British Journal of Pharmacology (2000) 130, 1241–1246

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Effect of cytochrome P-450 inhibitors econazole, bifonazole and clotrimazole on prostanoid formation

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1 The present study was carried out to clarify the effect of the imidazole antimycotics econazole, bifonazole and clotrimazole on prostanoid biosynthesis. Osteoblast-like MC3T3-E1 cells stimulated by endothelin-1, melittin, ionomycin or arachidonic acid showed diminished prostaglandin E_2 (PGE_2) production upon pretreatment with econazole. Following pretreatment with bifonazole, stimulation with ionomycin or arachidonic acid also resulted in decreased PGE_2 formation. Clotrimazole inhibited ionomycin but not arachidonic acid stimulated PGE_2 synthesis in MC3T3-E1 cells.

2 The results observed in osteoblast-like UMR-106 cells pretreated with econazole, bifonazole or clotrimazole and stimulated by arachidonic acid were similar with the exception of clotrimazole which was a more effective inhibitor of PGE_2 biosynthesis than in MC3T3-E1 cells.

3 Upon treatment with arachidonic acid thromboxane B_2 (TXB_2) production in human platelets was abolished completely at concentrations of the three imidazole antimycotics higher than 5 μM ($IC_{50} < 1$ μM).

4 These data were confirmed by a direct assay using purified ram seminal vesicle prostaglandin H_2 synthase-1 (PGHS-1), which clearly showed inhibitory properties of econazole (IC_{50} 4.7 ± 2.3 μM), bifonazole (IC_{50} 9.4 ± 0.8 μM) and clotrimazole (IC_{50} 4.4 ± 0.6 μM).

5 Summarizing, these results indicate an inhibitory effect of econazole, bifonazole and clotrimazole on PGHS-1, varying in its potency dependent on the cell system used. In addition TXB_2 formation is affected at doses even lower than those needed to suppress PGE_2 biosynthesis.
British Journal of Pharmacology (2000) **130,** 1241–1246

Keywords: Econazole; bifonazole; clotrimazole; prostaglandin H_2 synthase-1; thromboxane A_2 synthase; MC3T3-E1 cells; UMR-106 cells; human platelets

Abbreviations: AA, arachidonic acid; ET-1, endothelin-1; FCS, foetal calf serum; GC-NICI-MS, gas chromatography-negative ion chemical ionization mass spectrometry; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGH_2, prostaglandin H_2; PGHS-1, prostaglandin H_2 synthase-1; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2

Introduction

The imidazole compounds econazole (1-[2(2,4-dichlorophenyl)-2- (4-chlorobenzyloxy)-ethyl]-imidazole), bifonazole (1-[(4-biphenyl)-phenylmethyl]-imidazole) and clotrimazole (1-[1,1-diphenyl-1- (2-chlorophenyl)-methyl]-imidazole) are used as broad-spectrum antimycotics. Their mechanism of action includes inhibition of cytochrome P-450 which is essential for ergosterol biosynthesis at the step of lanosterol-14-demethylation (van den Bossche *et al*., 1989). However, inhibition is not restricted to fungal cytochrome P-450, since interaction with mammalian cytochrome P-450 has also been demonstrated (Lewis *et al*., 1989). Thus, these imidazoles have been shown to downregulate many cytochrome P-450 dependent processes in mammalian cells, including steroid aromatase activity in human placental microcosms (Mason *et al*., 1985) and cytochrome P-450-dependent pathways of arachidonate metabolism (Capdevila *et al*., 1988). It is claimed that these drugs represent efficient and widely used pharmacological tools to distinguish between epoxygenase and cyclo-oxygenase pathways of arachidonic acid metabolism *in vitro* as well as *in vivo*. A further hemoprotein-dependent metabolic reaction inhibited by clotrimazole and other N-substituted imidazoles is thromboxane biosynthesis in human blood mononuclear cells (Gordon *et al*., 1981).

In recent years econazole and clotrimazole have emerged as potent modulators of cytosolic calcium concentration. Both have been proposed to regulate voltage gated calcium channels in GH_3 cells and receptor operated calcium channels in human neutrophils and platelets (Villalobos *et al*., 1992). Furthermore imidazoles, such as econazole, are known inhibitors of hepatic microsomal calcium ATPase (Erickson *et al*., 1987), thus causing elevation of intracellular calcium probably *via* mitochondrial calcium release in vascular smooth muscle A7r5 cells (Hughes & Schachter, 1994). Econazole has also been shown to inhibit thapsigargin induced calcium influx by a mechanism different from cytochrome P-450 inhibition in human platelets (Vostal & Fratantoni, 1993). Modulatory properties of econazole on thapsigargin stimulated intracellular calcium elevation result in suppression of HIV expression in latently infected T-lymphocytic ACH-2 cells. This seems to represent a potential target for the pharmacological modulation of HIV expression (Papp & Byrn, 1995). Additionally econazole exerts an inhibitory effect on thrombin and ADP evoked protein-tyrosine phosphorylation in human platelets by unknown mechanisms (Sargeant *et al*., 1994). Another imidazole compound, SK&F 96365 (1-{β-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenylethyl}-1-*H*-imidazole hydrochloride), a blocker of receptor-operated calcium entry, has been shown to inhibit short term prostanoid formation in arachidonic acid stimulated osteoblast-like cell lines,

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MC3T3-E1 and UMR-106 as well as in human platelets, but did not show any effect on metabolites produced by the lipoxygenase pathway. These findings have been attributed to inhibition of prostaglandin H_2 synthase-1 (PGHS-1) activity (Leis *et al*., 1995).

With this background, the aim of the present study was to clarify the effects of the imidazole antimycotics econazole, bifonazole and clotrimazole on prostanoid metabolism distinct from their calcium and cytochrome P-450 modulating properties.

Methods

Cell culture

MC3T3-E1 and UMR-106 cells were routinely cultured in α-MEM containing 5% FCS in a humidified atmosphere of 5% CO_2 in 80 cm^2 flasks and transferred to 4 cm^2 12-well culture dishes before experiments. At confluency the medium (1 ml) was removed and the cell monolayer incubated with 1 ml of α-MEM containing 0.2% FCS, 4 mM Ca^{2+} and 20 mM HEPES. After preincubation with the indicated concentrations of econazole (bifonazole, clotrimazole) for 10 min, incubations with the appropriate stimuli or vehicle were carried out for 30 min. For prostaglandin measurement the incubation buffer was removed and processed as described below. Human platelets were prepared from human plasma as described by Mustard *et al*. (1989) and resuspended in 10 mM HEPES buffer containing (in mM): NaCl 121, D(+) glucose 10, KCl 5 and MgCl 0.2. One ml (5×10^7 cells) of the suspension was used and 1 mM Ca^{2+} added prior to experiments. Incubation was stopped by addition of 1 ml of ethanol and thromboxane B_2 (TXB_2) and prostaglandin E_2 (PGE_2) determined as described below.

PGHS-1 enzyme assay

Ten units (0.27 μg) PGHS-1 in 100 μl 30 mM TRIS/HCl buffer containing 1.3 mg ml^{-1} l-adrenaline were incubated for 10 min at 4°C with the appropriate concentrations of econazole, bifonazole or clotrimazole. Subsequently, prostaglandin H_2 (PGH_2) biosynthesis was stimulated with 4 μM of arachidonic acid. The reaction was allowed to progress for 15 min at 37°C and was terminated by 1 ml of 0.02% formic acid. PGH_2 is unstable in aqueous solution and therefore isomerizes under non-reducing conditions mainly to PGE_2 (Nugteren & Christ-Hazelhof, 1973), which can be measured as described below.

Prostaglandin determination

PGE_2 and TXB_2 were measured by gas chromatography-negative ion chemical ionization mass spectrometry (GC-NICI-MS) (Leis *et al*., 1987; Mayer *et al*., 1986; Malle *et al*., 1987). Briefly, PGE_2 and TXB_2 were converted to their pentafluorobenzyl ester-trimethylsilyl ether O-methyloxime derivatives. Quantitation was carried out by use of tetra-deuterated PGE_2 and ^{18}O-TXB_2 as internal standard. A Finnigan Voyager quadrupole mass spectrometer coupled to a Finnigan TRACE GC was used (Thermoquest, Vienna, Austria). GC was performed on a 15 m DB5-MS fused silica capillary column (Fisons Instruments). The temperature of the splitless Grob injector was kept at 280°C, initial column temperature was 160°C for 1 min, followed by an increase of 40°C min^{-1} to 310°C. NICI was carried out in the single ion recording mode with methane as moderating gas.

Materials

Econazole, bifonazole, clotrimazole, endothelin-1, ionomycin, arachidonic acid, melittin and HEPES buffer were from Sigma Chemical Co. (Munich, Germany). Ram seminal vesicle PGHS-1 was from Oxford Biomedical (Oxford, MI, U.S.A.). Foetal calf serum (FCS) was obtained from PAA (Linz, Austria) and α-minimum essential medium (α-MEM) from GibcoBRL *via* Life Technologies (Vienna, Austria). Trypsin-EDTA was purchased from Boehringer (Mannheim, Germany). Pentafluorobenzyl bromide (PFBBr), *bis*-(N,O-trimethylsilyl) trifluoroacetamide (BSTFA), silylation grade pyridine, acetonitrile and O-methoxyamine hydrochloride (MOX) were from Pierce Chemical Co. (Rockford, IL, U.S.A.). Culture dishes were from Falcon *via* Szabo (Vienna, Austria). MC3T3-E1 cells were kindly donated by Dr Klaushofer (Vienna, Austria) and UMR-106 cells were purchased from ATCC (Rockville, MD, U.S.A.). Deuterated PGE_2 was obtained through MSD Isotopes *via* IC Chemikalien GmbH (Munich, Germany). All other chemicals were from Merck (Darmstadt, Germany). ^{18}O-TXB_2 was prepared as described (Leis *et al*. 1986).

Results

Effects of econazole, bifonazole and clotrimazole on PGE_2 formation in MC3T3-E1 and UMR-106 cells

Econazole dose-dependently inhibited PGE_2 production in MC3T3-E1 cells stimulated by 50 nM of endothelin-1 (ET-1) or by 2 μM of melittin between 0.1 and 30 μM (Figure 1). An IC_{50} value for econazole of 1.4 ± 0.4 μM (ET-1) and 1.8 ± 0.3 μM (melittin) was obtained. Figure 2 compares the effect of various concentrations of econazole, bifonazole or clotrimazole on PGE_2 formation in MC3T3-E1 cells stimulated by 2 μM ionomycin. At a concentration of 20 μM, econazole caused complete and bifonazole caused 95.7% inhibition. Compared to econazole and bifonazole the inhibitory effect of clotrimazole (IC_{50} 9.3 ± 1.8 μM) was weaker but nevertheless evident from the present data.

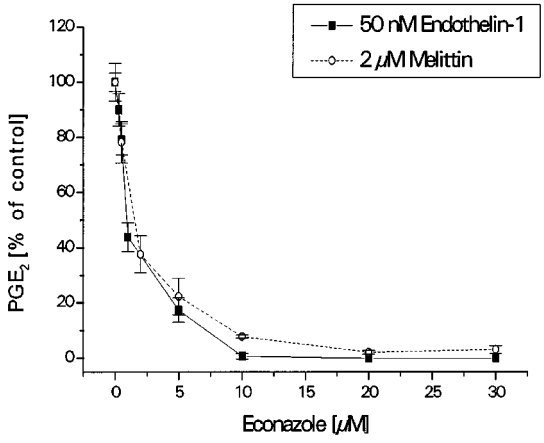

Figure 1 Inhibition of agonist-induced PGE_2 formation by econazole in the clonal murine osteoblast-like cell line MC3T3-E1. Cells were cultured as described in Methods and preincubated for 10 min with different concentrations of econazole in α-MEM containing 0.2% FCS, 4 mM Ca^{2+} and 20 mM HEPES. Subsequently, incubation with 50 nM endothelin-1 or 2 μM melittin was carried out for 30 min. Temperature was kept at 37°C. Points represent means ± s.d. of three determinations.

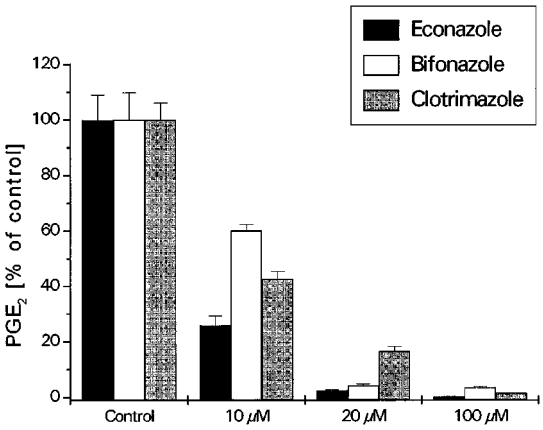

Figure 2 Inhibition of ionomycin-induced PGE_2 formation by econazole, bifonazole and clotrimazole in the clonal murine osteoblast-like cell line MC3T3-E1. Cells were preincubated for 10 min with different concentrations of econazole, clotrimazole or bifonazole and subsequently incubated for 30 min with 2 μM ionomycin. Experimental conditions are as described in Methods. Results represent means$\pm$s.d. of three determinations.

Next we tried to inhibit PGE_2 production in MC3T3-E1 cells induced by various concentrations of exogenous arachidonic acid (Figure 3a,b). The ability of econazole to reduce PGE_2 biosynthesis was markedly reduced by increasing concentrations of arachidonic acid, with almost no effect of the drug on PGE_2 formation evoked by 10 μM of arachidonic acid. Econazole at 20 μM inhibited PGE_2 formation (stimulated by 2 μM arachidonic acid) by 75.5% (IC_{50} 6.4$\pm$0.6 μM), whereas for PGE_2 formation stimulated with 10 μM arachidonic acid the inhibitory effect was only 47.0%. The results observed with

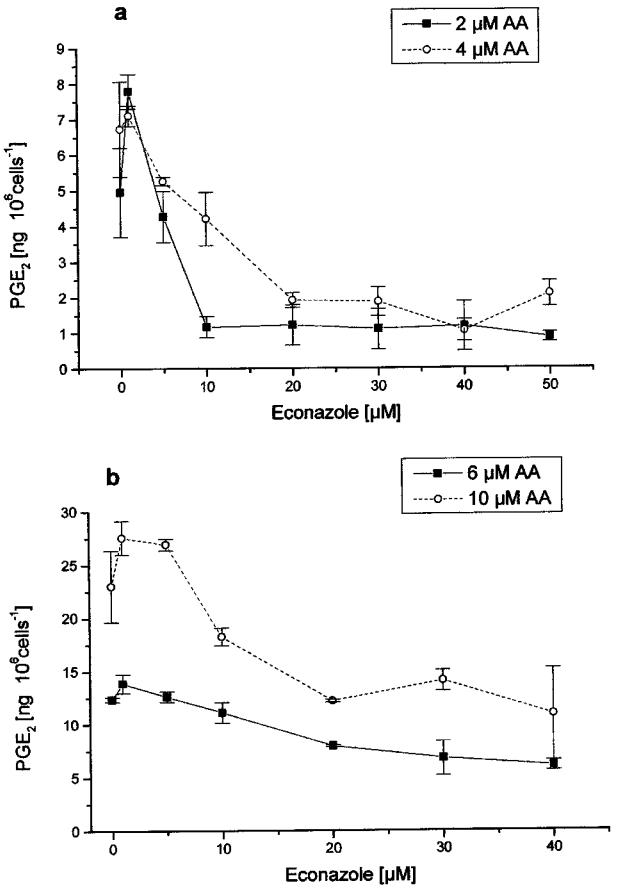

Figure 3 Effect of econazole on PGE_2 formation stimulated by various concentrations of arachidonic acid in the clonal murine osteoblast-like cell line MC3T3-E1. Cells were cultured as described in Methods and preincubated for 10 min with different concentrations of econazole in α-MEM containing 0.2% FCS, 4 mM Ca^{2+} and 20 mM HEPES. Subsequently, incubation with 2, 4 μM (a), 6 and 10 μM (b) arachidonic acid (AA) was carried out for 30 min. Temperature was kept at 37°C. Points represent means$\pm$s.d. of three determinations.

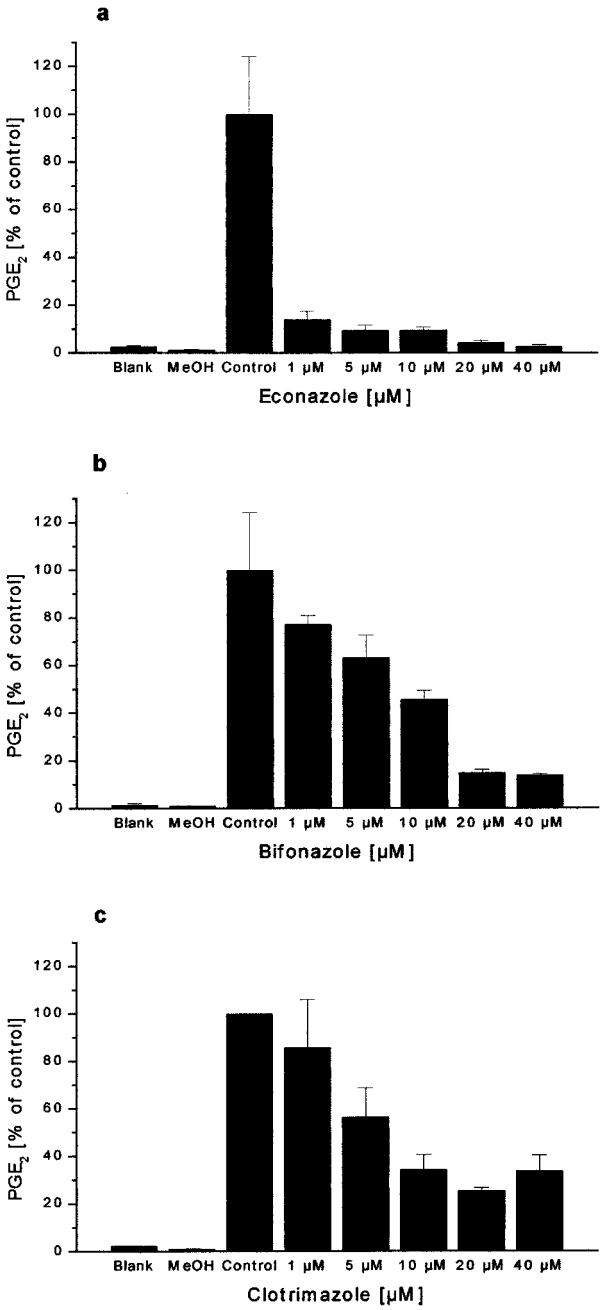

Figure 4 Inhibition of arachidonic acid-induced PGE_2 formation by econazole, bifonazole and clotrimazole in the rat osteosarcoma cell line, UMR-106. Cells were cultured as described in Methods and preincubated for 10 min with vehicle (methanol) or different concentrations of econazole (a), bifonazole (b) and clotrimazole (c) in α-MEM containing 0.2% FCS, 4 mM Ca^{2+} and 20 mM HEPES. Subsequently, incubation with 4 μM arachidonic acid was carried out for 30 min. Temperature was kept at 37°C. Results represent means$\pm$s.d. of three determinations.

bifonazole were similar to those obtained with econazole (data not shown). Bifonazole blocked PGE_2 formation induced by 2 and 4 μM arachidonic acid clearly better than at 6 or 10 μM of the agonist. Following treatment with 2 μM arachidonic acid bifonazole yielded an IC_{50} value of 1.5 ± 0.4 μM. No inhibitory effect on PGE_2 synthesis could be measured in arachidonic acid stimulated MC3T3-E1 cells pretreated by clotrimazole.

Additionally rat osteosarcoma UMR-106 cells were stimulated with arachidonic acid (4 μM) to produce prostaglandins. Econazole exhibited a far stronger inhibitory effect on PGE_2 biosynthesis in these cells than in MC3T3-E1 cells (Figure 4a). Increasing concentration of econazole caused dramatically decreasing quantities of PGE_2 ($IC_{50} < 1$ μM). Bifonazole (Figure 4b) and clotrimazole (Figure 4c) showed clearly weaker inhibitory potency than econazole, with IC_{50} values of 6.6 ± 2.0 μM (bifonazole) and 3.4 ± 1.2 μM (clotrimazole).

Effect of econazole, bifonazole and clotrimazole on purified PGHS-1

PGHS-1 activity was stimulated by 4 μM of arachidonic acid. All three imidazole antimycotics caused a dose-dependent decrease of PGE_2 (Figure 5). In these experiments more powerful inhibitory properties upon PGE_2 formation could be observed for econazole (IC_{50} 4.7 ± 2.3 μM) and clotrimazole (IC_{50} 4.4 ± 0.6 μM) than for bifonazole (IC_{50} 9.4 ± 0.8 μM). Econazole and clotrimazole, both at low doses, increased PGE_2 levels. At a drug concentration of 40 μM PGE_2 production was abolished totally by all of the used imidazole antimycotics. Furthermore no measurable prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) could be detected in this enzyme assay.

Effects of econazole, bifonazole and clotrimazole on PGE_2 and TXB_2 formation in isolated human platelets

In order to stimulate PGE_2 and TXB_2 biosynthesis, platelets were treated with 4 μM arachidonic acid. Controls showed a TXB_2/PGE_2 ratio of approximately 100 : 1. Preincubation with 5 μM econazole (Figure 6a) completely abolished TXB_2 production ($IC_{50} < 1$ μM). A compensatory stimulation of PGE_2 formation was evident. No PGE_2 was detected in controls, but econazole at low dose (1 μM) gave rise to measurable amounts of this metabolite of the eicosanoid cascade. If econazole was used at concentrations higher than 5 μM no endogenous PGE_2 was measurable.

Bifonazole totally abolished TXB_2 formation at 5 μM ($IC_{50} < 1$ μM). Compared to controls bifonazole initially increased endogenously produced PGE_2, reaching a maximum at 5 μM of the drug. This is in good correlation to the results

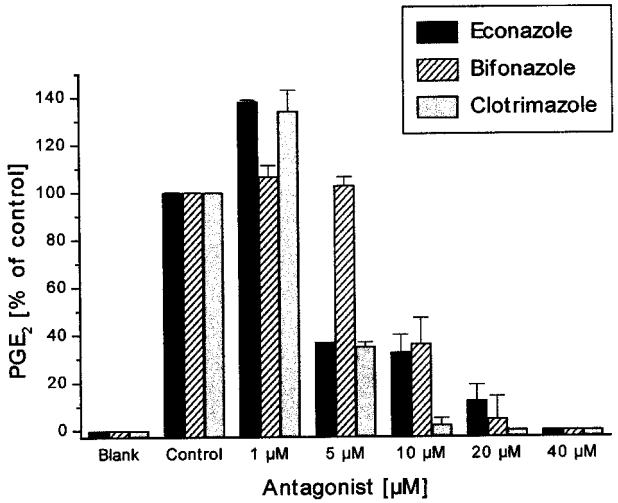

Figure 5 Inhibition of arachidonic acid-induced PGE_2 formation by econazole, bifonazole and clotrimazole using the PGHS-1 enzyme assay described in Methods. Ten units of PGHS-1 (0.27 μg protein) in 30 mM TRIS/HCl Buffer containing 1.3 mg ml^{-1} l-adrenaline were preincubated with various concentrations of econazole, bifonazole and clotrimazole for 10 min at 4°C. Subsequently, incubation with 4 μM arachidonic acid was carried out for 15 min at 37°C before terminating the reaction. Results represent means ± s.d. of three determinations.

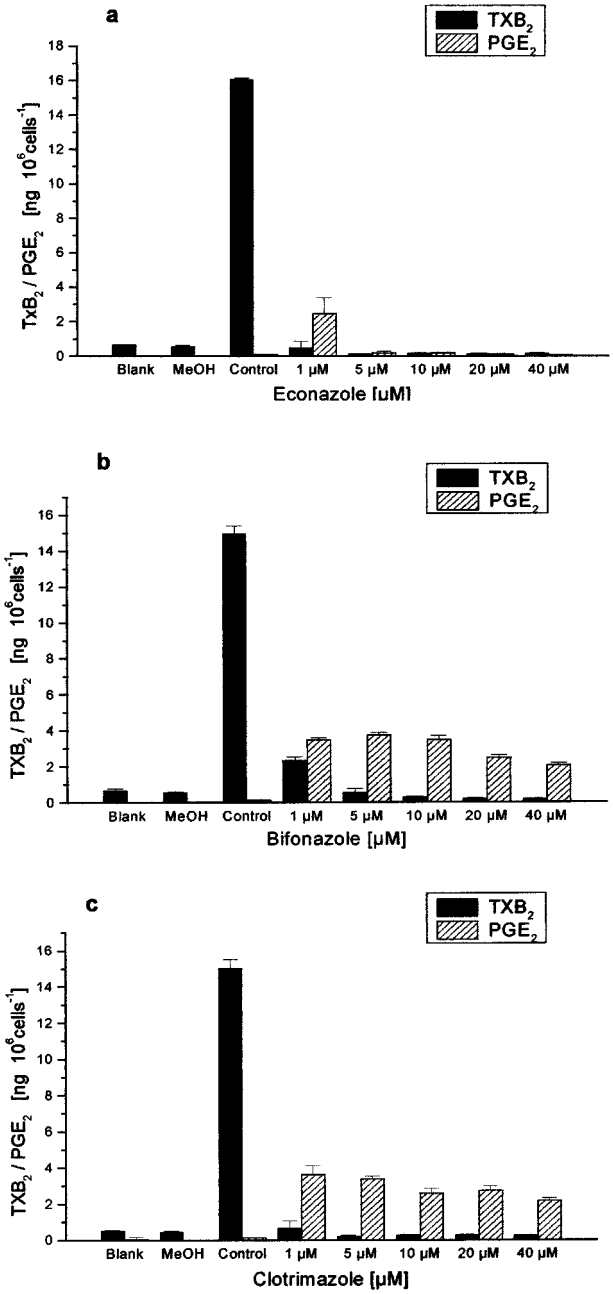

Figure 6 Effect of econazole, bifonazole and clotrimazole on TXB_2 and PGE_2 formation in isolated human platelets, using arachidonic acid (4 μM) as stimulant. Platelets were prepared as described in Methods and preincubated for 10 min with vehicle (methanol) or various concentrations of econazole (a), bifonazole (b) and clotrimazole (c) in 10 mM HEPES buffer containing 1 mM Ca^{2+}. Subsequently, incubation with 4 μM arachidonic acid was carried out for 30 min. Temperature was kept at 37°C. Results represent means ± s.d. of three determinations.

obtained with econazole, but, in contrast to econazole, this imidazole compound lacked the ability to completely inhibit PGE_2 formation, even at the highest doses used (Figure 6b).

Clotrimazole proved as inhibitor of TXB_2 biosynthesis at submicromolar doses, achieving complete suppression at 1 μM. There was no detectable PGE_2 formation in control experiments, but platelets showed enhanced PGE_2 levels after treatment with clotrimazole, which slightly decreased with increasing concentration of the drug. Similar to bifonazole, clotrimazole did not inhibit this compensatory increase in PGE_2 formation at concentrations up to 40 μM (Figure 6c).

Discussion

The results of the present study show that all three of the tested imidazole antimycotics exert distinct inhibitory effects on prostanoid biosynthesis in various cell systems as well as in enzyme assays with purified PGHS-1. Using ET-1 stimulated osteoblast-like MC3T3-E1 cells one might suppose that the suppression of PGE_2 formation by econazole could be secondary to impaired calcium influx, which is suggested to be an important step in the signal transduction cascade from ET_A receptor to PGE_2 formation in bone (Stern *et al*., 1995). To investigate this by avoiding receptor-mediated intracellular processes we used ionomycin, melittin and arachidonic acid, which are all known stimulants of prostanoid biosynthesis. The data obtained from these experiments indicate an inhibitory effect of econazole on PGE_2 formation which must not be attributable to effects on intracellular calcium. Bifonazole shows the same characteristics in MC3T3-E1 and UMR-106 cells stimulated by ionomycin or various concentrations of arachidonic acid. Arachidonic acid is directly metabolized by PGHS-1 and thus diminished PGE_2 biosynthesis gives an indication of impaired PGHS-1 or subsequent PGH_2-PGE_2 isomerase activity. Since the inhibitory effect of these drugs is also dependent on the concentration of arachidonic acid, a competitive, reversible mode of action is suggested. Experiments conducted with purified PGHS-1 distinctly point out that this inhibition takes place at the step of PGH_2 formation. Taking into account that PGHS-1 is a hemoprotein (Picot *et al*., 1994), this effect is not very surprising, since imidazole derivatives have been proposed to affect various proteins containing a heme moiety in their catalytical domain (Iizuka *et al*., 1981; Mason *et al*., 1985; Rodrigues *et al*., 1987). It can not be deduced from our results whether an additional suppressive effect on PGH_2-PGE_2 isomerase also contributes to inhibition of PGE_2 biosynthesis.

An interesting detail observed in many experiments was an increased production of PGE_2 at low doses of the used imidazole compounds. This happened in intact cells as well as in the assays using purified PGHS-1 enzyme. Thus it is suggested that econazole, bifonazole and clotrimazole at the low micromolar level might activate PGHS-1 directly, and therefore affect the enzyme activity in a biphasic manner. The precise mechanism leading to this observation requires further study.

Clotrimazole blocked PGE_2 synthesis in ionomycin treated MC3T3-E1 cells, but interestingly had no significant influence on PGE_2 produced by arachidonic acid stimulation. This is in contrast to the data observed in another osteoblast-like cell-line, UMR-106, and in the PGHS-1 enzyme assay. Such differences might be explained by the susceptibility of the drug for phospholipids and triglycerides containing unsaturated acyl chains (Yamaguchi, 1977). Clotrimazole and miconazole are proposed to form hydrophobic complexes with unsaturated phosphatidylcholine, thus lowering the effective concentration in medium (Yamaguchi, 1978). This fact could contribute to the failure of clotrimazole to affect PGE_2 biosynthesis in arachidonic acid supplemented MC3T3-E1 cells. Additionally it seems to be likely, that, regarding the structural analogy to clotrimazole and miconazole, econazole and bifonazole interact with phospholipids in a similar way. This might account for the variable IC_{50} value observed for the same compound in different cell-lines.

To provide further evidence for an inhibitory role of econazole, clotrimazole and bifonazole on PGHS-1 activity, experiments were conducted with arachidonic acid stimulated human platelets. The complete endogenous TXB_2 suppression between 1 and 5 μM of these drugs is likely due to direct inhibition of thromboxane A_2 synthase, since inhibition at the level of PGHS-1 would have also prevented PGE_2 release, and this was not the case (Figure 6). Nevertheless, inhibition of PGHS-1 is obvious, at least for econazole, at concentrations higher than 5 μM where neither TXB_2 nor PGE_2 and $PGF_{2\alpha}$ (data not shown) could be obtained in measurable amounts. In human platelets it has been demonstrated that PGH_2 is further metabolised to PGE_2 and $PGF_{2\alpha}$ if the predominant thromboxane A_2 (TXA_2) pathway is blocked (Hornberger & Patscheke, 1989). In bifonazole- and clotrimazole-treated platelets PGE_2, even though not completely reduced, never make up more than 30% of TXB_2 produced in control experiments. According to these data an inhibitory action on PGHS-1, although less than that on thromboxane A_2 synthase, is suggested for all imidazole antimycotics tested.

Calcium homeostasis is fundamental to many aspects of cell signalling and response (Berridge, 1997), including arachidonic acid liberation (Clark *et al*., 1995) and concomitant prostanoid formation. Therefore much effort has been spent on the investigation of intracellular- and plasma membrane calcium fluxes. Econazole and clotrimazole are widely used as modulators of cytosolic calcium in a concentration range between 10 and 50 μM (Benzaquen *et al*., 1995; Jan *et al*., 1999; Snajdrova *et al*., 1998; Thomas *et al*., 1999). Our data clearly indicate that these are concentrations which strongly affect prostanoid biosynthesis in an inhibitory way and diminished prostaglandin formation as well as further cellular responses may not solely be attributed to impaired calcium influx. Thus, we believe that it is very unwise to use econazole and clotrimazole as calcium modulating agents, unless this effect on arachidonate metabolism is taken into account.

Concentrations as high as 10 μM of econazole and clotrimazole have also frequently been utilized to selectively suppress cytochrome P-450-dependent pathways of arachidonic acid metabolism. This study demonstrates that at such quantities additional side effects on PGHS-1 mediated eicosanoid metabolism cannot be excluded. One should thus be particularly cautious in interpreting data obtained from such experiments.

In summary, we conclude that econazole, bifonazole and clotrimazole inhibit cyclo-oxygenase and thromboxane A_2 synthase in a dose-dependent manner in various cell systems, but that their efficacy varies according to the lipid profile of the cells and medium used.

This work was supported by grants from the Austrian Science Foundation, project number P-12589.

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(Received February 14, 2000
Revised March 30, 2000
Accepted April 12, 2000)