Inhibitory Effects of Trimetazidine Dihydrochloride on Aggregation, Serotonin Release and Malondialdehyde Production in Rabbit Platelets

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Abstract—Aggregation, serotonin release and malondialdehyde (MDA) production via cyclooxygenase and thromboxane A2 synthetase were investigated in rabbit platelets. Trimetazidine dihydrochloride (TMZ) attenuated the collagen-induced aggregation more strongly than the arachidonic acid (AA)-, thromboxane A2 agonist (U-46619)-, Ca2+-ionophore (A-23187)- and ADP-induced aggregation: IC50 values were 1.0±0.1, 4.4±0.3, 4.3±0.4, 4.1±0.7 and 3.3±0.2 mM, respectively. TMZ decreased dose-dependently the serotonin release induced by collagen and A-23187, but did not decrease that induced by AA. TMZ also decreased the MDA production induced by collagen and A-23187 (IC50: 0.3±0.03 and 1.0±0.1 mM, respectively), but did not decrease the production induced by AA. Furthermore, TMZ decreased dose-dependently the MDA production induced by exogenous phospholipase A2. On the other hand, indomethacin (10 μM) attenuated the aggregations induced by collagen and AA, but not by the other agents, and decreased the serotonin release and the MDA production induced by collagen, A-23187 and AA. The present results suggest that TMZ may inhibit the process preceding the cyclooxygenase pathway in the AA cascade, and subsequently may attenuate the aggregation and the serotonin release via thromboxane A2 production from endogenous AA.

TMZ was developed as an anti-anginal drug and has been used for a long time. It is known that TMZ is effective in preventing anginal attacks, reducing the need for nitroglycerin tablets and decreasing subjective complaints (1, 2). However, the mechanism of its anti-anginal action has not been understood fully. It has been reported that TMZ antagonized the effects of catecholamines in guinea-pig atria and perfused rabbit ear arteries (3, 4). TMZ also shows a weak Ca2+-antagonistic action in guinea-pig taenia-coli (4). Furthermore, TMZ, like nitroglycerin, is considered to reduce the preload of the heart since TMZ reduces the venous return in anesthetized dogs and dilates canine veins more potently than arteries (5, 6). In recent years, it has been reported that TMZ decreased the adhesiveness and aggregation of platelets in patients with ischemic heart disease (7). However, there have been few reports about the inhibitory effect of TMZ on platelet responses (aggregation and serotonin-release) with respect to AA metabolism.

In present work, the effect of TMZ on aggregation, serotonin release and MDA production induced by various agents were investigated to further characterize its anti-platelet action in rabbit platelets.

Materials and Methods

Animals: Male Japanese albino rabbits weighing 2–3 kg (Shizuoka Laboratory Animal Center) were used. Animals were housed at constant temperature (23±2°C)
and relative humidity (50–70%) and were allowed free access to food and water.

**Drugs:** All drugs were purchased. Trimetazidine dihydrochloride (TMZ) (Servier), serotonin-creatinine sulfate (Merck) and adenosine 5'-diphosphate disodium salt (ADP) (Kohjin) were dissolved in saline. Aspirin (Nakarai), arachidonic acid sodium salt (AA) (Sigma) and phospholipase A₂ from bee venom (Sigma) were dissolved in distilled water. 9,11-Dideoxy-9α,11α-methanoepoxy-prostaglandin F₂α (U-46619) (Cayman Chemical) and indomethacin (Sigma) were dissolved in 0.1 M Na₂CO₃ at 10⁻² M and diluted by saline. A-23187 (Calbiochem) was dissolved in dimethylsulfoxide. Collagen (Collagen Reagent "HORM", Hormon-Chemie) was dissolved and diluted in appended buffer.

**Preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP):** Blood samples were taken from the carotid arteries of rabbits under anesthesia with pentobarbital sodium (Pitman-Moore, 30 mg/kg, i.v.) into polyethylene tubes containing 0.1 volume of 3.8% sodium citrate. Collected blood was centrifuged at 1000 rpm for 10 min at 5°C, and PRP was obtained. The lower layer was recentrifuged at 3000 rpm for 10 min at 5°C, and the PPP was obtained.

**Preparation of washed platelet suspension (WPS):** WPS was prepared according to the method of Ardlie et al. (8). Blood was taken from common arteries of rabbits into the tubes containing 0.14 volume of ACD solution (85 mM sodium citrate, 64 mM citric acid, 110 mM glucose). PRP was prepared as described above and then centrifuged at 3000 rpm for 10 min at 5°C, and PRP was obtained. The lower layer was recentrifuged at 3000 rpm for 10 min at 5°C, and the PPP was obtained.

**Assay for platelet aggregation:** Aggregation was measured photometrically using an NKK-Hematracer (Niko Bioscience, Tokyo). TMZ, indomethacin or vehicle was added to the PRP 1 min prior to the addition of aggregating agents. Changes in light transmission were measured and recorded. Collagen (10 μg/ml), AA (0.3 mM), U-46619 (10 μM), A-23187 (10 μM) and ADP (10 μM) were used as aggregating agents. Maximal changes in light transmission of PRP treated with test compounds were expressed as relative values to that of PRP treated with vehicle.

**Assay for serotonin release:** Serotonin released from platelets was determined fluorometrically according to the method of Drummond and Gordon (9). TMZ, indomethacin or vehicle was added to PRP, and PRP was incubated for 5 min at 37°C under stirring at 1000 rpm; then collagen (10 μg/ml), AA (0.3 mM), U-46619 (10 μM), A-23187 (10 μM) or ADP (10 μM) was added. After a further 5 min incubation, the reaction was stopped by addition of ice-cold EDTA solution, and then the sample was centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was diluted by distilled water, and the sediment was dissolved in distilled water. Protein was precipitated by addition of trichloroacetic acid (TCA) (30%) in both samples, and removed by centrifuging at 3800 rpm. The protein-removed supernatant was boiled at 100°C for 10 min with o-phthalaldehyde solution and then cooled in an ice-cold water bath. After TCA was extracted by chloroform from the ice-cold samples, the fluorescence of the aqueous phase of samples was measured using a spectrofluorophotometer (Shimadzu, Kyoto) with activation and emission wavelength of 360 and 475 nm, respectively. Serotonin release ratio (SRR) was calculated according to the following formula: SRR = (amount of serotonin in PPP)/[(amount of serotonin in PPP)+(amount of serotonin in platelets)]. SRR values in PRP treated with TMZ or indomethacin were expressed as relative values to that in PRP treated with vehicle.

**Determination of MDA:** MDA produced in platelets was determined fluorometrically according to the method of Smith et al. (10). PRP or WPS treated with TMZ, indomethacin or vehicle was incubated at 37°C for 5 min under stirring at 1000 rpm; then collagen (10 μg/ml), AA (0.3 mM), U-46619 (10 μM), A-23187 (10 μM), ADP (10 μM) or phos-
phospholipase A₂ (10 U/ml) was added. PPP or Tyrode's solution was incubated under the same condition for blank determination. After a further 5 min incubation, reaction was stopped by addition of TCA and centrifuging at 4000 rpm for 15 min. TBA solution (0.12 M thiobarbituric acid, 0.26 M tris, pH 7.0) was added to the supernatant; then the sample was heated in boiling water for 15 min. After the sample was cooled to room temperature, the fluorescence was measured using a spectrofluorophotometer (Shimadzu, Kyoto) with activation and emission wave length of 510 and 553 nm, respectively. Amounts of MDA produced in PRP treated with test compounds were expressed as relative values to that in PRP treated with vehicle.

Statistical analyses: All results were expressed as the mean±S.E. Statistical significance was analyzed using Student's paired t-test.

Results

Effect of TMZ on aggregations of rabbit platelets: TMZ inhibited the collagen (10 μg/ml), AA (0.3 mM), U-46619 (10 μM), A-23187 (10 μM) and ADP (10 μM)-induced aggregations in a concentration-dependent manner, and the mean IC₅₀ values were 1.0, 4.4, 4.3, 4.1 and 3.3 mM, respectively (Fig. 1). Indomethacin (10 μM) inhibited markedly the collagen- and AA-induced aggregation, but not the other aggregations.

Effect of TMZ on serotonin release from rabbit platelets: Collagen (10 μg/ml), AA (0.3 mM) and A-23187 (10 μM) elicited the serotonin release, and the release ratio was 0.57±0.04, 0.29±0.04 and 0.84±0.03, respectively. U-46619 (10 μM) and ADP (10 μM) almost elicited no serotonin release. TMZ inhibited the serotonin release induced by collagen and A-23187, but not that by AA, in a concentration-dependent manner (Fig. 2). Indomethacin (10 μM) decreased the serotonin release induced by collagen, AA and A-23187 to 43.9±2.9% (n=4), 14.8±4.9% (n=4) and 66.0±5.1% (n=4), respectively.

Effect of TMZ on MDA production in rabbit platelets: Amounts of MDA produced by collagen (10 μg/ml), AA (0.3 mM) and A-23187 (10 μM) in PRP were 32.8±5.0, 88.1±6.2 and 81.8±9.9 nmole/10¹¹ cells, respectively. Detectable amounts of MDA were not produced by U-46619 (10 μM) and ADP (10 μM). TMZ inhibited the MDA production induced by collagen and A-23187 but not AA, and the mean IC₅₀ values were 0.3 mM and 1.0 mM, respectively (Fig. 3). The amount of MDA produced by exogenous phospholipase A₂ in WPS was 41.8±17.0 nmole/10¹¹ cells. TMZ inhibited the MDA production induced by phospho-
lipase A2 (Table 1). Indomethacin (10 \mu M) abolished the MDA production induced by collagen, AA, A-23187 and phospholipase A2.

Fig. 3. Effect of TMZ on the MDA production in rabbit platelets. Amount of MDA produced in PRP with the vehicle was taken as 100%. Each value represents the mean±S.E. Number of experiments is shown in parentheses. Collagen: 10 \mu g/ml, AA: 0.3 mM, and A-23187: 10 \mu M.

Discussion

Inhibitory effects of TMZ on aggregations induced by five aggregating agents were compared. The inhibitory effects of TMZ on the aggregations induced by AA, U-46619, A-23187 and ADP were less, but the inhibitory effect of TMZ on the aggregation induced by collagen was much more potent. The mechanism of action of these aggregating agents in rabbit platelets is considered to be as follows: AA is converted to thromboxane A2 (TXA2) by cyclooxygenase and TXA2 synthetase, and TXA2 elicits the aggregation (11); U-46619 acts as a TXA2 agonist (12); A-23187 is a Ca2+ ionophore; ADP induces the aggregation independent of TXA2 production (13); and collagen is thought to elicit the TXA2 production via liberation of endogenous AA from membrane phospholipids (14). Both collagen- and AA-induced aggregation are dependent of AA metabolism (TXA2 production), and in the present work, this was ascertained using indomethacin, a cyclooxygenase inhibitor. These raise the possibility that the inhibitory effect of TMZ on the collagen-induced aggregation may be due to blocking of the mechanism preceding the cyclooxygenase pathway in the AA cascade, rather than blocking of the cyclooxygenase pathway in rabbit platelets. Such a possibility was examined in the serotonin release.

In next series of experiments, the effect of TMZ on the serotonin release induced by collagen, AA and A-23187 was compared. The serotonin release was less in AA than in collagen and A-23187. Indomethacin decreased the serotonin release induced by collagen, AA and A-23187 to 44, 15 and 66%, respectively. TMZ decreased the serotonin release induced by collagen and A-23187 to about 50%, but did not decrease that induced by AA. From these results, it is assumed that TMZ may inhibit the serotonin release dependent on endogenous AA metabolism, but not on exogenous AA metabolism. As mentioned in the above section, these inhibitory effects of TMZ on serotonin releases support the above view that the inhibitory action of TMZ on platelets may be due to blocking of the process preceding the cyclooxygenase pathway in the AA cascade.

In order to determine whether TMZ affects the endogenous AA metabolism in platelets, the effect of TMZ on MDA production induced by various agents was examined. MDA is AA metabolite of the cyclooxygenase pathway, and the production of MDA and TXB2 is highly correlated in platelets (15). TMZ inhibited the MDA production induced by collagen and A-23187, but not AA. These results indicate that TMZ may inhibit the pro-

| Concentration of TMZ (mM) | 0 | 0.3 | 1 | 3 |
|---------------------------|---|-----|---|---|
| Phospholipase A2 (10 U/ml) | 41.8±17.0 | 32.6±9.8* | 29.0±8.4** | 21.8±4.7** |

Each value represents the mean±S.E. (nmole/10¹¹ cells), n=6, *P<0.05, **P<0.01.
cess preceding the cyclooxygenase pathway and may fail to affect cyclooxygenase and TXA_2 synthetase. Although TMZ rather increased the MDA production from exogenous AA at high concentration, this mechanism is unknown. TMZ inhibited the aggregation, the serotonin release and the MDA production at a similar concentration range. It is strongly suggested that TMZ inhibits the TXA_2 production from endogenous AA, and subsequently attenuates the aggregation and serotonin release. Indeed, TMZ also inhibited the MDA production induced by exogenous phospholipase A_2. Exogenously applied phospholipase A_2 produces prostanoids and thromboxane A_2 via the hydrolysis of AA-rich membrane phospholipids in rat platelets (16).

Therefore, the inhibitory effect of TMZ on phospholipase A_2 activity may be related to the inhibition of TXA_2 production from endogenous AA.

Alternatively, TMZ may inhibit indirectly the activation of phospholipase A_2. AA is considered to be liberated from membrane phospholipids by a Ca^{2+}-dependent hydrolase, possibly phospholipase A_2, and membrane acting agents including TMB-8, mepacrine and propranolol inhibit the AA liberation by blocking intracellular Ca^{2+} release (17). TMZ has been considered to be a so-called "membrane stabilizer" in platelets (18). Furthermore, TMZ shows Ca^{2+}-antagonistic action in smooth muscle (4). It is assumed that TMZ may act on the membrane and counteract the intracellular Ca^{2+} release required in the activation of phospholipase A_2.

From these results, it was suggested that the inhibitory effect of TMZ on the aggregation induced by collagen and the serotonin release induced by collagen and A-23187 might be due to blocking of the process preceding the cyclooxygenase pathway in the AA cascade. The demonstrated inhibitory effects of TMZ on platelet aggregation and serotonin release may be related to its antianginal action.

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