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Functional effects of econazole on inducible nitric oxide synthase: production of a calmodulin-dependent enzyme

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1 We performed experiments to examine the effects of an anti-fungal imidazole compound, econazole, on the regulation and effects of lipopolysaccharide-inducible nitric oxide synthase (iNOS) activity in rat aortic rings and cultured J774 murine macrophage cells.

2 In endothelium-intact rings of thoracic aorta, phenylephrine caused a concentration-dependent contraction with EC_{50} of $1.9 \pm 0.15 \times 10^{-8}$ M ($n=5$). Following incubation with lipopolysaccharide (LPS, 5 $\mu g\ ml^{-1}$) for 8 h there was a right-shift in the concentration-response curve (EC_{50} $3.1 \pm 0.28 \times 10^{-7}$ M, $P<0.05$) with a depression in the maximum contraction from 1.44 ± 0.25 g to 0.86 ± 0.26 g ($n=4$). Co-incubation of rings with econazole (1×10^{-5} M) partially inhibited the LPS-induced loss of reactivity to phenylephrine (EC_{50} $6.5 \pm 0.72 \times 10^{-8}$ M) and fully inhibited the reduction in maximum tension (1.49 ± 0.19 g; $n=5$).

3 In J774 cells, incubation with LPS (10 $\mu g\ ml^{-1}$, 24 h) resulted in significant nitrite production that was inhibited by co-incubation with econazole (IC_{50} $5.0 \pm 0.9 \times 10^{-6}$ M; $n=5$). In cells stimulated with LPS, production of L-[^{3}H]-citrulline from L-[^{3}H]-arginine was 6.41 ± 0.22 pmol mg^{-1} protein min^{-1} ($n=3$). This was inhibited by $92 \pm 6\%$ by addition of N^G-monomethyl-L-arginine (L-NMMA, 1×10^{-3} M; $n=3$) to the homogenate but not by econazole (1×10^{-5} M; $n=3$). In contrast pretreatment of cells with econazole (1×10^{-5} M) markedly reduced the LPS-induced [^{3}H]-citrulline production (0.86 ± 0.053 pmol mg^{-1} protein min^{-1}; $P<0.01$; $n=3$).

4 In cells treated with LPS and econazole, L-[^{3}H]-citrulline production was restored in a concentration-dependent manner by addition of calmodulin ($1 \times 10^{-8} - 3 \times 10^{-7}$ M) with an IC_{50} of $4.2 \pm 0.9 \times 10^{-8}$ M.

5 We have shown that econazole inhibits the functional and biochemical activity of iNOS in rat aortic rings and cultured J774 cells. Treatment of cells with econazole renders the NO synthase functionally inactive. In econazole-treated cells enzyme activity is restored by calmodulin suggesting that econazole may inhibit the binding of this essential co-factor to the enzyme following its production. These studies may have implications for the design of novel anti-inflammatory agents working through the L-arginine-nitric oxide pathway.

Keywords: Lipopolysaccharide; nitric oxide; econazole; anti-fungal imidazoles; calmodulin; inducible NO synthase

Introduction

Nitric oxide (NO) plays an important role in the regulation of cellular processes in the cardiovascular, nervous and immune systems (Moncada *et al.*, 1991). It has become clear that NO synthase occurs in at least three different enzyme isoforms known as endothelial type (eNOS), neuronal-type (nNOS) and inducible or macrophage-type (iNOS) NO synthase respectively (Vallance & Moncada, 1994). All three isoforms convert L-arginine to NO and a co-product L-citrulline, and are related to cytochrome *P*450 reductase (Bredt *et al.*, 1991). Under normal quiescent conditions iNOS is not expressed; however, in the presence of inflammatory cytokines (including certain interleukins and tumour necrosis factor), high levels of active iNOS are induced in a wide variety of cell types and this results in elevated NO production. Induction of NO synthesis through this pathway is important in the cardiovascular response to endotoxin (Wei *et al.*, 1995), in sepsis (Vallance & Moncada, 1993) and many other inflammatory conditions (Morris & Billiar, 1994).

Inhibition of iNOS expression by glucocorticoids or of enzyme activity by certain arginine analogues (e.g. N^G-monomethyl-L-arginine; L-NMMA) decreases NO generation (Moncada *et al.*, 1991) and this might have beneficial therapeutic effects. Recently however interest has focused on inhibitors of NO synthase that exhibit isoform selectivity. Such compounds may be particularly useful in the cardiovascular system where all three isoforms may contribute to the control of vascular tone. One approach has been to develop novel substrate analogues of L-arginine. Compounds such as aminoguanidine (Griffiths *et al.*, 1994), isothioureas (Southan *et al.*, 1995) and L-thiocitrulline (Joly *et al.*, 1995), have been reported to exhibit some isoform selectivity for iNOS. However, compounds based on inhibiting enzyme expression might also show some selectivity and we reported that anti-fungal imidazoles inhibit expression of the iNOS activity in murine macrophages (Bogle *et al.*, 1994). This effect might account for some of the anti-inflammatory actions of these compounds (Petri *et al.*, 1986). Our results suggested that anti-fungal imidazoles inhibited expression of NO synthesis at a level after production of mRNA for NO synthase but did not alter activity of the expressed enzyme. We hypothesized that anti-fungal imidazoles inhibit synthesis of NO either by inhibition of mRNA translation or by affecting a cofactor essential for the activity of the iNOS. It is known that iNOS requires tetrahydrobiopterin for full activity (Gross & Levi, 1992), and flavoproteins are also important co-factors (Stuehr *et al.*, 1991). In addition it appears that calmodulin is a subunit of iNOS from macrophages (Cho *et al.*, 1992). The enzyme binds calmodulin tightly so that in most cells iNOS activity appears

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calcium/calmodulin-independent. Since imidazoles are known to act as calmodulin antagonists (Hegemann *et al.*, 1993; Wolff *et al.*, 1993) it is possible that they may inhibit induction of iNOS via inhibition of calmodulin activity.

In the present study we have examined the effects of econazole on the functional expression of iNOS in rings of rat aorta and determined the mechanism of inhibition for these drugs using isolated preparations of iNOS. A preliminary account of some of this work has been presented at a meeting of the British Pharmacological Society (Bogle & Vallance, 1995a, b).

Methods

Rat thoracic aortic rings

Male Wistar rats (250–300 g) were stunned and killed by exsanguination. Rings, 4–5 mm wide, of thoracic aorta were suspended between two hooks connected to a transducer (Statham UC3, Gould Inc, Cleveland, Ohio, U.S.A.) for the measurement of isometric force. Endothelium-intact rings were used (> 70% relaxation of preconstricted rings in response to acetylcholine 1×10^{-6} M; data not shown). The preparations were suspended in 10 ml organ baths containing oxygenated (95% O_2/5% CO_2), warmed (37°C) Krebs solution in the presence of indomethacin (1×10^{-5} M). Basal tension was set at 1.5 g and rings were allowed to equilibrate until the resting tension was stable at 1 g (approximately 1 h); rings were washed every 20 min during this time. Tone was increased to approximately 70% maximal contraction by addition of phenylephrine ($1-3 \times 10^{-7}$ M). When responses had reached a plateau either lipopolysaccharide (LPS; 5 μg ml^{-1}), LPS and econazole (1×10^{-5} M) or vehicle (absolute ethanol; 0.1% v/v) were added and tone was monitored for 8 h. In some experiments after 4 h incubation with LPS, D-arginine (3×10^{-4} M) was added to the organ baths followed by L-arginine (3×10^{-4} M). In another series of experiments, unpreconstricted rings of rat aorta were incubated with either control vehicle (ethanol, 0.1% v/v), LPS (5 μg ml^{-1}) or LPS and econazole (1×10^{-5} M) under resting tension. After 8 h incubation concentration-response curves to phenylephrine ($10^{-9}-10^{-6}$ M) were constructed.

Murine macrophage cell culture

Cells of the murine monocyte/macrophage line J774 were maintained in continuous culture in tissue-culture flasks (75 cm^2 growth area) in Dulbecco's modified Eagle's medium supplemented with foetal calf serum (10% v/v), L-glutamine (4 mM), penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}). Cells were harvested by gentle scraping and passaged every 3–6 days by dilution of a suspension of the cells 1:10 in fresh medium. For use, cells were seeded into 96-well plates or 10 cm dishes and allowed to adhere for 2 h. After this, medium was replaced with fresh medium containing LPS (10 μg ml^{-1}) or LPS and econazole ($0.01-3 \times 10^{-5}$ M) and cells were incubated at 37°C for a further 20 h. Nitrite production in the culture medium was measured with the Greiss reagent as described below.

Measurement of nitrite production as an assay of NO release

Production of NO was assayed by measurement of the accumulation of nitrite in the culture medium by the Griess reaction (Green *et al.*, 1982; Bogle *et al.*, 1992). An aliquot (100 μl) of the culture medium was mixed with an equal volume of Griess reagent (sulphanilamide, 1% w/v; napthylethylenediamine dihydrochloride, 0.1% w/v; and orthophosphoric acid, 2.5% v/v) and incubated at room temperature for 10 min. The absorbance was read at 560 nm in a Titertek Multiskan II plate reader (Flow, High Wycombe, U.K.). Nitrite concentration in the medium was determined with sodium nitrite used as a standard.

Isolation and measurement of nitric oxide synthase activity from J774 cells

NO synthase activity was determined in crude homogenates of J774 cells. Cells were cultured in dishes (10 cm diameter; 5×10^6 cells) and incubated for 20 h with control medium, LPS (10 μg ml^{-1}) or LPS and econazole (1×10^{-5} M). Following incubation, cells were rinsed three times with ice-cold Dulbecco's phosphate-buffer saline and lysed by addition of 150 μl ice-cold homogenization buffer (composition: sucrose, 0.25 M; potassium phosphate, 0.1 mM; dithiothreitol, 0.1 mM EDTA, 1 mM, pH 7.4). Cellular material was removed from the culture plates with a cell scraper, collected and centrifuged at 10,000 *g* for 10 min at 4°C. The resulting supernatant was maintained on ice (up to 4 h) and used as a crude preparation of NO synthase as previously described (Salter *et al.*, 1991). For the assay, 70 μl of assay buffer (composition: HEPES 10 mM, EDTA 1 mM, NADPH 0.1 mM, L-[2,3-^{3}H]-arginine 220,000 d.p.m., pH 7.4) was mixed with 30 μl cell homogenate and incubated for 10 min at 37°C in microcentrifuge tubes. In some experiments cell lysates obtained from cells treated with LPS were co-incubated with assay buffer containing either L-NMMA (final concentration: 1×10^{-3} M) or econazole (final concentration: 1×10^{-5} M). In other experiments lysates obtained from cells treated with LPS and econazole were incubated with assay buffer containing increasing concentrations of calmodulin ($1 \times 10^{-8}-3 \times 10^{-7}$ M).

After incubation, 1 ml of Dowex AG40WX-8 resin (1:1; resin : H_2O) was added to each sample and centrifuged at 10,000 *g* for 5 min. Supernatants (400 μl) were mixed with 10 ml Optiphase Safe scintillation fluid and radioactivity determined by liquid scintillation spectroscopy. Portions of the assay buffer were included in each batch of samples counted. Background radioactivity was determined by including samples from which only the radiolabel was omitted. Disintegrations per minute were converted to citrulline production and expressed in pmol per mg protein per min.

Measurement of cell protein

Protein content was determined with the Coomassie blue protein reagent (Bradford, 1976) diluted 1:10 with distilled water before use. Samples (0.2 ml) were mixed with 0.8 ml protein reagent and incubated for 20 min at room temperature. Absorbance was measured in aliquots (200 μl) at 620 nm in a MultiSkan plate reader (Flow Laboratories, Irvine, U.K.). Bovine serum albumin was used as a standard.

Materials

The Krebs solution had the following composition, (mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, D-glucose 11). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (PBS 'A', composition, mM: NaCl 138, KCl 2.6, Na_2HPO_4 1.8, KH_2PO_4 1.5, pH 7.4) and foetal calf serum (heat-inactivated) were obtained from Flow Laboratories Ltd, Oxon, U.K. The following reagents were obtained from Sigma Chemical Co., Poole, U.K.: calmodulin (bovine brain), dithiothreitol, Dowex AG40WX-8 resin (Na^+ form), econazole nitrate, ethylenediamine-tetracetic acid (EDTA), N-[2-hydroxy ethyl] piperazine-*N'*-[2-ethanesulphonic acid] (HEPES), lipopolysaccharide (from *Escherichia coli*, serotype 055:B5), β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), phenylephrine and sulphanilamide. The following reagents were obtained from BDH Ltd, U.K.: sucrose, orthophosphoric acid, napthylethylenediamine hydrochloride and potassium phosphate. L-[2,3-^{3}H]-arginine (specific activity: 65 Ci mmol^{-1}) was obtained from NEN-DuPont (U.K.), Stevenage, Herts. Coomassie Blue

protein reagent was obtained from BioRad Ltd (Hemel Hempstead, U.K.). Optiphase Safe scintillation fluid was from LKB (Milton Keynes, U.K.).

Drugs were prepared fresh in Krebs buffer with the exception of econazole nitrate which was dissolved in ethanol to a concentration of 10 mM. The final concentration of ethanol in the organ bath was always 0.1% or less.

Data analysis and statistics

Results are shown as the means ± s.e.mean of n experiments. Statistical analysis was performed by a one-way analysis of variance and unpaired, two-tailed t tests with $P < 0.05$ considered statistically significant.

Results

Functional studies in rat aortic rings in vitro

In phenylephrine-constricted aortic rings, with intact endothelium, incubation in Krebs solution for 8 h resulted in a small decrease in tone (−20 ± 8%; $n = 4$, Figure 1a) and addition of L-NMMA (3×10^{-4} M) constricted these vessels (Figure 1a). Incubation of vessels with LPS (5 μg ml^{-1}) resulted in a significantly greater ($P < 0.05$) loss of tone which reached −88 ± 4% loss of tone by 8 h ($n = 5$, Figure 1b). The reduction in tone could be reversed by addition of L-NMMA (3×10^{-4} M; Figure 1b) but not by econazole (1×10^{-5} M; data not shown). Co-incubation of vessels with econazole (1×10^{-5} M) significantly ($P < 0.05$) inhibited the LPS-induced reduction in tone (Figure 2). After incubation with LPS for 4 h, addition of L-arginine (3×10^{-4} M) but not D-arginine (3×10^{-4} M) resulted in a rapid reduction in tone ($n = 4$, Figure 1c), the relaxation to arginine was not seen when rings were co-incubated with LPS and econazole ($n = 4$, Figure 1d), or when L-arginine was added to vessels not incubated with LPS ($n = 3$; data not shown).

In control rings phenylephrine caused a concentration-dependent increase in contraction (Figure 3). Incubation with LPS (5 μg ml^{-1}, 8 h) resulted in a right-shift in the concentration-response curve to phenylephrine and a depression of the maximum contractile response (Figure 3). Co-incubation with econazole (1×10^{-5} M) inhibited the LPS-induced changes in the response to phenylephrine (Figure 3). Calculated EC_{50} values and maximum contractile response for each part of the study are shown in Table 1.

Mechanisms of action of econazole on inducible nitric oxide synthase

In cultured J774 cells, incubation with LPS (10 μg ml^{-1}) resulted in increased nitrite production that was inhibited by co-incubation with econazole (IC_{50} 5.0 ± 0.9 × 10^{-6} M; $n = 5$). We have previously reported that the inhibitory effects of imidazoles are mediated at a site after messenger RNA production (Bogle *et al.*, 1994). Crude J774 cell extracts were obtained from either control cells or cells stimulated with either LPS (10 μg ml^{-1}; 20 h) or LPS and econazole (1×10^{-5} M; 20 h). Treatment of cells with LPS resulted in a large increase in production of L-[^{3}H]-citrulline from L-[^{3}H]-arginine by the cell extracts. This production of citrulline was inhibited by addition of L-NMMA (1×10^{-3} M; Figure 4) but not econazole (1×10^{-5} M; Figure 4). In contrast L-[^{3}H]-citrulline production was markedly reduced (> 90%) by cell extracts obtained from cells treated for 20 h with LPS and econazole (1×10^{-5} M; Figure 4). Addition of calmodulin ($1 \times 10^{-8} - 3 \times 10^{-7}$ M) to the cytosol prepared from J774 cells treated with LPS and econazole resulted in a full restoration of NO synthase activity

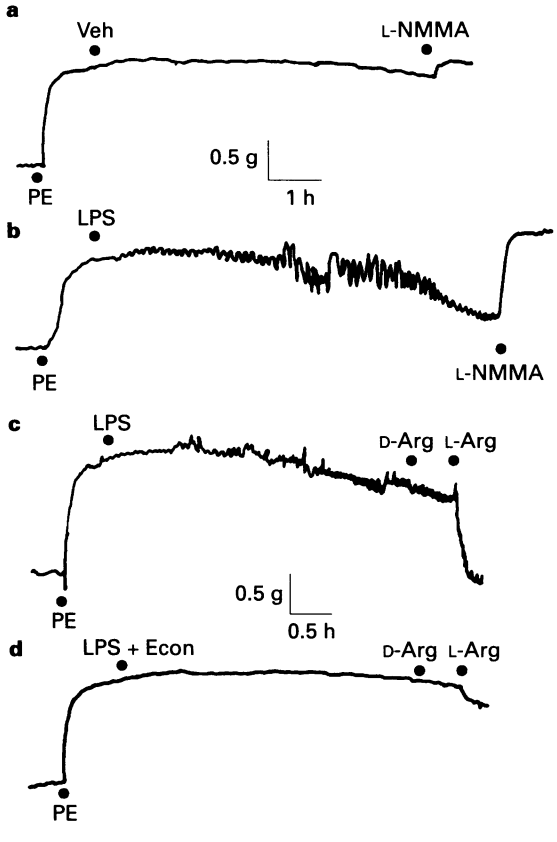

Figure 1 Induction of NO synthase in isolated vascular rings: representative traces obtained from rings of rat thoracic aorta mounted in organ baths and preconstricted with phenylephrine (PE) as described in the methods section. (a) Rings incubated with vehicle control for 8 h, followed by addition of L-NMMA (3×10^{-4} M); (b) rings incubated with LPS (5 μg ml^{-1}) for 8 h followed by addition of L-NMMA (3×10^{-4} M); (c) rings incubated with LPS (5 μg ml^{-1}; 4 h) followed by addition of D-arginine (3×10^{-4} M) and then L-arginine (3×10^{-4} M); (d) rings were incubated with LPS (5 μg ml^{-1}; 4 h) and econazole (1×10^{-5} M) followed by addition of D-arginine (3×10^{-4} M) and then L-arginine (3×10^{-4} M). Results show representative traces of data obtained in at least 3 other experiments.

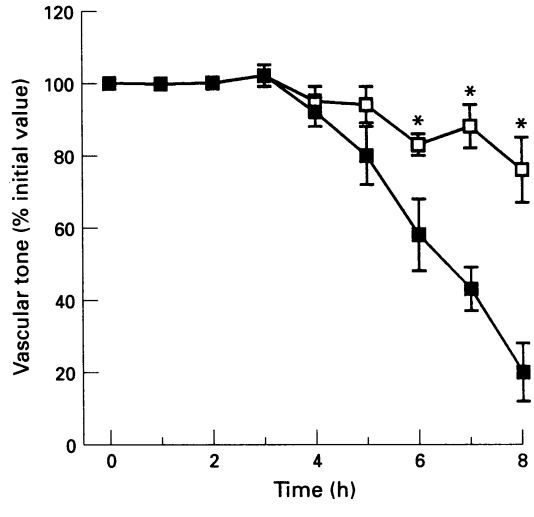

Figure 2 Econazole inhibits the time-dependent reduction of vascular tone in rat aortic rings *in vitro*: rings of aorta were mounted in 10 ml organ baths, preconstricted with phenylephrine and incubated with either LPS (5 μg ml^{-1}; ■) or LPS and econazole (1×10^{-5} M; □). Tone of the rings was monitored over the next 8 h. Results are shown as the means ± s.e.mean of data obtained in 4 separate experiments, *$P < 0.05$, unpaired t test.

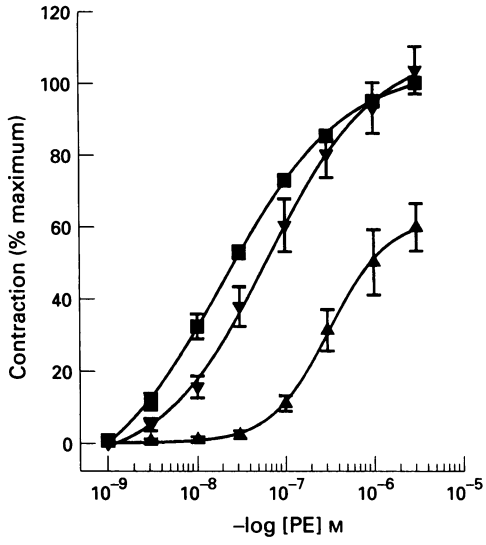

Figure 3 Effects of econazole on dose responses to phenylephrine (PE) in LPS-treated aortic rings: rings of aorta were mounted in organ baths and incubated with either Krebs solution alone (■), LPS ($5\,\mu g\,ml^{-1}$, ▲) or LPS and econazole (1×10^{-5} M, ▼). Following incubation for 8 h concentration-response curves to phenylephrine (1×10^{-9}–3×10^{-6} M) were constructed. Results shown are the means ± s.e.mean of 4–5 separate experiments.

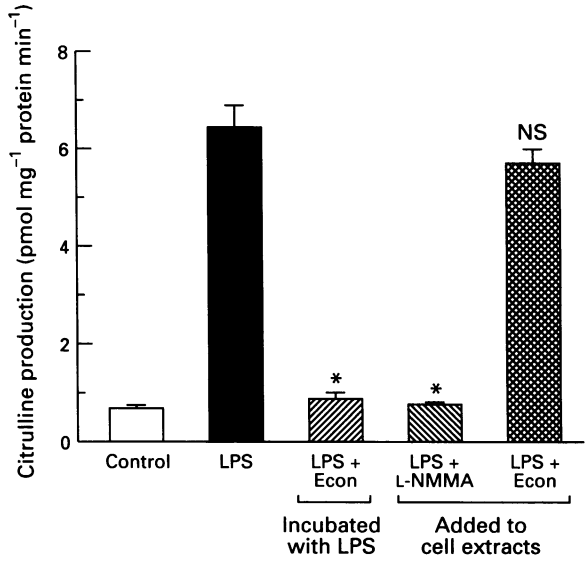

Figure 4 Effects of econazole (Econ) on isolated nitric oxide synthase activity: NO synthase activity was determined in cultured J774 cells treated for 20 h with either control vehicle (open column), LPS ($10\,\mu g\,ml^{-1}$; solid column) or LPS and econazole (1×10^{-5} M; hatched column). Alternatively, extracts from J774 cells treated with LPS alone ($10\,\mu g\,ml^{-1}$; 20 h) were co-incubated during the assay with either L-NMMA (1×10^{-3} M, hatched column) or econazole (1×10^{-5} M, cross-hatched column). Results are the means ± s.e.mean of data obtained from 3 batches of cells. $^*P<0.05$, unpaired t test.

Table 1 Prevention by econazole of the LPS-induced changes in vascular reactivity in rat aortic rings *in vitro*

Condition	EC_{50} (M)	*Maximum tension* (g)
Control	$1.9\pm0.15\times10^{-8}$	1.44 ± 0.27
LPS	$3.1\pm0.28\times10^{-7}$*	0.86 ± 0.26*
LPS + econazole	$6.5\pm0.72\times10^{-8}$*†	1.49 ± 0.19

Rings of thoracic aorta were incubated for 8 h with either control vehicle, LPS ($5\,\mu g\,ml^{-1}$) or LPS and econazole (1×10^{-5} M). Concentration-response curves to phenylephrine were then constructed and curves fitted using a logistic-sigmoid curve weighted for the standard deviation of each experimental value. Results are shown as the means ± s.e.mean of 4–5 observations; $^*P<0.01$ versus control, †$P<0.05$ vs LPS alone.

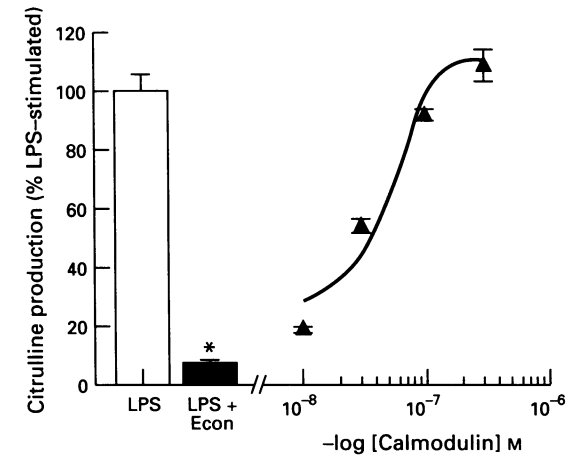

Figure 5 Calmodulin restores activity of isolated nitric oxide synthase from econazole treated cells: cultured J774 cells were treated for 20 h with either LPS ($10\,\mu g\,ml^{-1}$; open column) or LPS and econazole (1×10^{-5} M; solid column) and crude homogenates prepared as described in the methods section. Cell extracts ($30\,\mu l$) were incubated for 10 min at 37°C in the presence of increasing concentrations of calmodulin (1×10^{-8}–3×10^{-7} M) and L-[^{3}H]-citrulline production determined as previously described. Results are the means ± s.e.mean of results obtained in 3 separate experiments. $^*P<0.01$, unpaired t test.

(EC_{50} $4.2\pm0.9\times10^{-8}$ M; Figure 5). Calmodulin did not increase L-[^{3}H]-citrulline formation in cytosol obtained from J774 cells stimulated with LPS alone (data not shown).

Discussion

The results obtained in this study show that econazole prevents the decrease in vascular tone induced by LPS and inhibits the induction of an active NO synthase in murine macrophage J774 cells. The mechanism of action of econazole appears to be due to inhibition of the binding of active calmodulin to NO synthase since iNOS activity was restored *in vitro* by addition of exogenous calmodulin.

In isolated vascular rings we demonstrated that the slowly developing LPS-induced relaxation and hypo-responsiveness to phenylephrine was prevented when econazole was added to the vessels at the same time as LPS. After 4 h incubation with LPS vascular tone decreased only slightly, although there was a marked relaxant response to L-arginine but not D-arginine. Co-incubation with econazole also prevented this relaxant response to L-arginine in LPS-treated rings. Finally in vascular rings treated with LPS for 8 h, addition of econazole did not affect tone. Taken together these results are consistent with the prevention of induction of iNOS in vascular rings. Thus the inhibition of induction of a functionally active iNOS by anti-fungal imidazoles occurs in the vessel wall as well as macrophages (Bogle *et al.*, 1994) and thus might contribute to the anti-inflammatory effects of these compounds. Using J774 cells as an experimental model to study iNOS we have also identified a mechanism by which these effects appear to be mediated.

We have demonstrated previously that econazole and other anti-fungal imidazoles inhibit the generation of NO synthase in J774 cells when added at the same time as an inducing agent

(e.g. LPS), but are ineffective once NO synthase is expressed (Bogle *et al.*, 1994). These results suggested that anti-fungal imidazoles inhibited the induction rather than the activity of NO synthase. However, when mRNA levels for NO synthase were measured in imidazole-treated cells we found them to be similar to those found in cells that had been treated with LPS alone and were expressing fully active iNOS. These results could be explained in several ways. (1) Anti-fungal imidazoles inhibit the translation of NO synthase mRNA into protein; (2) anti-fungal imidazoles inhibit the synthesis or binding of a co-factor essential for activity of the expressed enzyme; or (3) anti-fungal imidazoles inhibit post-translational modification(s) necessary for the activity of NO synthase. Subsequently it has been shown that clotrimazole inhibits the induction of NO synthesis in rat hepatocytes without affecting mRNA or levels of the enzyme expressed (Kuo & Abe, 1995), indicating that translation of mRNA is not affected by these agents. In the present study we have shown that anti-fungal imidazoles are likely to act via inhibition of the activity or binding of calmodulin, which is required for activity of iNOS.

The suggestion that binding of calmodulin is the mechanism of action of the anti-fungal imidazoles is supported by the observation that these compounds inhibit calmodulin activity at low micromolar concentrations (e.g. IC_{50} 6.3×10^{-6} M for econazole; Hegemann *et al.*, 1993), similar to those concentrations required to inhibit expression of NO synthesis in our experiments in J774 cells (IC_{50} 5.0×10^{-6} M for econazole). Moreover the rank order of potency of the anti-fungal imidazoles in inhibiting NO synthase (Bogle & Vallance, 1995b) or calmodulin (Hegemann *et al.*, 1993) are similar, and other calmodulin antagonists including chlorpromazine (Palacios *et al.*, 1993), TMB-8 and W-7 (Sodhi & Kumar, 1994) and trifluoperazine (Bogle R.G., unpublished observations), also inhibit the induction of an active iNOS in macrophages. Finally we demonstrated that in econazole-treated macrophages iNOS was present and its activity could be restored by addition of excess calmodulin.

Anti-fungal imidazoles also inhibit activity of bovine brain Ca^{2+}/calmodulin-dependent NO synthase (Wolff *et al.*, 1993). This inhibition is attributable to interaction at two sites. At low concentrations (< 50 μM), anti-fungal imidazoles act as calmodulin antagonists, whilst at higher concentrations (> 50 μM) they reduce NO synthase activity directly by interaction at the haem site of the enzyme (Wolff *et al.*, 1993). Once it is actively expressed, iNOS is not inhibited by econazole (this study) or other anti-fungal imidazoles at concentrations up to 200 μM (Wolff & Bradley, 1994; Bogle & Vallance, 1995b). This is consistent with the observation that the iNOS does not undergo a reversible, Ca^{2+}-dependent association with calmodulin, but calmodulin is an integral subunit, tightly associated with the enzyme in an apparently Ca^{2+}-independent manner (Cho *et al.*, 1992). However, the present experiments suggest that inhibition of calmodulin activity of binding to iNOS during the process of induction results in expression of an inactive enzyme, the activity of which can be reconstituted by addition of excess calmodulin. Incubation with econazole (and presumably with other agents that remove or inhibit calmodulin during the process of induction) turns the normally calmodulin-insensitive NO synthase into a calmodulin-deficient enzyme that is calmodulin-dependent. It remains to be determined whether this observation accounts for the finding that an enzyme similar to, or identical with iNOS, appears to be Ca^{2+}/calmodulin-sensitive in some cells such as the rabbit chrondrocyte (Palmer *et al.*, 1992).

Recently there have been several reports suggesting that anti-fungal imidazoles have anti-inflammatory properties (Williams & Maier, 1992) and might be potential therapeutic agents for the treatment of septic shock (Slotman *et al.*, 1988; Yu & Tomasa, 1993). In septic shock there is induction of iNOS leading to widespread vasodilatation and reduced responsiveness to vasoconstrictors (Vallance & Moncada, 1993). Our results show that econazole prevents the induction of active NO synthase in response to LPS and indicate that the mechanism of action is likely to be dependent on inhibition of calmodulin. This might have implications for the development of novel anti-inflammatory agents.

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References

BOGLE, R.G., BAYDOUN, A.R., PEARSON, J.D., MONCADA, S. & MANN, G.E. (1992). L-arginine transport is increased in macrophages generating nitric oxide. *Biochem. J.*, **284**, 15–18.

BOGLE, R.G., SOO, S-C., WHITLEY, G. ST.J., JOHNSTONE, A.P. & VALLANCE, P. (1994). Effects of anti-fungal imidazoles on mRNA levels and nitric oxide synthesis in cultured murine macrophages. *Br. J. Pharmacol.*, **105**, 919–923.

BOGLE, R.G. & VALLANCE, P. (1995a). Econazole inhibits the induction of nitric oxide synthesis in lipopolysaccharide-treated rat aorta. *Br. J. Pharmacol.*, **114**, 130P.

BOGLE, R.G. & VALLANCE, P. (1995b). Ketoconazole and other anti-fungal imidazoles inhibit the induction of nitric oxide synthase. *Am. J. Resp. Crit. Care Med.*, **151**, A345.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

BREDT, D.S., HWANG, P.M., GLATT, C.E., LOWENSTEIN, C., REED, R.R. & SNYDER, S.S. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome *P*450 reductase. *Nature*, **351**, 714–718.

CHO, H.J., XIE, Q-W., CALAYCAY, J., MUMFORD, R.A., SWIDEREK, K.M., LEE, T.D. & NATHAN, C.F. (1992). Calmodulin is a subunit of nitric oxide synthase from macrophages. *J. Exp. Med.*, **176**, 599–604.

GREEN, L.C., WAGNER, D.A., GLOGOWSKI, K., SKIPPER, P.L., WISHNOK, J.S. & TANNENBAUM, S.R. (1982). Analysis of nitrate, nitrite and [^{15}N]nitrate in biological fluids. *Anal. Biochem.*, **126**, 131–138.

GRIFFITHS, M.J.D., MESENT, M., MACALLISTER, R.J. & EVANS, T.W. (1994). Aminoguanidine selectively inhibits inducible nitric oxide synthase. *Br. J. Pharmacol.*, **112**, 43–48.

GROSS, S.S. & LEVI, R.L. (1992). Tetrahydrobiopterin synthesis: An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle cells. *J. Biol. Chem.*, **26**, 25722–25729.

HEGEMANN, L., TOSO, S.M., LAHIJANI, K.I., WEBSTER, G.F. & UITTO, J. (1993). Direct interaction of antifungal azole-derivatives with calmodulin: A possible mechanism for their therapeutic activity. *J. Invest. Dermatol.*, **100**, 343–346.

JOLY, G.A., NARAYANAN, K., GRIFFITH, O.W. & KILBOURN, R.G. (1995). Characterisation of the effects of two new arginine/citrulline analogues on constitutive and inducible nitric oxide synthase in rat aorta. *Br. J. Pharmacol.*, **115**, 491–497.

KUO, P.C. & ABE, K.Y. (1995). Cytokine-mediated production of nitric oxide in isolated rat hepatocytes is dependent on cytochrome *P*-450III activity. *FEBS Lett.*, **360**, 10–14.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: Physiology, pharmacology and pathophysiology. *Pharmacol. Rev.*, **43**, 109–142.

MORRIS, S.M. & BILLIAR, T.R. (1994). New insights into the regulation of inducible nitric oxide synthesis. *Am. J. Physiol.*, **266**, E829–E839.

PALACIOS, M., PADRON, J., GLARIA, L., ROJAS, A., DELGADO, R., KNOWLES, R. & MONCADA, S. (1993). Chlorpromazine inhibits both the constitutive nitric oxide synthase and the induction of nitric oxide synthase after LPS challenge. *Biochem. Biophys. Res. Commun.*, **196**, 280–286.

PALMER, R.M.J., ANDREWS, T., FOXWELL, N.A. & MONCADA, S. (1992). Glucocorticoids do not affect the induction of a novel calcium-dependent nitric oxide synthase in rabbit chrondrocytes. *Biochem. Biophys. Res. Commun.*, **188,** 209–215.

PETRI, H., TRONNIER, H. & HAAS, P. (1986). Investigations into the anti-inflammatory effect of bifonazole. In *Advances in Topical Antifungal Therapy*. ed. Hay, R.J. pp. 26–31. Berlin: Springer Verlag.

SALTER, M., KNOWLES, R.G. & MONCADA, S. (1991). Widespread tissue distribution, species distribution and changes in activity of calcium-dependent and calcium-independent nitric oxide synthase. *FEBS Lett.*, **291,** 145–149.

SODHI, A. & KUMAR, R. (1994). Production of nitric oxide and its regulation in murine peritoneal macrophages treated with cisplatin and lipopolysaccharide. *Int. J. Immunopathol. Pharmacol.*, **7,** 65–77.

SOUTHAN, G.J., SZABÓ, C.S. & THIEMERMANN, C. (1995). Isothioureas: potent inhibitors of nitric oxide synthases with variable isoform selectivity. *Br. J. Pharmacol.*, **114,** 510–516.

SLOTMAN, G.J., BURCHARD, K.W., D'AREZZO, A. & GANN, D.S. (1988). Ketoconazole prevents acute respiratory failure in critically ill surgical patients. *J. Trauma,* **28,** 648–654.

STUEHR, D.J., CHO, H.J., KWON, N.S., WEISE, M.F. & NATHAN, C.F. (1991). Purification and characterisation of the cytokine-induced macrophage nitric oxide synthase: An FAD- and FMN-containing flavoprotein. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 7773–7777.

VALLANCE, P. & MONCADA, S. (1993). The role of endogenous nitric oxide in septic shock. *New Horizons*, **1,** 77–86.

VALLANCE, P. & MONCADA, S. (1994). Nitric oxide – from mediator to medicines. *J. R. Coll. Phys. Lond.*, **28,** 209–219.

WEI, X-q., CHARLES, I.G., SMITH, A., URE, J., FENG, G-j., HUANG, F-p., XU, D., MULLER, W., MONCADA, S. & LIEW, F.Y. (1995). Altered immune response in mice lacking inducible nitric oxide synthase. *Nature*, **375,** 408–411.

WILLIAMS, J.G. & MAIER, R.V. (1992). Ketoconazole inhibits alveolar macrophage production of inflammatory mediators involved in acute lung injury (adult respiratory distress syndrome). *Surgery*, **112,** 270–277.

WOLFF, D.J. & BRADLEY, B.J. (1994). Interferon-γ-inducible murine macrophage nitric oxide synthase: Studies on the mechanism of inhibition by imidazole agents. *Arch. Biochem. Biophys.*, **311,** 293.

WOLFF, D.J. DATTO, G.A. & SAMATOVICZ, R.A. (1993). The dual mode of inhibition of calmodulin-dependent nitric oxide synthase by antifungal imidazole agents. *J. Biol. Chem.*, **268,** 9430–9436.

YU, M. & TOMASA, G. (1993). A double blind, prospective, randomised trial of ketoconazole, a thromboxane synthetase inhibitor, in the prophylaxis of the adult respiratory distress syndrome. *Crit. Care Med.*, **21,** 1635–1642.

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