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Effects of Cyclobuxine D on the Biosynthesis of Prostaglandins in Vitro, Prostaglandins Production and Leukocyte Migration in Vivo

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ABSTRACT

Cyclobuxine D was extracted from *Buxus microphylla* var. *koreana* Nakai. The effects of cyclobuxine D on the biosynthesis of prostaglandins from arachidonic acid in guinea pig lung, prostaglandin production and leukocyte migration in carrageenin-induced inflammation was investigated. These effects of cyclobuxine D were compared with those of aspirin and dexamethasone. Cyclobuxine D does not inhibit significantly cyclooxygenase in guinea pig lung but reduces prostaglandin concentration and leukocyte migration in inflammatory exudates. These effects of cyclobuxine D differ from that of aspirin which inhibits biosynthesis of prostaglandin in vitro and has a relative small effect on leukocyte migration. Dexamethasone, which does not inhibit cyclooxygenase in vitro, has an effect similar to that of cyclobuxine D on leukocyte migration and prostaglandin production in inflammatory exudates.

Key Words: Cyclobuxine D, Prostaglandin, Cyclooxygenase, Lipoxygenase, Leukocyte Migration

INTRODUCTION

Since the demonstration of prostaglandin activity in carrageenin-induced inflammatory exudates, over whelming evidence has accumulated to support the theory that prostaglandins are important mediators of the vascular events in inflammation. Aspirin-like drugs inhibit the biosynthesis of prostaglandins (Vane, 1971; Ferreira *et al.*, 1971), and this explains their anti-inflammatory activity (Vane, 1976). The anti-oedema and anti-erythema action of the aspirin-like drugs correlate closely with their ability to inhibit the production of vasodilator prostaglandins in experimental inflammation (Higgs *et al.*, 1976). Additionally, some of these drugs have other effects. For example, indomethacin disrupts calcium flux across membranes, inhibits cyclic adenosine monophosphate-dependent protein kinase and phosphodiesterase (Braunwald *et al.*, 1978).

Indomethacin reduces the concentration of leukocytes in carrageenin-induced inflammatory exudates by up to 35 per cent (Higgs *et al.*, 1979; Di Rosa *et al.*, 1971), and the reduction of total numbers of leukocytes by cyclooxygenase (prostaglandin synthetase) inhibitors is proportional to the reduction in oedema volume (Blackham, 1975). At doses of indomethacin which completely inhibit prostaglandin synthesis, there is no reduction of leukocyte migration into polyester sponges implanted subcutaneously in rats (Eakins *et al.*, 1979). These results indicate that aspirin-like drugs have a relatively small effect on leukocyte chemotaxis in vivo at doses which are both anti-inflammatory and inhibit prostaglandin biosynthesis.

The fatty acid precursors of the prostaglandins can also be oxygenated by a lipoxygenase, which con-

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verts arachidonic acid to a hydroxy acid, 12-L-hydroxyeicosatetraenoic acid or HETE (Hamberg *et al.*, 1974); Noodleman *et al.*, 1986). HETE is a potent chemotactic agent for polymorphonuclear leukocytes (Turner *et al.*, 1975; Goetzl *et al.*, 1977) and has been detected in inflamed skin. The aspirin-like drugs do not prevent the generation of HETE from arachidonic acid (Hamberg *et al.*, 1974), and this may explain their failure to prevent the accumulation of leukocytes in inflammation.

The mechanism of anti-inflammatory action of the corticosteroids remains mysterious, although there is no lack of theories on this subject. The anti-inflammatory action has been attributed to suppression of migration of polymorphonuclear leukocytes, suppression of reparative processes and function of fibroblasts, reversal of enhanced capillary permeability, and lysosomal stabilization (Goth A, 1984). Anti-inflammatory corticosteroids reduce prostaglandin concentrations in inflammation (Greaves *et al.*, 1972; Flower *et al.*, 1979), prevent the release of prostaglandins from fat tissue (Lewis *et al.*, 1975), and reduce the availability of prostaglandin precursors (Hong *et al.*, 1975). Corticosteroids do not inhibit cyclooxygenase (Flower *et al.*, 1972) but their mechanism of action involves the inhibition of phospholipase A_2, and thus of arachidonic acid release, thus resulting in a reduction in both cyclooxygenase and lipoxygenase products (Flower *et al.*, 1979).

Buxus microphylla var. *koreana* Nakai is distributed widely in korea as an ornamental plant. Cyclobuxine D, steroidal alkaloid, was isolated from *Buxus microphylla* var. *koreana* Nakai. We investigated the effects of cyclobuxine D on the biosynthesis of prostaglandins in guinea pig lung, leukocyte migration and prostaglandins production in carrageenin-induced inflammation.

MATERIALS AND METHODS

Cyclobuxine D was extracted in our Lab. from *Buxus microphylla* var *koreana* Nakai, Carrageenin, arachidonic acid and PGE_2 were purchased from Sigma, $PGF_{2\alpha}$ was obtained from Soonchunhyang Hospital.

Effects on the Biosynthesis of Prostaglandins

Cell-free homogenates of guinea pig lung synthesize prostaglandin E_2 and $F_{2\alpha}$ from arachidonic acid and the following is based on the procedure of Änggard and Samuelson (1965). Lung from four adult guinea pigs (400-500g) were excised rapidly and washed in ice-cold medium (a modified Bucher medium containing 20 mM KH_2PO_4, 72 mM K_2HPO_4, 27.6 mM nicotinamide, and 3.6 mM $MgCl_2$: PH 7.4). The lung tissue was homogenized in a blade homogenizer (NISSEI) at 12000RPM for 2 min: medium ratio 1:4. The resultant suspension was further homogenized by 10 up and down strokes of the teflon pestle. The homogenate was then centrifuged at 900xg for 15 min and the supernatant fluid was used.

Arachidonic acid was dissolved in ethanol (0.1ml/mg) and diluted with 0.2% (w/v) sodium carbonate solution (0.9 ml/mg), thus giving a solution of arachidonic acid of 1 mg/ml. This was further diluted to 200 μg/ml, with the modified Bucher medium.

Flasks containing 10 μg of arachidonic acid (0.05ml) and lung homogenate (1 ml) were incubated aerobically at 37°C with gentle shaking for 30 min. A zero-time sample was taken. The reactions were stopped by heating the flasks in boiling water bath until the protein in the sample coagulated and then diluting five fold with 0.9% (w/v) saline. The samples were kept in ice until assayed.

Induction and Collection of Inflammatory Exudates.

Inflammatory exudates were induced and collected by the subcutaneous implantation of polyester sponges impregnated with 2% (w/v) carrageenin in saline in male rate (200 g) (Eakins, 1979). The sponges were removed after 24 hr, immersed in 5 ml of saline and kept in refrigerator below 4°C. Total leukocyte numbers in sponge exudates were estimated using hemacytometer and microscopy.

Prostaglandin Assays

Inflammatory exudates were diluted with water and the PH adjusted to 3.0 with 2N HCl. Extraction of the active material was carried out with ethyl ether (3 × 10 ml). Ethyl ether phase was dried with sod. sulfate anhydrous and evaporated. The residue was reconstituted in 0.9% saline for bioassay. PGE_2-like activity was assayed on isolated stomach strips from rat and activity was assayed on the rat colon in terms of $PGF_{2\alpha}$, superfused in series with Krebs solution containing a mixture of antagonists (Gilmore *et al.*, 1968) to make the assay more specific. Activity was assayed by bracketing the contractions induced by injections of diluted samples between smaller and larger contractions induced by the standards (PGE_2 and $PGF_{2\alpha}$)

Dosing Regime

Doses of cyclobuxine D were given three times during each experiment. The first dose was given 30 min before the implantation of the sponge. The second dose was given 7-8 hr after the first, and the third, 3-4 hr before removal of sponge. Each drug was given intraperitoneally in aqueous vehicle to at least three animals, and in each experiment the vehicle was given to a similar group of control rats.

Statistics

Statistical analysis of the data was performed in each case according to Student's t-test. Significance was taken as $p<0.1$

RESULTS

Effects on the Biosynthesis of Prostaglandins

Results are expressed as the generation of PGE_2 or $PGE_{2\alpha}$-like activity (30 min sample activity minus zero-time sample activity). To test for inhibition of prostaglandin synthesis, aspirin (10 μg/ml) dexamethasone (10 μg/ml) and cyclobuxine D (1 μg, 10 μg and 100 μg/ml) were added to the incubation flask in volume of 0.1 ml; inhibition of generation by a drug was expressed as the percentage inhibition of control generation. Active compound was tested two to five times at each of three concentrations.

Cyclobuxine D was inactive (that is 10% inhibition) up to 100 μg/ml on the genartion of PGE_2-like activity (Fig. 1). Aspirin was tested three times at 10 μg/ml, it gave a mean inhibition of 35% (range 24-46%) on the PGE_2-like activity. Dexamethasone (10 μg/ml) had no inhibitory activity (Fig. 2). Similar results were obtained when the activity of samples was assayed on ascending colon in term of $PGF_{2\alpha}$.

Effects on Leukocyte Migration and Prostaglandin Production in Carrageenin-Induced Inflammation

The mean concentration of PGE_2-like acitivity and the mean number of leukocyte /ml of inflammatory exudate were calculated for each group of tested animals and expressed as a percentage of control values for each experiment.

Cyclobuxine D caused a dose-dependent reduction in prostaglandin production and leukocyte migration in sponge exudates. Cyclobuxine D (20 mg/kg) inhibited leukocyte migration by 66 ± 11 per cent and reduced prostaglandin concentration by 80 ± 9 per cent (Fig. 3). Prostaglandin concentra-

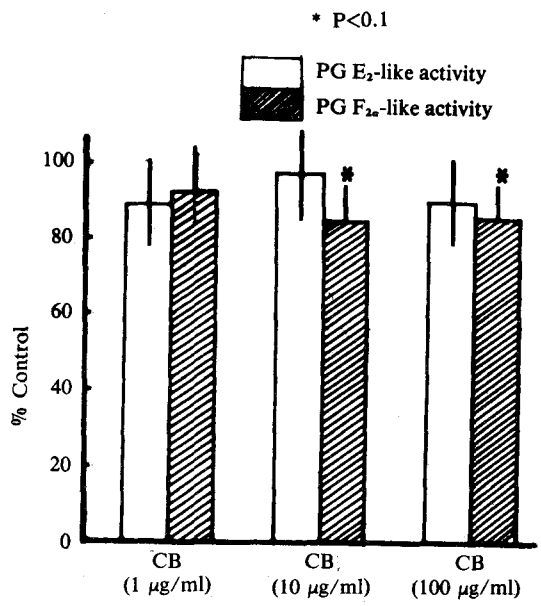

Fig. 1. Effects of cyclobuxine D (CB) on the biosynthesis of prostaglandins from arachidonic acid in guinea pig lung homogenates. Each value is the mean of 9 experiments and the bar represents ± S.E.M.

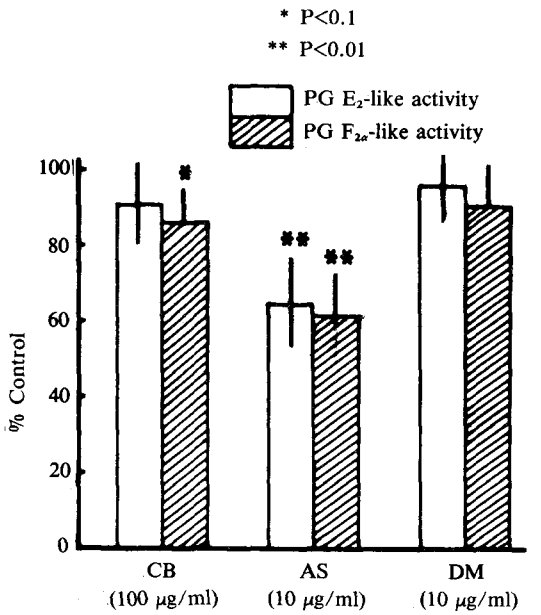

Fig. 2. Effects of cyclobuxine D (CB), aspirin (AS) and dexamethasone (DM) on the biosynthesis of prostaglandins from arachidonic acid in guinea pig lung homogenates. Each value is the mean of 9 experiments and the bar represents ± S.E.M.

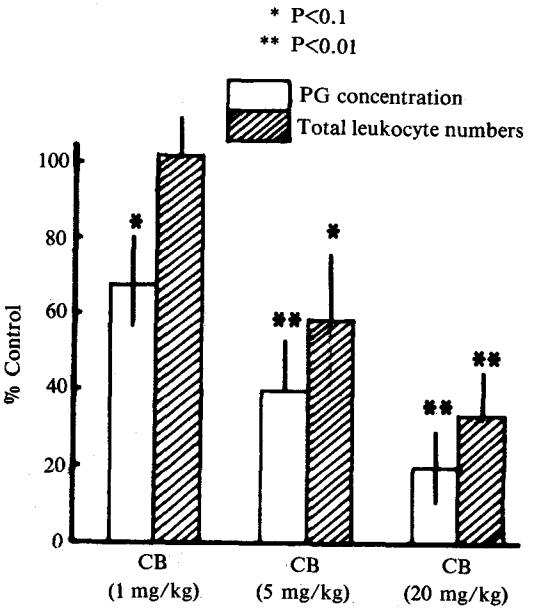

Fig. 3. Effects of cyclobuxine D (CB) on prostaglandin concentration and leukocyte migration in carrageenin-induced inflammation. Each value is the mean of 9 experiments and the bar represents ± S.E.M. Cyclobuxine D was administered intraperitoneally.

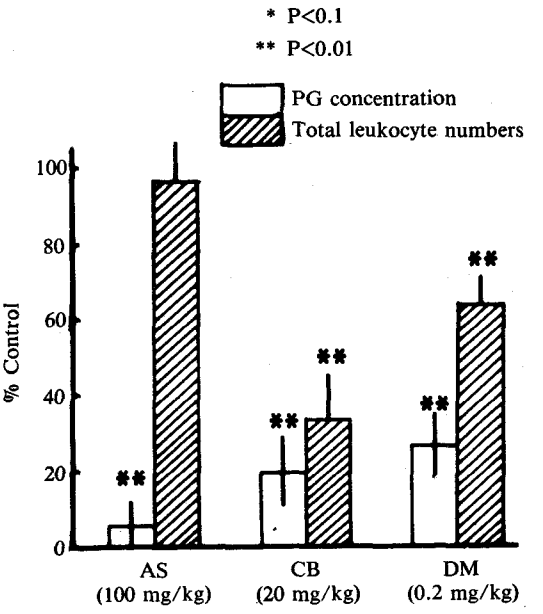

Fig. 4. Effects of cyclobuxine D (CB), aspirin (AS) and dexamethasone (DM) on prostaglandin concentration and leukocyte migration in carrageenin-induced inflammation. Each value is the mean of 9 experiments and the bar represents ± S.E.M. Each drug was administered intraperitoneally.

tion in sponge exudates was reduced by aspirin (100 mg/kg) by over 88 per cent, whereas aspirin has no effects on leukocyte migration (Fig. 4). Dexamethasone (0.2 mg/kg) reduced prostaglandin synthesis by 73 ± 8 per cent and leukocyte migration by 36 ± 7 per cent. Cyclobuxine D and dexamethasone had a significantly greater effect on leukocyte migration than did aspirin.

DISCUSSION

These results suggest that cyclobuxine D have an anti-inflammatory acitivity which differ from that of aspirin. Aspirin reduces prostaglandin concentration in vivo and in vitro by selective inhibition of cyclooxygenase but has relatively small effects on leukocyte migration. Hamberg *et al.* (1974) reported that there was another enzyme which involved in arachidonic acid transformation which is not inhibited by aspirin-like drugs. This is lipoxygenase which is related to biosynthesis of chemotactic factors (HETE, LTB_4 etc.) (Ford-Hutchingson *et al.*, 1984; Needleman *et al.*, 1986). Cyclobuxine D, which does not inhibit cyclooxygenase in vitro, has an effect similar to that of dexamethasone on leukocyte migration and prostaglandin production (Fig. 3 and Fig. 4). This activity may be explained by a reduction in the availability of arachidonic acid due to a reduction in phospholipase A_2 activity (Hong *et al.*, 1976; Nijkamp *et al.*, 1976) which would also account for the fall in cyclooxygenase and lipoxygenase products (Hammerstrom *et al.*, 1975). Corticosteroids reduce the release of arachidonic acid from the phospholipids of the cell and no inhibition of prostaglandin formation is observed when exogenously supplied arachidonic acid is used as substrate for the formation of prostaglandins by the cell (Hong *et al.*, 1976; Lewis *et al.*, 1975). In perfused lung, steroids induced the synthesis of a factor which blocks phospholipase A_2. Presumably, it is a peptide or protein, as its biosynthesis is prevented by cyclohexamide. The biosynthesis of this factor may be crucial to the anti-inflammatory effect of steroids (Flower *et al.*, 1979). Extract of *Buxus microphylla* var. *koreana* Nakai have been used as folk remidies of several disease, including malaria and venereal disease. Lee *et al.* (1986) reported that

cyclobuxine D exerted bradycardic effect and its actions are similar to that of quinidine sulfate in ECG of rats. In present study, cyclobuxine D showed an anti-inflammatory activity, simultaneous inhibition of both pathways of arachidonic acid oxygenation, which is similar to that of corticosteroids. Cyclobuxine D would also have advantage over the aspirin-like drugs in the treatment of chronic inflammation, by having a greater effect on leukocyte migration.

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=국문초록=

Cyclobuxine D의 prostaglandin 합성과 백혈구 유주에 미치는 영향

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이종화, 박영현, 조병헌, 김유재, 김종배, 김정목, 김천숙, 차영덕, 김영석

본 실험실에서는 회양목(*Buxus microphylla* var. *koreana* Nakai)으로 부터 steroid성 alkaloid인 cyclobuxine D를 분리하였고, 그의 여러 약리작용을 검색하고 있다. 본 연구에서는 cyclobuxine D의 guinea pig lung homogenate에서 prostaglandins의 합성에 미치는 영향과 carrageenin으로 유도한 염증에서 prostaglandin의 합성과 백혈구 유주에 대한 영향을 관찰하였다.

Cyclobuxine D (up to 100ug/ml)는 guinea pig lung homogenate에 의한 prostaglandins 합성에 대해서는 현저한 영향이 없었으나, 20mg/kg에서 carrageenin으로 유도된 염증에서 prostaglandin의 합성과 백혈구 유주에 대해 현저한 억제작용을 나타냈다. Aspirin은 vivo와 vitro에서 prostaglandin의 합성을 억제하나, 염증 삼출물에서 백혈구 유주에 대해서는 영향이 거의 없다. Dexamethasone은 vitro에서 외인성 arachidonic acid를 기질로 가했을때는 prostaglandin 합성에 대해 영향이 없었고 carrageenin으로 유도된 염증에서 prostaglandin의 합성과 백혈구 유주를 억제하였다.

이상의 결과로 cyclobuxine D의 항염증작용은 phospholipase A_2 activity를 저해하여 항염증 작용을 나타내는 것으로 사료되는 corticosteroid와 유사한 것으로 추정된다.