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Inhibition of leukotriene release in anaphylactic guinea-pig hearts by a 5-lipoxygenase inhibitor, CGS 8515

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1 Ovalbumen (100 μg)-induced coronary vasoconstriction and decrease in cardiac developed tension were studied in isolated perfused hearts from sensitized guinea-pigs. Leukotriene-like material released in the cardiac effluent was assayed against synthetic leukotriene C_4 (LTC_4).

2 LTC_4 was released in a time-dependent fashion, and release was enhanced when hearts were challenged in the presence of indomethacin (2.8 μM). The release was maximal at 2–3 min and detectable for as long as 10 min following ovalbumen challenge. Immunoreactive (ir) thromboxane-B_2 (TxB_2) was also detected in cardiac effluent which had been partially purified using C_{18} Sep-Paks.

3 CGS 8515 (0.03–1.0 μM), an inhibitor of 5-lipoxygenase, dose-dependently inhibited ovalbumen-induced coronary vasoconstriction and leukotriene-C_4 release. CGS 8515 inhibited ovalbumen-induced decreases in cardiac developed tension at 0.3 and 1.0 μM, but did not antagonize coronary vasoconstriction induced by synthetic LTC_4.

4 The release of cyclo-oxygenase products following ovalbumen challenge was not inhibited by CGS 8515, but was markedly inhibited by indomethacin (2.8 μM) pretreatment.

5 We conclude that leukotrienes have a major role in guinea-pig cardiac anaphylaxis, and that CGS 8515 has a cardio-protective action. The results obtained in these experiments *in vitro* show that CGS 8515 is a potent and selective 5-lipoxygenase inhibitor.

Introduction

Hypotensive shock and cardiac failure are clinical manifestations of systemic anaphylaxis (Austen, 1974). The anaphylactic reaction of isolated sensitized guinea-pig hearts shows resemblance to human anaphylaxis. It is characterized by the occurrence of typical symptoms such as coronary vasoconstriction, cardiac failure and arrhythmias (Hahn & Bernauer, 1970; Capurro & Levi, 1975; Liebig *et al.*, 1975; Levi & Burke, 1981). Slow reacting substance of anaphylaxis, now known to consist of leukotrienes (LTs) C_4, D_4 and E_4, has been detected in effluent of anaphylactic guinea-pig hearts and is believed to be responsible for the changes in cardiac function observed (Brocklehurst, 1960; Chakravarty, 1960). Indeed, partially purified SRS-A obtained immunologically from guinea-pig or synthetic LTC_4, LTD_4 and LTE_4 cause significant reductions in cardiac developed tension and coronary flow (Letts & Piper, 1982).

As 5-lipoxygenase products are among the important mediators released during cardiac anaphylaxis, one possible way of reducing the symptoms of cardiac dysfunction and hence the eventual cardiac failure secondary to cardiac anaphylaxis, may be by inhibiting the lipoxygenase enzymes. It will therefore be of interest to investigate the therapeutic effect of selective inhibitors of 5-lipoxygenase in cardiac anaphylaxis. There are, however, relatively few reports of specific 5-lipoxygenase inhibitors with activity both *in vivo* and *in vitro*. Evidence of inhibition of 5-lipoxygenase both *in vivo* and *in vitro* has been obtained with BW755c (Hammarstrom, 1977) but this compound is not specific for 5-lipoxygenase and also inhibits cyclo-oxygenase.

Most of the investigations carried out with this drug have been on the inhibition of various inflammatory responses. We have investigated the pharmacological activities of CGS 8515, a potent lipoxygenase inhibitor, on the release of LTC_4-like material from guinea-pig hearts during cardiac anaphylaxis and on the associated increase in coronary perfusion pressure and decrease in cardiac developed tension.

Methods

Sensitization

Male weanling guinea-pigs of Dunkin-Hartley strain (200–250 g) were sensitized with doses of crude egg albumen (Sigma grade II) 100 mg subcutaneously and 100 mg intraperitoneally.

Heart perfusion

Three to four weeks after sensitization, each animal was killed by cervical dislocation, the heart removed and placed in cold Tyrode solution. The aorta was cannulated retrogradely, the heart rapidly transferred to the modified Langendorf perfusion apparatus where it was immediately perfused with oxygenated Tyrode solution at a constant flow of $10\,ml\,min^{-1}$. Coronary perfusion pressure and cardiac developed tension were recorded continuously on a Watanabe linearcorder mark VII WR3101. After an equilibration period of 30 min, the heart was challenged with a bolus injection of ovalbumen given into the aorta. Cardiac effluent was collected on ice at 1 min intervals for measurement of the release of LTC_4, and thromboxane B_2 (TxB_2).

Drug infusions

Indomethacin, BW 755c and CGS 8515 were each added into the Tyrode solution to give the final concentrations shown below. Indomethacin dissolved in sodium carbonate was added to Tyrode solution to give a final concentration of $1\,\mu g\,ml^{-1}$ (2.8 μM). This concentration has been shown previously to inhibit almost completely the release of cyclo-oxygenase products during cardiac anaphylaxis (Anhut *et al.*, 1977). CGS 8515 was dissolved in N-N-dimethylacetamide at a concentration of 10 mM, which was then diluted in Tyrode solution to give concentrations of 0.03, 0.1, 0.3 and 1.0 μM. At the concentrations used, the vehicle N-N-dimethylacetamide had no measurable effect on the hearts. BW755c was dissolved in distilled water and the desired concentration was obtained by dilution in Tyrode solution as above.

Bioassay of leukotriene C_4

Leukotriene-like material in the cardiac effluent was assayed against synthetic LTC_4 on strips of longitudinal smooth muscle of the guinea-pig ileum (Rang, 1964), superfused at $5\,ml\,min^{-1}$ with oxygenated Tyrode solution warmed to 37°C, containing the following combination of antagonists: mepyramine ($0.1\,\mu g\,ml^{-1}$), hyoscine ($0.1\,\mu g\,ml^{-1}$), methysergide ($0.2\,\mu g\,ml^{-1}$), phenoxybenzamine ($0.1\,\mu g\,ml^{-1}$) and propranolol ($2\,\mu g\,ml^{-1}$). Changes in length of tissues were measured by means of auxotonic levers (Paton) connected to smooth muscle transducers (Harvard) and displayed on a pen recorder. Limits of LTC_4, detection were 1.0–3.0 pmol.

Radioimmunoassay

Immunoreactive TxB_2 in the heart effluent was determined by radioimmunoassays as described by Jose *et al.* (1976). Immunoreactive TxB_2 was measured in selected samples 1–10 min before and after challenge. Effluent from the heart was extracted twice with methanol, evaporated to dryness under vacuum and kept frozen until required. The extract was dissolved in standard buffer solution (pH 7) for radioimmunoassay. The assays were carried out using an antiserum to TxB_2. The limit of detection of TxB_2 was 0.01 pmol.

Materials

The following drugs were used: methyl-2-(3,4-dihydro-3,4-dioxo-1-napthalenyl)amino benzoate (CGS 8515, Ciba Geigy), indomethacin (Sigma), 3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2 pyrazoline (BW755c, Wellcome), 7-[3-(4-acetyl-3-hydroxy-2-propyl - phenoxy) - 2 - hydroxyproboxy] - 4 - oxo - 8 - propyl-4H-1-benzopyran-2-carboxylic acid (FPL 55712, Fisons Ltd.), platelet activating factor (Paf) (Bachem), LTC_4 (Miles Laboratories), hyoscine hydrobromide (BDH), mepyramine maleate (May & Baker), methysergide maleate (Sandoz), phenoxybenzamine hydrochloride (ICI), propranolol hydrochloride (ICI), TxB_2 antiserum to TxB_2, egg albumen grade II and III (Sigma).

Results

Effects of vehicle and CGS 8515 on hearts

Infusion of vehicle (0.01% N-N-dimethylacetamide) for CGS 8515 into the heart was without effect and did not significantly alter the resting level of the coronary perfusion pressure (CPP) and cardiac developed tension.

CGS 8515 at concentrations of 0.03, 0.1, and 0.3 μM exerted no significant effect on CPP in the perfused guinea-pig heart. At 1.0 μM CGS 8515 caused a marked increase in resting CPP ($P < 0.05$, $n = 4$) compared with the control hearts. In addition, it was also observed that CGS 8515 exerted a direct effect on the developed tension. At low doses it enhanced whereas at high doses it reduced the developed tension.

Effects of CGS 8515 pretreatment in anaphylactic hearts

Bolus injection of ovalbumen (100 μg) into the fluid perfusing the isolated sensitized guinea-pig hearts

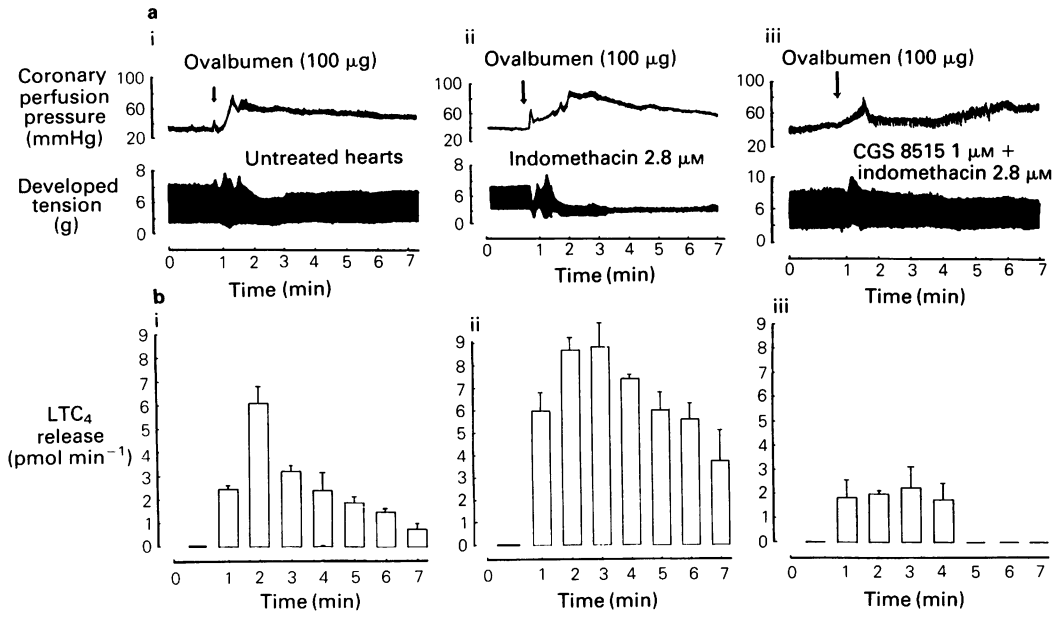

Figure 1 Effects of ovalbumen challenge (100 μg) on coronary perfusion pressure and cardiac developed tension. (a) Representative tracings from four separate experiments in untreated hearts (i), indomethacin treated hearts (ii), and hearts treated with CGS 8515 in the presence of indomethacin (iii). (b) Mean data for the experiments shown in a(i), a(ii) and a(iii).

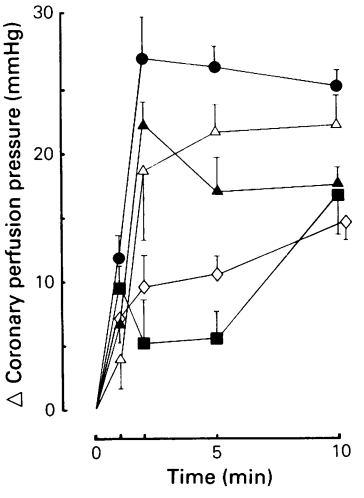

Figure 2 Indomethacin-induced potentiation of the anaphylactic increase in coronary perfusion pressure and its attenuation by different concentrations of CGS 8515 administered in the presence of indomethacin: indomethacin 2.8 μM (●); indomethacin plus CGS 8515: 0.03 μM (△), 0.1 μM (▲), 0.3 μM (◇) and 1.0 μM (■). Each point represents the mean of at least 4 experiments and vertical lines show s.e.mean ($P < 0.05$).

elicited an immediate increase in CPP and a decrease in cardiac developed tension (Figure 1a). The most severe changes occurred 1–2 min after antigen challenge. The peak increase in CPP occurred within 2 to 3 min and remained elevated above control levels for longer than 10 min. The decrease in cardiac developed tension also persisted over the 10 min observation period. Ovalbumen-induced increase in CPP and decrease in cardiac developed tension were altered by indomethacin and BW755c (40 μM) pretreatment. Indomethacin delayed the onset of the early phase of coronary vasoconstriction which occurred at 1 min after antigen challenge (Figure 1), and potentiated the delayed elevation of CPP ($P < 0.0025$, $n = 8$). It was also noted that the incidence of post-challenge atrial fibrillation occurred more frequently in the indomethacin-treated hearts (1 in 2 hearts) than in untreated hearts. Both CGS 8515 and BW755c when infused into indomethacin-treated hearts markedly inhibited both ovalbumen-induced increase in CPP and decrease in cardiac developed tension. The inhibition in the increase in CPP (Figures 2 and 3) was most marked in the early phase (0.5 min) after ovalbumen challenge. A dose-dependent inhibition was observed with CGS 8515 at concentrations between 0.03 and 1.0 μM. At these concentrations CGS 8515 also produced a dose-dependent inhibition of the cardiac developed tension ($P < 0.05$, $n = 4$, and

$P < 0.025$, $n = 4$). CGS 8515 at 0.03 and 0.1 μM did not significantly alter the decrease in cardiac developed tension. The inhibitory effects of CGS 8515 were markedly more potent than those obtained with BW755c (40 μM).

Effect of CGS 8515 on antigen-induced release of 5-lipoxygenase and cyclo-oxygenase products

Figure 1 shows that cysteinyl-containing leukotrienes assayed against LTC_4 were released during cardiac anaphylaxis in a time-dependent manner. Maximum release occurred within 2–3 min but output of leukotriene-like material could be detected for at least 8 min. Treatment of hearts with indomethacin (2.8 μM) (Figure 1, iia,b) substantially increased the release of leukotriene-like material. Perfusion of CGS 8515 either alone or in the presence of indomethacin (Figure 1, iii a,b, and Figure 2) produced a concentration-dependent inhibition of release of leukotriene-like substances. At concentrations of 0.1, 0.3 and 1.0 μM CGS 8515, the level of inhibition was 44%, 66%, and 78%, respectively. On the other hand the inhibition produced by BW755c was 67% (Figure 3). In these experiments the amount of leukotriene-like material released was significantly lower than in the indomethacin-treated hearts (Figures 1 and 5).

While the concentrations of TxB_2 in the hearts collected before challenge were close to or below the detection limit of radioimmunoassay, greatly increased amounts of this mediator were released from the hearts after ovalbumen challenge (13.7 ± 0.08 pmol min^{-1}). In the presence of indomethacin release of TxB_2 was markedly reduced (Figure 4), while, as shown above (Figure 1, ii a,b) release of cysteinyl-containing leukotrienes, assayed against synthetic LTC_4, was significantly enhanced. CGS 8515 (0.03–1.0 μM) did not inhibit antigen-induced release of the cyclo-oxygenase product TxB_2 (Figure 4), suggesting that CGS 8515 is a selective 5-lipoxygenase inhibitor.

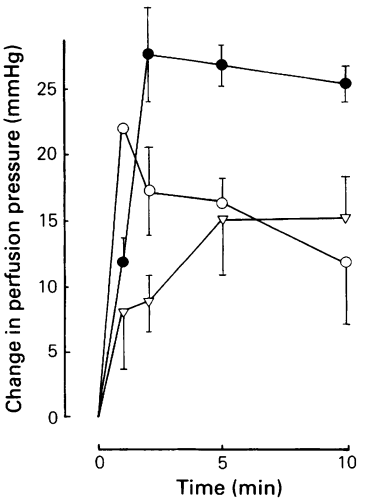

Figure 3 Enhancement of ovalbumen-induced increases in coronary perfusion pressure by indomethacin, 2.8 μM, (●) and its attenuation by BW755c, 40 μM, (▽); untreated heart (○). ($n = 4$–6, $P < 0.05$).

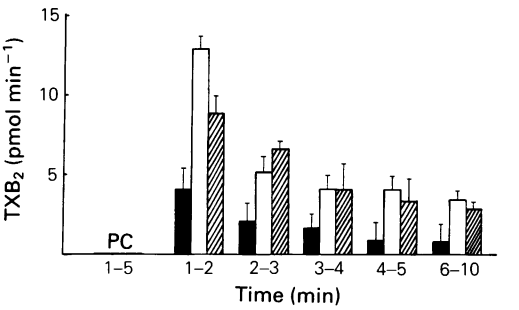

Figure 4 Generation of antigen-induced thromboxane B_2 (TxB_2) in untreated hearts (open columns), and hearts treated with indomethacin (2.8 μM) (solid columns) and with CGS 8515 (1 μM) (hatched columns). PC = prechallenge. Cardiac effluent was collected for periods shown on axis. ($n = 4$, $P < 0.05$).

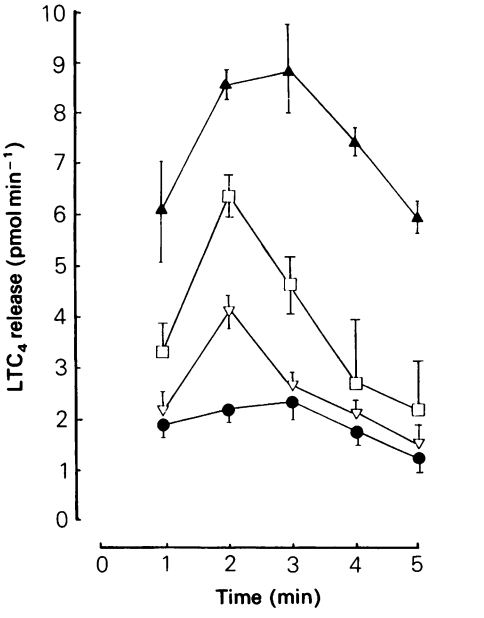

Figure 5 The effects of BW755c (40 μM) and CGS 8515 (1 μM) on the indomethacin-induced exaggeration in the anaphylactic release of leukotriene C_4 (LTC_4)-like material: indomethacin 2.8 μM (▲); untreated hearts (□); BW755c plus indomethacin (▽); CGS 8515 plus indomethacin (●). Cardiac effluent was sampled at 1 min intervals over 5 min.

The effect of CGS 8515 on LTC_4-induced coronary vasoconstriction

Infusions of LTC_4 (40–100 pmol) produced dose-dependent decreases in coronary flow and cardiac developed tension in guinea-pig isolated hearts. CGS 8515 (0.03–1.0 pmol) did not significantly attenuate these responses suggesting that this 5-lipoxygenase inhibitor has no antagonistic activity on leukotriene-receptors.

Discussion

Various mediators have been implicated in cardiac anaphylaxis. Technological advances during the past decade have revealed that lipoxygenation of arachidonic acid (AA) gives rise to a number of products with diverse biological activities and in particular, the discovery of leukotrienes as active constituents of SRS-A released immunologically from guinea-pig hearts (Aehringhaus *et al.*, 1984) has enabled us to elucidate further the mediators involved in cardiac anaphylaxis.

Allergens have been shown to be potent stimuli for the release of vasoactive arachidonic acid metabolites in hypersensitized subjects (Chakravarty, 1960). In the present experiments, guinea-pig hearts isolated from sensitized animals undergo a profound reaction to a bolus injection of ovalbumen resulting in sustained reduction in coronary flow coupled with dysrhythmias and marked impairment of cardiac function (Liebig *et al.*, 1975; Levi *et al.*, 1976). Irrespective of the presence or absence of indomethacin, the maximum release of leukotrienes occurred at 2–3 min after ovalbumen challenge during which time it was noted that the reduction in coronary flow and developed tension of the perfused hearts were maximal. It was of interest that the quantity and duration of leukotriene release significantly increased in the presence of indomethacin. These results suggest that leukotrienes may be partly responsible for cardiac dysfunction seen during anaphylactic shock.

In the present experiments the potency and activity of CGS 8515 against 5-lipoxygenase is manifested by its ability to inhibit ovalbumen-induced release of leukotrienes and the attenuation of the associated increase in CPP and reduction in cardiac developed tension.

CGS 8515 dose-dependently inhibited release of leukotriene-like material and concomitantly inhibited cardiac reactions to ovalbumen. In line with the above observations the effects of CGS 8515 were most marked at 1–5 min following ovalbumen challenge.

The potentiation of the anaphylactic release of SRS-A by indomethacin has been demonstrated in other laboratories (Liebig *et al.*, 1975); however, the significance of this effect on cardiac function is not known. The present experiments demonstrate that a cyclo-oxygenase inhibitor could not only alter mediator release during cardiac anaphylaxis but could also modify cardiac responses in response to antigen challenge. The delay of inhibition of the early phase of antigen-induced vasoconstriction by indomethacin suggests that cyclo-oxyegenase products are significantly involved in cardiac anaphylaxis. In addition, it is interesting to observe that pretreatment with indomethacin resulted in a higher than normal incidence of post-challenge atrial fibrillation. This is in apparent agreement with the previous observation that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) completely suppressed the antigen-induced arrhythmias while PGE_2 and PGI_2 decreased the incidence of arrhythmias in guinea-pig anaphylactic hearts (Aehringhaus *et al.*, 1984). Thus, the enhancement in the anaphylactic release of LTC_4 when sensitized hearts were challenged in the presence of indomethacin seen in the present experiments suggests two possibilities: firstly, cyclo-oxygenase products may exert a direct negative feedback on the anaphylactic release of leukotrienes and secondly, blocking the cyclo-oxygenase pathway resulted in a diversion of arachidonic acid metabolism away from cyclo-oxygenase towards the synthesis of lipoxygenase products. Based on experimental data from other laboratories, the latter possibility would explain our observation since it has been demonstrated that a range of cyclo-oxygenase metabolites has no effect on the anaphylactic release of LTC_4 in indomethacin-treated tissue; this included both vasodilator (PGE_2, PGI_2) and vasoconstrictor substances ($PGF_{2\alpha}$, PGD_2, and 11-9-epoxy-methano-PGH_2, TxA_2-mimetic) (Aehringhaus *et al.*, 1984).

The duration of increase in CPP following a bolus injection of exogenous LTC_4 (30 pmol) was 4 min. On the other hand, the observed increase in coronary perfusion pressure following ovalbumen challenge remained elevated for more than 10 min. This is consistent with the duration of release of LTC_4-like material seen in the present experiments, in which the anaphylactic release of LTC_4-like material was detectable up to 7–10 min. However, the reason for the disparity in the duration of release of LTC_4-like material and the protracted nature of the elevation of CPP following ovalbumen challenge is not known. It remains a possibility that the sustained elevation in perfusion pressure seen above is attributable to the action of vasoactive mediators other than leukotrienes. For instance, platelet activating factor (Paf), a putative mediator of inflammation has recently been implicated in cardiac anaphylaxis (Levi *et al.*, 1984). The protracted nature of Paf-induced increases in CPP and decreases in

cardiac developed tension, in addition to the demonstration that Paf-acether triggered the release of leukotrienes into the cardiac effluent (Piper & Stewart, 1986) makes Paf the most likely mediator responsible for the persistent coronary vasoconstriction seen during cardiac anaphylaxis. Furthermore, CGS 8515 at doses that produced significant attenuation of the increase in CPP only exhibited its marked inhibitory effects between 2–5 min following ovalbumen challenge.

The availability of a new lipoxygenase inhibitor allows participation of lipoxygenase-derived products in cardiac anaphylaxis and other pathophysiological conditions to be assessed. CGS 8515 has been reported to inhibit 5-HETE and LTB_4 synthesis in guinea-pig polymorphonuclear leukocytes ($IC_{50} = 0.1\ \mu M$) *in vitro* (Ku,*et al.*, 1988). In rats, CGS 8515 at oral doses of $5\ mg\ kg^{-1}$ inhibits the production of LTB_4 from whole blood *in vivo* by about 50% (Ku *et al.*, 1988). CGS 8515 has also been reported to inhibit leukocyte accumulation in carrageenin-impregnated rats (Ku *et al.*, 1988). The dose-dependent inhibition of the LTC_4-like material released by CGS 8515 at very low doses in the present experiments suggest that CGS 8515 is indeed a potent 5-lipoxygenase inhibitor. In addition, the amounts of BW755c and CGS 8515 required to inhibit approximately 66% of the LTC_4-like material released were 40 μM and 0.3 μM, respectively, showing that the potency of CGS 8515 is more than ten times greater than BW755c.

The absence of significant attenuation of the actions of infused synthetic LTC_4 in the perfused hearts by CGS 8515 suggests that the effects of CGS 8515 seen during cardiac anaphylaxis are not through antagonism of leukotriene receptors but through selective inhibition of the formation of 5-lipoxygenase products. Unlike BW755c, CGS 8515 is a selective 5-lipoxygenase inhibitor and our data show that it has a negligible effect on the release of ir-TxB_2. This supports the findings in other laboratories that CGS 8515 did not inhibit PGE_2 and 15-HETE formation in cell-free systems (Ku *et al.*, 1988).

At present, there are few selective 5-lipoxygenase inhibitors with activity both *in vivo* and *in vitro*. Various acetylenic analogues of arachidonic acid inhibit different lipoxygenases *in vitro*, but this type of compound is not active *in vivo* (Higgs & Mudgridge, 1982). Several compounds inhibit various lipoxygenases *in vitro*. These include nordihydroguiaretic acid (NDGA), benoxaprofen, baicalein, esculetin, some flavonoids and caffeic acid (Hamberg, 1976; Walker & Dawson, 1980; Sekiya & Okuda, 1982). There are however, relatively few reports of selective and potent 5-lipoxygenase inhibitors with activity *in vivo*. Benoxaprofen for instance, while inhibiting 5-lipoxygenase in isolated leukocytes (Harvey *et al.*, 1983) had no effect on LTB_4 generation in acute experimental inflammation (Salmon *et al.*, 1984). Thus, CGS 8515 showing the above properties and having activity both *in vivo* and *in vitro* may be a useful tool with which to elucidate further the role of 5-lipoxygenase products in pathological and physiological processes. Moreover, CGS 8515 has been shown to inhibit selectively the formation of pro-inflammatory agents, LTB_4 and 5-HETE without affecting the release of 15-HETE (Ku *et al.*, 1988). Thus, the results of these experiments, again emphasize the potential therapeutic significance of CGS 8515 in various allergic, inflammatory and pathological conditions.

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