Differential Inhibitory Effects of Isosorbide Dinitrate and its Mono-nitrate Metabolites on Platelet Aggregation and Thromboxane Formation

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Abstract: The effects of isosorbide dinitrate (ISDN) and its 2- and 5-monomonitrate metabolites (2-ISMN and 5-ISMN) against platelet aggregation and thromboxane release were investigated by analysis of platelet aggregation curves. ISDN, 2-ISMN and 5-ISMN (isosorbide nitrates, ISN) inhibited both ADP- and epinephrine (EPI)-induced platelet aggregation. ISN affected specifically the extent of ADP-induced aggregation and the velocity of EPI-induced effects. 2-ISMN was more potent against platelet aggregation compared to ISDN and 5-ISMN. The isosorbide nitrates were poor inhibitors of both arachidonic acid-induced aggregation and platelet TxB2 release. The differential inhibition by the three isosorbide nitrates of endogenous TxB2 release during ADP-induced aggregation further indicates that 2-ISMN is a significantly more potent platelet inhibitor than either ISDN or 5-ISMN. These studies suggest a role of the metabolites in modulating the pharmacological effects of ISDN on platelet activity.

The usual clinical manifestations of coronary-artery diseases are thought to result from vasospastic and thrombotic events, although the exact relationships between coronary spasm, coronary thrombosis, platelet-induced thromboembolism, and myocardial infarction are still under debate (1–5). Organic nitrates are widely used as vasodilators in the treatment of coronary heart disease, but the precise molecular mechanisms by which they act are uncertain (6, 7). In addition to their vasodilating properties, nitroglycerin, nitroprusside and isosorbide dinitrate (ISDN) are inhibitors of platelet aggregation in vitro and in vivo (8–11, 17). However, there is a marked disparity between concentrations which inhibit platelet aggregation in vitro and therapeutic plasma levels obtained in vivo, i.e., the minimal in vitro inhibitory levels can be several orders of magnitude greater than their in vivo counterparts (12, 13). Thus, if these agents inhibit platelet activity in vivo when used therapeutically, they must act indirectly. The same mechanism may also be responsible for the vasodilation. Organic nitrates stimulate the production of a second substance in vivo that in turn would decrease vascular tone and inhibit platelet activity. Prostaglandin I2 is a candidate for such a role (11, 14). On the other hand, it is conceivable that the active agent is not the drug itself but rather one or several of its metabolites. These studies were designed to test the latter hypothesis by comparing the inhibitory effects of ISDN and its mononitrate metabolites (2-ISMN and 5-ISMN) on platelet aggregation and thromboxane formation. We report here that 2-ISMN is a more potent inhibitor of platelet aggregation than ISDN and 5-ISMN.

Materials and Methods

Platelets were obtained from normal healthy male or female adult volunteer donors with no evidence of liver or hematological disease and no exposure to aspirin-like drugs or other medication for at least 2 weeks prior to venipuncture. Informed consent was obtained from each subject. Blood was drawn into citric acid/trisodium citrate (0.11 M) buffer, pH 7.4, using a Vacutainer and Hardpack multiple sample needle system, Becton-Dickinson and Co. Platelet-rich plasma (PRP) was prepared by centrifugation of blood at 150 x g for 20 min at 16°C. Platelet-poor plasma (PPP) was prepared by centrifugation of PRP or whole-blood at 6,000 x g for 15 min at 16°C. Washed platelets were obtained from blood drawn into EDTA-Vacutainer tubes by centrifugation of PRP at 4,000 x g for 15 min at 16°C. The pellet was suspended into Ca-free Krebs-Heinsleit buffer containing 5 mM Hepes and was washed twice under the same conditions. The resulting pellet was suspended in the same buffer.

Platelet aggregability was assayed with constant stirring at 1100 rpm in a dualchannel aggregometer (Coultronics) thermostated at 37°C. Platelet suspensions (PS) were prepared immediately before each series of studies by diluting PRP in autologous PPP to counts in the range of 3 to 3.5 x 105 platelets per microliter. Platelet counts in fresh PRP were above this range. Prior to the addition of 0.02 ml of aggregating agent solution, a 0.05 ml solution of the agent to be tested was added to 0.4 ml of PS in the aggregometer. In controls, the corresponding buffers were added. The results are given in terms of the change in light transmission (LT) expressed as a percentage of the difference in LT between PRP and PPP once a plateau was reached, thus defining the extent of the aggregation response. The slope of the aggregation response, defining the velocity, was the initial slope since thereafter platelet aggregation slowly reaches a plateau. Therefore, the velocity was the percentage of the difference in LT between PRP and PPP, thirty seconds after the addition of the aggregating agent.

Thromboxane (TxA2) production by platelets was measured by specific RIA of TxA2 as previously described (11, 16–18). Washed platelet suspensions (WPS) were diluted to 5 x 105 platelets/ml in Krebs-Heinsleit buffer containing 5 mM Hepes, final pH 7.4. Prior to the addition of 0.05 ml of aggregating agent solution, a 0.05 ml solution of the chemicals to be tested at the appropriate concentration was added to 0.4 ml of WPS. In controls, the corresponding buffers were added. Routinely, incubation time

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Abbreviations: ISN, isosorbide nitrates; ISDN, isosorbide dinitrate; 2-ISMN, 2-isosorbide mononitrate; 5-ISMN, 5-isosorbide mononitrate; PS, platelet suspension; LT, light transmission; WPS, washed platelet suspension; EPI, epinephrine; ARA, arachidonic acid.
was 5 min at 37°C in a shaking water-bath. Incubations were terminated by addition of 0.2 ml of citric acid solution (1M), and radioactive TxB2 was added in trace amounts to evaluate procedural losses. After diethyl ether extraction, the dried organic extracts were submitted to silicic acid chromatography. TxB2 was eluted from the column by 7 ml of benzene : ethyl acetate : methanol (6:4:0.3, vol/vol). RIAs were performed on buffer reconstituted residues from these fractions. Thromboxane production was expressed in nanograms of TxB2 produced by 10⁶ platelets/5 min at 37°C (11, 16–18).

The purity of ISDN, 2-ISMN and 5-ISMN standard solution was checked by GLC analysis (30) and was found to be over 98%. Similarly, the final concentration to be tested in these in vitro studies were confirmed by GLC.

Results were expressed as means ± standard deviation (SD). Statistical examination was carried out by one-way analysis of variance (ANOVA) or multiple ANOVA (MANOVA).

Results

The basic characteristics of platelet inhibition by 2-ISMN are illustrated by the aggregation curves shown in Fig. 1. Similar results were obtained for the effective concentration of ISDN and 5-ISMN. A marked difference in the inhibiting properties of isosorbide nitrates (ISN) was noted according to the nature of the aggregating agent: ISN lowered the extent but weakly affected the velocity of the aggregation response to ADP, while the reverse situation was observed when EPI was used as the aggregating agent. ISN affected both primary and secondary EPI-induced aggregation in a similar manner. ISN were found to be poor inhibitors of collagen and arachidonate-induced aggregation, since high concentrations (10⁻³ M) were needed to induce a 65% inhibition of platelet aggregation. Fig. 2 shows the effects of ISN on the extent or the velocity of platelet aggregation induced by ADP or epinephrine, respectively. Isosorbide nitrates when used alone are able to elicit an inhibitory response to both EPI- and ADP-induced aggregation. ISDN and 5-ISMN were found to affect platelet aggregation in a similar manner, and high concentrations (10⁻³ M) profoundly affected platelet aggregation. These effects were still statistically significant for 10⁻⁴ M ISDN or 5-ISMN concentration (p<0.05). In the presence of 10⁻⁵ M ISDN or 5-ISMN, platelet aggregation parameters returned to control levels. By contrast, 2-ISMN was found to be more potent than ISDN and 5-ISMN, since 1) at a concentration of 10⁻³ and 10⁻⁴ M 2-ISMN completely arrested EPI-induced aggregation and fully reversed ADP-induced aggregation, and 2) at a concentration of 10⁻³ M, 2-ISMN caused a 50% and 15% inhibition of EPI- and ADP-induced aggregation, respectively.

Attempts were made to investigate ISN effects on platelet TxB2 release as an inducer of platelet aggregation, when TxB2 was produced during arachidonic acid (ARA)-induced platelet aggregation, and as a biochemical marker of platelet aggregation, when TxB2 was produced from platelet endogenous arachidonate under exposure to aggregating agents. As shown in Fig. 3, thromboxane B₂ production by platelets under ARA-induced aggregation was inhibited by high ISN concentration (5×10⁻⁴ M), while 2×10⁻⁵ M ISN concentrations were ineffectual. No difference between the individual isosorbide nitrates was noted. These results are in agreement with light transmission studies demonstrating that ISN are poor inhibitors of ARA-induced aggregation. Under ADP-induced aggregation TxB2 release occurred from endogenously arachidonate, but a ten-fold lower TxB2 release was noted when ADP instead of ARA was used as an aggregating agent. At high (5×10⁻⁴ M) and low (2×10⁻⁵ M) concentrations of ISDN, 2-ISMN and 5-ISMN thromboxane-release inhibiting strengths were 20, 68 and 55%, and 0, 34 and 20%, respectively. Therefore, 2-ISMN is a more potent inhibitory agent of ADP-induced thromboxane release than 5-ISMN while ISDN is a poor inhibitor of arachidonate metabolism in ADP-stimulated platelets.

Discussion

The results of this study indicate that mononitrate metabolites of isosorbide dinitrate are inhibiting agents of both

![Fig. 1 Light transmission studies of 2-ISMN inhibitory effects on ADP- (left panel) and epinephrine- (right panel) induced platelet aggregation. The course of aggregation curves induced by 2-ISMN were recorded at various 2-ISMN concentration. 0.4 ml of PRP (3×10⁵ platelets per microliter) was incubated with 0.05 ml of 2-ISMN at the defined concentration immediately prior the addition of 0.02 ml of ADP or epinephrine solution (arrow). Note that the isosorbide nitrates affected the extent of ADP-induced aggregation and the initial velocity of epinephrine-induced aggregation.](image)