR is a novel antithrombotic target. (Circ Res. 2009;105:595-603.)

Key Words: platelet • kainate • glutamate • thrombosis • cyclooxygenase • thromboxane • mitogen activated kinase • p38

Thrombosis is driven by complex interactions between platelets, endothelial cells, and coagulation factors. Initial platelet activating events trigger intracellular signaling pathways necessary for rapid and efficient thrombus formation. These include changes in intracellular ion concentrations and pH, conformational changes in receptors such as Glycoprotein IIb/IIIa (GPIIb/IIIa), granule exocytosis, and secretion of vasoactive mediators. Many granule constituents are shared between platelets and neurons including Substance P, ADP, ATP, serotonin, and glutamate.1–3 Glutamate signaling is well explored in the brain, but glutamate effects on platelet function are less well studied and only beginning to be understood.

Ionotropic glutamate receptors are classified as kainate (KA) receptors (KAR), N-methyl-D-aspartate (NMDA) receptors (NMDAR), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPAR) based on their sensitivity to the ligand they are named for.

NMDARs mediate the influx of calcium, whereas AMPAR and KAR primarily mediate sodium influx on ligand binding. AMPAR and KAR are closely related in their tetrameric structure (4 subunits) but comprise distinct receptor subunits. KainateR subunits are commonly designated as GluR5, GluR6, GluR7, KA1, and KA2 (genes are also designated GRIK1 through 5, respectively)4 and each subunit type is the product of a distinct gene. The KAR was identified by its activation in response to the naturally occurring marine toxin kainic acid and has similar activity to AMPAR but is less well studied. GluR5–7 can form homomers (example, all GluR5) and heteromers (example, a receptor composed of both GluR5 and GluR6). KA1 and KA2 can only be part of functional receptors by combining with one of the GluR5–7 subunits.5,6 Like AMPAR, when activated the KAR allows sodium influx at a rate similar to that of the AMPAR, but KARs desensitize more rapidly after ligand binding.

Glutamate receptors are best described in the central nervous system, but are also expressed and functional in...
many tissues outside of the central nervous system. For example, AMPA can stimulate insulin release from β-cells in the pancreas, and the AMPAR subunit GluR3 has been described on circulating T-lymphocytes where it has a potential role in T-cell chemotaxis and adhesion. Other glutamate receptors, such as the NMDAR, have been described in osteoblasts, osteoclasts, and megakaryocytes. Most peripheral glutamate receptors when cloned and sequenced are identical to those of the central nervous system. We have previously demonstrated using a real-time glutamate sensitive enzymatic probe that glutamate reaches very high concentrations (greater than 400 μmol/L) within a developing thrombus. We have also demonstrated that glutamate signaling has an important role in efficient thrombus formation by amplifying platelet activation, in part through platelet expressed AMPARs.

Glutamate signaling induces mitogen activated protein kinase (MAPK) signaling pathways in neurons. MAPK messengers, including p38, are well described platelet signaling molecules. MAPK p38 is phosphorylated in agonist-stimulated platelets and dominant negative MAPK signaling inhibition reduces functional outcomes of agonist stimulation, such as GPIIb/IIIa activation. Platelet cyclooxygenase (COX) activation has been demonstrated to be downstream of MAPK in response to receptor stimulation. COX activation and the production of prostaglandins (such as thromboxane), is an important step in efficient thrombus generation. Kainate-induced seizures drive neuronal COX stimulation and KAR signaling can induce activation of MAPK pathways in neurons, including p38 pathways. This provides a background for a possible role of KAR signaling through MAPK pathways in platelet COX activation.

Figure 1. Kainic acid increases platelet activation. A, PRP was diluted in Tyrode buffer, pretreated with glutamate, and stimulated with U46619 (1 μmol/L). Platelet activation was determined by PAC-1 antibody binding (n=4±SD, *P<0.01 vs 0). B, Diluted PRP was pretreated with 250 μmol/L KA and stimulated with U46619 (1 μmol/L). Platelet activation was determined by FACS with PAC-1 antibody (n=5±SD, *P<0.01). C, KA dose response. Diluted PRP was treated with KA and stimulated with TRAP (5 μmol/L). Platelet activation was determined by FACS with PAC-1 antibody (n=5±SD, *P<0.01 vs 0).

Results

KAR Signaling Increases Platelet Activation

Our previous studies demonstrated that glutamate pretreatment increased agonist-induced platelet activation. Glutamate by itself does not directly alter the expression of surface markers of platelet activation, such as GPIIb/IIIa activation (PAC-1 antibody binding) and P-selectin expression. However, as shown in Figure 1A, pretreatment of platelets with production studies platelet-rich plasma (PRP) was pretreated with inhibitors for at least 20 minutes before stimulation. Platelets were pelleted after treating with indomethacin (20 μmol/L, Sigma). Plasma was isolated for the TxB2 ELISA and the platelet pellet lysed in p38 ELISA lysis buffer for measurement of P-p38. Plasma was further purified using a C-18 SPE cartridge (Amersham). All other materials and studies are completely described in the supplemental materials (available online at http://circres.ahajournals.org).

Methods

GluR6−/− mice are on a mixed 129 and C57Bl6J background. Littermate wild-type mice were used as controls. For thromboxane

Non-standard Abbreviations and Acronyms

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<th>Abbreviation</th>
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glutamate before agonist stimulation, such as with the thromboxane receptor agonist U46619 (1 μmol/L), greatly increased platelet activation as determined by increase in PAC-1 antibody binding (Figure 1A; MFI indicates mean fluorescent intensity). Our previous work has also shown that glutamate effects are in part mediated by AMPAR.1 The NMDAR has also been described on platelets, but pretreatment of platelets with NMDA does not increase agonist induced platelet activation (supplemental Figure I).

The other class of glutamate receptor that is similar in structure and function to AMPAR is KAR. To begin to explore the role of KAR in platelet activation, platelets were incubated with KA (250 μmol/L) before platelet activation with U46619 (1 μmol/L). Like glutamate, KA alone has no effect on PAC-1 antibody binding (Figure 1B; white bars). However, KA pretreatment increased platelet activation approximately 3 times that of control treated platelets (Figure 1B, black bars). A KA dose response curve was performed and as little as 100 μmol/L of KA significantly increased platelet activation (Figure 1C). These are physiologically relevant glutamate and KA concentrations, as we have shown in the past that the concentration of glutamate in a developing thrombus is greater than 400 μmol/L.1

KARs are made of subunits that assemble to form a tetrameric receptor complex. To demonstrate that platelets express KAR subunits, human platelets and mouse brain lysates were immunoblotted. Platelets express both GluR5 and GluR6 protein (Figure 2A, top). In addition, mRNA was isolated from rat primary neurons and the megakaryocyte cell line MEG-01, and RT-PCR was performed with primers specific for GluR5 and GluR6. MEG-01 cells also express GluR5 and GluR6 mRNA (Figure 2A, bottom).

Kainate, particularly at higher concentrations, can have some AMPAR affinity, although it is much less than its KAR affinity. To demonstrate that the KA induced increase in platelet activation was specific to KAR, we used both pharmacological and genetic methods. ATPA is an agonist that only acts on GluR5-containing receptors. At 100 μmol/L ATPA nearly doubled TRAP induced platelet activation, and as little as 10 μmol/L also significantly increased PAC-1 binding (Figure 2B, lower concentrations had no effect, not shown). To confirm KA-induced effects are mediated by KAR signaling, platelets were isolated from GluR6−/− mice and littermate control wild-type (WT) mice, incubated with KA (250 μmol/L), and then activated with 0.1 U/mL of thrombin (n=5±SD, *P<0.01 vs 0). KA pretreatment nearly doubled WT platelet activation, but had no effect on GluR6−/− platelets as measured by GPIIb/IIIa activation using JON/A antibody binding (Figure 2C).

To further demonstrate that KAR has an important role in platelet activation we performed platelet aggregation studies in the presence of control buffer (PBS) or the KAR antagonist UBP302. PRP was incubated with UBP302 and aggregation initiated with TRAP (n=7±SEM, *P<0.01 vs Control).
20 minutes, and then activated by addition of TRAP (2 μmol/L). At the same concentration of UBP302, KAR antagonism either reduced by about 50% (green line), or completely blocked (blue line), platelet aggregation as compared to control PBS treated platelets (Figure 2D, quantification at 5 minutes post TRAP).

**KAR Signaling Increases Platelet COX Activation**

Kainate induced seizures and neuronal loss are in part attributable to induction and expression of cyclooxygenases COX-1 and COX-2. Platelets primarily express COX-1 under normal physiological conditions, but COX-2 expression can be induced in pathophysiological processes. We determined whether or not KAR signaling in platelets stimulated COX. We first incubated platelets with the endogenous ligand for KAR, glutamate, to see whether this led to the production and release of thromboxane from platelets. Human PRP (approximately 1 × 10^8 total platelets per reaction) were treated with control buffer, glutamate alone, or glutamate in the presence of the COX inhibitor indomethacin (20 μmol/L) as a negative control. We first incubated platelets with the endogenous ligand for KAR, glutamate, to see whether this led to the production and release of thromboxane from platelets. Human PRP (approximately 1 × 10^8 total platelets per reaction) were treated with control buffer, glutamate alone, or glutamate in the presence of the COX inhibitor indomethacin (20 μmol/L) as a negative control. We first incubated platelets with the endogenous ligand for KAR, glutamate, to see whether this led to the production and release of thromboxane from platelets. Human PRP (approximately 1 × 10^8 total platelets per reaction) were treated with control buffer, glutamate alone, or glutamate in the presence of the COX inhibitor indomethacin (20 μmol/L) as a negative control. We first incubated platelets with the endogenous ligand for KAR, glutamate, to see whether this led to the production and release of thromboxane from platelets. Human PRP (approximately 1 × 10^8 total platelets per reaction) were treated with control buffer, glutamate alone, or glutamate in the presence of the COX inhibitor indomethacin (20 μmol/L) as a negative control. We first incubated platelets with the endogenous ligand for KAR, glutamate, to see whether this led to the production and release of thromboxane from platelets. Human PRP (approximately 1 × 10^8 total platelets per reaction) were treated with control buffer, glutamate alone, or glutamate in the presence of the COX inhibitor indomethacin (20 μmol/L) as a negative control. We determined whether or not KAR signaling in platelets stimulated COX.

We also determined the kinetics of KA-induced TxB2 production by incubating platelets with PBS or KA and measuring TxB2 after 5, 10, 20, and 30 minutes. At 5 minutes post KA there was no change in TxB2, but after 10 minutes a significant increase in TxB2 was found (Figure 3C). There was an additional increase noted after 20 and 30 minutes, but the greatest increase was seen between 5 and 10 minutes post KA (Figure 3C).

To confirm these pharmacological data we used platelets isolated from WT and GluR6−/− mice. Using methods to harvest platelets from WT and GluR6−/− mice and treat them with PBS or KA (250 μmol/L). TxB2 determined by EIA (n = 3 ± SD, *P < 0.01 vs Control).
similar to the human platelet studies, washed WT and GluR6−/− platelets were incubated with PBS or KA (250 μmol/L) and TxB2 measured by EIA. KA increased WT mouse thromboxane production (Figure 3D, left side) but had no effect on thromboxane production from GluR6−/− platelets (Figure 3D, right side). This provides complementary genetic evidence that KA induced COX activation is through the KAR.

KAR Signaling Activates COX in a MAPK Dependent Manner

MAPK signaling has been implicated as a pathway leading to COX activation in many cell types, including renal glomeruli, neurons, and platelets.13,14,22,24,25 KAR signaling is also known to activate MAPK pathways in neurons.22 We therefore explored whether KAR signaling drives thromboxane production in a MAPK dependent manner.

The MAPK p38 pathway is induced in response to numerous platelet agonists including vWF, collagen, and thrombin,14,24 and mice lacking p38 have prolonged time to thrombus formation.26 We determined whether KAR signaling activates platelet p38 MAPK pathway by incubating platelets with PBS, KA (250 μmol/L), or KA after the KAR specific antagonist UBP302. TRAP stimulation (0.5 μmol/L) was used as a positive control. p38 phosphorylation (P-p38) was quantified by ELISA. KA treatment increased P-p38 approximately twice that of resting platelet levels and this was blocked by KAR antagonism (Figure 4A). We confirmed these data using platelets from WT and GluR6−/− mice. WT and KO platelets express equal total p38 by Western blot (Figure 4B), and platelets were incubated with buffer or KA.

KA induced an increase in WT platelet P-p38, but KA had no effect on GluR6−/− platelet P-p38 (Figure 4C).

Although the downstream signaling effects of MAPK pathways are sustained, the actual phosphorylation of p38 is often transient. To determine the time course of KAR signaling–induced platelet P-p38, we incubated platelets with KA as above, and P-p38 was determined at multiple time points. Similar to TxB2 production, we found that P-p38 peaked at 10 minutes post KA addition (Figure 4D). This is a transient event, as P-p38 declined to baseline levels by 20 minutes post KA addition (Figure 4D), confirmed by Western blot (supplemental Figure VI).

To place p38 in the KAR-induced COX activation pathway we incubated platelets with the p38 inhibitor SB203580 before the addition of KA. The KAR antagonist UBP302 was used to pretreat samples before the addition of KA as a negative control. As expected KA increased platelet thromboxane production (Figure 4E, black versus white bars). This was blocked by preincubation of platelets with the p38 inhibitor (Figure 4E, black versus light gray bars), implicating KAR induced P-p38 as a mechanism to increase platelet COX activation. KAR signaling also induced phosphorylation of ERK1/2 (supplemental Figure VII), but ERK inhibition did not block KA induced thromboxane production (supplemental Figure VIII).

Taken together, these data demonstrate that KAR signaling drives low-level thromboxane production in a manner dependent on p38 MAPK pathway activation. The induction of MAPK activation may in part account for glutamate mediated increase in agonist induced platelet activation by “priming” signaling pathways, thereby amplifying agonist induced ac-
tivation. This is analogous to what has been noted in other important platelet signaling pathways; for example native low-density lipoprotein is known to sensitize platelets to other agonists in a manner that also involves MAPK.27,28

KAR Signaling Is a Mediator of Platelet Function In Vivo

It is often difficult to extrapolate in vitro platelet function tests to the in vivo relevance of a particular platelet signaling pathway. To address this we used an intravital microscopy model of arterial thrombosis with WT and GluR6−/− mice in which mesenteric arterioles were damaged with ferric chloride and the time to vessel occlusion determined by visualizing fluorescently labeled platelets (Figure 5A). WT mice on average form occlusive thrombi 500 seconds after vessel damage (Figure 5B). The time to vessel occlusion in GluR6−/− mice is approximately twice as long as WT mice (Figure 5B). To investigate whether KAR antagonists may have antithrombotic therapeutic potential, mice were injected intravenously with control buffer or the KAR antagonist UBP302 and bleeding time determined after amputating the distal 3 mm of the tail. We also determined the time to vessel occlusion using the intravital microscopy mesenteric thrombosis model. Mice treated with KAR antagonists have prolonged bleeding time (Figure 5C) and prolonged time to mesenteric arteriole occlusion following ferric chloride damage (Figure 5D). These data show that the KAR modulates thrombosis in vivo.

Gene Variants in KAR Subunits Modify Platelet Response to Aspirin Treatment

Laboratory experiments evaluating GluR5 (GRIK1) and GluR6 (GRIK2) expression and function were followed by gene association studies in humans. Using an established cohort of whites and blacks, we examined the association of single nucleotide polymorphisms (SNPs) in the GluR5 (located on chromosome 6q16.3-q21) genes, with platelet function after aspirin treatment. A block of highly correlated SNPs in intron 1 of GluR5 was associated with urinary excretion of 11-dehydro thromboxane B2 (Tx-M) in whites. Several of these SNPs were associated with platelet aggregation in blacks (Table, A, top). Significant associations were also observed after adjustments were made for baseline platelet function (for RS465566, \(P=3.5\times10^{-3}\) for Tx-M in whites, and \(P=0.004\) for collagen aggregation in blacks). Similar trends were observed for platelet aggregation in whites and Tx-M in blacks. The clinical relevance of these phenotypes is underscored by the fact that these measures are associated with greater risk of myocardial infarction (MI), stroke, and cardiovascular death in aspirin-treated patients with coronary artery disease (CAD).29–31 The adenine allele of a representative SNP in GluR5, rs465566, was associated with less residual platelet function after exposure to aspirin (ie, greater aspirin inhibition) for aggregation and Tx-M excretion (Table, B). A SNP in intron 13 of GluR6 was also associated with Tx-M in whites, but we were unable to reinforce this by finding an association in blacks (Table, A, bottom). Taken together, these data identify polymorphisms in KAR that are associated with important changes in platelet function.

**Discussion**

Our work demonstrates that platelets express functional KARs that have an important role in platelet activation and thrombosis. We have now defined a pathway in which glutamate released from activated platelets binds to KAR, driving MAPK pathway activation and thromboxane production. Mice lacking KAR subunits no longer respond to KA and exhibit delayed thrombus formation after vascular injury. Furthermore, we have identified polymorphisms in KAR that are associated with changes in platelet function, validating the potential physiological relevance of the KAR and platelet glutamate signaling pathways in human health and disease.
Glutamate has an important role in helping drive thrombus formation. Although glutamate does not directly increase the surface expression of markers of platelet activation such as P-selectin and active GPIIb/IIIa, it has a key function in amplifying the effects of agonist stimulation on platelet activation and aggregation. This and our previous studies demonstrate that glutamate receptor signaling makes thrombus formation more efficient in vivo. Mice lacking AMPAR or KAR subunits still form occlusive thrombi; however, it is significantly delayed. The function of glutamate in platelet activation can be seen as analogous to a rheostat in regulating the outcome of platelet agonist stimulation. Glutamate receptor signaling may in part serve to alter the “set point” for stimulation by other platelet agonists and thus augment and amplify platelet responses during thrombus generation. This is an important concept when considering that many of the important steps in platelet aggregation take place in close contact between platelets as a thrombus develops. The local increase in glutamate concentration as platelet activation proceeds serves to make other agonists such as thromboxane, thrombin, and ADP more effective. This may also help to focus thrombus generation locally to the site of vascular injury and lesion development.

One important downstream signaling event of KAR in platelets that may be important in reducing the set point to subsequent agonist stimulation is the production of low, subactivation threshold levels of thromboxane that on its own is not sufficient to increase measureable phenotypic markers of platelet activation. Rather, this low level p38 dependent thromboxane production makes platelets more sensitive to subsequent stimuli, thus amplifying agonist response and making thromboxane formation more efficient. This is similar to previous descriptions of other important platelet signaling pathways. For example, native low-density lipoprotein is known to sensitize platelets to other agonists in a manner that also involves MAPK. Serotonin, a small neurotransmitter similar to glutamate, is best understood in neurobiology. Serotonin also has little direct platelet stimulatory effects but can increase platelet response to other agonists in ways that are only beginning to be appreciated.

Table. Association of Variants in KAR Subunit Genes

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B. Platelet Function Data for Representative SNP rs465566

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not a “traditional” platelet agonist and serves primarily to amplify agonist-induced platelet response, mice lacking glutamate receptor subunits do not exhibit spontaneous bleeding or hemorrhagic phenotypes. Rather, thrombus formation in mice treated with glutamate receptor antagonists and mice lacking glutamate receptor subunits is left intact, although it is delayed and less efficient. Our mouse bleeding time studies with a KAR inhibitor however indicate that any potential use of these compounds in humans would have to be approached with caution to prevent adverse bleeding events. We do not yet fully appreciate the pharmacokinetics of these compounds. Nonetheless, glutamate antagonists make an attractive potential adjunctive therapy in the treatment of patients with acute coronary syndromes and strokes that warrants further consideration and study.

Our results have consistently demonstrated that glutamate amplifies platelet activation via the AMPAR and KARs, but acute NMDAR signaling has little effect on platelet activation. A major difference in these types of ionotropic glutamate receptors is that the KAR and AMPAR both mediate the influx of sodium whereas the NMDAR forms primarily a calcium permeable channel. Calcium signaling is obviously very important in platelet activation; however, there are multiple other pathways that provide for calcium influx during platelet activation thus blocking or triggering the NMDAR may not exert a significant effect on platelet activation. There are limited cellular mechanisms to rapidly depolarize platelet membranes, underscoring the notion that glutamate signaling through the KAR and AMPAR may have relatively greater effect on platelet activation in acute settings. This does not rule out the possibility that the NMDAR is important in other aspects of platelet biology. NMDAR has been shown to have a role in regulating platelet production and megakaryopoiesis and in the brain NMDAR signaling can also alter AMPAR and KAR densities and function. It is possible that because the effects we are observing are very acute, NMDAR has other important platelet functions that are unappreciated due to the constraints of our ex vivo and in vivo systems.

We examined the relationship between SNPs in 2 gene components of the human KAR (subunits GluR5 and GluR6) and platelet functional responses to aspirin. The goal of this analysis was to determine whether gene variants in components of human KAR have clinically relevant platelet functional phenotypes (such as in this case, aspirin response). These data demonstrated that there are important SNPs in KAR subunits that result in variability in aspirin response, a clinically defined and poorly understood phenomenon. Significant SNPs were approximately replicated in each ethnic group examined, adding great significance of the results. Mechanistic insight into how the block of SNPs, in GluR5 in particular, results in this interesting phenotypic change in platelet function is not within the scope of this initial description of the role of KAR in platelet activation and thrombus formation, but the data does lend itself to speculation for future study. The variant allele block (minor allele) is associated with less platelet aggregation and urinary thromboxane metabolite post aspirin in both whites and blacks. These data imply that the allelic variant confers greater aspirin induced suppression of platelet activation. Aspirin effectively blunts COX activity and our results define a KAR-mediated signaling pathway that augments platelet activation in part through COX stimulation. Does this mean that a reduction in KAR signaling in these individuals results in the loss of the KAR signaling pathway that leads to COX activation, thereby conferring greater platelet aspirin sensitivity? Does this imply an alternative pathway in COX stimulation through KAR in platelets that is yet to be understood? At this time any answer is purely speculative and will require further investigation. However, the importance of the genetic data are to demonstrate for the first time the functional and clinical relevance of glutamate receptor signaling in a large well described cohort. It should also be emphasized that because regulation of thrombosis is so critical, platelets have many redundant signaling pathways. Phenotypes in human populations where genes and protein products are not totally disrupted are often only noted under physiological stress or pharmacological treatment response. These data may emphasize this principal.

Platelet glutamate receptor signaling is an exciting and relatively unexplored area of platelet biology. Our data indicate it may have a significant role in platelet function that impacts vascular disease pathogenesis. Although much work remains to be done to fully validate the importance of the platelet glutamate receptor pathway, our study advances our current understanding of the novel role of glutamate receptors in platelet biology and in potential contribution to the variabilities in response to antiplatelet therapy.

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Disclosures
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23. Sun et al KAR Signaling Activates Platelets 603 by guest on April 5, 2017 http://circres.ahajournals.org/ Downloaded from
Platelet Kainate Receptor Signaling Promotes Thrombosis by Stimulating Cyclooxygenase Activation

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Materials and Methods

Reagents

Kainate, NMDA, ATP, thrombin, and U46619 were purchased from Sigma. ATPA, UBP301, UBP302, and SB203580 were purchased from Tocris. Antibodies to GluR5, GluR6, and p38 were purchased from Santa Cruz; PAC-1 and P-Selectin antibodies were purchased from BD Phamingen. TRAP-6 was purchased from Bachem. Thromboxane EIA was purchased from Cayman Chemicals and P-p38 ELISA from R and D Systems.

Platelet Isolation and Ex Vivo Experiments

Human platelets were isolated from healthy volunteers who had not taken aspirin or NSAID within 10 d, under a protocol approved by The Johns Hopkins University School of Medicine Institutional Review Board (JCCI). Blood was collected into citrate anticoagulant, platelets were isolated as platelet rich plasma (PRP) by centrifugation at 180 g for 15 min, and then diluted in Tyrode’s buffer 1:20 for activation flow cytometry studies. Mouse platelets were isolated by collection into heparinized Tyrode’s buffer and isolated by centrifugation as we have described. 1 Washed platelets were resuspended in Tyrode's buffer.

Immunoblotting

Platelets purchased from HemaCare. Platelet and mouse brain lysates were prepared in NP-40 lysis buffer and supernatants fractionated on a 4-15% gel (BioRad). Transferred proteins were immunoblotted.
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Aggregation

Human platelet rich plasma (PRP) was isolated as described above and normalized to a concentration of $2 \times 10^8$/mL using platelet poor plasma (PPP). PRP was incubated with control buffer (approximately $0.2 \times 10^{-3}$ N NaOH final concentration) or UBP302 (final concentrations, $50 \mu$M in $0.2 \times 10^{-3}$ N NaOH). Aggregation was induced by the addition of TRAP (2 µM), and measured by change in optical density using an aggregometer (BioData PAP-4) and reported as total aggregation 5 min post TRAP.

RT-PCR

MEG-01 cells were purchased from ATCC and primary rat neurons a gift of Richard Huganir (Johns Hopkins). To generate cDNA RNA was isolated using Trizol and cDNA made using an Invitrogen kit and random hexamers. The PCR reaction was performed with primers specific for GluR5 or GluR6.

Thromboxane and MAP Kinase experiments

300 µL of PRP (approximately $6 \times 10^7$ platelets) was incubated with buffer or KA for 10 mins. PRP was pre-treated with inhibitors for at least 20 mins prior to stimulation. Platelets were pelleted after treating the PRP with indomethacin (20 µM, Sigma) to inhibit additional COX activity. Plasma was isolated for the TxB$_2$ ELISA and the platelet pellet lysed in p38 ELISA lysis buffer for measurement of P-p38. Plasma was further purified using a C-18 SPE cartridge (Amersham) prior to TxB2 measurement by EIA.
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Based on manufacture recommendations, MAPK inhibitor compounds were first dissolved in mild HCl to 20 mM, such that they went into solution and reached a neutral pH. We then diluted the compounds further in PBS for working concentrations.

In Vivo Studies

All mouse experiments were performed as approved by The Johns Hopkins University School of Medicine Animal Care and Use Committee.

For tail bleeding studies 6 week old male mice were anesthetized with ketamine and xylazine (80/13 mg/kg). UBP302 was resuspended at 25 mM in 1N NaOH and diluted to 1 mM in Tyrodes’s buffer for storage. Mice were injected intravenous (IV) with either dilution buffer or 1 mg/kg UBP302 final concentration in a total volume of 100 µL (Tyrodes again used as volume dilution). Twenty minutes later the distal 3 mm of the tail was amputated, immersed in 37°C saline and the time to visual cessation of bleeding recorded with a maximum time of 15 mins.

For intravital microscopy studies platelets were isolated from mice as above and resuspended in Tyrode’s at a concentration of 1x10^8/100 µL, fluorescently labeled with 10 µM calcein-AM, and 100 µL injected intravenously into a mouse anesthetized with ketamine and xylazine. The mesentery was externalized, thrombosis initiated by the addition of a 5 mm² piece of Whatmann’s paper soaked in 15% FeCl3 to the vessel surface for 45 sec, and thrombosis recorded using a digital imaging camera and software (Retiga, QCapture Pro). Control and treated mice used in KA inhibitor studies were C57Bl6/J (Jackson Labs). GluR6^-/- mice are on a mixed 129 and C57Bl6/J background. WT mice were littermate controls.
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Data analysis

Data are expressed as means ± standard deviation unless otherwise stated. Statistical comparisons between two groups were performed using Student's t-test.

Kanic Acid Receptor Gene Variants and Platelet Function in a Human Population

Genotype-phenotype relations were examined in 1239 Caucasians (mean age 44.6 ± 13.2; 55.0% female) and 822 African Americans (mean age 43.4 ± 12.4, 61.6% female) recruited into the Genetic Study of Aspirin Responsiveness (GeneSTAR). Full details on the study population and study protocol are reported elsewhere. Briefly, individuals without clinically apparent cardiovascular disease were recruited from white and black families with a history of premature coronary artery disease (CAD, age of onset <60 yrs). Optical platelet aggregation to collagen (5 µg/ml) and urinary excretion of 11-dehydro thromboxane B2 (Tx-M) were assessed before and after treatment with aspirin 81 mg/day for 14 days. The primary phenotypes of interest were platelet aggregation and Tx-M after aspirin treatment because these phenotypes are associated with increased risk of myocardial infarction, stroke, and cardiovascular death in aspirin-treated patients with cardiovascular disease. Genotyping was performed using the Illumina bead array platform; 148 SNPs in GluR5 and 215 SNPs in GluR6 were chosen to optimize gene coverage at 2 kb density from those available on the Illumina 1 million SNP chip, without regard to intronic or exonic status of variants. Correlation among selected SNPs was examined by linkage disequilibrium analysis using HaploView. Of the selected GluR5 SNPs, 125 fell into 43 independent LD blocks in the Caucasian sample and 116 fell into
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58 independent LD blocks in African Americans. Of the selected GluR6 SNPs, 175 fell into 74 independent LD blocks in Caucasians and 166 fell into 93 LD blocks in African Americans. Genotype-phenotype associations were determined using the likelihood ratio test implemented by the “ASSOC” subroutine of the program Merlin. The additive model was used for all tests, and all associations were adjusted for cardiac risk factors (age, sex, hypertension, current smoking, BMI, diabetes, LDL-C, and fibrinogen levels). To account for multiple comparisons, we adopted a two-part strategy: First, a conservative Bonferroni correction was applied for all SNPs in each gene; and, second, correction was made for the number of independent SNPs within each gene (i.e. number of independent LD blocks + SNPs outside any LD block). In Caucasians, significance thresholds for GluR5 were \(<0.0003\) (Bonferroni-corrected) and \(<0.001\) (LD-corrected), and for GluR6 thresholds were \(<0.0002\) (Bonferroni-corrected) and \(<0.001\) (LD-corrected). SNP’s that reached a significance threshold in Caucasians were examined for a genotype-phenotype association in African Americans. A replication threshold of \(P <0.05\) was considered significant in African Americans.

Online Supplement References


Figure 1. Platelets were treated with NMDA and stimulated with TRAP (A. 5 μM). Platelet activation was determined by FACS using PAC-1 antibody. NMDA signaling does not increase platelet activation.

Figure 2. Platelets were isolated from WT and GluR6-/- and stimulated with thrombin. Platelet activation was determined by FACS with JON/A antibody (n=5).

Figure 3. Washed platelets were treated with KA or glutamate (250 μM) or glutamate after indothacin and TbxB2 determined by EIA (n=4 ± S.D. *P<0.01).
Figure 4. Glutamate Receptor Signaling Directly Induces Platelet COX Activation. PGE2 production from platelets measured by EIA (n=4 ± S.D. *P<0.01).

Figure 5. Platelets were incubated with control PBS or AMPA (250 μM) with and without the AMPA receptor blocker CNQX (n=4 ± S.D. *P<0.01).

Figure 6. P-38 phosphorylation in response to KA. Platelets were incubated with 250 μM of KA and 0, 10, and 20 mins post KA addition platelets were lysed and P-p38 immunoblotted. Despite an overloading of time point 0, there is an increase in P-p38 at 10 mins that declines by 20 mins post KA.
Figure 7. KAR signaling increased P-ERK. Platelets were treated with KA (250 μM) or low dose thrombin (0.05 U/mL) and P-ERK was measured by ELISA (n=4 ± S.D. *P<0.01 vs Control).

Figure 8. Erk inhibitor does not blunt KA induced TbxB2 production. Platelets were treated with KA (250 μM) or KA after Erk inhibitor U0126 and TbxB2 was measured by EIA (n=3 ± S.D. N.S=not significant).

Figure 9. LD Plots.