Mechanism of Epinephrine-Induced Platelet Aggregation

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Abstract—We report that a genetic polymorphism of the α2-adrenergic receptor (A2AR) encoded by chromosome 10 is associated with hypertension and an increase in epinephrine-mediated platelet aggregation in humans. The mechanism responsible for this heritable contrast in sensitivity to epinephrine is unknown. We tested our hypothesis that epinephrine-induced platelet aggregation is mediated by activation of chloride transport. We measured epinephrine-mediated increases in optical density of gel-filtered platelets suspended in a bicarbonate-buffered physiological salt solution. Compared with platelets incubated in the control buffer (130 mmol/L NaCl), platelets incubated with either bumetanide, a Na/K/2Cl cotransport inhibitor; anthracene-9-carboxylic acid, a chloride channel blocker; or acetazolamide, an agent that blocks ATP-dependent chloride transport had significantly decreased aggregation responses to epinephrine. When measured fluorometrically, epinephrine significantly increased intraplatelet chloride concentrations. Chloride-dependent modifications of epinephrine-induced platelet aggregation were not attributable to changes in A2AR ligand binding characteristics or to the concentration of platelet cAMP. Finally, subthreshold concentrations of epinephrine also potentiated thrombin-induced platelet aggregation, and blockade of chloride transport diminished this synergistic action of epinephrine on thrombin-stimulated platelet aggregation. Heritable differences in epinephrine-mediated platelet aggregation may be attributable to genetic differences in chloride transport in platelets. Furthermore, because we observed a necessary role for chloride transport in epinephrine-mediated platelet aggregation, pharmacological agents that block chloride transport, such as diuretics, may provide salutary protection against vascular thrombosis in patients with hypertension independent of the effect of these drugs on blood pressure. (Hypertension. 1998;31:603-607.)

Key Words: adrenergic receptor ■ diuretics ■ thrombosis ■ chloride ■ humans ■ polymorphism ■ blacks ■ genetics

The mechanism by which epinephrine induces platelet aggregation is unknown. In some tissues, such as the respiratory epithelium, activation of the A2AR increases transcellular sodium and chloride cotransport.1 We reasoned that epinephrine-induced platelet aggregation could also be mediated by A2AR-dependent sodium and chloride cotransport. Accordingly, we tested our hypothesis by measuring epinephrine-mediated platelet aggregation under experimental conditions that would inhibit A2AR-mediated sodium chloride transport, and we measured epinephrine-mediated changes in intracellular chloride concentrations fluorometrically in platelets. Because platelet aggregation may also be dependent on A2AR-mediated changes in the accumulation of intracellular cAMP, we determined the effect of chloride transport inhibition on postreceptor signal transduction by cAMP.

Methods

Subject Recruitment

We recruited healthy, fasting, male and female college-age subjects who were taking no prescription medications or over-the-counter drugs. These protocols were approved by our institutional committee for the protection of humans in research, and each volunteer gave informed consent.

Platelet Aggregation Measurements

All reagents were obtained from Sigma Chemical Co unless otherwise indicated. Platelet aggregation was measured by standard aggregometry techniques based on optical density. Briefly, 45 mL of whole blood was collected from resting subjects in a citrate anticoagulant (final ratio, 9:1 whole blood/citrate). PRP was obtained after centrifugation of the whole blood at 200g for 15 minutes. PPP served as the appropriate blank, and it was obtained by centrifugation of an aliquot of the PRP at 17 000g for 3 minutes. The remainder of the PRP was washed over a Sepharose 2B gel column with a PSS of the following composition (in mmol/L): NaCl 130, KCl 4.7, NaHCO3 14.9, KH2PO4 1.18, MgSO4·7H2O 1.17, and CaCl2 1.6, pH=7.4. In some experiments, the chloride concentration of the PSS was decreased by substitution of chloride with an iso-osmotic concentration of sodium gluconate. To study the role of chloride/bicarbonate countertransport, experiments were also performed in which we replaced HEPES buffer (10 mmol/L, pH 7.4) with sodium bicarbonate PSS. Chloride transport was also blocked in some experiments by preincubation of the platelets for at least 10 minutes in PSS to which either 0.5 mmol/L acetazolamide, 5 μmol/L anthracene-9-carboxylic acid, or 50 μmol/L bumetanide had been added. In every case, each subject served as his or her own control. Platelet aggregation induced by 3 μmol/L and 10 μmol/L epinephrine was measured in 390 μL of PRP after 5 minutes in an aggregometer (Chronolog Co) at 37°C. These concentrations of epinephrine represented ED50 and ED100 doses, respectively, for platelet aggregation under these experimental conditions.7 Platelet aggregation was defined as the difference in light

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transmission measured in PPP and PRP. Finally, because epinephrine could potentiate aggregation induced by other agonists, changes in optical density in response to gamma thrombin were also measured in PRP that had been incubated with subthreshold concentrations of epinephrine (0.3 μmol/L).

**Determinations of Platelet [Cl]i**

Venous blood from healthy volunteers was collected as described above except that the EDTA was used as the anticoagulant. PRP was then incubated with 2.5 mmol/L N-ethoxycarbonylmethyl-6-me-thoxyquinolinium bromide at 37°C for 2 hours. The PRP was centrifuged at 1000 g, and the resulting pellet was washed with 5 mL of calcium-free PSS and resuspended in 6 mL of bicarbonate PSS. Fluorometric data were obtained with a spectrophotometer (Spex Fluorolog, Spex Industries) at 37°C. An excitation wavelength of 359 nm and an emission wavelength of 464 nm were used with monochromator slits set at 3 nm. The platelets were diluted to 1:6 and incubated in PSS to which CaCl2 (1.6 mmol/L), epinephrine (10 μmol/L), and/or acetazolamide (0.5 mmol/L) was added. The ratio of fluorescence at time 0 and over 5 minutes was measured and compared with the ratios obtained with a known [Cl]i determined from a calibration curve with a double-ionophore technique.

**Platelet A2AR Binding Studies**

A2AR receptor binding and number were determined as described previously4 with Scatchard analysis of [3H]yohimbine binding to platelets incubated in different buffers. Briefly, platelets were isolated after centrifugation of 10 mL of PRP. Aliquots of platelets were then washed and suspended in equal volumes of an isotonic buffer containing 50 mmol/L Tris-HCl, 100 mmol/L NaCl, and 5 mmol/L EDTA (pH 7.5) or in a Tris-HCl buffer in which the NaCl was replaced with 100 mmol/L choline chloride or 100 mmol/L sodium gluconate. Platelets were then incubated with increasing concentrations of [3H]yohimbine (1.0 to 20.0 nmol/L, Amersham) with a specific activity of 84.5 mCi/mol. Phentolamine (2 mmol/L, Regi-tine, CIBA) was used to block specific binding. The reaction was allowed to proceed at room temperature for 45 minutes, and then the reaction was stopped by the addition of ice-cold buffer and rapid filtration. The amounts of bound and free ligand were calculated, and the saturation binding isotherms were derived by Scatchard analysis as described.

**Platelet cAMP**

Whole blood (20 mL) was collected in EDTA, and PRP again was obtained by low-speed centrifugation. Platelet concentrations were adjusted to 109 platelets/μL with a calcium-free PSS. Epinephrine-mediated inhibition of forskolin-stimulated (10 μmol/L) adenylate cyclase activation was measured in the presence of 1 mmol/L isobutylmethylxanthine in 500-5000 μL aliquots of platelets incubated for 1 hour in the bicarbonate-buffered PSS, HEPES/bicarbonate-free PSS, or bicarbonate-buffered PSS with 1 mmol/L acetazolamide at 37°C. The reaction was terminated with the addition of ice-cold ethanol, and platelet cAMP was extracted and measured with a commercially available radioimmunoassay (Amersham) following the directions of the manufacturer.

**Statistical Analysis**

Statistical analysis was performed using Excel 5.0 (Microsoft). A Student’s t test was used to compare mean and median responses between treatment groups, and Bonferroni adjustments were made for multiple comparisons. In cases in which variances between comparison groups were unequal, the nonparametric Wilcoxon rank sum test was used. All values are expressed as mean±SEM, and P = .05 was considered significant.

**Results**

Attenuation of chloride transport by relatively low concentrations of various pharmacological agents significantly decreased epinephrine-mediated platelet aggregation. As demonstrated in Fig 1, inhibition of chloride transport by antagonists relatively specific for various chloride transporters such as anthracene-9-carboxylic acid, bumetanide, or acetazolamide, or inhibition of the chloride/bicarbonate exchanger by substitution of a bicarbonate buffer with HEPES, all decreased A2AR-mediated platelet aggregation. Furthermore, epinephrine markedly increased [Cl]i, as shown in Fig 2; this A2AR-dependent change in platelet [Cl]i, was calcium-dependent. In the absence of physiological concentrations of extracellular calcium, baseline [Cl]i was much higher in the unstimulated platelets (110±6 mmol/L) than in platelets incubated in 1.6 mmol/L calcium (53±9 mmol/L, P<.05), and the addition of epinephrine caused no increase in the intraplatelet chloride concentration over the baseline value (data not shown).

We next used [3H]yohimbine binding to quantify A2AR and binding affinity in platelets. When platelets were incubated in sodium-free PSS, A2AR binding was completely inhibited (data not shown); however, incubation of platelets in a buffer in which chloride was replaced iso-osmotically with gluconate modestly increased the number of receptors available for ligand binding (Fig 3), and this manipulation tended to increase the affinity of this receptor for its ligand. Replacement of NaCl with sodium gluconate increased A2AR number (values expressed in binding sites/platelet±SEM) from 66±10 to 88±12 (P<.05) without any significant change in the affinity binding constant (values expressed in mmol/L±SEM): 1.27±0.20 versus 0.91±0.07 (P=NS). Neither bumetanide nor acetazol-
amidine had any effect on \(^{[3H]}\)yohimbine binding to platelets (data not shown).

A2AR-mediated platelet aggregation can be dependent on changes in the intraplatelet concentration of cAMP. As demonstrated in Fig 4, inhibition of chloride/bicarbonate exchange by incubation of the platelets in a bicarbonate-free HEPES buffer not only significantly attenuated forskolin-stimulated increases in cAMP, but this maneuver also decreased epinephrine-mediated reductions in cAMP in forskolin-stimulated platelets. On the other hand, inhibition of chloride transport by acetazolamide had no discernible effect on epinephrine-induced changes in cAMP concentrations in forskolin-stimulated platelets (Fig 4).

Localized increases in endogenous epinephrine could be directly responsible for the precipitation of thrombotic events in humans. However, it is equally likely that epinephrine potentiates the proaggregatory effect of other autacoids, such as thrombin. Indeed, we found that subthreshold concentrations of epinephrine dramatically augmented aggregation in washed platelets that were stimulated with \(\gamma\)-thrombin; this potentiation by epinephrine was dependent on chloride, and this synergistic action of epinephrine was blocked by yohimbine, a specific A2AR antagonist (Fig 5).

**Discussion**

The gene for the A2AR encoded by chromosome 10 is polymorphic, and Southern blotting with a cDNA probe after restriction enzyme digestion of this gene results in fragments of either 6.7 or 6.3 kb in size.\(^2\) In a population-based study of urban Detroit blacks, we reported a strong association between homozygosity for the 6.3-kb allele of the C10 A2AR gene and hypertension.\(^2\) Subsequently, we noted that normotensive individuals carrying at least one allele of the 6.3-kb C10 A2AR had increased epinephrine-mediated platelet aggregation compared with individuals homozygous for the 6.7-kb allele, and

transfection studies with these genotypes demonstrated that this polymorphism was functional.\(^2\) We theorized that the increased frequency of this 6.3-kb allele in blacks compared with whites can place blacks at risk for stroke or coronary thrombosis, and we postulated that increased platelet aggregation may be the result of genetic variation and heritability in epinephrine-mediated platelet aggregation that occurs independently of pathological elevations in blood pressure in this ethnic group.\(^2\) Accordingly, we deemed it necessary to determine the mechanism by which epinephrine mediates platelet aggregation.

The mechanism by which A2AR ligands induce platelet aggregation was unknown. We found that manipulations designed to inhibit chloride transport in platelets were associated with attenuation of epinephrine-mediated platelet aggregation. Specifically, inhibition of Na\(^{+}/K^{+}/2Cl^{-}\)cotransport with bumetanide, blockade of anion channels with anthracene-9-carboxylic acid, or inhibition of Cl\(^{-}/\text{HCO}_3\) exchange by substituting bicarbonate buffer with HEPES all diminished A2AR-mediated platelet aggregation. Acetazolamide most clearly inhibited epinephrine-mediated platelet aggregation. Acetazolamide is believed to block ATP-dependent chloride transport.\(^7\) Alternatively, when carbonic anhydrase is inhibited by acetazolamide, there is a decrease in the intraplatelet bicarbonate concentration. This decrease in [HCO\(_3^-\)], can result in a loss of bicarbonate/chloride exchange and a subsequent reduction in [Cl\(^{-}\)]. Furthermore, inhibition of carbonic anhydrase with acetazolamide results in the equimolar loss of a strong acid (H\(^+\)) and comparatively weak base (HCO\(_3^-\)), and a net intracellular alkalinization ensues. It remains to be seen whether the acetazolamide-induced increase in intraplatelet pH diminishes platelet responsiveness to epinephrine and
thrombin. Indeed, when measured fluorometrically, acetazolamide increased intraplatelet pH by ≈0.2 pH units (data not shown). It is likely that epinephrine-mediated increases in intraplatelet chloride concentration contribute, in part, to A2AR–dependent aggregation; our data support this hypothesis.

The interrelationship among $[\text{Cl}^-]_i$, chloride movement through conductance channels, and ATP-dependent chloride transport in post-A2AR receptor signal transduction in platelets is uncertain. Although it has been reported that a reduction in cellular calcium concentration of renal mesangial cells, it is possible that chloride fluxes in platelets contribute to the control of the intracellular calcium milieu in thrombocytes, and changes in intraplatelet calcium may ultimately control A2AR–dependent platelet aggregation. Alternatively, it could also be argued that the dependence of epinephrine-mediated increases in chloride on extracellular calcium is attributable to an effect of epinephrine on calcium–dependent chloride transport. The exact nature of the interaction between intracellular calcium and chloride in modulating platelet responsiveness remains unclear. The argument for a primary role for chloride in platelet aggregation, however, is supported by the observation that U46619, a synthetic thromboxane agonist, also induces platelet aggregation through a chloride-dependent mechanism.

It has been reported that epinephrine serves primarily to increase platelet aggregation induced by other autacoids. Our data on the effect of epinephrine on potentiating thrombin-induced platelet aggregation were striking. It remains to be determined whether epinephrine acts synergistically with other autacoids in addition to thrombin in facilitating thrombosis. Furthermore, it is exciting to speculate that other vasoactive effects of thrombin, such as the mitogenic property of this autacoid, could be potentiated by the A2AR. Should this prove to be the case, it would be of interest to determine whether increases in $[\text{Cl}^-]_i$, in endothelial and vascular smooth muscle cells are also dependent on the net flux of chloride into the cell. We have found that A2AR–mediated vascular contraction is dependent on the presence of extracellular chloride in preparations of isolated vascular smooth muscle (unpublished observations), and others have reported that the increased vascular reactivity found in deoxycorticosterone/salt hypertension is attributable to abnormalities in ATP-dependent chloride transport in blood vessels.
Recently, debate has focused on the selection of first-line agents in the treatment of hypertension. The goal in the treatment of these patients should not be limited to the reduction in blood pressure but, instead, to reduce the morbidity associated with hypertension, such as the increased prevalence of strokes and coronary thrombosis. Agents that specifically block chloride transport may decrease thrombosis independent of their effect of blood pressure.

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References
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