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A STUDY OF THREE VASODILATING AGENTS AS SELECTIVE INHIBITORS OF THROMBOXANE A_2 BIOSYNTHESIS

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SUMMARY: Three clinically efficacious vasodilatory drugs were found to be selective inhibitors of thromboxane A_2 biosynthesis. Hydralazine, dipyridamole, and diazoxide inhibited platelet aggregation at 1×10^{-4} M, 1.75×10^{-4} M, and 2×10^{-3} M respectively. Their relative inhibitory potencies on thromboxane B_2 production in human platelet microsomes were examined and found to be similar to that observed for their inhibition on human platelet aggregation. At 10^{-3} M, hydralazine, dipyridamole, and diazoxide inhibited thromboxane B_2 formation by 65 percent, 27 percent and 18 percent respectively. These compounds were examined in the sheep vesicular gland system, and they were shown not to be inhibitors of the cyclooxygenase enzyme. Thus, the inhibition of thromboxane A_2 biosynthesis by these three drugs in human platelet microsomes appeared to be specific at the thromboxane synthetase level.

INTRODUCTION

Thromboxane A_2 (TxA_2) is a potent platelet aggregator (1) and is the vasoconstrictor originally described as rabbit aorta contracting substance (RCS) (2). Its formation results from the enzymatic metabolism of the prostaglandin (PG) endoperoxides by an enzyme referred to as thromboxane synthetase. TxA_2 biosynthesis to date has been observed in platelets (2), white cells (3), umbilical arteries (4), brain (5), spleen (6), and kidney (7). In general, PGs are potent vasoactive substances, and because of their myriad of effects on the cardiovascular system, they may play a role in the pathogenesis of cardiovascular diseases. The enzymatic conversion of arachidonic acid to the endoperoxides by a cyclooxygenase is the pivotal step in the biosynthesis of all these vasoactive compounds. Vane (8) originally

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noted that PG biosynthesis could be inhibited by non-steroidal antiinflammatory agents such as aspirin and indomethacin. These agents block the initial cyclooxygenase reaction. Such an inhibition would lead to unpredictable physiological effects since it affects the production of the potentially beneficial arachidonate metabolites as well as the detrimental ones. Therefore, it is of great importance to identify agents which can selectively modify the PG biosynthetic pathway. Such manipulations will hopefully yield greater insight to the understanding of the normal and pathophysiological function of the PGs. Currently, it has been reported that imidazole (9), endoperoxide analogues (10-13), burimamide (14), L-8027 (15), N-0164 (16) and benzydamine (17) are inhibitors of TxA_2 formation. However, benzydamine appears to be a non-selective PG inhibitor because it also inhibits cyclooxygenase function. N-0164 lacks specificity in that it is also a PG receptor antagonist in isolated smooth muscle preparation (18). Recently, we reported that diazoxide is an inhibitor of platelet aggregation (19). Preliminary data indicated that diazoxide suppressed TxA_2 formation but not the other PGs. This has led us to undertake a detailed study of other similarly effective smooth muscle vasodilators in hope of examining their ability to serve as inhibitors for TxA_2 biosynthesis. Such information may contribute to the current understanding of the mechanism of action of this class of therapeutic agents.

MATERIALS AND METHODS

Arachidonic acid was purchased from Nu-Chek Prep Inc. (Elysian, Minn.) and [1-^{14}C] arachidonic acid (55 Ci/mol) was purchased from New England Nuclear (Boston, Mass.). L-tryptophan and methemoglobin were purchased from Sigma Chemical Co. (St. Louis, Mo.). We thank Dr. W. Benson of the Boehringer Ingelheim Company for the kind gift of dipyridamole, Dr. A. Barnett of the Schering Corporation for the sample of diazoxide, Mr. C. Brownley Jr. of the Ciba Pharmaceutical Company for hydralazine, and Dr. A. Scriabine of Merck Sharp and Dohme for indomethacin. PGE_2, $PGF_{2\alpha}$ and TxB_2 were kind gifts of Dr. J. Pike of the Upjohn Company. Outdated human platelet concentrations were supplied by the Ohio State University Hospital's Blood Bank.

Platelet Aggregation: Blood samples were drawn from healthy male volunteers who had not taken aspirin or any other medication for at least two weeks. Platelet rich plasma (PRP) was prepared by the method of Panganamala et al (20). The final platelet count was adjusted to 300,000 platelets/µl. Five µl of drugs were added to 0.5 ml of PRP and incubated at 37°C for 2 min.

Aggregation was induced by 0.5mM arachidonic acid and measured with an aggregometer (Chrono-Log, Havertown, Pa.)

Preparation of human platelet microsomes and sheep vesicular gland microsomes (SVGM): Human platelet microsomes were prepared by the method of Ho et al (21). The platelet microsomes were suspended in tris-HCl buffer at pH 7.4 and stored in 0.5 ml aliquots at -90°C until use. SVGM was prepared by the method of Takeguchi et al (22). SVGM were suspended in phosphate buffer at pH 7.5 and stored in 1.0 ml aliquots at -90°C until use. Protein concentrations were determined by the method of Lowry (23).

Enzyme assay for PG and thromboxane synthesis: The vasodilatory drugs were added in 5 µl of vehicle and preincubated with enzyme for 5 min. PG and thromboxane biosynthesis was initiated by incubating 2 µM of [1-^{14}C] arachidonic acid with 100 µg of platelet microsomal protein. Five mM L-tryptophan and 2 µM methemoglobin were used as cofactors in a final volume of 0.5 ml reaction media. The reaction was carried out for 15 minutes at 37°C and terminated by the addition of 150 µl of 1N HCl. As a control, vehicle alone was preincubated with enzyme and the reaction was carried out as previously described. To initiate the cyclooxygenase reaction in SVGM, 2 µM of [1-^{14}C] arachidonic acid was incubated with 154 µg of microsomal protein.

Radiochemical analysis of PG and TxB_2: The acidified reaction mixture was first extracted with 9 ml of ethyl acetate. The solvent was taken to dryness under reduced pressure and the PGs were reconstituted in chloroform/methanol (1/1). The sample was spotted on Whatman LK6D thin layer chromotographic plates (Whatman Ind. Clifton, N.J.). The plates were developed in chloroform/methanol/acetic acid (180/10/10). Zones corresponding to PG and TxB_2 standards, visualized by I_2 vapors, were scraped and counted for radioactivity by a liquid scintillation spectrometer (Packard Instr. Co., Downers Grove, Ill.). PG production was expressed as percent of total radioactivity on the TLC plate.

RESULTS AND DISCUSSION

We initially studied these vasodilatory compounds on platelet aggregation. It has been reported by Hamberg and Samuelsson that TxA_2 plays a critical role in platelet aggregation (1). Later it was discovered that selective inhibitors of TxA_2 biosynthesis would inhibit aggregation (15). As shown in Figure 1, 2×10^{-3} M of diazoxide inhibited platelet aggregation induced by arachidonic acid. Dipyridamole elicits a similar response at 1/10 of that concentration, while hydralazine shows a comparable inhibition at 1/20 of that amount. Consistant with our findings, Mustard has reported that dipyridamole, at a concentration of 2.5×10^{-4} M inhibited thrombin or collagen induced human platelet aggregation (24).

From these studies, we have demonstrated that these drugs are indeed inhibitors of platelet aggregation induced by arachidonic acid. However, it is not clear as to whether this phenomenon could be attributed to inhibition

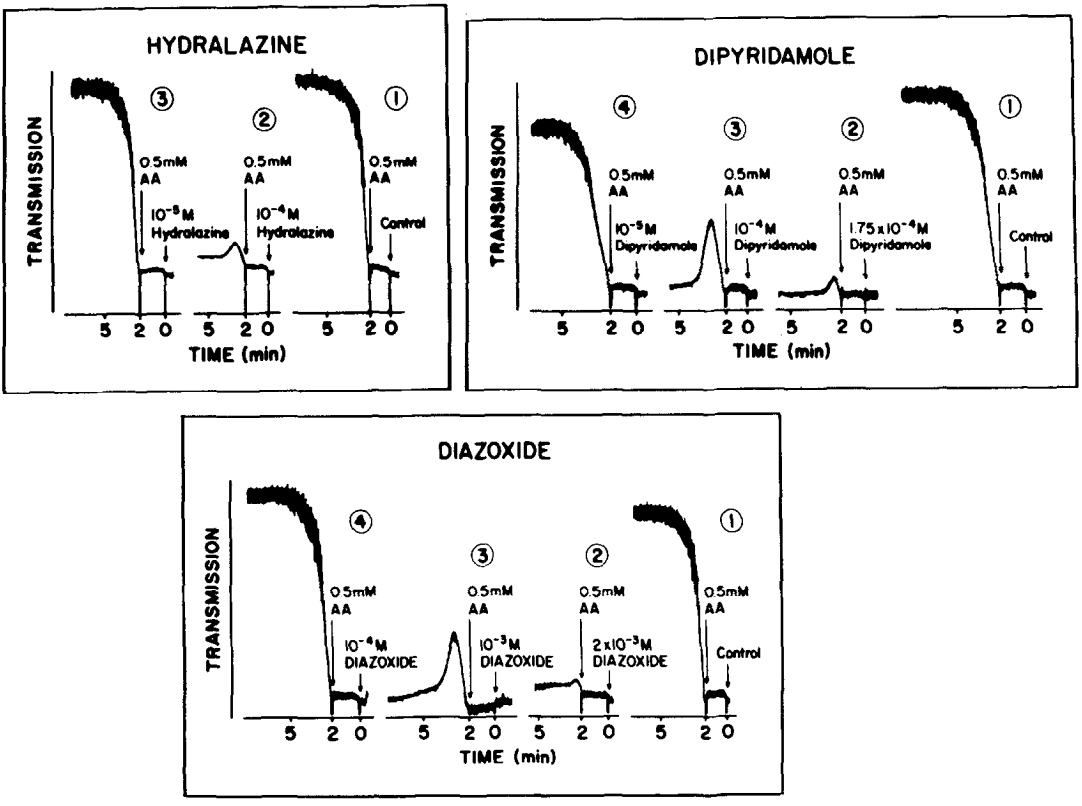

Figure 1: Effects of hydralazine, dipyridamole and diazoxide on aggregation of human PRP.

of TxA_2 synthetase or cyclooxygenase enzyme, or inhibition by a non-PG mediated mechanism. To answer this question, we studied the effect of these drugs on thromboxane production in the platelet microsomal system. In the control experiments, incubation of human platelet microsomes with 2 μM of arachidonic acid for 15 min at 37°C yielded 18% of TxB_2, 7% of PGE_2 and 3% of $PGF_{2\alpha}$. Incubations in the presence of increasing doses of hydralazine (Fig. 2) produced a dose dependent decrease in the amount of TxB_2 formed. In the same experiments, no change was noted in the recovery of unreacted arachidonic acid or PGE_2. These data suggested that hydralazine did not affect cyclooxygenase function in human platelet microsomes. On the other hand, a dose dependent increase in $PGF_{2\alpha}$ formation was evident. A maximal 5-fold production of $PGF_{2\alpha}$ was noted at 10^{-3} M hydralazine. These data are consistent with the recent work of

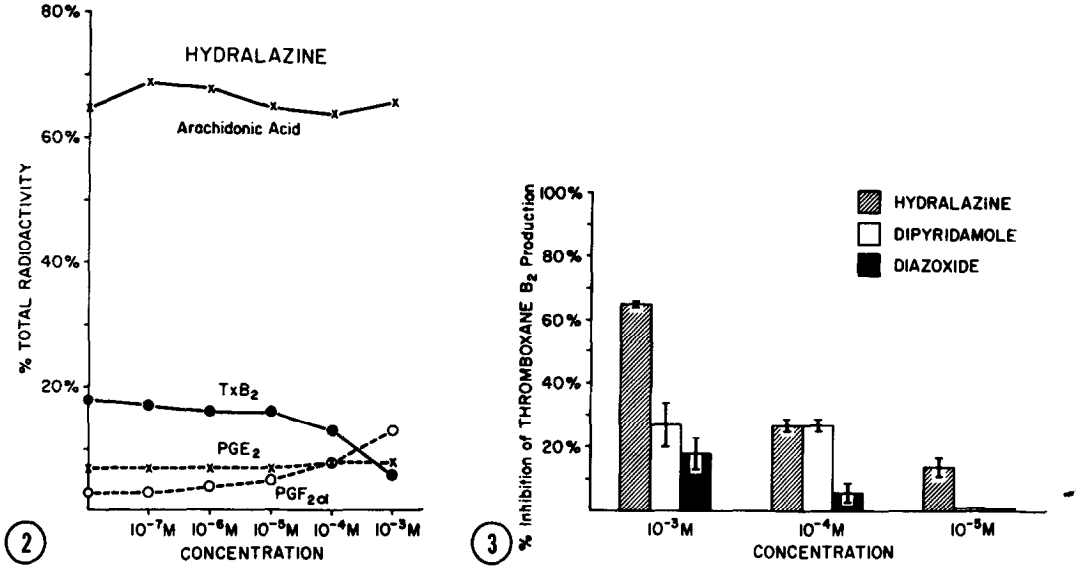

Figure 2: A plot of arachidonic acid metabolism in human platelet microsomal preparation versus hydralazine concentration. Each data point is an average of 3 determinations.

Figure 3: Relative potencies of hydralazine, dipyridamole, and diazoxide as inhibitors of thromboxane B_2 production in human platelet microsomes. Values presented are means ± S.E.M.

Vane (25) who also noted a 6-fold increase in $PGF_{2\alpha}$ formation in human platelet homogenates after selective inhibition of TxA_2 formation with imidazole.

The ability of diazoxide and dipyridamole to inhibit TxB_2 formation was also examined. Figure 3 represents the relative inhibitory potencies of these drugs on TxB_2 formation. At 10^{-3} M, hydralazine shows a 65 % inhibition of TxB_2 formation. This is approximately a 2-fold greater inhibition than that seen with a similar dose of dipyridamole, and a 4-fold greater inhibition than that of diazoxide. Using the same microsomal system we also compared the potencies of these compounds with indomethacin. At 10^{-4} M, indomethacin showed a maximal 65 % inhibition of TxB_2 formation. This suggests that there is a basal production of TxB_2 even in the presence of an inhibitor.

In a third set of experiments, hydralazine, dipyridamole, and diazoxide were compared with indomethacin in their ability to inhibit the SVGM cyclo-oxygenase reaction. In order to evaluate whether hydralazine, dipyridamole and

diazoxide inhibit cyclooxygenase, SVGM were preincubated with these drugs at 10^{-3}M concentration before initiation of reaction with [1-^{14}C]arachidonic acid. Unreacted arachidonic acid recovery was 15.7% ± 1.7% (mean ± S.D.) in control experiments and in the presence of drugs the recovery was 11.7% ± 1.2% (mean ± S.D.). When incubations were carried out with indomethacin (10^{-5} M), 90% of the unreacted fatty acid was recovered.

These data are consonant with our hypothesis that hydralazine, dipyridamole, and diazoxide, three clinically efficacious vasodilatory drugs are inhibitors of TxA_2 formation. The order of potency of these three drugs, i.e. hydralazine > dipyridamole > diazoxide, on the inhibition of platelet aggregation as well as on the suppression of TxB_2 formation in human platelet microsomes follows the same sequence. The SVGM experiments further confirms that these drugs inhibit thromboxane synthetase rather than cyclooxygenase. Recently, Ally *et al* (26) have suggested that dipyridamole is a possible inhibitor of TxA_2 synthetase in vascular smooth muscle. Our results support this hypothesis. Johnson and co-workers (27) reported the ability of the oral administration of imidazole to rabbits inhibited TxA_2 biosynthesis in platelets by 36 percent, while it stimulated prostacyclin (PGI_2) synthesis in aortic rings by 215 percent. Likewise, hydralazine, dipyridamole, and diazoxide may not only inhibit TxA_2 formation, but may also stimulate PGI_2 production since the endoperoxides are common substrates for TxA_2 and PGI_2. We are currently investigating such a hypothesis. The ability of these drugs to inhibit the synthesis of a potent vasoconstrictor lends new insight to elucidating their mechanisms of action as arterial smooth muscle vasodilators.

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REFERENCES

1. Hamberg, M., Svensson, J., Samuelsson, B. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2994-2998.
2. Svensson, J., Hamberg, M., Samuelsson, B. (1975) Acta Physiol. Scand. 94, 222-228.
3. Higgs, G.A., Bunting, S., Moncada, S., Vane, J.R. (1976) Prostaglandins 12, 749-757.
4. Tuvemo, T., Strandberg, K., Hamberg, M., Samuelsson, B. (1976) Acta Physiol. Scand 96, 145-149.
5. Wolfe, L.S., Rostworoski, K., Marion J. (1976) Biochem. Biophys. Res. Commun. 70, 907-913.
6. Gryglewski, R., Vane, J.R. (1972) Brit. J. Pharmacol. 43, 37-47.
7. Morrison, A.R., Nishikawu, K., Needlemen, P. (1977) Nature 267, 259-260.
8. Vane, J.R., (1971) Nature New Biol. 231, 232-235.
9. Needleman, P., Raz, A., Ferrendelli, J.A., Minkes, M., (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1716-1720.
10. Sun, F.F., (1977) Biochem. Biophys. Res. Commun. 74, 1432-1440.
11. Tai, H.H., Yuan, B. (1977) Fed. Proc. 36, 309.
12. Gorman, R.R., Bundy, G.L., Peterson, D.C., Sun, F.F., Miller, O.V., Fitzpatrick, R.A. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4007-4011.
13. Diczfalusy, U., Hammarstrom, S. (1977) Febs Lett. 82, 107-110.
14. Allan G., Eakins, K.E. (1978) Prostaglandins 15, 659-662.
15. Gryglewski, R.J. (1977) Naunyn-Schmiedeberg's Arch. Pharmacol. 297. S85-S88.
16. Kulkarni, P.s., Eakins, K.E. (1976) Prostaglandins 12, 465-469.
17. Moncada, S., Needleman, P., Bunting, S., Vane, J.R. (1976) Prostaglandins 12, 323-336.
18. Eakins, K.E., Rajadhyaska, V., Schroer, R. (1976) Brit. J. Pharmacol. 58, 333-336.
19. Greenwald, J., Rao, M., Wong, L.K. (1978) The Pharmacologist 20. 234.
20. Panganamala, R.V., Miller, J.S., Gwebu , E.T., Sharma, H.M., Cornwell, D.G., (1977) Prostaglandins 14: 261-271.
21. Ho, P.P.K., Walters, C.P., Sullivan, H.R., (1976) Prostaglandins 12: 951-969.
22. Takeguchi, C., Kohno, E., Sih, C.J., (1971) Biochemistry 10: 2372-2376.
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) J. Biol Chem. 193, 265-275.
24. Cucuianu, M.P., Nishizawa, E.E., Mustard, J.F. (1971) J. Lab. Clin. Med. 77, 958-974.
25. Nijkamp, F.P., Moncada, S., White, H.L., Vane, J.R. (1977) Eur. J. Pharmacol. 44, 179-186.
26. Ally, A.I., Manku, M.S., Horrobin, R.O., Morgan, R.O., Karmazin, M., Karmali, R.A. (1977) Prostaglandins 14, 607-609.
27. Johnson, M., Reece, A.H., Wale, J., Jessup, R. (1978) Abstracts of papers presented at the 7th International Congress of Pharmacology, Paris, July, 1978, pp. 342.