Vol. 80, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

January 13, 1978 Pages 236-242

ON THE INHIBITORY POTENCY OF IMIDAZOLE AND ITS DERIVATIVES ON THROMBOXANE SYNTHETASE

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Received December 5,1977

SUMMARY: The relative inhibitory potency of imidazole and derivatives on thromboxane synthetase from human platelets was found to be increased by substitution of the 1-position and abolished in other positions. The potency of 1-substituted imidazoles was increased as the side chain became more hydrophobic. Among the imidazole derivatives tested 1-nonyl-imidazole and 1-(2-isopropyl phenyl)-imidazole showed the highest potency with I_{50} in the range of 10^{-8} M. Inhibition by imidazole and its derivatives appeared to be very specific for thromboxane synthetase since other enzymes in prostaglandin endoperoxide metabolism were not affected. Kinetic studies indicated that inhibition was competitive with respect to prostaglandin endoperoxide substrate.

INTRODUCTION

Hamberg and Samuelsson(1) first reported the novel transformation of prostaglandin endoperoxides into a highly unstable intermediate in human platelets which was extremely effective in inducing platelet aggregation and smooth muscle contraction. The unstable intermediate, later termed thromboxane A_2 (TXA_2), was readily converted into the stable product thromboxane B_2 (TXB_2) after incorporation of one mole of water. The enzyme which catalyzes the formation of this potent and yet unstable intermediate from prostaglandin endoperoxides has been identified and named thromboxane synthetase (2). Factors regulating the enzyme activity have also been reported. These include prostaglandin endoperoxide analogs (3-6), 2-isopropyl-3-nicotinyl-indole (7), imidazole (8,9) and the prostaglandin antagonist, N-0164 (10). Among these inhibitors

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0006-291X/78/0801-0236$01.00/0

imidazole appears to be the most selective but least potent inhibitor of thromboxane synthetase. We have investigated several derivatives of imidazole and found that substitution of the 1-position of imidazole but not of other positions with a hydrophobic side chain provides the most potent and selective inhibitors of thromboxane synthetase.

MATERIALS AND METHODS

Arachidonic acid, 1-methyl imidazole, 2-methyl imidazole, methimazole, histamine, hemoglobin, L-epinephrine, DL-isoproterenol, GSH, bovine γ-globulin, bovine serum albumin, and tyrosine methyl ester (TME) were purchased from Sigma Chemical Company. Benzimidazole and 1-benzylimidazole were obtained from Aldrich Chemical Company. Imidazole was supplied by Fisher Scientific Company. Other imidazole derivatives were kindly donated by Dr. C. F. Wilkinson of Cornell University. PGE_2 and TXB_2 were kind gifts of Dr. U. Axen of the Upjohn Company. Outdated human platelet concentrates were kindly supplied by the American Red Cross (Rochester, N.Y.). The microsomal fraction of sheep lung was prepared according to the previously published method (11). Prostaglandin endoperoxide, PGH_2, was prepared according to the method of Hamberg and Samuelsson (12).

Preparation of microsomal fraction from human platelets: Human platelet concentrates were centrifuged at 200 x g for 10 min to remove contaminating red blood cells. The platelets were collected by centrifugation at 2,000 x g for 15 min. The pellet was washed with one original volume of saline once and recentrifuged at 2,000 x g for 15 min. The pellet was resuspended in 0.05M Tris-HCl buffer, pH 7.5 containing 1 mM EDTA equivalent to 3 volumes of platelet wet weight. The platelet suspension was homogenized with a polytron operated at a full speed for 2 min. Disrupted platelet was centrifuged at 5,000 x g for 15 min. The supernatant was further centrifuged at 105,000 x g for 60 min. The resultant pellet was homogenized in the same buffer equivalent to one third volume of platelet wet weight and stored in small aliquots at -40°C.

Enzyme assay for thromboxane synthetase: Since TXA_2 is extremely short-lived and readily converted into TXB_2 in aqueous medium, the enzyme activity was assayed by measuring the formation of TXB_2 immunoreactivity from substrate PGH_2. The reaction mixture contained: PGH_2, 5.0 n moles; human hemoglobin, 1 n mole; L-epinephrine, 1 μ mole and microsomal fraction in a final volume of 1 ml of 0.05 M Tris HCl buffer, pH 7.5. The reaction was initiated with PGH_2 and the mixture was incubated at 22°C for 3 min. The reaction was terminated by the acidification with 0.05 ml of 1 N HCl. The mixture was then neutralized with 0.05 ml of 1 M Tris base and diluted with the radioimmunoassay buffer for further measurement of TXB_2 immunoreactivity. The difference in TXB_2 immunoreactivity between o min and 3 min samples was taken as the amount of TXB_2 being synthesized during 3 min of incubation.

Radioimmunoassay: The details of the radioimmunoassay have been previously described by Tai and Yuan (13). Briefly, antibodies, labeled antigen, TXB_2 standards and samples were diluted in the standard radioimmunoassay buffer, 0.05 M Tris HCl, pH 7.5 containing 0.1% gelatin. The incubation mixture (0.4 ml) contained: 0.2 ml of standards or samples, 0.1 ml of diluted antibodies (final resultant dilution 1 to 5,000) and 0.1 ml of [^{125}I]-TXB_2-TME conjugate (10,000 cpm). The incubation was carried out at room temperature

for one hour. Separation of bound from free antigen was achieved by γ-globulin plus dextran coated charcoal. The supernatant and the pellet obtained after centrifucation were counted separately in a Packard gamma counter. The bound-to-total ratio was calculated for each sample and the concentration of each sample was determined from a standard curve after logit transformation as described previously (14).

RESULTS AND DISCUSSION

Moncada et al. (8) and Needleman et al. (9) first discovered that imidazole was a selective inhibitor of human platelet thromboxane synthetase. A variety of imidazole derivatives were also screened for inhibitory effect and found to be relative inactive except for 1-methyl-imidazole. We have confirmed these findings with the microsomal thromboxane synthetase from both human platelets and sheep lung (15). We further investigated the positional derivatives of imidazole and observed that substitution at 1 position retained the inhibitory action of imidazole, while substitutions at other positions rendered the compound inactive (see Table 1). Furthermore, the inhibitory potency of 1-substituted imidazoles was increasing with increasing chain length, passing through a maximum, and decreasing again in the higher homologs. Among the 1-alkyl and 1-aryl imidazoles we examined, 1-nonyl imidazole and 1-(2-isopropylphenyl) imidazole possessed the highest potency with I_{50} of 1×10^{-8}M and 4.0×10^{-8}M respectively. These compounds appeared to be the most potent inhibitors of thromboxane synthetase yet described. The low potency of imidazole itself and the increasing potency of a homologous series of 1-sutstituted imidazoles strongly suggests that the substituent group plays an important role in the inhibitory action and might indicate the importance of hydrophobic binding to some site close to the active site of the enzyme. The fact that the inhibitory potency of 1-alkyl imidazoles exhibits an optimum value indicates that the inhibitor must bind to a hydrophobic pocket which can only accommodate a certain number of methylene groups. The inactivity of those compounds substituted at the 2 and 1,4 positions suggests that the 3 position nitrogen must be sterically unhindered for inhibitory activity.

TABLE I. Inhibitory Power of Imidazole and Its Derivatives on Thromboxane Synthetase of Human Platelets

Inhibitors	I_{50}(μM)
Imidazole	46
1-Methyl imidazole	52
2-Methyl imidazole	>500
4-Ethylamine imidazole (Histamine)	>500
4,5-Dimethyl imidazole	>500
1-Methyl-2-thio-imidazole (Methimazole)	>500
1-Methyl-4-phenyl-imidazole	>500
4,5-Diphenyl imidazole	>500
Benzimidazole	>500
1-Ethyl imidazole	8.4
1-Propyl imidazole	2.0
1-Butyl imidazole	0.5
1-Pentyl imidazole	0.19
1-Nonyl imidazole	0.01
1-Decyl imidazole	0.05
1-Dodecyl imidazole	3.15
1-Benzyl imidazole	0.25
1-(2-Isopropylphenyl) imidazole	0.04

The assay mixture was essentially the same as described in the Methods section except that six different concentrations of each inhibitor were employed to determine the inhibitor concentration which gave 50% inhibition. The amount of microsomal protein used per assay was 83 μg.

The effects of imidazole and its derivatives appeared to be instantaneous and reversible as evidenced by the lack of time dependent inhibition and the relief of inhibition after dilution of the inhibited enzyme preparation. The nature of the action of imidazole and its derivatives was further investigated by kinetic studies. Inhibition of thromboxane synthetase activity was carried out with a series of concentrations of imidazole at three different fixed concentrations of substrate PGH_2. The Dixon plot, as shown in Fig. 1, shows a competitive inhibition pattern indicating that imidazole is competing with the substrate PGH_2 to interact with the enzyme at the same site. A similar pattern was also observed with 1-(2-isopropyphenyl)-imidazole as the inhibitor.

Imidazole and its 1-alkyl and 1-aryl derivatives have previously been shown to be inhibitory towards a variety of microsomal drug oxidation reactions

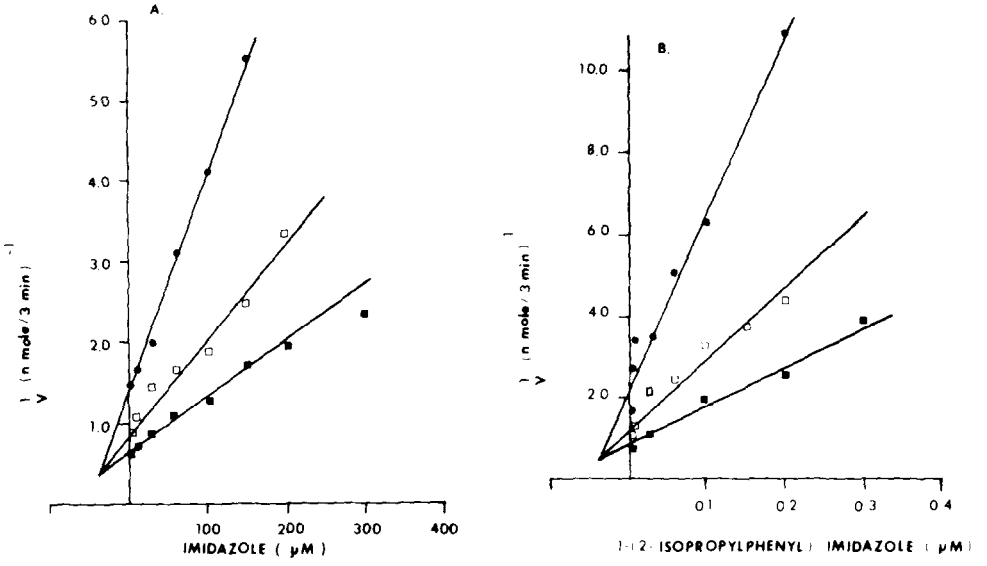

Fig. 1: Inhibition of human platelet thromboxane synthetase by A. Imidazole B. 1-(2-Isopropylphenyl) imidazole at three different concentrations of PGH_2. The amount of microsomal protein used was 124 μg.

—●—●— 3 μM, —□—□— 5 μM, —■—■— 10 μM.

in rat liver (16-18). The inhibitory activity was thought to be related to the ability of these nitrogeneous compounds to bind to the fifth or sixth ligand of the heme moiety of cytochrome P-450. Although the nature of isomerization catalyzed by thromboxane synthetase is totally unknown, the possibility that a heme containing cofactor such as coenzyme B_{12} might be involved in the rearrangement of the bicyclic rings of prostaglandin endoperoxide to form thromboxane A_2 appears to be an interesting one.

The specificity of inhibition by imidazole and its derivatives was also examined. The microsomal fraction of sheep lung has been shown to exhibit appreciable thromboxane, prostaglandin E and prostacyclin synthetase activities when assayed in the presence of a catacholamine and GSH (11). This provides a unique system to test the effect of inhibitors on various enzyme systems. Table II shows that imidazole inhibits thromboxane synthesis but not prostaglandin E_2 and 6-keto-$PGF_{1\alpha}$ synthesis from arachidonic acid. The inhibitor

TABLE II. Effect of Imidazole on 6-Keto-$PGF_{1\alpha}$, PGE_2 and TXB_2 Biosynthesis by Sheep Lung Microsomes.

Imidazole concentrations (μM)	6-Keto-$PGF_{1\alpha}$	PGE_2 (n moles / 5 min)	TXB_2
0	1.92	5.80	3.00
30	2.22	6.72	2.76
60	2.16	7.45	2.44
100	2.23	7.68	2.02
200	2.41	8.14	1.80
400	3.23	8.50	1.28

The incubation mixture contained: Arachidonic Acid (33 n moles, 60,000 cpm), DL-isoproterenol (1 μ mole), GSH (1 μ mole), sheep lung microsomes (2.6 mg of protein) and indicated concentrations of imidazole in 1 ml of 0.05 M Tris-HCl buffer, pH 7.5. The incubation was carried out at 37°C for 5 min and the reaction was terminated by acidification to pH 3.0. The mixture was extracted with 2 x 3 ml of ethyl acetate. The organic extract with added standards after evaporation was chromatographed on a silica gel G plate (2 x 20 cm) developed in the solvent system of benzene/dioxane/acetic acid (20:10:1) to a height of 15 cm. Bands corresponding to each product were visualized in iodine vapor and scrapped off for radioactivity determination.

also did not show an inhibitory effect on prostaglandin endoperoxide synthetase activity since the total amount of products formed was not decreased. Similar observations were obtained with 1-(2-isopropylphenyl)-imidazole as the inhibitor. Imidazole and its derivatives appear to be rather specific inhibitors of thromboxane synthetase.

Although thromboxane A_2 is known to induce irreversible platelet aggregation and to contract arterial smooth muscle, the role of thromboxanes in many other physiological systems still remains unknown. The discovery of potent, specific and reversible inhibitors of the biosynthesis of thromboxanes will undoubtedly aid in elucidating the role of thromboxanes in many physiological and pathophysiological processes.

ACKNOWLEDGMENTS

We are indebted to Dr. C. F. Wilkinson of Cornell University for the generous supply of various imidazole derivatives, and to Dr. U. Axen of the Upjohn

Company for the gift of prostaglandin E_2 and thromboxane B_2. This study was supported in part by a USPHS Grant (GM-21588).

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