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SELECTIVE INHIBITORY ACTIONS OF SODIUM-*p*-BENZYL-4-[1-OXO-2-(4-CHLOROBENZYL)-3-PHENYL PROPYL] PHENYL PHOSPHONATE (N-0164) AND INDOMETHACIN ON THE BIOSYNTHESIS OF PROSTAGLANDINS AND THROMBOXANES FROM ARACHIDONIC ACID

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1 Sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenyl propyl]phenyl phosphonate (N-0164) selectively inhibited the formation of thromboxane-A_2 from prostaglandin endoperoxides by human platelet microsomes in a dose-dependent manner (IC_{50} 2.2×10^{-5} M or 11.6 μg/ml).

2 N-0164 was approximately 15 to 20 times as potent as indomethacin as an inhibitor of thromboxane-A_2 formation. In contrast, indomethacin was 20 times as potent as N-0164 as an inhibitor of prostaglandin endoperoxide formation from arachidonic acid (IC_{50} 2.6×10^{-5} M or 9.4 μg/ml).

3 Spiral strips of dog coronary arteries relaxed in the presence of prostaglandin endoperoxides and were contracted by prostaglandin E_2 and thromboxane-A_2 and were therefore used to distinguish between prostaglandins and their intermediate precursors, the endoperoxides.

4 Neither indomethacin nor N-0164 (both 50 μg/ml) significantly inhibited the formation of prostaglandin-like activity from the endoperoxides following incubation with indomethacin-pretreated rabbit kidney medulla microsomes.

5 It is not known whether this action of N-0164 is related to its ability to antagonize certain actions of prostaglandins (and related compounds) or whether N-0164 can penetrate the cell membrane to inhibit thromboxane formation in the intact cell.

6 Selective inhibition of thromboxane formation by drugs such as N-0164 may be useful both clinically and as a pharmacological tool to elucidate the patho-physiological roles of the thromboxanes.

Introduction

The prostaglandin endoperoxides (prostaglandin G_2 and H_2) can be converted by thromboxane synthetase, an enzyme present in platelets, to the potent but very unstable rabbit aorta contracting substance, thromboxane A_2, which is known to be released during platelet aggregation (Hamberg, Svensson & Samuelsson, 1975; Bunting, Moncada, Needleman & Vane, 1976; Needleman, Moncada, Bunting, Vane, Hamberg & Samuelsson, 1976b).

Sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenyl propyl]phenyl phosphonate (N-0164) has been found to antagonize selectively certain actions of prostaglandins (Eakins, Rajadhyaksha & Schroer, 1976). In addition, in a preliminary series of experiments, this compound in higher concentrations was also found to inhibit the conversion of prostaglandin endoperoxides to thromboxane-A_2-like activity by human platelet microsomes (Kulkarni & Eakins, 1976). Only one other compound, benzydamine, has been shown to inhibit selectively thromboxane generation from prostaglandin endoperoxides at this time (Moncada, Needleman, Bunting & Vane, 1976).

In the present study, we have further investigated this action of N-0164 and compared it with indomethacin, a known prostaglandin synthetase inhibitor (Vane, 1971) on other pathways in the metabolism of arachidonic acid to prostaglandin E_2.

Methods

Synthesis of prostaglandin endoperoxides

Sheep seminal vesicle microsomes were prepared as described by Takeguchi, Kotina & Sih (1971) and

resuspended in phosphate buffer (50 mM, pH 7.5–8). This suspension was then redistributed in 0.5 ml quantities in smaller tubes and the protein content of each sample determined by the method of Lowry, Rosebrough, Farr & Randall (1951). These microsomal preparations plus arachidonic acid were used as a prostaglandin endoperoxide generating system as described by Bunting *et al.* (1976). Seminal vesicle microsomes (2–300 μg protein) were incubated without cofactors with sodium arachidonate (2 μg) at room temperature (22°C) for 1 min, 50 μl samples of this mixture were then tested for biological activity as described below.

Preparation of human platelet microsomes

Batches of expired platelet-rich human plasma were incubated with indomethacin (2 μg/ml) for 16 h then centrifuged at 2000 *g* for 15 minutes. The resultant platelets were resuspended in ice-cold 0.9% w/v NaCl solution (saline) and lysed by shell freezing and thawing (five times). Cell debris was then removed by centrifugation at 8000 *g* for 15 minutes. The supernatant was then centrifuged at 100,000 *g* for 45 minutes. The resultant microsomal pellet was resuspended in 5 ml phosphate buffer (50 mM, pH 7.5–8) and redistributed in 0.5 ml quantities and the protein content of each sample measured as before.

Preparation of rabbit kidney medulla microsomes

Kidneys were excised from white albino rabbits and the kidney medulla separated from the cortex. The medulla was then cut into small pieces, homogenized in ice-cold 0.15 M KCl containing 5 μg/ml indomethacin and centrifuged at 2000 *g* for 15 minutes. The supernatant was then re-centrifuged at 100,000 *g* for 45 min and the resultant microsomal pellet resuspended in phosphate buffer (50 mM, pH 7.5–8). This suspension was then redistributed in 0.5 ml quantities in small tubes and the protein content of each sample determined as before.

Synthesis of thromboxane-A_2-like activity

In some experiments, thromboxane-A_2-like activity was produced by further incubation of the prostaglandin endoperoxide suspension with the indomethacin-treated human platelet microsomes (150–250 μg protein) for 2 min at 0°C; 50 μl aliquots were then immediately tested for biological activity.

In other experiments, the authentic prostaglandin endoperoxide, prostaglandin H_2 was incubated with the indomethacin-treated human platelet microsomes as described above.

Assay of biological activity

Bioassays were made either on a rabbit aorta (Piper & Vane, 1969) or in some experiments, on a dog coronary artery (Kulkarni, Roberts & Needleman, 1976) superfused at 10 ml/min with Krebs solution at 37°C containing a mixture of pharmacological antagonists (Gilmore, Vane & Wyllie, 1968) to block acetylcholine, 5-hydroxytryptamine, catecholamines and histamine, plus indomethacin (1 μg/ml), to inhibit prostaglandin biosynthesis by the assay tissues (Vane, 1971). Angiotensin II was used to standardize the assay tissues.

Drugs

The following drugs were used: arachidonic acid, sodium salt (Sigma Chemical Company, St. Louis, Mo.); indomethacin (Merck Sharp and Dohme, Rahway, New Jersey); methysergide maleate (Sandoz, Hanover, New Jersey); diphenhydramine hydrochloride (Parke Davis); atropine sulphate (Burroughs Wellcome); propranolol (Ayerst Laboratories); phenoxybenzamine hydrochloride (Smith, Kline and French); angiotensin amide (Ciba-Geigy Laboratories); N-0164 (Nelson Research and Development Company).

Stock solutions of N-0164 up to a maximum concentration of 250 μg/ml were freshly prepared each day by dissolving the compound in warm phosphate buffer. Particular care was taken to ensure that the drug remained in solution, any signs of precipitation disappeared with further warming.

Results

When sodium arachidonate was incubated without cofactors with sheep seminal vesicle microsomes at room temperature for 1 min, a product was formed which resembled the prostaglandin endoperoxides, prostaglandin G_2 or H_2. It contracted the rabbit aorta, had a half life of 2–4 min in aqueous solution at 37°C and, in addition, was converted to a more powerful rabbit aorta contracting substance when incubated at 0°C for 2 min with indomethacin-treated human platelet microsomes and immediately tested (Figure 1f, g). In other experiments, a similar activity was produced when authentic prostaglandin H_2 was incubated with human platelet microsomes under identical conditions (Figure 1a, b). The characteristics of this rabbit aorta contracting substance were found to be the same as those described previously (Bunting *et al.*, 1976; Needleman *et al.*, 1976b).

Addition of N-0164 (5–50 μg/ml) to the human platelet microsomes for 2 min inhibited the subsequent conversion of prostaglandin endoperoxides to thromboxane-A_2 in a dose-dependent manner.

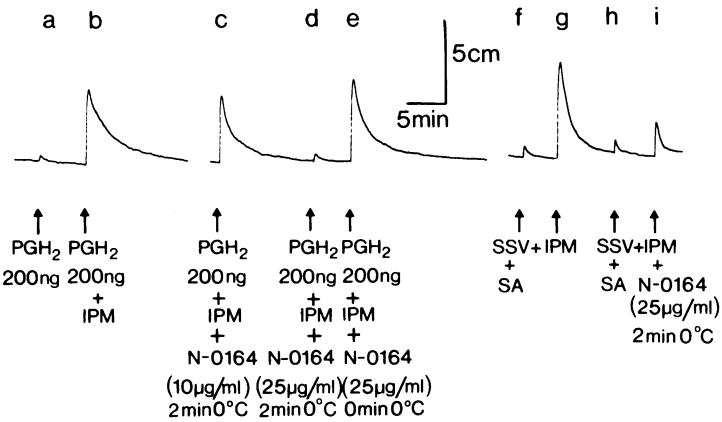

Figure 1 Inhibition of thromboxane-A_2-like (TxA_2-like) activity by N-0164. Bioassay of rabbit aorta contracting substances: (a) prostaglandin H_2 (PGH_2), 200 ng; (b) PGH_2 (200 ng) was incubated at 0°C for 2 min in 50 μl phosphate buffer (50 mM, pH 8) with indomethacin-treated human platelet (IPM) microsomes (200 μg protein) to yield TxA_2-like activity; (c and d) addition of N-0164 (10 μg/ml and 25 μg/ml) to the IPM incubate for 2 min at 0°C inhibited formation of TxA_2-like activity; (e) addition of N-0164 (25 μg/ml) to IPM incubate immediately before testing did not prevent the appearance of TxA_2-like activity; (f) sheep seminal vesicle (SSV) microsomes (250 μg protein) were incubated with 2 μg sodium arachidonate (SA) in 100 μl phosphate buffer for 1 min at 22°C and 50 μl tested; (g) further incubation of this sample at 0°C for 2 min with IPM (200 μg protein) to yield TxA_2-like activity; (h) same as (f); (i) addition of N-0164 (25 μg/ml) to IPM incubate for 2 min at 0°C inhibited formation of TxA_2-like activity.

Inhibition of the formation of thromboxane-A_2 from authentic prostaglandin H_2 is seen in Figure 1c and d. It was possible that these concentrations of N-0164 could antagonize the contraction produced by thromboxane-A_2 on the rabbit aorta. However, this was shown not to be the case since addition of the higher concentration of N-0164 (25 μg/ml) immediately before testing (zero minute incubation) failed to inhibit the contractions induced by thromboxane-A_2 (Figure 1e). A similar inhibition of thromboxane-A_2 formation was obtained when sheep seminal vesicles and sodium arachidonate were used as the source of prostaglandin endoperoxides (Figure 1i).

In the next series of experiments, the inhibitory effects of N-0164 were compared with indomethacin on both the prostaglandin endoperoxide and the thromboxane-A_2 generating systems. Dose-response curves were obtained for each drug as inhibitors of prostaglandin endoperoxide formation from the sheep seminal vesicle plus sodium arachidonate incubates (Figure 2a), and as inhibitors of thromboxane-A_2 formation produced by the subsequent incubation with platelet microsomes (Figure 2b). In addition, thromboxane-A_2 formation by platelet microsomes from authentic prostaglandin H_2 was also examined. It can be seen that indomethacin was a potent inhibitor of endoperoxide formation, the concentration required to produce a 50% inhibition of endoperoxide formation (IC_{50}) being 2.6×10^{-5} M (9.4 μg/ml); at the IC_{20} level of inhibition, indomethacin was 20 times as potent as N-0164 on this preparation. However, indomethacin was much less effective than N-0164 as an inhibitor of thromboxane-A_2 formation. N-0164 was approximately 10 to 15 times as potent against thromboxane-A_2 formation (IC_{50} 2.2×10^{-5} M or 11.6 μg/ml) as it was against prostaglandin endoperoxide formation. When authentic prostaglandin H_2, instead of the sheep seminal vesicle incubate, was incubated with the platelet microsomes to generate thromboxane-A_2 activity and the inhibitory effects of N-0164 retested, the IC_{50} remained virtually unchanged at 10 μg/ml.

In the final series of experiments, an attempt was made to assess the activity of N-0164 as an inhibitor of the conversion of prostaglandin endoperoxides to prostaglandin. In these experiments, bioassays were made on spiral strips of dog coronary artery which relax in the presence of prostaglandin endoperoxides generated by the sheep seminal vesicle plus arachidonate incubate and contract in the presence of thromboxane-A_2 and prostaglandin E_2 (Figure 3a, b, c). Further incubation of the endoperoxides with indomethacin-treated rabbit kidney medulla microsomes for 4 min at 22°C yielded a product, probably prostaglandin E_2, which contracted the coronary artery strips (Figure 3e). Equivalent quantities of boiled kidney medulla microsomes failed to produce this contractile activity. Addition of either N-0164 or indomethacin, both 50 μg/ml, to the kidney medulla microsomes for 4 min did not interfere with

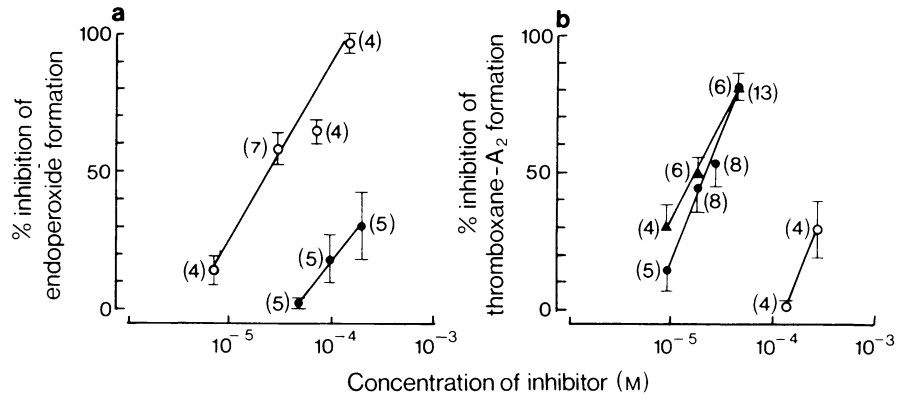

Figure 2 A comparison of the inhibitory effects of N-0164 and indomethacin on the biosynthesis of prostaglandin endoperoxides (a) and thromboxane-A_2 (b). (a) Dose-response curves for indomethacin (○) and N-0164 (●) as inhibitors of prostaglandin endoperoxide formation from sheep seminal vesicle plus sodium arachidonate incubates. (b) Dose-response curves for indomethacin (○) and N-0164 (●) as inhibitors of thromboxane-A_2 formation produced by subsequent incubation of the sheep seminal vesicles plus arachidonate with platelet microsomes. (▲) Dose-response curve for N-0164 as inhibitor of thromboxane-A_2 formation produced by incubation of platelet microsomes with authentic prostaglandin H_2. Number of observations at each point is shown in parentheses.

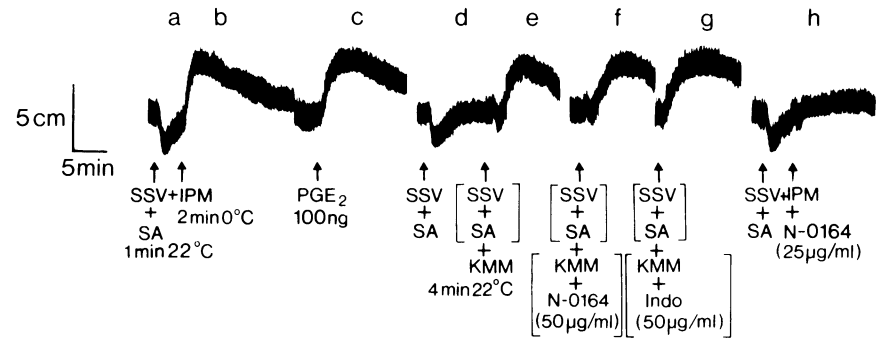

Figure 3 N-0164 and indomethacin do not inhibit the conversion of prostaglandin endoperoxides to prostaglandin-like activity. Bioassay on dog coronary artery. (a) Sheep seminal vesicle (SSV) microsomes (246 μg protein) were incubated with 1 μg sodium arachidonate (SA) in 100 μl phosphate buffer (50 mM, pH 8) at 22°C for 1 min to generate prostaglandin endoperoxides (PGG_2 and PGH_2) which induced relaxation. (b) Further incubation with indomethacin-treated human platelet microsomes (IPM) at 0°C for 2 min generated thromboxane-A_2 (TxA_2) resulting in contraction. (c) Authentic prostaglandin E_2 (PGE_2), 100 ng, caused contraction. (d) Same as (a). (e) Same as (a) but incubated with indomethacin-treated rabbit kidney medulla microsomes (KMM) (30 μg protein) at 22°C for 4 min to generate prostaglandin-like activity. (f) Addition of N-0164 (50 μg/ml) to KMM incubate for 4 min failed to inhibit the appearance of prostaglandin-like activity. (g) Addition of indomethacin (Indo, 50 μg/ml) to KMM incubate also failed to inhibit the formation of prostaglandin-like activity. (h) Addition of N-0164 (25 μg/ml) to IPM incubate for 2 min at 0°C inhibited formation of TxA_2-like activity.

the formation of this prostaglandin E_2-like substance (Figure 3f and g), although half this concentration of N-0164 (25 μg/ml) was found to inhibit considerably the formation of thromboxane-A_2-like activity on this bioassay tissue (Figure 3h).

Discussion

N-0164 (0.5–5 μg/ml) has previously been found to be a potent, partially selective, antagonist of the contractile actions of both E and F prostaglandins on

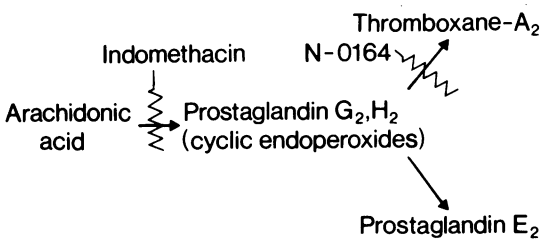

Figure 4 Schematic representation of the major sites of actions of indomethacin and N-0164 on pathways in the biosynthesis of thromboxane-A_2 and prostaglandin E_2 from arachidonic acid.

isolated preparations of gerbil, rat and guinea-pig gastrointestinal muscle (Eakins *et al.*, 1976). However, it should be noted that N-0164 failed to antagonize selectively the relaxant action of prostaglandin E_2 on the rabbit isolated coeliac artery (Kulkarni & Eakins, unpublished observations). The results of the present study indicate that, in addition, higher concentrations of N-0164 (10–50 μg/ml) can selectively inhibit the formation of thromboxane-A_2-like activity from prostaglandin endoperoxides by human platelet microsomes.

The inhibitory action of N-0164 was studied at three possible sites of action on the pathways of arachidonic acid metabolism as outlined in Figure 4. Thus the inhibitory effect of N-0164 was determined on the conversion of arachidonic acid to prostaglandin endoperoxide by the fatty acid cyclo-oxygenase (Hamberg & Samuelsson, 1973), on the formation of thromboxane-A_2 from the endoperoxide by thromboxane synthetase (Hamberg *et al.*, 1975; Needleman *et al.*, 1976b) and finally on the conversion of prostaglandin endoperoxides to prostaglandin E_2 which involves the endoperoxide isomerase (Hamberg, Svensson & Samuelsson, 1976).

Bunting *et al.* (1976) have described the use of seminal vesicle microsomes plus arachidonate as an endoperoxide generating system coupled with platelet microsomes plus prostaglandin endoperoxides to generate thromboxane-A_2. The rabbit aorta contracting activity formed from the endoperoxides by the platelet microsomes in the present experiments was very labile, disappearing within 1 min in aqueous solution at 37°C. Furthermore, under control conditions, equivalent quantities of boiled platelet microsomes failed to produce the rabbit aorta contracting activity. From these characteristics, which are essentially the same as those described previously (Bunting *et al.*, 1976; Needleman *et al.*, 1976b), it was assumed that the rabbit aorta contracting activity generated in the present experiments was thromboxane-A_2.

Indomethacin, a known prostaglandin synthetase inhibitor (Vane, 1971), was approximately 20 times as potent as N-0164 as an inhibitor of prostaglandin endoperoxide formation from arachidonic acid. In contrast, N-0164 was approximately 10 to 15 times as potent as indomethacin in preventing the formation of thromboxane-A_2 from prostaglandin endoperoxides by thromboxane synthetase. The similarity between the IC_{50} obtained for N-0164 as an inhibitor of thromboxane-A_2 generation by platelet microsomes from the authentic prostaglandin endoperoxide (10 μg/ml), compared to that derived from the sheep seminal vesicle incubate (11.6 μg/ml) provides additional confirmation of the validity of the method described by Bunting *et al.* (1976). The mechanism of this action of N-0164 is not known at present, but it may be related to the ability of the compound to block the contractile actions of prostaglandin and prostaglandin endoperoxides on certain smooth muscles (Kulkarni & Eakins, unpublished observations). It should also be noted that thromboxane synthetase is associated with the microsomal fraction in cells (Needleman *et al.*, 1976b), and it is not clear at this time whether N-0164 can penetrate and inhibit thromboxane synthetase within the intact cell.

The conversion of prostaglandin endoperoxides both to prostaglandin and thromboxane-A_2 was studied using the dog coronary artery as the assay tissue. Indomethacin-treated rabbit kidney medulla microsomes were used as a source of endoperoxide isomerase (Moncada, personal communication). Spiral strips of dog coronary arteries relax in the presence of prostaglandin endoperoxide but contract in response to either prostaglandin E_2 or thromboxane-A_2. Thus this preparation responds in a similar manner to human and bovine coronary arteries (Needleman, Kulkarni & Raz, 1977) and may therefore be used to distinguish between prostaglandins and their intermediate precursors, the endoperoxides. The fact that neither N-0164 nor indomethacin (both 50 μg/ml) had a significant effect on the formation of prostaglandin-like activity from the endoperoxides, although N-0164 (25 μg/ml) markedly inhibited the formation of thromboxane-A_2, suggests that neither drug is an effective inhibitor of the prostaglandin endoperoxide isomerase.

The importance of thromboxane-A_2 in pathological conditions is not well established at the present time. However, Vane (1972) has suggested that the actions of prostaglandin synthetase on arachidonic acid could lead to a whole cascade of substances, each of which may have some contribution to the inflammatory response. Furthermore, the identification of the 'rabbit aorta contracting substance' released during anaphylaxis from sensitized guinea-pig lungs (Piper & Vane, 1969) with thromboxane-A_2 (Hamberg *et al.*, 1975) suggests a role for these substances in anaphylactic bronchoconstriction. Thromboxanes are also released during platelet aggregation (Hamberg *et al.*, 1975; Needleman *et al.*, 1976b; Needleman,

Minkes & Raz, 1976a) and may play a role in arterial vasoconstriction. Specifically it has been suggested that thromboxanes may be involved in both coronary and cerebral arterial spasm (Ellis, Oelz, Roberts, Payne, Sweetman, Nies & Oates, 1976; Needleman *et al.*, 1977).

Selective inhibition of thromboxane formation may thus be useful in treating myocardial ischemia and other conditions in which thromboxanes are involved. Finally, N-0164 may be useful as a pharmacological tool to elucidate the patho-physiological roles of the thromboxanes.

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