MECHANISM OF ANTI-PLATELET AGGREGATING ACTION OF DILAZEP

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(Received June 8, 1984)

In vitro effect of Dilazep on the release and metabolism of arachidonic acid (AA) in human platelets was studied. Dilazep reduced in a dose-dependent manner platelet aggregation and thromboxane B_2 (TXB_2) formation when stimulated by adenosine diphosphate, collagen and epinephrine.

Dilazep decreased thrombin-induced release of [1^4C]arachidonic acid ([1^4C]AA) from platelets prelabeled with [1^4C]AA. The conversion of [1^4C]AA to cyclooxygenase metabolites was reduced by the addition of Dilazep, while that to 12-lipoxygenase metabolite was rather increased. Adenosine 3',5'-cyclic monophosphate and guanosine 3',5'-cyclic monophosphate levels in washed human platelets were not affected by the addition of Dilazep. These results suggest that the decreased TXB_2 formation by Dilazep may be ascribed to the impairment of AA release from platelet membrane phospholipids and the reduced conversion of released AA to TXA_2.

**Keywords** — platelet aggregation; arachidonic acid; thromboxane A_2; cyclooxygenase; 12-lipoxygenase

INTRODUCTION

It has been reported that Dilazep, [tetrahydro-1H-1,4-diazepine-1,4(5H)-dipropanol bis (3,4,5-trimethoxy benzoate) dihydrochloride monohydrate], a coronary vasodilator, has a potent anti-aggregating effect in in vitro and in vivo studies. However the detailed study of the effect of Dilazep on arachidonic acid metabolism in platelets has not been reported yet.

Arachidonic acid (AA) released from platelet membrane phospholipids has been said to be metabolized by two different enzymatic pathways 1) cyclooxygenase and 2) 12-lipoxygenase. Thromboxane A_2 (TXA_2) derived via cyclooxygenase is a potent aggregating agent while 12-hydroperoxyeicosatetraenoic acid (12-HPETE) is said to be rather anti-aggregating one.

Accordingly the present investigation was performed to study the in vitro effect of Dilazep on arachidonic acid cascade in human platelets obtained from normal healthy subjects in order to give further insight into the mechanism of anti-platelet aggregating action of dilazep.

MATERIALS AND METHODS

Platelet Aggregation — Citrated venous blood was obtained from healthy volunteers who had not taken any drugs for at least 2 weeks. Blood samples were centrifuged at 150 × g for 10 min at room temperature to obtain platelet rich plasma (PRP) and 1800 × g for 15 min to obtain platelet poor plasma (PPP).

The platelet count of PRP was adjusted to 4 × 10^9/μl. Platelet aggregation study was carried out using a Sienco dual channel aggregometer...
DP-247E as previously reported. The base line for 0% transmission was set with PRP and for 100% with PPP. The change in light transmission after the addition of aggregants was record-
ed as a percent value to determine the maximal aggregation. PRP was incubated with the addi-
tion of various concentrations of Dilazep (Kowa Pharmaceutical Co., Ltd., Tokyo, Japan) for 3 min and then stimulated by an aggregator at 37.5 °C. The following aggregants were used: Two μg/ml of collagen (Hormonchemie, München, West Germany), 2.0 μg/ml of epinephrine (Sigma Chemical Company, St. Louis, Miss, U.S.A.) and 2.0 μM of adenosine diphosph-
ate (ADP, Sigma Chemical Company, St. Louis, Miss, U.S.A.).

**Thromboxane B₂ Formation** — Thromboxane B₂ (TXB₂) formation in PRP was determined by a radioimmunoassay in conjunction with a plate-
et aggregation study as previously reported. One hundred μl of reaction mixture was trans-
ferrred to a plastic tube containing 200 μl of 50 mM Tris-HCl buffer, pH 7.5 with 1% gelatin. This tube was immediately dipped into liquid nitrogen for 10 s and stored at −80 °C until the assay of TXB₂ by radioimmunoassay (TXB₂ [³H] RIA kit, New England Nuclear, Boston, U.S.A.). The extraction of TXB₂ from the reaction mix-
ture was performed by the method of Green et al. with minor modifications. The recovery of TXB₂ was around 75%. The cross reactivity of the RIA kit antibody was 0.2% for prostaglandin (PG) E₂ and less than 0.2% for PGA₂, PGF₂α and 6-keto PGF₁α.

**Release and Metabolism of Arachidonic Acid in Thrombin-Stimulated Human Platelets Prelabeled with [¹⁴C]Arachidonic Acid** — Citrated venous blood was obtained from healthy volunteers who had not taken any drugs at least for 2 weeks. Platelets prelabeled with [¹⁴C]arachidonic acid ([¹⁴C]AA) (50 mCi/mm, New England Nuclear, Boston, U.S.A.) were prepared as previously reported. Two hundred and seventy μl of suspension of platelets prelabeled with [¹⁴C]AA (prelabeled platelets) was preincubated with 30 μl of Dilazep (1 or 5 mg/ml 50 mM Tris-
HCl buffer pH 7.5) for 3 min at 37.5 °C in silico-
nized aggregometer cuvettes placed in the aggreg-
gometer under mixing at 1000 rpm. Then, 33 μl of thrombin (5 U/ml, Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) was added and incubated for 5 min at 37.5 °C. Lipids were extracted and separated by thin layer chromatography (TLC) on Silica gel G plates (60F254, Merck, West Germany) in the upper phase of ethyl acetate-isooctane-acetic acid-water (110:50:20:100, v/v/v/v). The areas corresponding to phos-
pholipids, AA and its metabolites were detected by autoradiography, scraped off and counted in a liquid scintillation counter (LKB, Rack β, Wallac, Turku, Finland).

**Metabolism of Exogenous [¹⁴C]Arachidonic Acid by Washed Human Platelets** — Blood samples were obtained from healthy volunteers who had not taken any drugs at least for 2 weeks before blood sampling. Washed platelets were prepared according to the method of Minks et al. with minor modifications as previously reported. The platelet count of platelet sus-
pensions was adjusted to 10⁹/μl. Two hundred and sixty μl of washed platelet suspension was preincubated with 30 μl of various concentra-
tions of Dilazep for 3 min at 37.5 °C and further incubated with 5 μl of [¹⁴C]AA (40 μCi/ml Eth-
hanol) and 5 μl of unlabeled AA (99% pure, 1 μg/μl, ethanol, Nuchek, Elysian, U.S.A.). The reaction was carried out at 37.5 °C for 5 min and terminated by the addition of 150 μl of 2M citric acid. [¹⁴C]AA and its metabolites were extracted twice with ice-cold ethyl acetate. The extract was separated by TLC Silica gel G plates in a sol-
vent system as described above. The areas corres-
ponding to [¹⁴C]AA and its metabolites were detected by autoradiography, scraped off and counted in a liquid scintillation counter.

**AMP and GMP Levels in Washed Human Platelets** — A part of the washed platelets prepared for the aggregation study mentioned above was used for the determination of adenosine 3',5'-cy-
clic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) levels in washed platelets.
Four hundred and fifty μl of washed platelet suspension (10^6 platelets/μl) was mixed with 50 μl of Dilazep (0, 1, 2.5 or 5 mg/ml) and was incubated at 30 °C for 30, 60, 120 or 300 s. Then 500 μl of precooled 0.1N HCl was added. After freezing and thawing 3 times, the reaction mixture was centrifuged at 1800 × g at 4 °C for 30 min. cAMP and cGMP in the supernatant were succinylated quantitatively and were then measured by use of commercially available kits (Yamasa Shoyu Co., Ltd., Choshi, Japan) according to the method of Ui et al. 18) Assays were performed in triplicates.

Statistical Analysis — Comparisons were made using the non-paired Student’s t-test.

RESULTS
1) Effect of Dilazep on Platelet Aggregation and Thromboxane B₂ Formation Induced by ADP, Collagen and Epinephrine

<table>
<thead>
<tr>
<th>Aggregant</th>
<th>Dilazep (μg/ml)</th>
<th>Aggregation (%)</th>
<th>Thromboxane B₂ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (2 μM)</td>
<td>0</td>
<td>63 ±6</td>
<td>67 ±3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>53 ±4</td>
<td>36 ±6 a</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>47 ±1 a</td>
<td>15 ±4 a</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>26 ±4 a</td>
<td>5 ±2 b</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0 b</td>
<td>5 ±1 b</td>
</tr>
<tr>
<td>Collagen (2 μg/ml)</td>
<td>0</td>
<td>63 ±3</td>
<td>64 ±3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>55 ±5</td>
<td>44 ±6</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>35 ±3 a</td>
<td>20 ±3 a</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0 b</td>
<td>8 ±2 b</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0 b</td>
<td>5 ±1 b</td>
</tr>
<tr>
<td>Epinephrine (2 μg/ml)</td>
<td>0</td>
<td>66 ±5</td>
<td>97 ±3</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>60 ±2</td>
<td>66 ±6 a</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>48 ±5</td>
<td>28 ±5 a</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0 b</td>
<td>12 ±8 a</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0 b</td>
<td>5 ±1 b</td>
</tr>
</tbody>
</table>

Platelet rich plasma (4× 10^5/μl) was preincubated with Dilazep for 3 min before the stimulation. After stimulation, platelet aggregation and thromboxane B₂ formation were determined as described in “Materials and Methods.” Each value was obtained from 3 experiments and represents mean ± S.D. Significant difference from control stimulation represents a) p < 0.01, b) p < 0.001.

Dilazep inhibited dose-dependently platelet aggregation and thromboxane B₂ (TXB₂) formation induced by ADP, collagen and epinephrine (Table I). When 2 μM of ADP was used as an aggregant, the addition of Dilazep (100–500 μg/ml) resulted in a significant inhibition of the aggregation. Dilazep inhibited also ADP-induced TXB₂ formation in a dose-dependent manner. When 2 μg/ml of collagen was used as an aggregant, the addition of Dilazep (75–250 μg/ml) resulted in a marked inhibition of the aggregation. TXB₂ formation was also reduced in parallel with the inhibition of the aggregation. When 2 μg/ml of epinephrine was used as an aggregant, the addition of Dilazep (250–500 μg/ml) resulted in a marked inhibition of the aggregation. TXB₂ formation was also reduced by the addition of Dilazep (75–500 μg/ml).

2) Effect of Dilazep on Release and Metabolism of [14C]Arachidonic Acid in Thrombin-Stimulated
Human Platelets Prelabeled with $[^{14}C]$Arachidonic Acid

Thrombin (0.5 U/ml) induced aggregation of washed human platelets was inhibited by Dilazep (100–500 μg/ml). The addition of a high dose of Dilazep (500 μg/ml) resulted in a complete inhibition of the aggregation (Table II).

A marked loss of radioactivity in phospholipids was observed when prelabeled platelets were stimulated by thrombin (0.5 U/ml) for 5 min in the absence of Dilazep (Table II). The addition of Dilazep suppressed thrombin-induced loss of radioactivity from phospholipids in a dose-dependent manner. Stimulation of prelabeled platelets by thrombin (0.5 U/ml) for 5 min resulted in a release of $[^{14}C]$AA and its metabolites of cyclooxygenase pathway [TXB₂, PGD₂, PGE₂ and 12-hydroxyheptadecatrienoic acid (HHT)] and 12-lipoxygenase pathway [12-hydroxyeicosatetraenoic acid (12-HETE)]. Dilazep reduced the release of $[^{14}C]$AA and its metabolites from prelabeled platelets in a dose-dependent manner (Table II). The addition of low dose of Dilazep (100 μg/ml) resulted in one-half decrease in radioactivity in 12-HETE, compared with the control (thrombin-stimulated platelets), while the radioactivity in HHT and TXB₂ decreased one-third and one-fifth, respectively. The addition of high dose of Dilazep (500 μg/ml) resulted in one-third decrease in radioactivity in 12-HETE in comparison with the control, while the radioactivity in HHT and TXB₂ decreased one-fifth and one-seventh, respectively. The radioactivity in PGD₂ and PGE₂ was not detectable when Dilazep was added. The ratio of 12-lipoxygenase metabolite (12-HETE) versus cyclooxygenase metabolites (TXB₂+PGD₂ +PGE₂+HHT) was significantly increased in a dose-dependent manner by the addition of Dilazep.

3) Effect of Dilazep on Metabolism of Exogenous $[^{14}C]$Arachidonic Acid by Washed Human Platelets

The conversion of $[^{14}C]$AA to 12-HETE by washed platelets was increased dose-dependently by the addition of Dilazep, while that to TXB₂ and HHT was decreased (Table III). The ratio of

### Table II: Effect of Dilazep on Thrombin-Induced Platelet Aggregation, Release of Arachidonic Acid and Its Metabolites from Human Platelets Prelabeled with $[^{14}C]$Arachidonic Acid

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Platelet aggregation (%)</th>
<th>$[^{14}C]$ radioactivity (dpm)</th>
<th>12-HETE</th>
<th>HHT</th>
<th>TXB₂</th>
<th>PGD₂</th>
<th>PGE₂</th>
<th>12-HETE : Total CO metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no stimulation)</td>
<td>0</td>
<td>93713 ± 855</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Thrombin (0.5 U/ml)</td>
<td>65 ± 2</td>
<td>77608 ± 905</td>
<td>769 ± 107</td>
<td>5229 ± 267</td>
<td>4940 ± 31</td>
<td>4790 ± 184</td>
<td>450 ± 232, 408 ± 184</td>
<td>N.D.</td>
</tr>
<tr>
<td>Thrombin + dilazep (0.5 U/ml 100 μg/ml)</td>
<td>35 ± 3 ³</td>
<td>88774 ± 1017³</td>
<td>487 ±</td>
<td>7³</td>
<td>2335 ± 102³</td>
<td>1749 ± 73³</td>
<td>948 ± 207³</td>
<td>N.D.</td>
</tr>
<tr>
<td>Thrombin + dilazep (0.5 U/ml 500 μg/ml)</td>
<td>0³</td>
<td>90823 ± 3107³</td>
<td>394 ±</td>
<td>97³</td>
<td>1723 ± 118³</td>
<td>1032 ± 186³</td>
<td>647 ± 31³</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

$[^{14}C]$AA prelabeled platelets (10⁶/μl) were prepared as described in “Materials and Methods.” Platelets were stimulated by thrombin (0.5 U/ml). Five minutes after stimulation, phospholipids and AA metabolites were extracted and analyzed. Radioactive bands corresponding to phospholipids, arachidonic acid (AA), 12-hydroxyeicosatetraenoic acid (12-HETE), 12-hydroxyheptadecatrienoic acid (HHT), thromboxane B₂ (TXB₂), prostaglandin D₂ (PGD₂) and prostaglandin E₂ (PGE₂) were counted. Total cyclooxygenase (CO) metabolites is the total radioactivity recovered from areas corresponding to HHT, TXB₂, PGD₂ and PGE₂. Reported data are mean ± S.D. for triplicate samples. Significant difference from stimulation by thrombin represents α) p < 0.001.
12-HETE versus TXB$_2$+HHT was increased by the addition of Dilazep in a dose-dependent manner.

4) Effect of Dilazep on AMP and GMP Levels in Washed Human Platelets

Washed human platelets were incubated with different concentrations of Dilazep at 30 °C for 30, 60, 120 or 300 s, and cAMP and cGMP levels in platelets were determined. cAMP level in platelets did not change during 5 min incubation in the absence of Dilazep, while cGMP level increased during incubation. The addition of Dilazep did not affect cAMP and cGMP levels in washed platelets during 5 min incubation.

DISCUSSION

In vitro addition of Dilazep decreased dose-dependently platelet aggregation and TXB$_2$ formation when stimulated by ADP, collagen and epinephrine. This result in quite in accordance with those previously reported by several investigators, who showed Dilazep suppressed platelet aggregation by the same aggregants.\textsuperscript{1-3}

In order to investigate further in detail the mechanism of the reduced TXB$_2$ formation by Dilazep, we studied in vitro effect of Dilazep on the release of AA from phospholipids and its metabolism in thrombin-stimulated prelabeled platelets.

It was noted that the radioactivities of released AA and its metabolites decreased dose-dependently in prelabeled platelets pretreated with Dilazep. It was also observed that Dilazep suppressed loss of [\textsuperscript{14}C]AA from platelet phospholipids in a dose-dependent manner. These results may indicate that Dilazep impaired the release of AA from platelet phospholipids.

Agents which activate adenylate cyclase (PGI$_2$ or PGE$_2$) or guanylate cyclase (Molsidomine), have been reported to suppress AA release from platelet phospholipids and thereby reduce TXA$_2$ formation through the accumulation of cAMP or cGMP contents in platelets.\textsuperscript{16,17} In the present study, Dilazep did not affect cAMP and cGMP levels in washed human platelets. Therefore it seems to be unlikely that cAMP or cGMP may play an essential role in Dilazep-induced impairment of AA release from platelet phospholipids.

Molecular structure of Dilazep is quite similar to TMB-8 [\textsuperscript{8}-(N,N-diethylamino)-octy1-3, 4, 5-trimethoxybenzoate], which is said to reduce AA release from platelet phospholipids by the potent intracellular Ca$^{2+}$ antagonistic activity.\textsuperscript{18} Furthermore Tamura et al. reported that Dilazep itself has a Ca$^{2+}$ antagonistic activity.\textsuperscript{19} From these evidences, it might be inferred that Dilazep-induced impairment of AA release from platelet phospholipids could be closely

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[\textsuperscript{14}C]Radioactivity (dpm)</th>
<th>12-HETE</th>
<th>HHT</th>
<th>TXB$_2$</th>
<th>CO metabolites ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µg/ml</td>
<td>10656 ± 467</td>
<td>6131 ± 104</td>
<td>21897 ± 368</td>
<td>0.380 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>100 µg/ml</td>
<td>10798 ± 579</td>
<td>5613 ± 69b</td>
<td>19816 ± 473a</td>
<td>0.430 ± 0.025a</td>
<td></td>
</tr>
<tr>
<td>250 µg/ml</td>
<td>12627 ± 730b</td>
<td>4463 ± 83c</td>
<td>13540 ± 328c</td>
<td>0.702 ± 0.006c</td>
<td></td>
</tr>
<tr>
<td>500 µg/ml</td>
<td>14379 ± 729b</td>
<td>3357 ± 72c</td>
<td>10218 ± 121c</td>
<td>1.147 ± 0.028c</td>
<td></td>
</tr>
</tbody>
</table>

Washed platelets (10$^8$/µl) were incubated with [\textsuperscript{14}C]AA and unlabeled AA. Five minutes after incubation, AA metabolites were extracted and analyzed as described in "Materials and Methods." Cyclooxygenase (CO) metabolites is the total radioactivity recovered from areas corresponding HHT and TXB$_2$. Reported data are mean ± S.D. for triplicate samples. Significant difference from control (Dilazep 0) represents a) p < 0.02, b) p < 0.01, c) p < 0.001.
related to its Ca\(^{2+}\) antagonistic activity, though precise mechanism is still undetermined yet.

An interesting finding is that Dilazep increased dose-dependently the ratio of 12-HETE to cyclooxygenase metabolites in thrombin-stimulated prelabeled platelets. Similar result was obtained when we studied the effect of Dilazep on the conversion of exogenously added [\(^{14}\)C]AA by washed human platelets. These results suggest that Dilazep may shift released AA toward 12-lipoxygenase pathway. Though it is still undetermined yet, whether this effect of Dilazep may be ascribed to suppression of cyclooxygenase pathway and/or stimulation of 12-lipoxygenase pathway, the shift of AA toward 12-lipoxygenase pathway might certainly contribute to the antiplatelet aggregating action of Dilazep by the reduction of AA available for TXA\(_2\) formation.

An increased ratio of 12-lipoxygenase metabolite (12-HETE) to cyclooxygenase metabolites by Dilazep could also contribute to its antiaggregatory action, because 12-HETE, a precursor of 12-HETE, has been reported to have rather anti-aggregatory action on platelets.\(^8,9\)

In conclusion, the findings presented here lead us to think that the reduced TXB\(_2\) formation by Dilazep may be ascribed to a decreased availability of free AA for TXA\(_2\) formation, most probably by the suppression of AA release from platelet phospholipids and by the shift of free AA from cyclooxygenase pathway to 12-lipoxygenase pathway.

REFERENCES


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