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Inhibitory effect of econazole on the release of thromboxanes

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Abstract

The effect of econazole on the release of thromboxanes was investigated. It was found that econazole inhibited concentration-dependently the aggregation of guinea pig platelets stimulated with arachidonic acid. The compound also reduced significantly the LTB_4-induced contraction of guinea pig lung parenchyma strips and the contraction of rabbit aorta to the effluent of LTD_4-stimulated guinea pig lungs, both effects mediated mostly by thromboxane generation. The concentration of TXB_2 in the effluents from LTD_4 stimulated lungs, assayed by EIA, was significantly reduced following pretreatment of the lungs with 10^{-4} *M* and 10^{-5} *M* of econazole, whereas the levels of PGE_2 were increased. These results demonstrate that econazole is a selective inhibitor of thromboxane synthesis.

Introduction

Econazole (1-[2(2,4-dichlorophenyl)-2- (4-chlorobenzyloxy)-ethyl]-imidazole), synthesized by Godefroi in 1969 [1], is an effective broad spectrum antimycotic agent which is widely used in the treatment of several mycoses. The general pharmacology of this compound as well as the possible mechanism of its antifungal activity has been fully described [2, 3]. Regarding its effect on the inflammatory reaction, it was reported that econazole reduces carrageenan-induced paw edema [3]. Although the mechanism of this anti-inflammatory effect is not known, a closely related compound, miconazole nitrate, was shown to inhibit platelet aggregation induced by collagen and sodium arachidonate [4]. The authors reported that miconazole inhibited the formation of TXB_2 and PGE_2, possibly by inhibiting platelet cyclooxygenase.

In the present study we investigated the effect of econazole on three reactions mediated by thromboxane formation: arachidonic acid-induced platelet aggregation, contraction of lung parenchyma strips to LTB_4 and contraction of rabbit aorta to the effluent of LTD_4-stimulated lungs. Furthermore, we investigated the effect of this compound on the levels of TXB_2 and PGE_2 present in the effluent from the lungs perfused with LTD_4.

Materials and methods

Animals:

Male albino guinea pig weighing between 300–450 g and male New Zealand rabbits, 2–3 month old were used.

Platelet aggregation studies

Arterial blood samples were collected from the abdominal aorta of anesthetized guinea pigs using citric acid-citrate-dextrose (ACD) as anticoagulant

(6 volumes of blood: 1 volume of ACD). Aggregation assays were performed with washed platelets prepared according to the method of Mustard et al. [5] and measured by the turbidometric method of Born [6] using an aggregometer (Payton Scientific, USA, Model 402).

Platelet inhibition was measured by incubating the washed platelet preparations for 3 min with different concentrations of econazole before addition of 10 μM of arachidonic acid. As control, platelets were preincubated with the solvent for econazole (see later) alone and stimulated with arachidonic acid in the same conditions. Aggregation was recorded over 10 min. The amplitude of the aggregation curves was measured and inhibition calculated as follows:

$$\left[\frac{\text{(cm aggregation control)}-\text{(cm aggregation treated)}}{\text{(cm aggregation control)}}\right]\times 100$$

Assay of LTB_4 on guinea pig lung parenchyma strips

Guinea pig lungs were removed and strips of parenchyma (3 × 3 × 30 mm) were cut along the edges of the lobes according to the technique described previously [7]. After dissection, the tissues were installed in the organ baths of a cascade superfusion system and perfused with oxygenated (95% O_2:5% CO_2) Krebs solution (5 ml/min, 37 °C). A tension of 2 g was applied to each tissue. After a stabilization period of 1 h, the agonists (LTB_4 and histamine) were injected as a bolus (0.1 ml) in the superfusion fluid, and the responses recorded with Grass FT 03 C isometric transducers coupled to a Beckman polygraph. The tissues were allowed to rest for 75 min between each addition of LTB_4 to avoid tachyphylaxis. After tissues had stabilized, control responses to a range of doses of LTB_4 (10–250 ng) and histamine (1–100 µg) were obtained. Subsequently, econazole 10^{-4}, 10^{-5} and 10^{-6} M was infused over the tissues for 30 min before and continuously during the measurement of a second set of responses to the agonists.

Perfusion of guinea pig lungs and assay of the effluent

Guinea pigs were killed by cervical dislocation, the thorax was cut open and the lungs removed and transferred to a perfusion apparatus and perfused continuously via the pulmonary artery as previously described [8]. The effluent of the lungs was superfused over strips of rabbit aorta. After a stabilization period of 1 h, the tissues were stimulated with standard doses of LTD_4 (50 ng) and of the thromboxane analogue U-44069 (50 ng), given directly to the tissues. The isometric responses were recorded as described above. Following this, the standard dose of LTD_4 was given into the perfusate entering the pulmonary artery and the response of the aorta strips (mainly to the TXA_2 released from the lung [11]) was recorded. For the inhibition studies, the lungs were pre-treated with econazole (10^{-4} M and 10^{-5} M) for 90 min before another injection of 50 ng of LTD_4 into the lungs. To measure the effect of econazole on the amounts of TXB_2 and PGE_2 released from LTD_4-stimulated lungs, the following protocol was designed: samples of the lung effluents were collected during 1 min before stimulation to assess the basal levels and after injection of 50 ng of LTD_4 into the lungs for 5 minutes. Econazole was then infused into the lungs as described above. Samples were collected before (basal levels) and after another injection of 50 ng of LTD_4 into the same lungs. This experiment was repeated 4 times for each concentration of econazole. The concentration of PGE_2 and TXB_2 in the samples was determined by EIA.

Assay of TXB_2 and PGE_2

The concentration of TXB_2 and PGE_2 on the cell-free supernatants from the lung effluents was determined by specific EIA according to Pradelles et al. [9].

Briefly, 50 µl of different dilutions of the samples were mixed with 50 µl of conjugated eicosanoid-acetylcholinesterase and 50 µl of specific antiserum in 96-well plates pre-coated with anti-rabbit IgG antibodies. After an overnight incubation at 4 °C, the plates were washed and the enzyme substrate (Ellman's reagent) was added for 60–120 min at 25 °C. The optical density of the samples was determined at 412 nm in a microplate reader (Titertek Multiscan, Flow Labs). The concentration of each eicosanoid was determined according to a standard curve. Results were expressed as picograms or nanograms of eicosanoid released per ml of effluent.

Drugs used

Histamine hydrochloride and arachidonic acid were purchased from Sigma Chem., Co., USA; leukotrienes B_4 and D_4 from Merck Frosst, Canada; econazole nitrate was kindly supplied by Formil Quimica, Brazil. Antibodies to PGE_2 and TXB_2 labeled with acetylcholinesterase, the enzyme and anti-rabbit IgG antibodies were from Cayman Chem. Co., USA. The thromboxane analogue U-44069 and the standard PGE_2 and TXB_2 were from Upjohn Co. USA.

Stock solutions of leukotriene B_4 were made in a small volume of absolute methanol. Further working dilutions were made in water, after evaporation of methanol. Econazole nitrate was dissolved in a small volume of ethanol and further diluted in PBS. LTD_4 methyl ester was hydrolyzed with a solution of Na_2CO_3 (5%) for 1 h at room temperature and its concentration assayed spectrophotometrically.

Statistical Methods

Statistical evaluation of data was carried out by analysis of variance and sequential differences among means tested according to Tukey contrast analysis at $p<0.05$.

Results

Effect of econazole on arachidonic acid-induced platelet aggregation

Addition of 10 μM of AA to washed guinea pig platelets induced aggregation which was prevented by preincubation of the platelets with econazole for 3 min. The effect of the drug was concentration dependent and the maximum inhibition was achieved with 10^{-5} M, as illustrated in Fig. 1. Each concentration of econazole was tested with platelets from 6–9 animals and the results are summarized in Table 1. It is noteworthy that in 55% of the experiments, the inhibition induced by the higher concentration of econazole reversed spontaneously around ten min after the addition of AA.

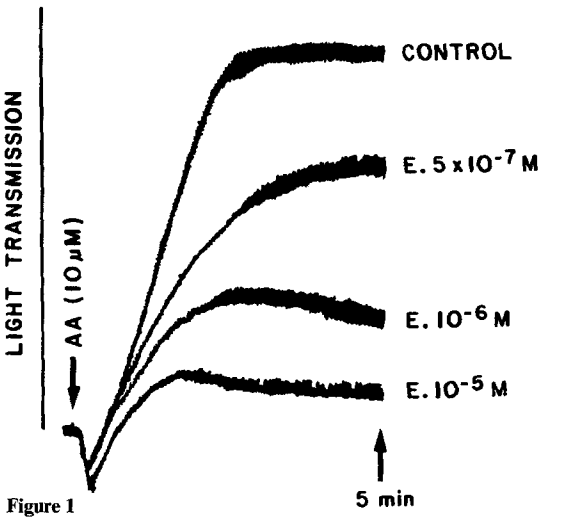

Figure 1
Representative tracings of arachidonic acid-induced aggregation of guinea pig platelets in the absence (control) or presence of econazole (E). Platelets were incubated with increasing concentrations of econazole for 3 min at 37 °C before addition of arachidonic acid (10 μM).

Table 1
Effect of econazole on arachidonic acid-induced aggregation of guinea pig platelets.

Concentration of econazole	Amplitude cm	% Inhibition aggregation
Control	13.6±0.7	– (9)
5×10^{-7} M	8.9±0.3*	33 (6)
1×10^{-6} M	6.2±1.5*	63 (7)
5×10^{-6} M	2.7±0.8*	85 (9)
1×10^{-5} M	0.2±0.1*	98 (9)

Washed guinea pig platelets were stimulated with 10 μM of arachidonic acid. Platelets were incubated with econazole or its vehicle for 3 min before addition of arachidonic acid. * The amplitude of the aggregation curves was measured and expressed as mean +SEM. The inhibition was calculated as described in the methods. Significant values are marked * ($p<0.05$). Number of animals in brackets.

Effect of econazole on LTB_4-induced contraction of guinea pig lung parenchyma strips

The contraction of guinea pig lung parenchyma strips to LTB_4 is mediated mostly by thromboxane formation by the lung tissue [10]. In our experiments the lung parenchyma strips responded dose-dependently to bolus injections of LTB_4 (10–250 ng). The responses of the tissue were sig-

nificantly reduced by (48%) 10^{-5} *M* of econazole (Fig. 2) and at this concentration, econazole did not affect the response of the tissues to histamine (1–100 μg). At a 10 fold higher concentration (10^{-4} *M*) econazole inhibited the response of the tissues to LTB_4 (82%) but also reduced the response to histamine by about 30%. At 10^{-6} *M*, the drug had no significant effect in the response to either agonists (data not presented).

Effect of econazole on the release of thromboxane from LTD_4-stimulated lungs

Isolated guinea pig lungs perfused with LTD_4 release significant amounts of TXA_2 [11] which can be detected by its contractile effect on rabbit aorta strips. In our experiments, the rabbit aorta strips responded well to the thromboxane analogue U-44069 given directly to the tissues (Fig. 3). However, the standard dose of LTD_4 given directly was without effect but when given via the lung, caused a strong contraction of the aorta strips (Fig. 3, second and third responses). When the lungs were infused for 90 min with econazole (10^{-5} *M*) prior to LTD_4 injection, the myotropic activity of the lung effluent was reduced (Fig. 3, last response), and with 10^{-4} *M* of econazole, the inhibition was more pronounced. At either concentration of econazole, the responses to U-44069 were not affected. Furthermore, infusion of econazole directly over the aortas did not inhibit responses to U-44069 or to LTD_4 injected via the lung. These responses were not quantitated but qualitatively similar results were obtained from 6 separate guinea pig lungs.

Release of TXB_2 and PGE_2 from lung following LTD_4 stimulation was quantitated by EIA. Samples of the lung perfusate were collected during 1 min before, to assess the basal levels, and after an intra arterial injection of 50 ng of LTD_4, during 5 min. As shown in Fig. 4, the injection of LTD_4 strongly stimulated the release of TXB_2 and PGE_2 from the lung into the lung effluent. Econazole was then infused into the lungs for 90 min when the samples were repeated before and after injection of LTD_4 into the lungs. It can be seen in Fig. 4A that treatment of the lung with 10^{-5} *M* of econazole significantly inhibited the release of TXB_2 in the samples collected 1 and 2 minutes after LTD_4 stimulation (80.5% and 52.3% respectively). At the concentration of 10^{-4} *M* (Fig. 4B) significant inhibition of TXB_2 release was observed in all samples: 89.6% (1 min); 72.6% (2 min); 54.8% (3 min); 36.6% (4 min); 29.0% (5 min). Inhibition of thromboxane was accompanied by increase of PGE_2 release. The levels of PGE_2 were significantly elevated in the samples (2–5 min) from lungs treated with both concentration of econazole; this increase being more pronounced in the later samples. The concentration of PGE_2 in the last sample (5 min) collected from lungs treated with 10^{-5} and 10^{-4} *M* was increased 4 and 16 times respectively when compared to the non-treated lungs.

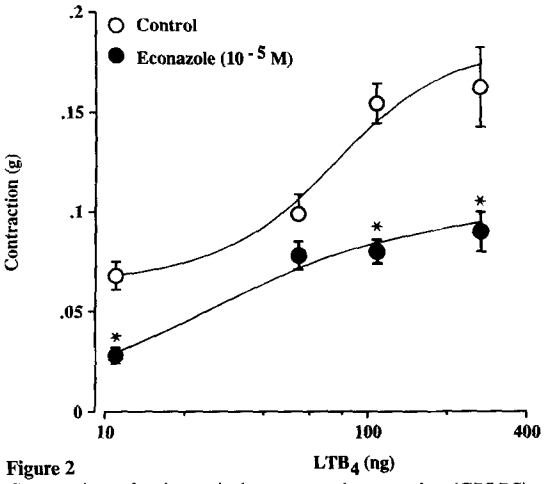

Figure 2
Contraction of guinea pig lung parenchyma strips (GPLPS) to LTB_4 in the absence (open circles) or presence (closed circles) of 10^{-5} *M* of econazole. Data represent the mean ± SEM from 6 experiments. * $p<0.01$.

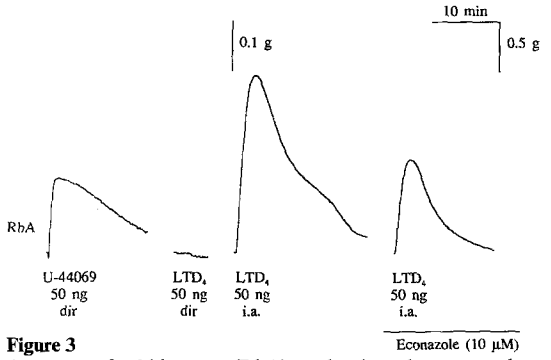

Figure 3
Response of rabbit aorta (RbA) to the thromboxane analogue (U-44069) and LTD_4 given directly (dir) or intra arterialy (i.a.) into guinea pig lungs, before and after infusion of 10^{-5} *M* of econazole. Representative tracings of 6 individual experiments.

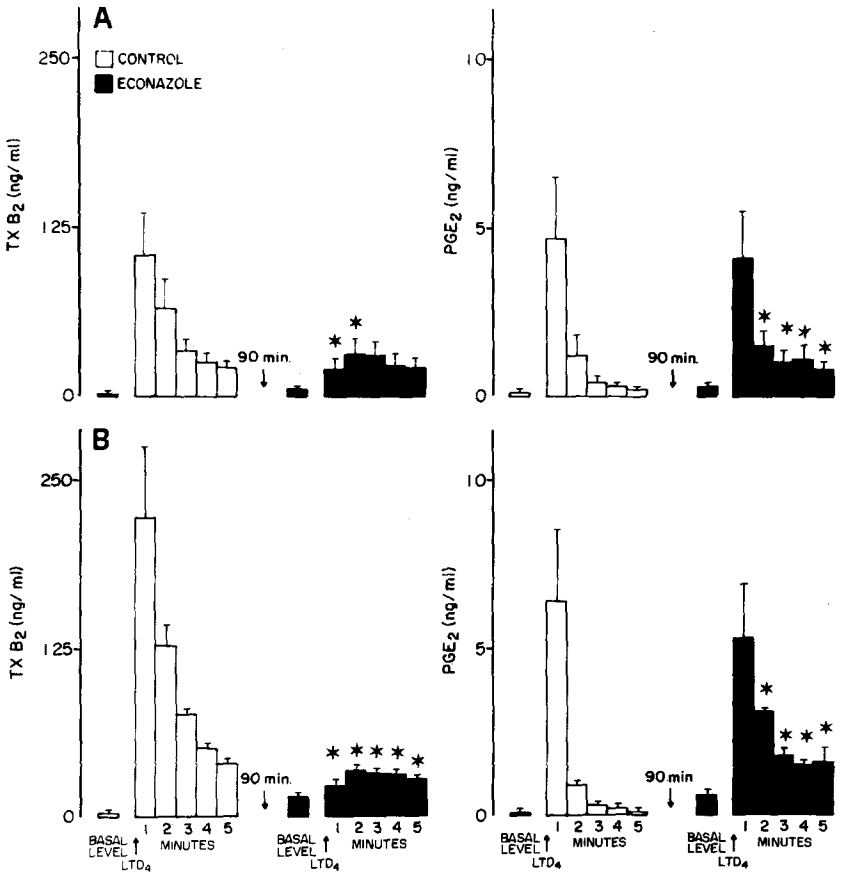

Figure 4
Concentrations of TXB_2 and PGE_2 in lung effluent before and after a bolus injection of LTD_4 (50 ng) into the pulmonary artery in the absence (open bars) or presence of econazole (closed bars). Panel A: 1×10^{-5} *M* econazole; Panel B: 1×10^{-4} *M* econazole. The concentration of eicosanoids was determined by specific EIA. Data represent the mean ± SEM of 4 separate experiments.

Discussion

Arachidonic acid induces platelet aggregation which is mostly attributed to its transformation into TXA_2 and this aggregation can be prevented by aspirin and other NSAID which inhibit cyclooxygenase [12, 13]. The results presented here show that econazole inhibits AA-induced aggregation of guinea pig platelets. This effect might be due to inhibition of either cyclooxygenase or thromboxane synthetase enzymes. Inhibition of thromboxane synthesis is not always effective to inhibit AA-induced platelet aggregation. One of the possible explanations is that inhibition of this enzyme may redirect AA-metabolism towards anti-aggregating products. Bertelé et al. [14], showed that the anti-aggregating effect of dazoxiben can be overcome by prostaglandins formed in excess as consequence of the blockade of thromboxane synthesis in platelets from "non-responder" individuals. In humans, a proportion of individuals have platelets that are resistant to thromboxane synthetase inhibitors [15, 16]. In our experiments, we observed that in about 30% of the platelet samples tested, econazole was not effective in blocking AA-induced aggregation.

To clarify further the mechanism of action of econazole we studied its effect on other reactions that are mediated by TXA_2. The contraction of guinea pig lung strips to LTB_4 given directly is mostly mediated by thromboxane generation, since it is inhibited by selective thromboxane syn-

thetase inhibitors [10]. In our studies econazole significantly inhibited the contraction of the lung strips to LTB_4. It is also known that stimulation of guinea pig lungs with LTD_4 induces release of TXA_2 [11]. In our studies, the effluent from guinea pig lungs perfused with LTD_4 induced contraction of rabbit aorta, which is the classical assay for thromboxane [12]. This effect was also reduced by econazole. More direct evidence was provided when samples of the effluent from LTD_4 stimulated lungs were analyzed for their content of TXB_2 and PGE_2. These studies confirmed that LTD_4 induced generation of high amounts of TXB_2 and also PGE_2 but in much lower amounts. When the lungs were pretreated with econazole before LTD_4 injection, TXB_2 levels in the effluent were significantly reduced and PGE_2 levels increased and this effect was dose-dependent. Overall, these results clearly demonstrate that econazole inhibits TXA_2 generation, without causing a general inhibition of all cyclooxygenase products, possibly by selectively blocking the thromboxane synthetase enzyme, as reported for other imidazole derivatives [17].

Econazole was also effective in inhibiting thromboxane generation *"in vivo"*. In a model of antigen-induced arthritis in rabbits, 20 mg/kg given intravenously, significantly decreased the level of TXB_2 without affecting PGE_2, LTB_4 and peptidoleukotrienes in the synovial exudate. Similarly, in rat carragenin pleurizy, 200 mg/kg give per oz, reduced TXB_2 below basal levels whereas PGE_2 increased in the pleural exudates. Although it is beyond the scope of this paper to discuss the effect of thromboxane blockade in pathological conditions, it is interesting to mention that in both models, econazole significantly inhibited oedema and infiltration of inflammatory cells into the synovial and pleural cavities (data not shown).

Whether this pharmacological action of econazole is involved in the anti-fungal properties of the drug is not known. However, the inhibitory effect of econazole on thromboxane synthesis may open new vistas about the mechanism of the biological action or side effects of this drug.

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