Enhanced Platelet Cyclic AMP Response to Prostaglandin £\textsubscript{1} in Essential Hypertension

THERÈSE J. RESINK, ERNST BÜRGISER, AND FRITZ R. BÜHLER

SUMMARY Platelets provide an accessible and homogeneous cellular system for investigative studies on hypertension. Hypertension-associated abnormalities of cyclic adenosine 3',5'-monophosphate (AMP) metabolism were studied in human platelets. Platelets from hypertensive subjects had an enhanced cyclic AMP accumulation response to prostaglandin £\textsubscript{1} (twofold increase in prostaglandin £\textsubscript{1} sensitivity). The degree of adenylate cyclase activation in response to both prostaglandin £\textsubscript{1} (receptor-mediated) and forskolin (non-receptor-mediated) was greater in hypertensive than normotensive subjects, and prostaglandin £\textsubscript{1}-stimulated and forskolin-stimulated adenylate cyclase activity correlated directly ($r = 0.71$, $p<0.001$, $n = 26$). This finding suggests that the catalytic subunit of the enzyme is the rate-limiting step of this hormonal information transduction. Platelets from hypertensive subjects were more sensitive to epinephrine-induced inhibition of the stimulatory effects of prostaglandin £\textsubscript{1} on both cyclic AMP accumulation (fourfold) and activation of cyclic AMP-dependent protein kinase. These findings suggest that the enhanced cyclic AMP metabolic response to prostaglandin £\textsubscript{1} in platelets from subjects with established essential hypertension may function as a negative feedback mechanism to protect the cells against calcium overload and to reduce their stimulated participation in hemostatic and thrombotic processes. (Hypertension 8: 662-668, 1986)

KEY WORDS • hypertension • human platelets • cyclic AMP • cyclic AMP-dependent protein kinase • prostaglandin £\textsubscript{1}

BOTH cyclic nucleotides and calcium play substantial roles in blood pressure regulation. A generalized defect in cellular Ca\textsuperscript{2+} handling is involved in the failure of blood pressure regulation.\textsuperscript{1,2} Abnormalities in the vascular metabolism of cyclic nucleotides in both humans and experimental animals have also been observed in hypertension, although several discrepancies exist between studies.\textsuperscript{3} Controversial observations regarding both direction and magnitude of anomalies have been made with respect to tissue concentrations of cyclic adenosine 3',5'-monophosphate (AMP),\textsuperscript{4,5} and the activities of the enzymes adenylate cyclase,\textsuperscript{5,6} and cyclic nucleotide phosphodiesterase,\textsuperscript{5,7,8} which control cyclic AMP levels. Controversy also exists concerning the activity of the target enzyme of cyclic AMP, cyclic AMP-dependent protein kinase.\textsuperscript{9,10} These qualitative and quantitative discrepancies in vascular cyclic AMP metabolism are partly due to difficulties in working with tissues of heterogeneous cell type and variations in experimental cellular environmental conditions, while further complications arise through factors associated with age and type of hypertension.\textsuperscript{3}

Blood platelets are an easily accessible and homogeneous model for studying cellular reactions to external stimuli. The hormonal regulation of both smooth muscle and platelet function is mediated by two functionally opposing “second messenger” systems, namely, cyclic AMP (inhibitory)\textsuperscript{11,12} and calcium (stimulatory).\textsuperscript{13,14} These second messengers also display opposing influences on their relative concentrations. For example, calcium itself is a potent inhibitor of adenylate cyclase,\textsuperscript{15} whereas the effects of calcium are overcome by conditions that elevate intracellular cyclic AMP.\textsuperscript{11,16}

Platelets from hypertensive subjects have been shown to have an elevated basal cytosolic free calcium concentration that is directly correlated with the height of blood pressure.\textsuperscript{17,18} In view of the bidirectional control of platelet function by calcium and cyclic AMP, the abnormality in cytoplasmic free calcium concentration may be associated with anomalies in platelet...
cyclic AMP metabolism in persons with essential hypertension. We have therefore investigated adenylate cyclase activities in platelet membranes from normotensive and hypertensive subjects. The hormone responsiveness of washed intact platelets from these groups also was examined in terms of cyclic AMP accumulation and activation of cyclic AMP-dependent protein kinase.

**Methods**

**Materials**

The [8-3H]cyclic AMP (25 Ci/mmole), [α-32P]adenosine 5'-triphosphate (ATP; 300 Ci/mmole), and [α-32P]ATP (50 Ci/mmole) were purchased from Amer- sham International, Amersham, England. Tris basic so- dium citrate (3.64%) was obtained from CIBA-Geigy AG, Basel, Switzerland. Epinephrine (I-adrenaline-dhydrogen tartrate) was obtained from Fluka AG, Buchs, Switzerland; forskolin (β-acetyloxy-8,13α-epoxy-1α,β,δ,9α-hydroxy-abd-14-en-11-one) from Calbiochem-Behring, La Jolla, CA, USA; prostagladin E1 (PGE1) from Serva, Heidelberg, Germany. Dowex AG 50W-X4 (200–400 mesh) was bought from BioRad Laboratories, CA, USA, and neutral alu- minum WN-3 from Sigma Chemical, St. Louis, MO, USA. All other chemicals were of analytical grade and were purchased from Merck, Darmstadt, Germany, or from Sigma.

**Subjects**

The first study was composed of nine healthy normotensive volunteers (4 women, 5 men; mean age, 29 ± 4 years) with a resting blood pressure (Korotkoff phase V) of 140/85 mm Hg or less (average, 126 ± 4/79 ± 3 mm Hg) on at least three different occasions and nine subjects with established essential hypertension (3 women, 6 men; mean age 46 ± 4 years) and blood pressure averaging 149 ± 4/97 ± 2 mm Hg. Platelet intracellular free Ca2+ concentrations ([Ca2+]i) were determined using the fluorescent quin-2 dye technique and were significantly higher in the hypertensive than in the normotensive subjects (147 ± 6 vs 99 ± 5 nM; p < 0.001).

A second study was composed of 15 healthy normotensive volunteers (4 women, 11 men, mean age, 36 ± 4 years) with a resting blood pressure of 140/85 mm Hg or less (average, 126 ± 4/81 ± 3 mm Hg) and 11 subjects with established essential hypertension (6 women, 5 men; mean age, 46 ± 5 years) and blood pressure averaging 168 ± 8/105 ± 4 mm Hg. Platelet [Ca2+]i was 107 ± 6 nM for normotensive subjects and 167 ± 10 nM for hypertensive subjects (p < 0.001).

There was no overlap in essential hypertensive (EHT) subjects between study groups, whereas six normotensive (NT) subjects from the first study were included in the second study. The NT volunteers were not taking any medication and had no known family history of hypertension. The EHT subjects had not received any (antihypertensive) therapy for at least 6 weeks before the study. Secondary forms of hyperten-
Measurement of Cyclic AMP–Dependent Protein Kinase Activity

Platelets (5 × 10⁶ cells/ml) were incubated at 37°C in Buffer A and treated for 30 seconds without additions, with PGE₁ alone (10⁻⁶-10⁻⁴ M), or with PGE₁ (10⁻⁶ M) plus epinephrine (10⁻⁵-10⁻⁴ M). Thereafter, 50-μl platelet samples were treated with 200 μl of ice-cold lysis buffer containing 200 mM morpholineethanesulfonic acid NaOH (pH 6.9), 1 mM EDTA, and 0.2% Triton X-100 and immediately placed in liquid nitrogen. After thawing at 4°C, samples were assayed for protein kinase activity essentially using the phosphocellulose filter method described by Roskoski.²² The reaction mixture (final volume, 50 μl) contained 50 mM morpholineethanesulfonic acid NaOH (pH 6.9); 2.5 mM magnesium acetate; bovine serum albumin, 1 mg/ml; 0.1 mM EDTA; 0.1 mM Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly; Sigma); 0.1 mM [α-³²P]ATP (200,000 cpm/nmol); and where necessary, 0.01 mM cyclic AMP. The reaction was initiated by the addition of lysed extract (10 μl; 0.1–0.15 mg/ml), and samples were incubated for 10 minutes at 37°C. Following incubation, 35-μl aliquots were withdrawn, spotted onto Whatman P81 phosphocellulose squares (Clifton, NJ, USA), and immersed in 75 mM phosphoric acid to terminate the reaction.²³ The squares were washed repeatedly in 75 mM phosphoric acid and dried, and radioactivity was determined by scintillation spectrometry (Quicksintz, Zinss Analytic, Frankfurt, Germany). Appropriate controls were performed to correct for endogenous phosphorylation. The activity ratio, which expresses the activity measured in the absence of cyclic AMP divided by the activity measured in the presence of cyclic AMP, is proportional to the percentage of free catalytic subunit in a sample.²³ Freezing and thawing had no appreciable effect on activity ratios. One unit of protein kinase activity is defined as that amount that catalyzes the incorporation of 1.0 nmol [³²P]phosphate from [α-³²P]ATP into substrate per minute in the above assay.

Determination of Adenylate Cyclase Activity

Adenylate cyclase activity was measured by the method of Salomon²⁴ with the following modifications. Washed platelet membranes (20 μg protein) were added to an incubation mixture (final volume, 60 μl) containing 1 μCi of [α-³²P]ATP, 0.1 mM ATP, 0.1 mM cyclic AMP, 10 mM MgCl₂, 1 mM EDTA, 2 mM dithiothreitol, 2.8 mM phosphoenolpyruvate, pyruvate kinase (5.2 μg/ml), and myokinase (10 μg/ml) in 50 mM Tris HCl (pH 7.4 at 37°C). Incubations were performed in either the absence or presence of hormones (10⁻³ M epinephrine, 10⁻³ M forskolin, 10⁻³ M PGE₁) at 37°C for 15 minutes. The reaction was terminated at 0°C by adding 1 ml of an ice-cold solution containing 0.2 mg ATP, 0.1 mg cyclic AMP, and [³H]cyclic AMP (20,000 cpm). Samples were stored overnight at −20°C, and after thawing at 4°C, the tubes were centrifuged at 1600 g for 15 minutes at 4°C to remove membrane protein precipitates. Cyclic AMP was isolated by sequential Dowex and alumina chromatography,²⁴ and corrections were made for recovery using [³H]cyclic AMP as an internal standard. Then, 3.5 ml of the eluate was mixed with 6 ml of scintillation fluid (Atomlight, New England Nuclear), and the radioactivity of the two isotopes was counted in a scintillation spectrometer.

Determination of Protein Concentration

Protein concentrations were determined colorimetrically²⁵ using bovine serum albumin (A₂₈₀ = 6.5) as standard.

Statistical Analysis

The p values were obtained from the unpaired Student’s t test. Sigmoidal log dose-response curves for each subject were analyzed according to a logistical four-parametric function as described by De Lean et al.²⁶ using a weighted nonlinear regression computer program.²⁷ The EC₅₀ denotes the concentration of agent inducing half-maximal stimulatory effects; IC₅₀ denotes the concentration of agent inducing half-maximal inhibitory effects.

Results

Similar basal levels of cyclic AMP were observed in isolated washed platelets from NT and EHT subjects (Table 1). Platelets were more sensitive to stimulation by PGE₁ in EHT than in NT subjects (Figure 1; see Table 1. Cyclic AMP Concentration Response to Prostaglandin E₁ and Cyclic AMP–Dependent Protein Kinase Activities in Platelets from Normotensive and Hypertensive Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Cyclic AMP concentrations (pmol/10⁶ cells)</th>
<th>Cyclic AMP–dependent protein kinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal (±10 μM PGE₁)</td>
<td>PGE₁ EC₅₀ Basal (±10 μM PGE₁) Activity ratio</td>
</tr>
<tr>
<td>Normotensive (n = 9)</td>
<td>1.16 ± 0.34</td>
<td>0.731 ± 1.60</td>
</tr>
<tr>
<td>Hypertensive (n = 9)</td>
<td>1.07 ± 0.25</td>
<td>0.371 ± 0.054*</td>
</tr>
</tbody>
</table>

Total cyclic AMP concentrations and cyclic AMP–dependent protein kinase activities were determined following incubation of intact platelets at 37°C, for 30 seconds in the absence or presence of PGE₁ (10⁻⁶-10⁻⁴ M) as detailed in Methods. Specific activities for cyclic AMP–dependent protein kinase represent activities in the presence of 10 μM cyclic AMP. Values are means ± SEM of nine separate experiments for each subject group.

PGE₁ = prostaglandin E₁; EC₅₀ = effective concentration, 50%.
*p < 0.01, compared with values in normotensive subjects.
CYCLIC AMP AND PLATELETS IN HYPERTENSION

Prostaglandin

FIGURE 1. Concentrations of cyclic AMP in response to various doses of prostaglandin E, (PGE,) in platelets from normotensive and hypertensive subjects. Total cyclic AMP concentrations were determined after treatment of isolated platelets with various concentrations of PGE, for 30 seconds at 37°C, as described in Methods. Data represent typical dose-response curves for a single normotensive (○) and a single hypertensive (●) subject. Calculated EC50 values for PGE, in both groups are given in Table 1.

Table 1). At saturating concentrations of PGE, no difference in cyclic AMP accumulation between NT and EHT subjects was observed (see Table 1). The relative amounts of cyclic AMP–dependent protein kinase (U/mg) were similar in platelets from NT and EHT subjects (see Table 1). Neither the basal activity ratios (− cyclic AMP/+ cyclic AMP) nor the activity ratios at saturating concentrations of PGE, were different between platelets from NT and EHT subjects (see Table 1). The calculated EC50 values for PGE, stimulation of cyclic AMP–dependent protein kinase were comparable in the two groups (NT, 17 ± 5 nM vs EHT, 15 ± 8 nM; complete data not shown).

In platelets from both NT and EHT subjects, epinephrine produced a dose-dependent inhibition of the stimulatory effects of PGE, on cyclic AMP concentrations and cyclic AMP–dependent protein kinase activity ratios (Figure 2). However, the platelets from EHT subjects exhibited an enhanced sensitivity to inhibition by epinephrine with respect to both cyclic AMP (see Figure 2A) and cyclic AMP–dependent protein kinase (see Figure 2B). Calculated IC50 values for epinephrine inhibition of PGE,–induced cyclic AMP production were different between NT and EHT subjects (0.168 ± 0.082 vs 0.043 ± 0.0016 μM; p < 0.01). Similarly, the IC50 for epinephrine inhibition of PGE,–induced activation of cyclic AMP–dependent protein kinase in EHT subjects was lower than that for NT subjects (0.061 ± 0.021 vs 0.224 ± 0.027 μM; p < 0.01). Under the given experimental conditions there was no measurable epinephrine effect on basal (non-PGE,–stimulated) cyclic AMP concentrations and protein kinase activity (data not shown).

In platelet membranes, the receptor-mediated activation of adenylate cyclase by PGE, and the non–receptor-mediated stimulation of cyclase by forskolin (which thus far is believed to act directly on the catalytic subunit) were enhanced in EHT as compared with NT subjects (p < 0.05 for PGE, and p < 0.001 for forskolin; Table 2). Non–receptor-stimulated activation of adenylate cyclase by forskolin correlated with PGE,–receptor-stimulated adenylate cyclase activity (n = 26, r = 0.71, slope = 1.55, p < 0.001; Figure 3). There was also a direct correlation between epinephrine-induced inhibition of basal adenylate cyclase activity and inhibition of PGE,–stimulated activity (n = 26, r = 0.89, slope = 0.89, p < 0.001; Figure 4).

Discussion

Our study provides evidence that platelet cyclic AMP metabolism is altered in EHT subjects. In intact platelets the cyclic AMP accumulation response to both stimulatory (PGE,) and inhibitory (epinephrine) hormones was enhanced in platelets from EHT subjects compared with those from NT controls. In the cell-free system, platelet membrane adenylate cyclase from EHT subjects exhibited an enhanced response to both receptor-mediated (PGE,) and non–receptor-
TABLE 2. Activation of Membrane Adenylate Cyclase by Prostaglandin E\textsubscript{1} and Forskolin

<table>
<thead>
<tr>
<th>Group</th>
<th>Adenylate cyclase activity ratio (stimulated/basal)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PGE\textsubscript{1} (10\textsuperscript{-5} M)</td>
</tr>
<tr>
<td>Normotensive (n=15)</td>
<td>7.87 ± 0.43</td>
</tr>
<tr>
<td>Hypertensive (n=11)</td>
<td>9.30 ± 0.37*</td>
</tr>
</tbody>
</table>

Adenylate cyclase activity was measured in platelet membranes as detailed in Methods. Data are means ± SEM. Activation of adenylate cyclase by prostaglandin E\textsubscript{1} (PGE\textsubscript{1}) and forskolin is expressed as an activity ratio that represents activity measured in the presence of stimulating agent divided by the activity measured in the absence of any additions (basal).

*\(p < 0.05\), †\(p < 0.001\), compared with values in normotensive subjects.

mediated (forskolin) stimulation. The data support a likely involvement of the catalytic subunit of adenylate cyclase in the pathophysiology of hypertension.

The enhanced cyclic AMP accumulation response to PGE\textsubscript{1}, confirms earlier observations for platelets from spontaneously hypertensive rats (SHR).\textsuperscript{3, 32} Hamet et al.\textsuperscript{32} have reported a transient epinephrine-induced decrease in cyclic AMP concentrations in platelets from control rats but not from SHR, although we were unable to measure an inhibitory effect of epinephrine on basal cyclic AMP concentrations in platelets from either NT or EHT subjects. These results are not necessarily discrepant but rather reflect the sensitivity limitations of assay procedures under our experimental conditions. Alternatively, the discrepancies may be due in part to differences in \(\alpha\)-adrenergic properties between rat and human platelets.\textsuperscript{32}

Simultaneous treatment of platelets with PGE\textsubscript{1} and epinephrine indicated an enhanced epinephrine inhibition of PGE\textsubscript{1}-stimulated cyclic AMP synthesis in EHT subjects. This finding confirms earlier reports from this laboratory\textsuperscript{18, 19} on increased human platelet [Ca\textsuperscript{2+}], responsiveness to \(\alpha\)-adrenergic stimulation in platelets from EHT subjects. One hypothesis for altered epinephrine responsiveness relates to receptor density or affinity, or both. However, radioligand binding studies have indicated that neither platelet \(\alpha\)-adrenergic receptor number nor affinity is altered in borderline hypertensive subjects\textsuperscript{30} or in treated and untreated subjects with established essential hypertension.\textsuperscript{31} Since both epinephrine and PGE\textsubscript{1} exert their effects by way of adenylate cyclase (inhibition and stimulation, respectively),\textsuperscript{32} an alternative hypothesis explaining modified hormone responsiveness might relate to receptor-cyclase coupling.

Hormone-sensitive adenylate cyclase is composed of at least three separate proteins: the hormone receptor; the catalytic subunit, which converts ATP into cyclic AMP; and the N proteins (N\textsubscript{1}, N\textsubscript{2}), which are modulated by guanine nucleotides and serve as a shuttle between the hormone receptor and the catalytic subunit.\textsuperscript{33} Our observation of enhanced cyclase responsiveness to PGE\textsubscript{1} and forskolin in EHT subjects suggests that enhanced catalytic subunit activity may
account for the phenomenon of increased cyclic AMP accumulation of platelets from EHT subjects. Increased cyclase responsiveness to PGE, has also been demonstrated in platelets from SHR.38

Although PGE, and epinephrine operate through distinct guanyl nucleotide binding proteins (stimulatory N, and inhibitory N, respectively), they share a common catalytic subunit.34 In this study, a direct correlation between epinephrine-induced inhibition of basal and PGE-stimulated adenylyl cyclase of platelet membranes was found. This finding, together with the direct correlation between non-receptor-mediated (forskolin) and receptor-mediated (PGE,) stimulation of cyclase, suggests that the catalytic subunit of cyclase is the rate-limiting component of the receptor-effector system. Recent studies38 on the receptor-cyclase coupling protein of erythrocytes have indicated that N protein activity, as assayed after reconstitution into cye^- S49 membranes, is not different between EHT and NT subjects. Although purification and reconstitution studies (see References 33 and 34 for reviews) indicate a high degree of conservation of N proteins in different species and cell types, we cannot completely exclude altered N protein activity in platelets from EHT subjects. Nevertheless, the present data support a more likely involvement of the catalytic subunit of adenylyl cyclase in the pathophysiology of hypertension.

Intracellular cyclic AMP levels are also controlled by cyclic nucleotide phosphodiesterase,3 and the activities of this enzyme have been found to be unaltered,3 elevated,3 or decreased3 in the aorta and heart of SHR. The possibility of anomalous platelet phosphodiesterase activity in EHT subjects was not investigated in this study but does not appear to be primarily responsible for the observed cyclic AMP concentration differences between EHT and NT subjects, since neither basal nor maximally stimulated or inhibited cyclic AMP levels were significantly different between groups.

A fraction of the intracellular cyclic nucleotides generated in response to hormones is known to be released from the stimulated cells, and it serves to lower cellular cyclic nucleotides and provide a source of extracellular cyclic nucleotides.34,37 In this study, measurements of platelet cyclic AMP concentrations included both the intracellular and possibly extruded extracellular nucleotides. In turkey erythrocytes and WI-38 fibroblasts, hormone-mediated egression of cyclic AMP was shown to occur much later (10–20 minutes) than the initial (30–60 seconds) intracellular accumulation of this cyclic nucleotide.37 Thus, under the experimental conditions described in the present report, the measurements of platelet cyclic AMP concentrations probably reflect only intracellular levels of accumulation. An increase in plasma cyclic AMP has been correlated with an increase in systolic blood pressure in subjects injected with epinephrine.38 Prostaglandins have also been shown to inhibit cyclic AMP egression in many cell types.34,37 The relevance of these observations to platelet cyclic AMP metabolism in persons with essential hypertension is unclear. A study of the effects of long-term exposure to hormones such as PGE, and epinephrine is needed to determine whether modulation of platelet cyclic AMP egression plays a role in hypertension.

It is also necessary to consider whether the observed alterations in cyclic AMP metabolism are relevant to the activity of cyclic AMP-dependent protein kinase, since this enzyme plays a major role in regulating platelet Ca^2+ flux and contractile protein function.11 An increase10 and a reduction,3 as well as no change,39 in cyclic AMP–dependent protein kinase activity have been reported for the aorta of SHR. In the present study, the response of this enzyme to hormone-stimulated increases in cyclic AMP was comparable between platelets from NT and EHT subjects, and levels of cyclic AMP–dependent protein kinase were not different between groups. Thus, any involvement of cyclic AMP–dependent protein kinase in altering platelet function in persons with essential hypertension would appear to be indirect and dependent on alterations in the relative concentrations of circulating stimulatory and inhibitory humoral factors.

The increased platelet responsiveness to epinephrine in essential hypertension found in this study and elsewhere17,18 suggests an enhanced platelet activation potential, which may contribute to enhanced stimulated vasoconstriction and an increased risk for coronary infarction and cerebrovascular accidents in hypertension.40 We propose that the enhanced adenylyl cyclase stimulation and cyclic AMP accumulation response to PGE, in platelets from hypertensive persons reflects a potentiated negative feedback controlling mechanism. Such an adaptive mechanism may function to protect the platelets against calcium overload and to dampen their participation in hemostatic and thrombotic processes.

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