Effects of Angiotensin II and Endothelin-1 on Platelet Aggregation and Cytosolic pH and Free Ca\(^{2+}\) Concentrations in Essential Hypertension

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The aims of this study were to determine the relations between platelet free calcium concentrations ([Ca\(^{2+}\)]), intracellular pH (pH\(_i\)), and aggregation and to assess the effects of angiotensin II (Ang II) and endothelin-1 on these platelet parameters in normotensive subjects and hypertensive patients. Seventeen normotensive subjects, 25 untreated hypertensive patients, and 34 treated hypertensive patients were studied. Platelet cytosolic free [Ca\(^{2+}\)], and pH\(_i\) were measured spectrofluorometrically using specific fluorescent probes (fura 2-AM and BCECF-AM, respectively) in unstimulated and Ang II- and endothelin-1-stimulated platelets. Aggregation was measured by a turbidometric technique. Basal [Ca\(^{2+}\)]\(_i\) (141±11 nmol/L) and pH (7.16±0.01) were higher (P<.05) in the untreated hypertensive group compared with the normotensive (118±9 nmol/L, 7.11±0.01, respectively) and treated hypertensive (121±11 nmol/L, 7.12±0.01, respectively) groups. In the combined normotensive and hypertensive groups, there were significant correlations between [Ca\(^{2+}\)]\(_i\) and mean arterial pressure (r=.75, P<.01), pH\(_i\), and mean arterial pressure (r=.72, P<.01), [Ca\(^{2+}\)]\(_i\), and pH\(_i\) (r=.71, P<.01), [Ca\(^{2+}\)]\(_i\), and aggregation (r=.69, P<.02), and pH\(_i\), and aggregation (r=.56, P<.05). Ang II stimulation significantly increased [Ca\(^{2+}\)]\(_i\) and pH\(_i\) in the untreated hypertensive and normotensive groups. The net change in [Ca\(^{2+}\)]\(_i\), induced by Ang II was significantly higher (P<.05) in the untreated hypertensive group compared with the other groups (67±6 nmol/L for the untreated hypertensive group versus 54±5 and 29±8 nmol/L for the normotensive and treated hypertensive groups, respectively). In the presence of Ang II, thrombin-induced aggregatory responses were increased in all three groups, but the maximal response was significantly higher in the untreated hypertensive group compared with the other groups (P<.05). Endothelin-1 increased pH\(_i\) through endothelin A-receptors (effect blocked by the specific antagonist BQ-123) but had no significant effect on [Ca\(^{2+}\)]\(_i\), or aggregation. However, endothelin-1 blunted thrombin-induced platelet aggregation in normotensive subjects but not in hypertensive patients. In conclusion, increased Ang II-stimulated [Ca\(^{2+}\)]\(_i\) and pH\(_i\) in platelets of essential hypertensive patients may be associated with increased aggregatory responses. The stimulatory effect of endothelin-1 on pH\(_i\), but not on [Ca\(^{2+}\)]\(_i\), or aggregation suggests that in platelets endothelin-induced signaling pathways other than phospholipase C may be involved. The significant correlations between platelet aggregation, [Ca\(^{2+}\)]\(_i\), pH\(_i\), and blood pressure may suggest a possible link between altered platelet cation status and platelet function in hypertension.

Key Words • platelet aggregation • hydrogen ion concentration • calcium • angiotensin II • endothelins • hypertension, essential

Functional disturbances of the cell membrane resulting in altered regulation of intracellular cations and pH have been implicated in the pathogenesis of essential hypertension.\(^1\)\(^2\) Cytosolic free calcium regulates many cellular processes, including tone, contractility, and reactivity in vascular smooth muscle cells and activation, aggregation, and adhesion in platelets.\(^3\)\(^4\) Intracellular pH (pH\(_i\)), which is partially regulated by the Na\(^{+}\)-H\(^{+}\) exchanger, also controls vital cellular functions\(^5\) and provides a metabolic milieu in which the actions of other effectors are integrated.\(^6\)\(^7\) The pH changes with cell activation.\(^7\) Vasoactive peptides such as angiotensin II (Ang II), endothelin-1, and arginine vasopressin acting on membrane-bound receptors increase intracellular calcium concentrations ([Ca\(^{2+}\)]\(_i\)) and pH by stimulating phosphoinositide hydrolysis and Na\(^{+}\)-H\(^{+}\) exchange, respectively.\(^8\)\(^9\) In vascular smooth muscle these effects manifest as increased tone and may result in increased cell mass. Vasoactive peptides, in addition to altering vascular contractility, may induce or modulate the activity of platelets. The platelet-activating effects of such agents could be important in hypertension, because aggregating platelets are a source of vasoactive and mitogenic agents that may contribute to the exaggeration of vasoconstriction and play a role in the remodeling of blood vessels in hypertension.

In essential and experimental hypertension, defects in the regulation of cytosolic calcium and pH have been...
identified. Cytosolic calcium overload has been demonstrated in many cell types, including platelets, from hypertensive patients and genetic and experimental hypertensive rats. Increased Na⁺−H⁺ exchange and intracellular alkalization have been described in lymphocytes and vascular smooth muscle cells from spontaneously hypertensive rats and in platelets and leukocytes from hypertensive patients. Although most studies have reported increased cytosolic pH in hypertension, an early study demonstrated erythrocyte acidification in experimental and essential hypertension. These cellular abnormalities in hypertension may result from different mechanisms, including altered reactivity of cell membrane receptors to vasoactive agents. Increased stimulus-effect coupling in response to Ang II and endothelin-1 may be associated with the elevated [Ca²⁺], and alkalization that have been described in hypertension. Hypersensitivity to the pressor effects of Ang II as well as abnormal stimulated calcium responses have been described in patients with pregnancy-induced and essential hypertension. Although the effects of Ang II on platelet aggregation are unclear, studies have suggested that endothelin-1 has antiaggregatory properties.

The exact relations between vasoactive peptides, intracellular ions, and platelet aggregation in essential hypertension remain obscure. Platelets were examined in this study because they are an easily accessible cell model that may reflect abnormalities occurring in vascular smooth muscle cells. Also, of greater importance, platelets themselves may play a role in high blood pressure, as platelets from hypertensive patients display exaggerated aggregation, which may contribute to vascular abnormalities in hypertension. We investigated the in vitro influence of Ang II and endothelin-1 on platelet free calcium and pH as well as on thrombin-induced aggregatory responses in normotensive subjects and hypertensive patients.

Methods

Reagents

Chemicals were of the highest grade available and were obtained from Sigma Chemical Co, St Louis, Mo; Fisher Scientific Co, Fair Lawn, NJ; and BDH Inc, Darmstadt, Germany. Fura 2-AM, BCECF-AM, nigericin, and pluronic F127 were from Molecular Probes Inc, Eugene, Ore. Human Ang II, [Sar¹, Ile⁸]Ang II, bovine thrombin, human endothelin-1, and BQ-123 were from Peninsula Laboratories Inc, Belmont, Calif. 5-N,N-(Hexamethylene)amiloride was from Research Biochemicals Inc, Natick, Mass. Sarafotoxin S6c was obtained from Bachem Inc, Torrance, Calif.

Subjects and Patients

Seventy-six subjects were studied. Seventeen normotensive control subjects, 25 untreated mild to moderate essential hypertensive patients, and 34 treated hypertensive patients from the Hypertension Clinic of the Clinical Research Institute of Montreal (Canada) entered into this study. The blood pressure of the normotensive subjects was consistently less than 140/90 mm Hg. The hypertensive patients had a systolic blood pressure greater than or equal to 145 mm Hg and a diastolic blood pressure greater than or equal to 95 mm Hg on at least two separate measurements. Blood pressure was measured in the dominant arm with the patient sitting. A standard mercury sphygmomanometer was used, and the mean of three measurements was taken for analysis. Treated patients were taking one or more of the following types of agents: β-blockers, calcium channel blockers, diuretics, and/or angiotensin converting enzyme inhibitors and had been on medication for at least 1 month.

Fifteen to 20 mL of venous blood was drawn from each donor and collected into polypropylene tubes containing 75 mmol/L trisodium citrate, 42 mmol/L citric acid, and 139 mmol/L dextrose, pH 6.8, as anticoagulant (1 part to 6 parts blood). Ten milliliters of heparinized blood was used for analysis of serum biochemistry.

Determination of Intracellular Free Calcium in Intact Platelets

Platelet free [Ca²⁺], was measured by a nondisruptive fluorescent dye technique based on that described by Tsien et al. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood samples at 160g for 15 minutes at room temperature. The PRP was divided into aliquots, one each for [Ca²⁺], pH, and aggregation studies. For [Ca²⁺], measurements the PRP was incubated with 5 μmol/L fura 2-AM (diluted in 0.02% pluronic in anhydrous dimethyl sulfoxide [DMSO]) at 37°C for 30 minutes. Extrinsic dye was removed by washing the loaded platelets in a buffer containing (mmol/L) NaCl, 145; KCl, 5; MgSO₄, 1; Na₂HPO₄, 0.5; glucose, 5; and HEPES, 10, pH 7.4 at 25°C. The platelets were washed twice by centrifugation at 450g for 10 minutes at 25°C and then were diluted to 1 to 2x10⁷ cells per milliliter in the HEPES buffer. Extracellular calcium was adjusted with 1 mmol/L CaCl₂.

Fluorescent measurements for [Ca²⁺], were performed with a CAF-100 spectrofluorometer (Jasco, Japan Spectroscopic Co Ltd, Tokyo). The instrument was set at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm. Recordings were made on a Grass model 7 polygraph. The platelets were stirred continuously with a magnetic stirrer at 1000 rpm in thermostated (37°C) 1-mL cuvettes. Maximum [Ca²⁺], was achieved by addition of Triton X-100 (final concentration of 0.1%). This corresponded to complete complexation of fura 2 with Ca²⁺. Minimal [Ca²⁺], was obtained in the presence of EGTA (final concentration, 3 mmol/L). [Ca²⁺], was calculated according to the formula of Grynkiewicz et al: [Ca²⁺] = Ka(R - Rmin/Rmax - R)b, with a Ka of fura 2 for calcium taken as 224 nmol/L at 37°C, and where R is the ratio of fluorescence of the sample at 340 and 380 nm; Rmax and Rmin are the ratios for fura 2-free acid at these wavelengths in the presence of saturating Ca²⁺ (with Triton X-100) and EGTA, respectively; and b is the ratio of fluorescence at 380 nm in the presence of EGTA to the fluorescence at 380 nm in the presence of Triton X-100. To account for autofluorescence, the background fluorescence was subtracted from each reading. The intra-assay coefficient of variation for [Ca²⁺], was less than 4.4%, and the day-to-day variation in one subject was less than 3%.
TABLE 1. Clinical Characteristics and Serum Biochemistry of Normotensive and Hypertensive Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normotensive</th>
<th>Untreated Hypertensive</th>
<th>Treated Hypertensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (men/women)</td>
<td>17 (7/10)</td>
<td>25 (13/12)</td>
<td>34 (22/12)</td>
</tr>
<tr>
<td>Ql, kg/m²</td>
<td>26.0±1.0</td>
<td>26.6±1.2</td>
<td>26.1±1.8</td>
</tr>
<tr>
<td>Age, y</td>
<td>48±15</td>
<td>52±16</td>
<td>51±11</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>130±11</td>
<td>172±23*</td>
<td>136±7</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>80±5</td>
<td>101±10*</td>
<td>88±8</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>96±10</td>
<td>125±16*</td>
<td>104±8</td>
</tr>
<tr>
<td>Pulse rate, bpm</td>
<td>69±5</td>
<td>77±11</td>
<td>76±11</td>
</tr>
<tr>
<td>Serum Na⁺, mmol/L</td>
<td>140±0.48</td>
<td>140±0.91</td>
<td>140±1.82</td>
</tr>
<tr>
<td>Serum K⁺, mmol/L</td>
<td>4.4±0.26</td>
<td>4.2±0.31</td>
<td>4.1±0.31</td>
</tr>
<tr>
<td>Serum Ca²⁺, mmol/L</td>
<td>2.35±0.03</td>
<td>2.30±0.09</td>
<td>2.34±0.09</td>
</tr>
<tr>
<td>Serum GGT, U/L</td>
<td>18±7</td>
<td>22±16</td>
<td>17±13</td>
</tr>
<tr>
<td>Serum cholesterol, mmol/L</td>
<td>5.8±0.86</td>
<td>5.0±0.79</td>
<td>5.2±0.4</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>1.34±0.42</td>
<td>1.58±0.80</td>
<td>1.70±0.71</td>
</tr>
<tr>
<td>Serum creatinine, μmol/L</td>
<td>92±9</td>
<td>88±21</td>
<td>89±11</td>
</tr>
</tbody>
</table>

Ql indicates quetelet index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; bpm, beats per minute; and GGT, γ-glutamyltransferase.

*P<.05 vs other groups.

Platelet Intracellular pH Measurement

Cytosolic pH of platelets was measured with the fluorescent indicator 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) as its tetra-acetoxymethyl ester (BCECF-AM), which is a membrane-permeable intracellularly trappable pH indicator. One part PRP was incubated at 37°C for 25 minutes with 2 μmol/L BCECF-AM (from 1 mmol/L stock solutions prepared in DMSO). The final concentration of DMSO was less than 0.02%. A platelet pellet was obtained by centrifuging the PRP at 160g for 10 minutes at 20°C. The loaded platelets were washed twice in HEPES buffer (mmol/L: NaCl, 145; KCl, 5; MgCl₂, 0.5; glucose, 5; HEPES, 10, pH 7.4 at 20°C). The cells were resuspended in the HEPES buffer containing 1 mmol/L CaCl₂ and corrected to a count of 1 to 2 x 10⁸ cells per milliliter. Fluorescence was measured immediately after resuspension.

Cells (250 mL) suspended in thermostated (37°C) 1-mL cuvettes were stirred continuously at 1000 rpm in the spectrofluorometer. Fluorescence signals were recorded at excitation wavelengths of 500 and 450 nm, with an emission wavelength of 540 nm and slit of 5 nm. After establishment of a stable baseline, the agents were added. Although the platelets did not exhibit significant autofluorescence in the BCECF wavelength, the background fluorescence was subtracted from each reading. The pH was directly calculated from the ratio of excitation fluorescence intensities at 500 and 450 nm.

Calibration of pH versus fluorescence intensities was performed with the K⁺ ionophore nigericin, which sets [K⁺]/[K⁺]o, so that if cells are suspended in media in which [K⁺]o=[K⁺], [H⁺], will equal [H⁺]o. With the use of nigericin, pH can be manipulated by adjusting the external pH (pHₓ) to specific values. The fluorescence of platelets recorded at various known pHₓ (=pHᵥ) values provided a calibration curve that was used to determine platelet basal and stimulated pHᵥ. The ratio technique of Thomas et al²⁸ was used to construct the calibration curve. The difference at set pH values was correlated with fluorescence ratios (R=F500/F450) obtained when cells were incubated in K⁺-rich HEPES (mmol/L: NaCl, 20; KCl, 115; MgCl₂, 0.5; HEPES, 10; glucose, 5) before and after addition of 2 μmol/L nigericin. The change in ratio was calculated as follows:

| Table 2. Effects of Angiotensin II, Endothelin-1, Saralasin, and Amiloride on Platelet Free Calcium Concentrations in Normotensive and Hypertensive Groups |
|------------------------------------------|----------------|----------------|----------------|
| Agent                                    | Normotensive   | Untreated Hypertensive | Treated Hypertensive |
| Basal                                    | 118±9*         | 141±11**          | 121±11          |
| Ang II (1 nmol/L)                        | 172±10         | 208±10†           | 150±14          |
| Endothelin-1 (1 nmol/L)                  | 120±12         | 136±9            | ...            |
| Ang II (1 nmol/L) plus [Sar₁,Leu⁶]Ang II (10 μmol/L) | 123±10*        | 140±9*†          | ...            |
| Ang II (1 nmol/L) plus amiloride (10 μmol/L) | 133±22*        | 160±8*†          | ...            |

Ang II indicates angiotensin II.

*P<.05 vs Ang II-stimulated Ca²⁺ in the same group.
†P<.02 vs other groups.
TABLE 3. Effects of Vasoactive Peptides on Platelet pH in Normotensive and Hypertensive Groups

<table>
<thead>
<tr>
<th>Agent</th>
<th>Normotensive Basal</th>
<th>Untreated Hypertensive Basal</th>
<th>Treated Hypertensive Basal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>7.11±0.01†</td>
<td>7.16±0.01††</td>
<td>7.12±0.01</td>
</tr>
<tr>
<td>Ang II (1 nmol/L)</td>
<td>7.15±0.01</td>
<td>7.21±0.02‡</td>
<td>7.14±0.01</td>
</tr>
<tr>
<td>Endothelin-1 (1 nmol/L)</td>
<td>7.17±0.02</td>
<td>7.22±0.03‡</td>
<td>7.15±0.02</td>
</tr>
<tr>
<td>Ang II (1 nmol/L) plus [Sar(^{1}),Ile(^{8})]Ang II (10 (\mu)mol/L)</td>
<td>7.12±0.02</td>
<td>7.17±0.02‡</td>
<td>...</td>
</tr>
<tr>
<td>Ang II (1 nmol/L) plus amiloride (10 (\mu)mol/L)</td>
<td>7.12±0.01†</td>
<td>7.15±0.01*</td>
<td>...</td>
</tr>
<tr>
<td>Endothelin-1 (1 nmol/L) plus amiloride (10 (\mu)mol/L)</td>
<td>7.12±0.01†</td>
<td>7.15±0.02†</td>
<td>...</td>
</tr>
<tr>
<td>Endothelin-1 (1 nmol/L) plus BQ-123 (0.1 (\mu)mol/L)</td>
<td>7.11±0.02†</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Sarafotoxin S6c (0.1 (\mu)mol/L)</td>
<td>7.12±0.02†</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Ang II indicates angiotensin II.
*P<.05 vs Ang II-stimulated pH in the same group.
†P<.05 vs endothelin-1-stimulated pH in the same group.
‡P<.05 vs other groups.

The resting pH of the platelets (the pH at which nigericin yields no change in fluorescence) was 7.10±0.07 (\(n=6\), mean±SD). The day-to-day variation in one subject was less than 2%.

Exposure of Platelets to Angiotensin II, Endothelin-1, Saralasin, Amiloride, BQ-123, and Sarafotoxin S6c

Platelet calcium responses to varying concentrations of Ang II and pH responses to varying concentrations of endothelin-1 were determined in 6 normotensive subjects and 4 hypertensive patients. The effects of Ang II and endothelin-1 on \([Ca\^{2+}]\) and pH were determined by evaluating the maximal responses after the addition of the agonist. Maximal cellular responses to the agents occurred within 50 seconds after exposure to the stimulant. Plateau or baseline values were achieved within 4 minutes after application. The effects of a specific Ang II antagonist, \([Sar^{1},Ile^{8}]\)Ang II (saralasin) (0.1 \(\mu\)mol/L), on \([Ca^{2+}]\) and pH, were determined in 8 subjects (4 normotensive and 4 hypertensive). Cells from 6 normotensive control subjects were also incubated with the endothelin A-receptor antagonist BQ-123 (0.1 \(\mu\)mol/L) and the endothelin B-receptor agonist sarafotoxin S6c (0.1 \(\mu\)mol/L). In addition, the effects of 5-N,N-(hexamethylene)amiloride (10 \(\mu\)mol/L), a potent inhibitor of Na\(^+\)-H\(^+\) exchange, on \([Ca^{2+}]\) and pH, were assessed in 10 subjects (6 normotensive and 4 hypertensive).

Platelet Aggregation Study

Aggregation studies were performed in 26 subjects (10 untreated hypertensive patients: 5 men, 5 women; 10 treated hypertensive patients: 6 men, 4 women; and 6 normotensive subjects: 3 men, 3 women). Platelet aggregation was analyzed according to the method of Born and Cross.\(^{29}\) The change in optical density in plasma was measured using the same instrument as for \([Ca^{2+}]\) and pH, except that the setting was adjusted for aggregation measurements. Calibration was performed with PRP as zero aggregation, and platelet-poor plasma (obtained by centrifugation of an aliquot of the same blood sample for another 10 minutes at 1000g) as maximal aggregation. The instrument was recalibrated after each experiment. Aggregation was measured in continuously stirred (1000 rpm) unstimulated and agonist-stimulated platelets at 37°C.

The final concentration of thrombin used was 0.05 U/mL, which was the lowest concentration that elicited sustained aggregation. The PRP was preincubated for 30 seconds in the aggregation tube at 37°C during continuous stirring. Ang II (1 nmol/L) or endothelin-1 (1 nmol/L) was then added and incubated for 5 minutes. Aggregation was measured for the complete incubation period. If no aggregation developed within 5 minutes, the response was considered negative. Thereafter, thrombin (0.05 U/mL) was added, and the effects of Ang II or endothelin-1 on thrombin-induced aggregation were determined.

Serum Biochemical Analyses

Serum Na\(^+\), Ca\(^{2+}\), K\(^+\), creatinine, \(\gamma\)-glutamyltransferase, cholesterol, and triglyceride levels were determined by automated methods.

Statistical Analysis

Data are presented as mean±SD. Differences between means were evaluated by analysis of variance followed by Bonferroni's correction to compensate for multiple testing procedures. Linear dependencies were determined by regression analysis. Statistical significance was taken at a value of \(P<.05\).

Results

Patient Characteristics

The demographics of the control subjects and hypertensive patients studied are presented in Table 1. The untreated hypertensive patients had significantly higher blood pressures compared with the normotensive and treated hypertensive groups. There were no significant differences in serum biochemistry among the groups (Table 1).

Platelet Cytosolic \([Ca^{2+}]\) and pH,

Basal concentrations of \([Ca^{2+}]\) and pH, were significantly higher in the hypertensive compared with the normotensive and treated hypertensive groups (\(P<.02\)) (Tables 2 and 3). There were no significant differences in basal \([Ca^{2+}]\) and pH, between the normotensive and
Fig 1. Plots show linear regression correlations between platelet pH, and platelet cytosolic free [Ca^{2+}]; (r=.71, n=76, P<.01) (top), platelet cytosolic free [Ca^{2+}], and mean arterial pressure (MAP) (r=.75, n=76, P<.01) (middle), and platelet pH, and MAP (r=.72, n=76, P<.01) (bottom) in combined normotensive and hypertensive groups. In the groups combined, there were positive correlations between [Ca^{2+}] and pH (r=.71, P<.01) (Fig 1, top), [Ca^{2+}], and mean arterial pressure (r=.75, P<.01) (Fig 1, middle), and pH, and mean arterial pressure (r=.72, P<.01) (Fig 1, bottom).

Effects of Angiotensin II and Endothelin-1 on Platelet [Ca^{2+}], and pH.

To assess what concentrations of Ang II and endothelin-1 to use, we performed dose-response studies. For Ang II, platelet [Ca^{2+}], responses were determined in six normotensive subjects and four hypertensive patients. For endothelin-1, platelet pH responses were determined in four normotensive subjects. The effects of increasing Ang II concentrations on platelet [Ca^{2+}], are presented in Fig 2. At the lower Ang II concentrations (10^{-12} to 10^{-8} mol/L), responses were significantly greater in the hypertensive group. Concentrations of 1 nmol/L were subsequently used in this study. Ang II (1 nmol/L) significantly increased [Ca^{2+}], by 67±6, 54±5, and 29±8 nmol/L in the untreated hypertensive, normotensive, and treated hypertensive groups, respectively (Figs 3 and 4). The Ang II–stimulated [Ca^{2+}], increase was significantly higher in the untreated compared with the other groups (P<.05) (Table 2). Ang II increased pH, in all three groups, but significance was only reached in the normotensive and untreated hypertensive groups (7.11 versus 7.15 in normotensive subjects, 7.16 versus 7.21 in hypertensive patients, basal versus stimulated, P<.05) (Table 3, Fig 5). All the Ang II–related responses were abolished in the presence of the Ang II antagonist [Sar¹, Ile⁸]Ang II (n=8) (Tables 2 and 3, Fig 4).

Fig 2. Line graph shows concentration-response curves to angiotensin II by cytosolic free calcium in platelets from normotensive subjects (n=6) and hypertensive patients (n=4).

Fig 3. Bar graph shows angiotensin II–induced platelet cytosolic free [Ca^{2+}], changes in normotensive (NT), untreated hypertensive (UnRx-HT), and treated hypertensive (Rx-HT) groups. *P<.05 vs other groups.
FIG 4. Tracings show effects of angiotensin II (Ang II) on free $[Ca^{2+}]_i$ in platelets from normotensive (NT) subjects and hypertensive (HT) patients. $[Sar^{1},Ile^8]$angiotensin II abolished the effects of angiotensin II. Tracings are representative of multiple independent experiments.

The effects of increasing endothelin-1 concentrations on platelet pH, are presented in Fig 6. A concentration of 1 nmol/L endothelin-1 was subsequently used in this study. Endothelin-1 had no significant effect on $[Ca^{2+}]_i$, in any of the groups (Table 2). However, it did significantly increase pH in the normotensive and untreated hypertensive groups (7.11 versus 7.17 in normotensive subjects, 7.16 versus 7.22 in hypertensive patients, basal versus stimulated) (Table 3, Fig 5). To determine whether the alkalinization responses to Ang II and endothelin-1 occur via the Na$^+-$H$^+$ exchanger, platelets from 10 subjects were preincubated for 5 minutes with a specific Na$^+-$H$^+$ antiport blocker $[5-N,N-(hexamethylene)amiloride]$, and the effects of the vasoactive peptides were determined. Preincubation of platelets with $5-N,N-(hexamethylene)amiloride$ abolished the effects of endothelin-1 and decreased the effects of Ang II on platelet pH, and $[Ca^{2+}]_i$ (Table 3, Fig 5). The endothelin-1 effects on pH were abolished by the endothelin A-receptor antagonist BQ-123, whereas sarafotoxin S6c, an endothelin B-receptor agonist, had no effect on cellular pH (Table 3).

Effect of Angiotensin II and Endothelin-1 on Platelet Aggregation

The platelet aggregation responses induced by thrombin and Ang II in unstimulated and thrombin-stimu-

lated cells were greater in the hypertensive patients than in the normotensive control subjects and the untreated hypertensive patients (Table 4, Fig 7). Preincubation of platelets with Ang II significantly increased thrombin-induced platelet aggregation in all three groups. Endothelin-1 added to platelets did not modify the optical density of the suspended platelets. When preincubated with platelets at 37°C for 5 minutes, endothelin-1 reduced thrombin-induced platelet aggregation in the normotensive group only. In the combined normotensive and hypertensive groups, aggregation was positively correlated to platelet $[Ca^{2+}]_i$ ($r=.69, P<.02$) (Fig 8) and platelet pH ($r=.56, P<.05$).

Discussion

The aims of this study were threefold. The first objective was to measure resting platelet $[Ca^{2+}]_i$ and pH; the second was to measure platelet aggregatory responses to determine the relations between aggregation and $[Ca^{2+}]_i$, and pH; status; and the third was to investigate the effects of Ang II and endothelin-1 on $[Ca^{2+}]_i$, pH, and aggregation in normotensive subjects and treated and untreated essential hypertensive patients. From the results of the dose-response studies, we chose to study the effects of 1 nmol/L Ang II and endothelin-1. At this subpharmacologic concentration, significant responses were elicited.

This study demonstrates that platelet free calcium and pH are significantly increased in untreated essential hypertensive patients. Although most studies have documented increased intracellular calcium in hypertension, data from the literature are conflicting with respect to the cellular pH in hypertension, with studies demonstrating increases, decreases, or no change in essential and experimental hypertension compared with normotensive control values. $12,13,15,20,32$ Most studies that have investigated pH in platelets from hypertensive patients have reported cytosolic alkalinization. $32,33$ Intracellular alkalinization is an important regulator of many cell functions, including modification of calcium-calmodulin interactions, enhancement of inositol triphosphate effects, activation of phospholipase A$_2$, and stimulation of mitogenesis. $34$ These effects contribute to hyperplasia and hypertrophy of vascular smooth muscle and to activation and aggregation of platelets. $3,7,8$ Plate-
Platelet activation is involved in the development and maintenance of atherosclerosis, and platelet-endothelium interactions may result in vasoconstriction and proliferation of vascular smooth muscle cells in the media and intima. These factors may be important in the pathogenesis of hypertension. Many studies have reported altered platelet function in hypertensive patients, with increased responsiveness to aggregating agents and elevated levels of β-thromboglobulin (a platelet-specific protein released into plasma when platelets are activated). In our study thrombin-induced aggregation was significantly greater in the untreated hypertensive group compared with the other groups. Altered platelet function may be related to variations in cytosolic ions, and in this study we found significant correlations between aggregation, Ca²⁺, pH, and blood pressure. These correlations, which are not necessarily cause-and-effect associations, suggest a possible link between platelet function, cytosolic calcium, pH, and hypertension.

Intracellular events and platelet aggregatory responses may be linked to common regulatory pathways. Vasoactive ligands, such as Ang II and endothelin-1, bind to specific G protein-coupled membrane-bound receptors, resulting in phospholipase C activation and consequent phosphoinositide hydrolysis. These intracellular responses may lead to increased cytosolic calcium concentrations and alkalinization. Ang II, the principal effector peptide of the renin-angiotensin system, increases [Ca²⁺] in vascular smooth muscle cells from spontaneously hypertensive rats and in platelets from hypertensive patients. In this study we have demonstrated that Ang II increases platelet cytosolic calcium and pH and that the maximal responses achieved were greater in the untreated hypertensive group compared with the other groups. In addition, data from the dose-response studies demonstrate that Ang II (in concentrations from 10⁻⁴ to 10⁻¹² mol/L) induces greater calcium responses in hypertensive patients than in normotensive subjects. These results may be largely attributable to higher basal platelet [Ca²⁺] and pH. Because the net change in [Ca²⁺] was significantly greater in the untreated hypertensive group compared with the other groups, increased platelet membrane responsiveness to Ang II, as has been reported previously, may possibly play a role in increased platelet calcium responses in hypertension. The calcium responses are less than those reported for vascular smooth muscle cells and may be due to the lower number of Ang II binding sites on platelets compared with the number on vascular smooth muscle cells. In the presence of the specific Ang II antagonist [Sar¹, Ile⁸]Ang II, the Ang II responses were abolished, implying that the effects were indeed receptor mediated.

Endothelin-1, another modulator of intracellular events, is a 21-amino acid peptide synthesized and released from endothelial and other cells and endowed with potent pressor activity. Platelet activation may promote local production of endothelin-1 by endothelial cells, and endothelins in turn may influence platelet function. The relation between endothelin-1 and platelets in hypertension is unclear. In this study we have demonstrated that endothelin-1 had no significant direct effect on platelet calcium. The lack of calcium response to this peptide in unstimulated platelets has already been reported by other investigators. Unlike calcium, platelet pH was increased by endothelin-1. BQ-123, which blocks endothelin A-receptors, inhibited the effects of endothelin-1 on cellular pH. Sarafotoxin S6c, an endothelin B-receptor agonist, had no effect on pH. These data suggest that the effects of endothelin-1 on platelet pH are receptor mediated, probably by endothelin A-receptors. The fact that endothelin-1 influences platelet pH and not calcium suggests that a pathway other than phospholipase C activation may be involved. Phospholipase D, which is known to be activated by endothelin-1 and which will generate diacylglycerol and thus activate protein kinase C independently of phospholipase C, could play a role in

![Graph showing concentration-response curve of platelet pH to endothelin-1](image)

**Table 4. Effects of Angiotensin II and Endothelin-1 on Aggregation In Unstimulated and Thrombin-Stimulated Platelets In Normotensive and Hypertensive Groups**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Normotensive</th>
<th>Untreated Hypertensive</th>
<th>Treated Hypertensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang II (1 nmol/L)</td>
<td>60±7</td>
<td>74±5*</td>
<td>66±4</td>
</tr>
<tr>
<td>Thrombin (0.05 U/mL)</td>
<td>68±4</td>
<td>81±8*</td>
<td>70±7</td>
</tr>
<tr>
<td>Ang II (1 nmol/L) plus thrombin (0.05 U/mL)</td>
<td>79±6†</td>
<td>89±7†</td>
<td>78±5†</td>
</tr>
<tr>
<td>Endothelin-1 (1 nmol/L) plus thrombin (0.05 U/mL)</td>
<td>55±7†</td>
<td>76±9</td>
<td>71±6</td>
</tr>
<tr>
<td>Endothelin-1 (1 nmol/L)</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
</tr>
</tbody>
</table>

Ang II indicates angiotensin II. Values are expressed as percentage of light transmission.

*P< .05 vs other groups.
†P< .05 vs thrombin effect in the same group.
modulating platelet pH without significantly altering cytosolic calcium.43

Intracellular alkalinization induced by Ang II and endothelin-1 are probably related to stimulation of the Na\(^+\)-H\(^+\) exchanger. We have shown that in the presence of a highly specific Na\(^+\)-H\(^+\) exchange blocker, the normal alkalinization responses to Ang II and endothelin-1 were abolished. Under these conditions, the vasoactive peptides induced a slight acidification. These data confirm the presence of an Na\(^+\)-H\(^+\) antiporter in platelets and establish its involvement in regulating pH in agonist-stimulated platelets. In addition to decreasing pH, the amiloride analogue used also decreased cytosolic calcium responses to Ang II. This suggests that activation of Na\(^+\)-H\(^+\) exchange influences the calcium rise in Ang II-stimulated platelets, possibly via Na\(^+\)-Ca\(^{2+}\) exchange. Similar results have been reported for thrombin-induced intracellular calcium increases and have been attributed to increased protein kinase C activity.44

There are little data on the relation between Ang II and platelet aggregation, and to our knowledge this is the first study to examine the effects of Ang II on platelet aggregation in relation to platelet free calcium and pH in essential hypertensive patients. In normotensive volunteers, Ang II increased collagen-induced platelet activation, and this increase was significantly greater when subjects were on a high-salt diet compared with a low-salt diet.45 Our results for the normotensive control subjects confirm those of Swartz and Moore.45 We have also demonstrated that thrombin-induced platelet aggregation is increased in essential hypertension and that Ang II-stimulated aggregatory responses are higher in untreated hypertensive compared with treated hypertensive patients and with normotensive subjects. This may be related to higher basal calcium levels in platelets of hypertensive subjects.

Unlike Ang II, the effects of endothelin-1 on platelet aggregation have been extensively studied.21,22,46,47 Although some studies have failed to demonstrate any effect of endothelin-1 on platelet aggregation, others have shown that endothelin-1 inhibits platelet aggregation when measured both in vivo and in vitro.53,47 This effect is associated with increased platelet cyclic AMP and seems to be mediated by the endothelial release of prostaglandin I\(_2\) into the circulation.21 Because indomethacin, a cyclooxygenase inhibitor, only partially blocks these effects, other mechanisms, such as altered responsiveness to agonists or changes in pH, and [Ca\(^{2+}\)], might be involved. These studies were performed in normotensive subjects. The relation between endothelin-1 and platelet aggregation in hypertension is unclear. In unstimulated platelets from normotensive subjects and hypertensive patients, we have demonstrated that endothelin-1 affects neither [Ca\(^{2+}\)], nor aggregation. These data confirm those of Astarie-Dequeker et al.48 When platelets were stimulated with thrombin, endothelin-1 decreased the calcium transient and aggregation in the normotensive group only (results not shown). These results suggest that the antiaggregatory properties of endothelin-1 may be mediated via
changes in \([\text{Ca}^{2+}]\). The exact mechanisms, however, need to be determined. In the hypertensive group, endothelin-1 had no effect on thrombin-stimulated calcium or aggregation, implying that the possible antiaggregatory property of endothelin-1 may be absent or defective in hypertension. Interestingly, endothelin-1–induced constriction of resistance arteries is blunted in human essential hypertension.\(^{40}\) Thus, effects of endothelin-1 on thrombin-induced platelet aggregation and on vasoconstriction are altered in a similar direction in untreated mildly hypertensive patients. Whether the mechanisms involved in both findings are similar remains to be investigated.

With antihypertensive therapy, platelet free calcium concentration, pH, and aggregation were normalized, and the effects of Ang II and endothelin-1 were blunted. This might be related to drug effects on the platelet membrane. In hypertension the functional integrity of the cell membrane may be altered. Indeed, changes in membrane fluidity have been described in cells from hypertensive patients.\(^{40}\) As multiple classes of antihypertensive agents were used in our study, the platelet effects do not appear to be drug specific. These findings have important clinical implications, because normalization of platelet calcium and pH may be associated with reduced platelet aggregation. Thus, hypertensive drugs may have an antiaggregatory effect in addition to reducing blood pressure. Previous studies have documented that antihypertensive agents reduce platelet free calcium concentrations and platelet aggregation in hypertension.\(^{30,37,51,52}\)

In conclusion, this study demonstrates that \([\text{Ca}^{2+}]\), pH, and aggregatory responses are increased in platelets from essential hypertensive patients. These parameters return to baseline values when blood pressure is normalized with different antihypertensive drugs. The response of platelets from hypertensive patients to Ang II was greater than that of platelets from normotenstive control subjects and may be associated with higher basal \([\text{Ca}^{2+}]\) and pH. The role of endothelin-1 on platelet function appears to be different from that of Ang II. Endothelin-1 had no effect on intracellular calcium or aggregation in unstimulated cells and exerted an effect only in the presence of thrombin and then only in normotenstive subjects and not in hypertensive patients. However, endothelin-1 significantly increased platelet pH in hypertensive patients. Although the exact implications of these responses are unclear, they may be associated with calcium-independent pathways of platelet activation. Also, if platelet alterations are representative of those in vascular smooth muscle cells, these data suggest that endothelin-1 may play a greater role in pH-modulated cell growth than in vascular contractility per se. This study demonstrates significant correlations between platelet calcium, pH, aggregation, and blood pressure and for the first time links platelet biochemical events with functional effects in hypertension. These findings may partly explain the underlying platelet hyperreactivity, which may contribute to the accelerated vascular disease, that occurs in essential hypertension.

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